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From the regulation of peptidoglycan synthesis to bacterial growth and morphology

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Abstract

How bacteria grow and divide while retaining a defined shape is a fundamental question in microbiology, but technological advances are now driving a new understanding of how the shape-maintaining bacterial peptidoglycan sacculus grows. In this Review, we highlight the relationship between peptidoglycan synthesis complexes and cytoskeletal elements, as well as recent evidence that peptidoglycan growth is regulated from outside the sacculus in Gram-negative bacteria. We also discuss how growth of the sacculus is sensitive to mechanical force and nutritional status, and describe the roles of peptidoglycan hydrolases in generating cell shape and of D-amino acids in sacculus remodelling.

Bacteria come in a range of shapes (such as cocci and rods), and their internal volume ranges from ~10^{-2} to ~10^{6} μm^3 (REF 1). Importantly, however, cells of any given species are rather uniform in shape and size during vegetative growth. Therefore, growing bacteria must have robust mechanisms to maintain their shape and pass it on to their progeny. How bacteria achieve this remains a fundamental question in microbiology.

The peptidoglycan `sacculus` maintains cell shape and provides mechanical strength to resist osmotic challenges[2]. The mesh-like sacculus surrounds the cytoplasmic or inner membrane and is composed of glycan chains crosslinked by short peptides. Growth of the sacculus is a dynamic process requiring synthases to make peptidoglycan and attach it to the existing sacculus, and presumably hydrolases to cleave the sacculus to allow insertion of the

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newly synthesized material[3]. Because these enzymes are several orders of magnitude smaller than the sacculus itself, their activity must be spatiotemporally controlled so that insertion of new material maintains the cell shape. This Review focuses on our current understanding of sacculus growth, primarily in Gram-negative bacteria, and its relationship to bacterial growth and morphogenesis.

Peptidoglycan synthesis and hydrolysis

Synthesis of peptidoglycan occurs in three overall stages (FIG. 1). First, soluble, activated nucleotide precursors (UDP-*N*-acetylglucosamine and UDP-*N*-acetylmuramyl pentapeptide) are synthesized in the cytoplasm[4]. Second, at the inner leaflet of the inner membrane, the nucleotide precursors are assembled with undecaprenyl phosphate to form the lipid-anchored disaccharide-pentapeptide monomer subunit (lipid II), and are flipped across the membrane[5,6]. Third, lipid II is polymerized, releasing undecaprenyl pyrophosphate, and the resulting glycan chains are inserted into the sacculus. Peptidoglycan synthesis and insertion are guided by distinct elements of the `bacterial cytoskeleton` at different phases in the cell cycle. With the cooperation of `actin`-like rod-shapedetermining protein MreB, newly divided cells elongate by inserting peptidoglycan into multiple sites in the lateral wall of the cell (‘dispersed’ elongation). Later, the `tubulin`-like cell division protein FtsZ localizes to the midcell to guide a ‘preseptal’ phase of cell elongation, followed by ‘constrictive’ septum synthesis, which enables cell division and daughter cell separation. TABLE 1 summarizes the established peptidoglycan enzymes and cell morphogenesis proteins of *Escherichia coli.*

Peptidoglycan synthases

Peptidoglycan synthesis requires glycosyltransferases (GTases) to polymerize the glycan chains and DD-transpeptidases (DD-TPases) to crosslink the peptides[7] (FIG. 1). TPases, also called `penicillin-binding proteins` (PBPs), were initially identified because of their ability to covalently bind penicillin[8]. There are three types of these peptidoglycan synthases: bifunctional GTase–TPases (the class A PBPs), monofunctional TPases (the class B PBPs) and monofunctional GTases[7]. *E. coli* has three bifunctional synthases (PBP1A, PBP1B and PBP1C), a GTase (MgtA) and two TPases (which are essential either for cell elongation (PBP2) or for cell division (PBP3; also known as FtsI)). PBP1A and PBP1B are partially redundant; the cell requires one of them for viability[9]. PBP1C may be used in host cells[10]. PBP1A, which is thought to have a role in cell elongation, interacts with the elongation TPase PBP2 (M.B. and WV., unpublished observations). PBP1B interacts with two essential division proteins (FtsN and the division TPase PBP3) and is enriched at the septum[11].

The TPase and GTase activities of bifunctional synthases have been reconstituted *in vitro* with their lipid II substrate[12,13]. Under conditions that favour PBP1B dimerization, this synthase produces glycan chains of ~28 disaccharide units on average, crosslinking about 40–50% of the peptides. PBP1A produces shorter glycan chains (~20 disaccharide units) and crosslinks ~22% of the peptides. Importantly, although glycan chain polymerization occurs in the absence of transpeptidation (for example, in the presence of penicillin), efficient transpeptidation requires ongoing GTase reactions[12,13]. It was suggested, based on the crystal structure of PBP1B, that the growing glycan chain produced by the GTase domain moves towards the TPase domain, enabling the pentapeptide of the glycan chain to enter the active site of the TPase domain and serve as a donor for transpeptidation[14]. Surprisingly, crystal structures of peptidoglycan TPases do not show any obvious binding site for the acceptor peptide, indicating that transpeptidation, the target of `β- lactam antibiotics`, is not fully understood at the molecular level[15].

Peptidoglycan hydrolases

Simply attaching new material to the sacculus by transpeptidation thickens the sacculus without elongating it, suggesting that peptidoglycan growth requires the cleavage of covalent bonds in the sacculus to allow the newly attached material to insert into the layer without increasing its thickness. Peptidoglycan cleavage is also required for reductive cell division and cell separation. *E. coli* has at least 13 periplasmic peptidoglycan hydrolases (`autolysins`), which can collectively cleave almost any glycoside and amide bond[16] (FIG. 1). Studies in a range of bacteria have indicated that hydrolases sculpt the shape, size and thickness of peptidoglycan and are essential for separation of daughter cells during cell division or after it is completed (see below). However, possibly owing to high redundancy, no single hydrolase gene knockout prevents growth of *E. coli*, and multiple hydrolase genes have to be deleted for chains of non-separated cells to form. Amidases have a prominent role in septum cleavage[17], but lytic transglycosylases and endopeptidases also contribute to cell separation[18,19], and their role is probably understated owing to their greater redundancy. In *E. coli*, as much as 40–50% of the total peptidoglycan material is removed every generation by the action of peptidoglycan hydrolases, and lytic transglycosylases are major contributors to this process[20]. The soluble fragments that are removed from the sacculus are reused via an efficient peptidoglycan-recycling pathway[21].

Sacculus growth

Biophysical and `electron cryotomography` (ECT) data on isolated *E. coli* sacculi support a singlelayer model of peptidoglycan architecture, with somewhat disordered glycan chains running perpendicular to the long axis of the cell, on average[22]. Enlargement of the peptidoglycan requires new glycan chains to attach and insert while peptidoglycan thickness and cell shape are maintained. Labelling experiments have confirmed that peptidoglycan grows by the insertion of newly synthesized glycan chains, or patches of them, into the existing sacculus[23,24]. The absence of unattached oligomeric intermediates of nascent glycan chains in the cell suggests that the polymerization of new glycan chains and their attachment to the sacculus by transpeptidation occur simultaneously[25], which fits with the existence of bifunctional enzymes or enzyme complexes that perform both reactions. The attachment of newly made peptidoglycan to sacculi by transpeptidation has been reconstituted *in vitro* with the bifunctional synthase PBP1A[13]. The newly synthesized peptidoglycan is rich in pentapeptides and has glycan chains with an average length of ~50–60 disaccharides. Shortly after synthesis, the pentapeptides are trimmed to tetrapeptides by DD-carboxypeptidases; some tetrapeptides are further shortened to tripeptides by LD-carboxypeptidases. In addition, lytic transglycosylases reduce the average length of glycan chains to ~35–40 disaccharides, and LD-TPases covalently attach major outer-membrane lipoprotein Lpp (also known as Braun's lipoprotein) to the sacculus[24] (FIG. 1).

The mechanism for insertion of new glycan chains remains unsettled. During cell division, when peptide crosslinks are formed exclusively between newly synthesized glycan chains, about one-third of the new septal peptidoglycan is removed[26], consistent with the model that peptidoglycan grows by simultaneously inserting three new chains and removing one old one (the ‘3 for 1’ model[3]). It is less clear whether this model applies to lateral cell wall growth during cell elongation, when peptide crosslinks seem to form predominantly between new and old chains and peptidoglycan turnover is low[21,23,26,27].

The cytoskeleton and peptidoglycan growth

Our understanding of the interplay between bacterial cytoskeletal elements and peptidoglycan growth is evolving (FIG. 2). For instance, there is an increasing appreciation of the diversity of bacterial cytoskeletal elements and the parts that they play in force generation and sensing cellular states. For more information on bacterial cytoskeletal elements, the reader is referred to an excellent recent review[28].

MreB and peptidoglycan synthesis during cell elongation

The actin-like protein MreB is used by many rod-shaped bacteria in their elongation mode of peptidoglycan synthesis. MreB forms filaments[29–31] and interacts with the conserved inner-membrane proteins MreC, MreD and RodZ[32–36], as well as with the lipid II synthesis enzymes MraY and MurG[37]. The MreB filament is tethered to the inner membrane via an interaction with the cytoplasmic domain of RodZ[38]. *E. coli* MreB also binds directly to the inner membrane through an N-terminal amphipathic helix[39]. When MreB is depleted, or filament formation is specifically inhibited (for example, with A22 (*S*-(3,4-dichlorobenzyl)isothiourea)), cells rapidly stop elongating, increase their diameter and grow with spherical morphology, indicating that MreB is required for rod-shaped growth[40–42].

MreB was initially thought to form dynamic helical structures spanning the length of the cell[30,43]. Preliminary evidence of treadmilling-type movement, with polymerization at one end of the helix and depolymerization at the other end, led to the suggestion that polymerization dynamics guide filament movement[44–46]. However, recent studies have challenged this view. High-resolution imaging indicates that MreB filaments do not span the cell length either in *E. coli* or in the Gram-positive bacterium *Bacillus subtilis.* Instead, MreB forms small filament patches that move around the cell circumference perpendicularly to its long axis[47–49]. Perturbing the ATP cycle of MreB, either by adding the inhibitor A22 (REF. 49) or by mutation of *mreB*[48], does not affect MreB motion, indicating that polymerization might not be the driving force for its movement. By contrast, antibiotic-mediated inhibition of cell wall synthesis rapidly stops filament movement[47–49]. Interestingly, inhibition or depletion of the specific elongation class B TPases (PBP2 in *E. coli*, and PBP2A and PBPH in *B. subtilis)* eliminates filament movement in both organisms, suggesting that peptidoglycan synthesis itself is the motor that drives filament movement[47–49]. Consistent with this idea, both MreB and the peptidoglycan synthesis complexes move with comparable velocities in roughly similar circumferential directions[47–48], mirroring the position of glycan chains in the sacculus[22]. Thus, MreB is integral to shape determination, as it directly or indirectly recruits and/or positions peptidoglycan biosynthesis machineries[31,50,51], but it cannot move without ongoing peptidoglycan synthesis. Instead, both MreB filaments and peptidoglycan complexes move as a coordinated unit in the direction of the glycan chains, at least in a short range, and are functionally interdependent. It remains to be determined whether such short-range coordinated movements contribute to a coherent rod shape. Interestingly, ovococci, such as *Streptococcus pneumoniae*, lack MreB but retain MreC and MreD, which are essential; recent genetic evidence suggests that MreC and MreD control peripheral peptidoglycan synthesis by affecting the activity or localization of PBP1A[52].

FtsZ and peptidoglycan synthesis

FtsZ, a tubulin-like protein, is the master regulator of bacterial cell division. Prior to cell division, FtsZ forms a dynamic ringlike structure at midcell, called the Z ring. The Z ring consists of arches of bundles of FtsZ filaments that rapidly polymerize and depolymerize on binding and hydrolysis of GTP, a process that is regulated by numerous effectors[53]. More than ten essential cell division proteins localize to the Z ring to form the divisome[54]. Cells lacking functional FtsZ are unable to divide and instead grow as filaments.

In *E. coli*, the first step of divisome assembly begins before constriction, when early cell division proteins, including FtsZ, FtsA and ZipA, localize to the future division site[55] (FIG. 2), and ZipA and the actin-like protein FtsA interact with and stabilize the Z ring at the inner membrane. Enzymes for lipid II synthesis, and presumably peptidoglycan synthesis, are recruited to the Z ring to carry out preseptal elongation[7,56]. Notably, this stage of synthesis is independent of the TPase PBP3, an essential late divisome protein required for constrictive peptidoglycan synthesis[57]. Preseptal peptidoglycan synthesis contributes substantially to cell elongation in *Caulobacter crescentus* and less so in *E. coli*[56,57].

When assembly is complete, the divisome synthesizes the new cell poles, including the peptidoglycan layer of the daughter cells (FIG. 2). PBP3, a core member of the divisome, is essential for septal peptidoglycan synthesis. It is recruited to the septum by the lipid II flippase FtsW[6,58] and interacts with the FtsQLB complex[59,60], PBP1B[11] and FtsN[61]. Although FtsN has a central role in triggering septation and stabilizing the divisome ring[62], a hyperactive FtsA can bypass some of its effects[63]. The carboxy-terminal peptidoglycan-binding SPOR domain of FtsN may contribute to its midcell localization via specific recognition of septal peptidoglycan[64,65]. The essential function of FtsN is mediated by a short (35 amino acid) periplasmic region, which does not include its SPOR domain. It is tempting to speculate that the essentiality of FtsN is related to its interactions with the peptidoglycan synthases PBP1B and/or PBP3 (REF 66).

Although most core divisome components are widely conserved, their order of assembly may differ across species. For example, in *C. crescentus*, FtsA arrives at midcell well after FtsZ and the FtsEX complex, and shortly before FtsN, FtsQ, PBP3, FtsK (the DNA translocase) and FtsL. Septation occurs only after FtsW and FtsB arrive last at midcell[67].

The role of peptidoglycan hydrolases in septation is discussed below. How the cell controls the switch from dispersed to preseptal peptidoglycan elongation and from preseptal to septal peptidoglycan synthesis is unknown, but it probably involves changes in the localization and dynamics of the cytoskeletal-like elements.

Additional classes of cytoskeleton-like elements

Cytoskeletal elements with similarity to eukaryotic `intermediate filaments` (IFs) have been recently identified via ECT[68,69] and seminal *C. crescentus* studies[70,71]. Crescentin (CreS) is anchored in the inner membrane through MreB[70] and has a role in generating curvature in *C. cresentus*[72]. The IF-like protein, FilP, is required for hyphal mechanical strength in the Gram-positive bacterium *Streptomyces coelicolor*[73].

Bactofilins are a new class of cytoskeletal element. In *C. crescentus*, they form a sheet-like polymer that lines the inner membrane of the stalked cell pole and recruits the synthase PBPC to nucleate stalk biogenesis. Bactofilins are widely conserved and associate spontaneously in ribbon-or rod-like filaments *in vitro*[74]. Bacterofilins participate in diverse processes: cell division in *Shewanella oneidensis*[74], social motility and colony morphology in *Myxococcus xanthus*[74,75], and helical shape formation in *Helicobacter pylori* (specifically, the protein curved cell morphology A (CcmA; encoded by the locus HPG27_1480)[76]). The extent to which these cytoskeletal elements are scaffolds for coordinating peptidoglycan synthesis and hydrolysis remains to be addressed.

Cytoskeletal elements and mechanical control of growth

When transient mechanical forces bend a rod-shaped bacterial cell, the bent cell straightens after the force is removed[77]. However, the outcome is quite different when cells experience constant mechanical force from one side during growth. Filamentous *E. coli* cells growing in a curved cell shape along microchamber walls retain their bent cell shape when removed from the microchamber. Thus, a long-term mechanical force on one side of the cell makes the cells produce a curved peptidoglycan sacculus during growth[78]. This experiment demonstrates that mechanical force can affect the topology of peptidoglycan synthesis and bacterial morphogenesis, just as mechanical force can affect growth and shape of eukaryotic organisms[79]. Here, we consider how forces exerted by cytoskeletal elements alter the mechanical properties of the cell and affect peptidoglycan growth (FIG. 3).

CreS localizes exclusively to the inner cell curvature by an unknown mechanism involving MreB[70,71] (FIG. 3a). By reducing the physical strain on the inner side of the cell wall, CreS reduces the peptidoglycan growth rate on the inner relative to outer side of the sacculus, inducing curvature. CreS is necessary and sufficient for curvature[72]. A mechanical role for CreS is supported by the fact that the straight rod phenotype of the *creS* mutant is suppressed by external mechanical force when cells elongate along the walls of a microchamber, and by the observed relaxation of the CreS filament on antibiotic-induced membrane detachment *in vivo*[72] (FIG. 3a). Heterologously expressed CreS also bends *E. coli* cells[72]. The rate of straightening of *C. crescentus* cells following disruption of the CreS filament is related to the processivity of peptidoglycan synthesis enzymes[80]. Without mechanical force, processive peptidoglycan synthesis in the direction of the glycan chains perpendicular to the long axis may provide a robust mechanism for straight growth of rod-shaped cells[80]. A biophysical model simulating cellular growth dynamics indicates that robust cell wall synthesis, which is insensitive to local peptidoglycan density, is a key additional contributing feature to rod-shape maintenance[81].

Other cytoskeletal elements also exert forces. The cell division protein FtsZ generates a constriction force at the division site of the inner membrane, possibly through bending of the FtsZ protofilaments (FIG. 3b). A membrane-attached version of FtsZ generates constrictions in tubular liposomes in the presence of GTP. Thus, when FtsZ becomes membrane attached, other cellular components are not required to generate force[82]. Helical MreB filaments also affect the mechanical properties of *E. coli*. When MreB polymers are disassembled with the antibiotic A22, the bending stiffness of cells, as measured by optical traps, decreases (FIG. 3c). Reformation of the MreB cytoskeleton restores the stiffness[77], indicating that force generation by MreB may contribute to cellular mechanical properties. It is possible that the forces generated by the FtsZ-and MreB-containing cytoskeletal structures cause local stretching of the sacculus, promoting peptidoglycan synthesis during cell division and elongation.

Cytoskeleton-like elements provide metabolic inputs

Recent reports suggest that information on the metabolic status of the cell can be conveyed through cytoskeletal elements to the peptidoglycan synthesis machinery. UgtP, a *B. subtilis* processive diacylglycerol glucosyltransferase, prevents FtsZ assembly when UDP-glucose levels are high. This allows cells in carbon-rich media to grow bigger before they divide[83]. YvcK, a novel putative *B. subtilis* metabolic enzyme, builds helical structures that are required for the normal rod-shaped cell morphology and for localization of PBP1 (REF. 84). PBP1 localization is also dependent on MreB, and *mreB* mutants swell and lyse unless they are supplemented with Mg^{2+} (REF 50). Interestingly, YvcK and MreB helices (or, more accurately, dynamic patches) build independently of each other and do not overlap, but slight overexpression of either YvcK or MreB compensates for the loss of the other protein, restoring PBP1 localization and cell shape. The physiological role of YvcK is connected with gluconeogenic growth, when it becomes essential for PBP1 localization and the rod shape[84]. YvcK is also conserved in spherical Gram-positive bacteria and is essential in *Staphylococcus aureus*[85], begging the question of its role in such organisms. Finally, CTP synthase (CtpS; also known as PyrG) forms filaments both in *E. coli* and *C. crescentus.* These filaments interact with CreS and inhibit curvature in *C. crescentus*. Although the CTP synthase activity of CtpS is not required for filament formation, its glutamine amidotransferase activity is absolutely essential[86], suggesting that filament formation conveys information on the cellular metabolic status to influence sacculus growth.

Peptidoglycan hydrolases sculpt the cell

Peptidoglycan hydrolases have intimate roles in peptidoglycan growth, cell division and bacterial shape. Although these hydrolases are usually highly redundant, recent studies in a wide range of bacteria are beginning to uncover their diverse roles in the cell.

Role of DD-carboxypeptidases

E. coli has at least six DD-carboxypeptidases, but PBP5 (also known as DacA) is the most active. Cells lacking PBP5 have slightly altered morphology; when additional PBPs are removed, the cells branch, kink and bend[87,88]. Guided by its membrane anchor and the availability of its pentapeptide substrate, PBP5 localizes to sites of peptidoglycan synthesis[89], where it removes terminal D-Ala residues from pentapeptides and reduces the number of donors for transpeptidation (FIG. 1). DD-carboxypeptidases are important shape regulators in *E. coli* but have only a minor role in other species, such as *C. crescentus*[90], which must possess peptidoglycan growth mechanisms that permit the enlargement of a pentapeptide-rich sacculus.

Roles of the amidases and M23–LytM endopeptidases

The three *N*-acetylmuramyl-L-Ala amidases in *E.* coli, AmiA, AmiB and AmiC, are important for septation and cell separation. Double and triple amidase mutants form cell chains in which individual cells constrict their inner membrane and peptidoglycan but not their outer membrane, resulting in abnormally large periplasmic spaces[17]. These cells also exhibit unusually high outer-membrane permeability[19]. Although AmiB and AmiC are septally localized, AmiA is not[91]. Interestingly, cells with only AmiA can still partially separate[17].

The endopeptidases of the M23–LytM family are also implicated in septation in *E. coli*, as a quadruple knockout of all *E. coli* LytM paralogues (EnvC, NlpD, YgeR and YebA) is defective in cell separation[92]. The two characterized members, EnvC and NlpD, are catalytically inactive but, instead, are the first proteins shown to regulate amidase activity. NlpD activates AmiC, and EnvC activates AmiA and AmiB, both by as-yet-unknown mechanisms[93]. Transient interaction of AmiA with EnvC, coupled with its activation, is likely to be sufficient for septal cleavage, thereby explaining why non-septally localized AmiA can function as the sole amidase. Interestingly, EnvC and NlpD localize to the septum earlier than their cognate amidases, which require active PBP3 for localization, thereby ensuring that peptidoglycan synthesis initiates before the amidases concentrate at the septum[94].

In contrast to the regulatory role of the characterized *E. coli* LytM family peptidases, in *C. crescentus* the LytM peptidase, DipM, is essential for cell constriction[95–97]. DipM locates to midcell through an interaction with FtsN and is then likely to bind septal peptidoglycan via its four LysM peptidoglycan-binding domains[96]. The LytM peptidase domain is required for septum cleavage[96,97] and may have peptidoglycan hydrolase activity itself[96]. Remarkably, compared with wild-type cells, *dipM* mutants have a peptidoglycan layer of 2–3-fold greater average thickness and exhibit outer-membrane `blebbing`[95], indicating that the hydrolase activity of DipM is involved in a mechanism to maintain constant peptidoglycan thickness in *C. crescentus*.

The *H. pylori* LytM paralogues, cell shape determinant 1 (Csd1), Csd2 and Csd3, are all required for the helical shape of these cells, as mutants grow as slightly (in the case of *csd1* or *csd2* mutants) or highly (in the case of *csd3* mutants) curved rods, rather than helically[76].

The increased crosslinkages in the sacculi of these mutant strains supports the proposal that Csd proteins have an endopeptidase activity. Csd3 exhibits both DD-endopeptidase and DD-carboxypeptidase activities, consistent with the higher number of crosslinks and pentapeptides in csd3-mutant cells[76,98]. Hence, the Csd proteins use their endopeptidase activity to achieve controlled local relaxation of peptidoglycan crosslinks, thereby introducing curvature and twist that leads to the helical cell shape of *H. pylori.*

Regulation of hydrolase activity

On inhibition of peptidoglycan synthesis, uncontrolled hydrolase activity results in rupture of the sacculus and cell lysis, making it imperative to control hydrolase activity. It is likely, as proposed by Höltje[3], that hydrolase activity is controlled by incorporation into multi-enzyme complexes that span the periplasm, extending from inner membrane-anchored synthases to the hydrolases, many of which are localized to the outer membrane. As part of a complex, hydrolases would be localized only to sites of peptidoglycan synthesis, thereby preventing them from hydrolysing peptidoglycan elsewhere. Intact complexes have not yet been isolated, possibly because they are dynamic, held together by weak interactions and/or tend to dissociate on breakage of the sacculus during cell lysis. However, protein interaction data[7,99,100] and the fact that each major bifunctional synthase has an outer-membrane protein regulator[101,102] support the existence of such complexes.

Hydrolase regulation is likely to be widespread. Above, we describe one situation in which hydrolases (or non-catalytic hydrolase variants) bind to and activate other hydrolases[93], and other examples of regulation have been reported recently[100]. The *Pseudomonas aeruginosa* protein inhibitor of vertebrate lysozyme (Ivy) illustrates a different regulatory paradigm. Originally thought to be a defence mechanism against only host `lysozyme`, Ivy also works against bacterial hydrolases, inhibiting membrane-bound lytic murein transglycosylase B (MltB)[103]. Such studies imply that bacteria have specific inhibitors to control their hydrolases. Indeed, some `type VI secretion systems` (T6SSs) involved in bacterial interspecies interactions use peptidoglycan hydrolases as effectors[104]. Interestingly, periplasmic immunity proteins protect the producer cell by binding to and inhibiting hydrolases delivered by neighbouring cells[104].

Control of sacculus growth from the outside

Recently, outer membrane-anchored lipoproteins that reach through the sacculus were found to control peptidoglycan synthases, complementing synthase dependence on cytoskeletal elements located in the sacculus. PBP1A and PBP1B each require a dedicated lipoprotein regulator for function *in vivo*[101,102]. Indeed, deleting the outer-membrane PBP activator lipoproteins, LpoA and LpoB, recapitulates the *in vivo* phenotypes of deleting PBP1A and PBP1B, respectively[101,102]. Each Lpo protein stimulates the TPase activity of its cognate PBP *in vitro* by an unknown mechanism[102]. LpoB has an additional small effect on the GTase rate of PBP1B *in vitro*, possibly at the initiation step[101]. Further studies are necessary to elucidate the basis for Lpo essentiality *in vivo.*

The Lpo proteins are limited to gammaproteobacteria (LpoA) or even to enterobacteria (LpoB), although they control universally conserved enzymes. LpoA and LpoB arose independently and have no sequence homology. Interestingly, LpoA and LpoB bind to portions of their cognate PBPs that are present in bacterial lineages roughly coincident with those harbouring the Lpo proteins themselves, suggesting that the Lpo proteins and their interacting domains co-evolved[102]. The additional level of regulation provided by Lpo proteins may enable niche-specific adaptation.

Other bacterial groups may have proteins with regulatory roles similar to those identified in Lpo proteins. In favour of this idea, class A PBPs in many bacterial clades exhibit diversified clade-specific non-catalytic regions near their termini or sandwiched between the GTase and TPase domains (FIG. 4). Importantly, in some cases, these regions or domains are functionally linked by genomic context or co-occurrence to outer-membrane lipoproteins or a peptidoglycan-related enzyme. For example, *P. aeruginosa* PBP1B has two non-catalytic domains that are highly conserved in members of the order Pseudomonadales (FIG. 4) and are functionally linked to an adjacent *Pseudomonas* spp.-specific outer-membrane lipoprotein containing an SPR domain, PA4699 (REF. 105). Similarly, *Agrobacterium tumefaciens* PBPC (encoded by the locus Atu0103) has unique domains that are not found outside the order Rhizobiales (FIG. 4) but are functionally linked to three M23–LytM family endopeptidases: Atu1832, Atu4178 and the outer-membrane lipoprotein Atu1700 (REF 105). Synthase diversity is also evident in Gram-positive organisms. *B. subtilis* PBP1 has a C-terminal region of ~220 amino acids that is present only in this organism (FIG. 4); closely related *Bacillus* spp. retain at least part of this domain. The recent crystal structure of *Acinetobacter baumannii* PBP1A shows an additional oligonucleotide–oligosaccharide-binding (OB)-fold domain inserted into the TPase domain[106].

Cell division in Gram-negative organisms involves simultaneous invagination of the outer membrane and constriction of the peptidoglycan and inner-membrane layers, an issue not yet discussed. The Tol–Pal complex participates in outer-membrane constriction[107] and is essential in the alphaproteobacterium *C. crescentus*[108]. However, the Tol–Pal complex is not essential in *E. coli* and other gammaproteobacteria, raising the possibility that other proteins facilitate outer-membrane constriction in gammaproteobacteria. Removing PBP1B or LpoB from *E. coli* cells lacking the Tol–Pal complex results in synthetic sickness or lethality and severe division defects, suggesting that the PBP1B–LpoB complex participates in the process[102]. This unique role for PBP1B–LpoB in outer-membrane invagination is consistent with its preferential localization at the divisome[102]. How the two systems coordinate their actions remains to be determined.

Why regulate peptidoglycan synthesis from outside the sacculus?

Using outer-membrane regulators of peptidoglycan synthases ensures that these multiprotein synthetic complexes stretch from the inner membrane to the outer membrane. If the outer-membrane proteins also anchored the hydrolases, this would provide the kind of complex envisioned by Höltje to coordinate peptidoglycan synthesis and turnover, providing a rationale for the existence of outer-membrane regulators. Alternatively, or in addition, requiring the Lpo proteins to traverse the peptidoglycan sacculus might enable the physical properties of the sacculus to directly regulate peptidoglycan synthesis. Outer membrane-anchored LpoB must reach through pores in the peptidoglycan layer to interact with its docking domain in PBP1B[102] (FIG. 5a). Several interesting regulatory scenarios can be imagined. First, the maximal molecular length of the Lpo activators might provide a molecular ruler to limit peptidoglycan thickness to a single layer; a thick, multilayered peptidoglycan might prevent access of the outer-membrane regulator to its synthases. Second, the method of activating peptidoglycan synthesis through the pores could be a robust, autoregulatory mechanism to adjust the peptidoglycan growth rate to the overall cellular growth rate based on peptidoglycan stretching (FIG. 5b). Unlike the much stiffer walls of algae and higher plants, which are only slightly strained under normal `turgor`[109], the *E. coli* peptidoglycan net is elastic and can be stretched by turgor *in vivo*[110–112] or by mechanical force *in* vitro[113,114], resulting in larger pore size. As turgor is high in fast-growing cells or those in low-osmolality medium[110], such cells might have a stretched peptidoglycan with bigger pores, thereby facilitating Lpo access to its synthases and activation of peptidoglycan synthesis. This homeostatic strategy would mechanically couple the peptidoglycan synthesis rate to the growth rate via peptidoglycan pore size (FIG. 5c). Moreover, the periplasmic volume and dimensions change during steady-state growth in different osmolality conditions and/or osmotic shifts[110,115], and this could influence Lpo-mediated PBP activation by changing the distance between Lpo proteins and PBPs. Finally, it is important to note other mechanisms that might couple peptidoglycan synthesis and stretching. At high turgor, the net is likely to be more ‘ordered’, possibly resulting in a less flexible or less variable orientation of glycan chains and peptides. Optimally positioning the synthases and their sacculus substrates could increase peptidoglycan synthesis[116]. Thus, internal turgor can have a mechanical impact on peptidoglycan growth and thereby determine cell growth and morphology[117].

Peptidoglycan remodelling

The peptidoglycan composition of stationary-phase *E. coli* cells differs from that of exponentially growing cells. The average glycan chain length decreases by ~30%, whereas crosslinkage (especially 3–3 crosslinks) and covalent attachment of Lpp increase[118]. These changes may reflect peptidoglycan maturation, as the rate of insertion of new material into the sacculus slows down in stationary phase. Remodelling of peptidoglycan occurs more frequently and to a larger extent in Gram-positive bacteria. The glycan chains are *O*-acetylated and/or *N*-deacetylated for lysozyme resistance; the peptides are amidated and have cell wall proteins and anionic surface polymers, such as teichoic acids or capsular polysaccharides, covalently attached[2,119].

E. coli can covalently link certain `D-amino acids` (for example D-Cys, D-Met or D-Trp) to its peptidoglycan. Indeed, *in vivo* labelling of peptidoglycan with exogenous D-Cys is a valuable tool for the study of peptidoglycan growth and segregation in various species[56,57]. Although D-amino acids are produced by some eukaryotes and bacteria for non-ribosomal peptide synthesis, they were not thought to have extensive roles in bacteria and their attachment to peptidoglycan was considered to be a side reaction of peptidoglycan enzymes. This view has changed with the discovery of distinct amino acid racemases for the synthesis of D-Met, D-Leu, D-Tyr and D-Phe in *Vibrio cholerae* and *B. subtilis* and the release of these D-amino acids into the growth medium, where they accumulate at millimolar concentrations[120]. The D-amino acids are incorporated into peptidoglycan and, in the case of

V. cholerae, cause a rod-to-sphere transition in a strain lacking PBP1A, even in vegetatively growing cells. Subsequent experiments showed that D-amino acids are produced by many bacteria, accumulate mainly during stationary phase and are incorporated into peptidoglycan in several ways[120,121]. LD-TPases can add D-amino acids to position 4 of peptidoglycan tetrapeptides in *E. coli* and *V. cholerae*, in addition to forming 3–3 crosslinks or attaching Lpp (FIG. 1). Moreover, in *V. cholerae*, two enzymes involved in synthesis of the activated nucleotide precursors, D-Ala–D-Ala ligase (Ddl) and UDP-*N*-acetylmuramyl tripeptide–D-alanyl-D-Ala ligase (MurF), can use D-amino acids to give rise to modified peptidoglycan pentapeptides[121]. It is interesting that the *E. coli* TPase PBP1A can incorporate D-amino acids into pentapeptides of peptidoglycan fragments *in vitro*[122], although D-amino acid-modified pentapeptides have not been detected yet *in vivo*.

What is the physiological role of modification with D-amino acids? These amino acids could have regulatory roles in cell wall remodelling during stationary phase and/or act as extracellular regulatory signals in a similar way to muropeptides, which have been shown to trigger developmental decisions in bacteria[123]. Astonishingly, a mixture of four D-amino acids at nanomolar concentrations inhibits biofilm formation in *B. subtilis* and causes disassembly of already formed biofilms. D-amino acids are also able to disperse biofilms of *S. aureus* or *P. aeruginosa*, but the molecular mechanisms underlying these processes have yet to be elucidated[124].

Future directions

Despite the substantial progress made in recent decades, we are far from fully understanding peptidoglycan synthesis and its control, because of the complexity of this process, the functional redundancy of players involved and the multiple interconnections with other central processes. New technologies are gradually revealing the contours of this process (BOX 1). Fuelled by improved imaging techniques, the increasing diversity of bacteria investigated, more sophisticated genetics, and the entrance of biophysicists, modellers and engineers to the field, peptidoglycan research is currently experiencing a renaissance period. Perhaps for the first time, we have the ability to address fundamental questions about the entire process.

The recent identification of key molecules in peptidoglycan synthesis illustrates that this process is not completely described. The new technologies now available will be able to identify the missing players, determine their physiological roles and reconstitute the relevant complexes *in vitro* to study both mechanism and structure.

How peptidoglycan synthesis is tied to overall cellular growth and coordinated with other processes is under intense investigation. Equally important is to understand how peptidoglycan synthesis is coordinated with the synthesis and transport of other envelope macromolecules. These studies must address how the growing cell changes size while maintaining its integrity and shape. Some of the species-specific non-catalytic domains found in peptidoglycan synthesis enzymes (FIG. 4) may be used as docking domains to other processes or pathways that facilitate coordination between peptidoglycan growth and remodelling and envelope biogenesis.

The recent spate of articles seeking to understand the driving force behind peptidoglycan growth clearly indicates the diversity of cutting-edge technologies addressing this issue. These technologies will undoubtedly be employed to explore related general issues, such as the role of mechanical force and cell wall stresses in cell wall growth, and to identify the minimal physical parameters required for shape determination in bacteria.

The overarching principles coupling cell growth to cell shape and cell shape to peptidoglycan synthesis are likely to be broadly conserved. However, the diversity of cell shapes, developmental programmes and ecological niches that exists suggests that there are species-specific ways of tailoring these processes and/or that completely new mechanisms have arisen to facilitate coordination. Studying morphogenesis in differently shaped bacteria with more complicated cell cycle programmes will not only broaden our understanding of the mechanisms underlying cell growth and morphology, but also pinpoint strategies used to make such mechanisms robust.

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Glossary

Sacculus

A bag-like macromolecule that is made of peptidoglycan chains crosslinked by short peptides. The sacculus completely encases the cytoplasmic membrane in most bacteria, and isolated sacculi retain the shape of the bacterial cell

Bacterial cytoskeleton

A filamentous and often dynamic cytoplasmic structure that includes bacterial structural homologues of actin, tubulin or intermediate filaments and is essential for bacterial growth, motility, cell division, morphology and DNA segregation

Actin

A eukaryotic cytoskeletal protein with ATPase activity. MreB and ParM, two bacterial proteins involved in cell elongation and plasmid partitioning, respectively, are distant actin homologues

Tubulin

A cytoskeletal protein that forms microtubules in eukaryotes;the bacterial tubulin-like protein FtsZ, is a GTPase and forms dynamic filaments to drive cell division.

Penicillin-binding proteins (PBPs)

A protein family involved in the synthesis (the class A and class B PBPs) or hydrolysis (the class C PBPs) of D-amino acid–D-amino acid peptide bonds. They contain an active-site Ser residue that participates in the transfer of an acyl compound to an amino group or water.

PBPs are the targets of β-lactam antibiotics (such as penicillin). Pathogen resistance to β-lactams can be caused by low-affinity PBPs

β-lactam antibiotics

An important class of antibiotics, members of which contain a β-lactam ring and inhibit peptidoglycan synthesis by covalent binding to the active-site Ser of penicillin-binding proteins

Autolysins

Proteins that are located in the periplasm of Gram-negative bacteria or in the cell wall of Gram-positive bacteria and can lyse the cell using their peptidoglycan-hydrolysing activity. Autolysins can have muramidase, glucosaminidase, amidase and/or endopeptidase activity

Electron cryotomography (ECT)

An electron microscopy technique that provides high-resolution pictures of an object from different angles, permitting its three-dimensional reconstitution; plunge-freezing of the samples prevents staining and fixation artefacts. In the case of the bacterial sacculus, ECT has yielded a nanometre-scale three-dimensional representation of the fine structure.

Intermediate filaments

Filaments formed by coiled-coil-rich cytoskeletal proteins, such as keratin. Crescentin is a bacterial version of an intermediate filament and is required for the bent cell shape of *Caulobacter crescentus.*

Blebbing

The release of vesicles from the outer membrane of Gram-negative bacteria. Blebbing occurs during normal growth and is enhanced in certain mutants that are impaired in cell division

Lysozyme

An antibacterial enzyme that is produced in animals, plants, fungi and even bacteria and is capable of lysing sensitive bacteria by hydrolysing the *N*-acetylmuramic acid–*N*-acetylglucosamine bonds in peptidoglycan chains

Type VI secretion systems (T6SSs)

A recently discovered secretion apparatus that is widely distributed in Gram-negative bacteria. Some of its components are similar to phage injection systems.The T6SS punctures both eukaryotic and bacterial cells, often injecting toxic effector proteins into them

Turgor

The osmotic pressure of a compartment (here, the bacterial cytoplasm) that is due to the lower activity of water

D-amino acids

Rare chiral forms (mirror structures) of the abundant L-amino acids that build proteins. D-amino acids are present in peptidoglycan and in some non-ribosomally synthesized antibiotics

Atomic force microscopy (AFM)

A microscopy technique that uses a cantilever tip to scan the surface of a probe, either in direct contact or in oscillation mode, to produce topography images with nanometre-scale resolution

Total internal reflection fluorescence microscopy (TIRF microscopy)

A fluorescence microscopy technique that uses an evanescent wave to selectively excite a fluorophore in a small area of a specimen adjacent to a glass–water interface to reduce background fluorescence. This technique provides a superior axial resolution

Photoactivated localization microscopy (PALM)

A super-resolution fluorescence microscopy technique based on the controlled activation and sampling of subsets of photoconvertible fluorescent molecules in the sample. This technique can achieve 10–20 nm resolution

Solid-state NMR spectroscopy

NMR spectroscopy of insoluble polymers. The technique requires rapid spinning of the sample at a certain 'magic' angle. It provides information on the structural flexibility of a polymer and the interactions of chemical entities within it (for example, amino acids or sugars in peptidoglycan sacculi)

Förster resonance energy transfer (FRET)

A technique that detects and characterizes the interaction between two molecules coupled to two fluorophores, by measuring the excitation of one fluorophore by the light emitted from the other. A positive FRET signal indicates a distance of less than 10 nm between the fluorophores

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Box 1

New technologies will help to address long-standing questions

High-throughput genetic screens

Genetic screens have become extremely powerful for dissecting the physiological roles of partially redundant gene functions. Unbiased, high-throughput reverse-genetics screens probe and quantify high numbers of genetic interactions in *Escherichia* coli[125,126], and chemical genetic screens quantify the fitness of a large number of mutants under numerous environmental and chemical stresses[127]. These two methods, along with targeted genetics and proteomics, led to the identification of the Lpo proteins[101,102,127] and provided additional leads about other peptidoglycan-related functions (C.G., A.T. and W.V., unpublished observations).

New imaging technology

New, high-resolution imaging techniques are being increasingly used to understand sacculus growth and organization. Visualization of cell machineries and the ultrastructure by electron cryotomography (ECT) has revealed the orientation of glycan chains and facilitated the discovery of new cytoskeletal elements[22,71,86]. `Atomic force microscopy` (AFM), which provides nanoscale images of native cell surfaces of living bacteria[128,129], revealed the unexpected complexity of the peptidoglycan architecture in *Bacillus subtilis* and *Staphylococcus* aureus[130,131] and is used to study assembly dynamics of cytoskeletal elements *in vitro*[132]. `Total internal reflection fluorescence microscopy` (TIRF microscopy) and `photoactivated localization microscopy` (PALM) methodologies[45] are also increasingly employed. Indeed, TIRF measurements underlie the recent proposal that peptidoglycan synthesis drives MreB filament movement[47,48].

Modelling and biophysical techniques

Computational models are being used to explain data and guide future experimentation[49,80,81,133], and a suite of high-resolution biophysical analyses is also proving transformative. Optical traps are being used to assess the forces that are important in peptidoglycan maintenance[77]. The structural dynamics of entire sacculi, as well as of sacculus–protein interactions, are being identified using solid-state NMR spectroscopy[134,135], and Förster resonance energy transfer (FRET) is being applied to identify protein interactions *in* vivo[136].

In vitro reconstruction

In vitro reconstitution studies are increasingly providing insights into molecular mechanisms, demonstrating that FtsZ has the intrinsic ability to mediate constriction when present in lipsomes[137] and that the transpeptidase and glycosyltransferase activities of the bifunctional synthases are interdependent[12,13].

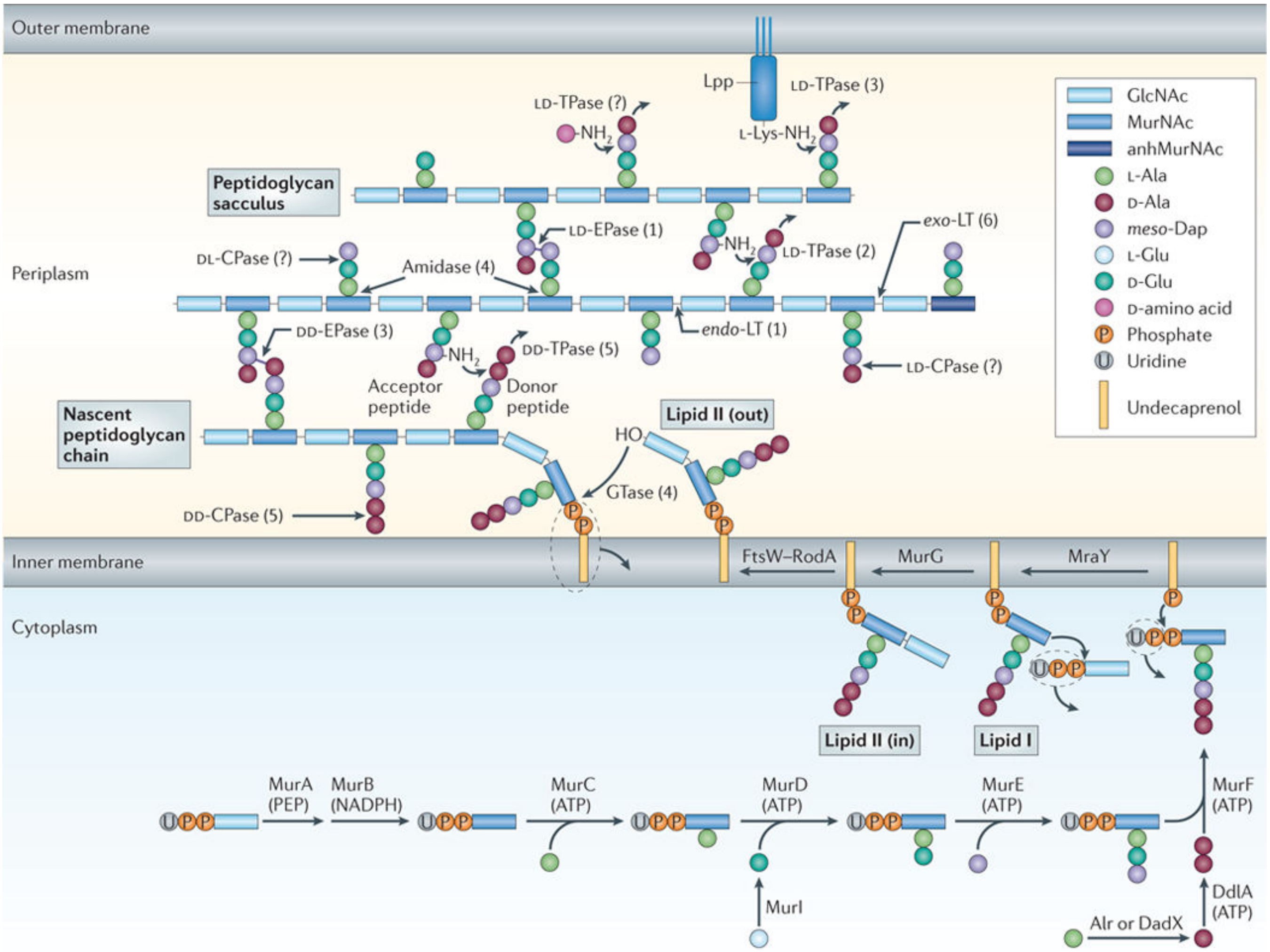

Figure 1. Peptidoglycan synthesis and cleavage

The synthesis and attachment of a new peptidoglycan strand to the existing sacculus, with particular emphasis on the different synthetic and degrading enzymes. Precursors are synthesized in the cytoplasm, linked to the transport lipid (undecaprenyl phosphate) and flipped accross the inner membrane by FtsW–RodA. A glycosyltransferase (GTase) catalyses polymerization of a nascent peptidoglycan chain from lipid II precursor at the inner membrane, followed by attachment of the new chain to the sacculus by a DD-transpeptidase (DD-TPase). Peptides are trimmed by DD-, LD-and DL-carboxypeptidases (CPases), and crosslinks are cleaved by the DD-and LD-endopeptidases (EPases). Amidases remove peptides from glycan chains, and *exo*-or endo-specific lytic transglycosylases (LTs) cleave in the glycan chain to form 1,6-anhydro-*N*-acetylmuramic acid (anhMurNAc) residues, which are the hallmark of glycan chain ends. LD-TPases are responsible for the formation of LD-crosslinks, the attachment of the major outer-membrane lipoprotein (Lpp), which is anchored in the outer membrane, and the binding of unusual D-amino acids. The number of known *Escherichia coli* enzymes for each group is shown in brackets, but this is probably an underestimate, as even in *E. coli* not all players are known and/or characterized. Alr, Ala racemase, biosynthetic; DadX, Ala racemase, catabolic; DdlA, D-Ala–D-Ala ligase

A; GlcNAc, *N*-acetylglucosamine; *meso*-Dap, *meso*-diaminopimelic acid; MraY, UDP-MurNAc-pentapeptide phosphotransferase; MurA, UDP-GlcNAc enolpyruvyl transferase; MurB, UDP-MurNAc dehydrogenase; MurC, UDP-MurNAc–L-Ala ligase; MurD, UDP-MurNAc-L-Ala–D-Glu ligase; MurE, UDP-MurNAc-L-Ala-D-Glu-*meso*-Dap ligase; MurF, UDP-MurNAc-tripeptide–D-alanyl-D-Ala ligase; MurG, UDP-GlcNAc-undecaprenoyl-pyrophosphoryl-MurNAc-pentapeptide transferase; Murl, Glu racemase; PEP, phosphoenolpyruvate.

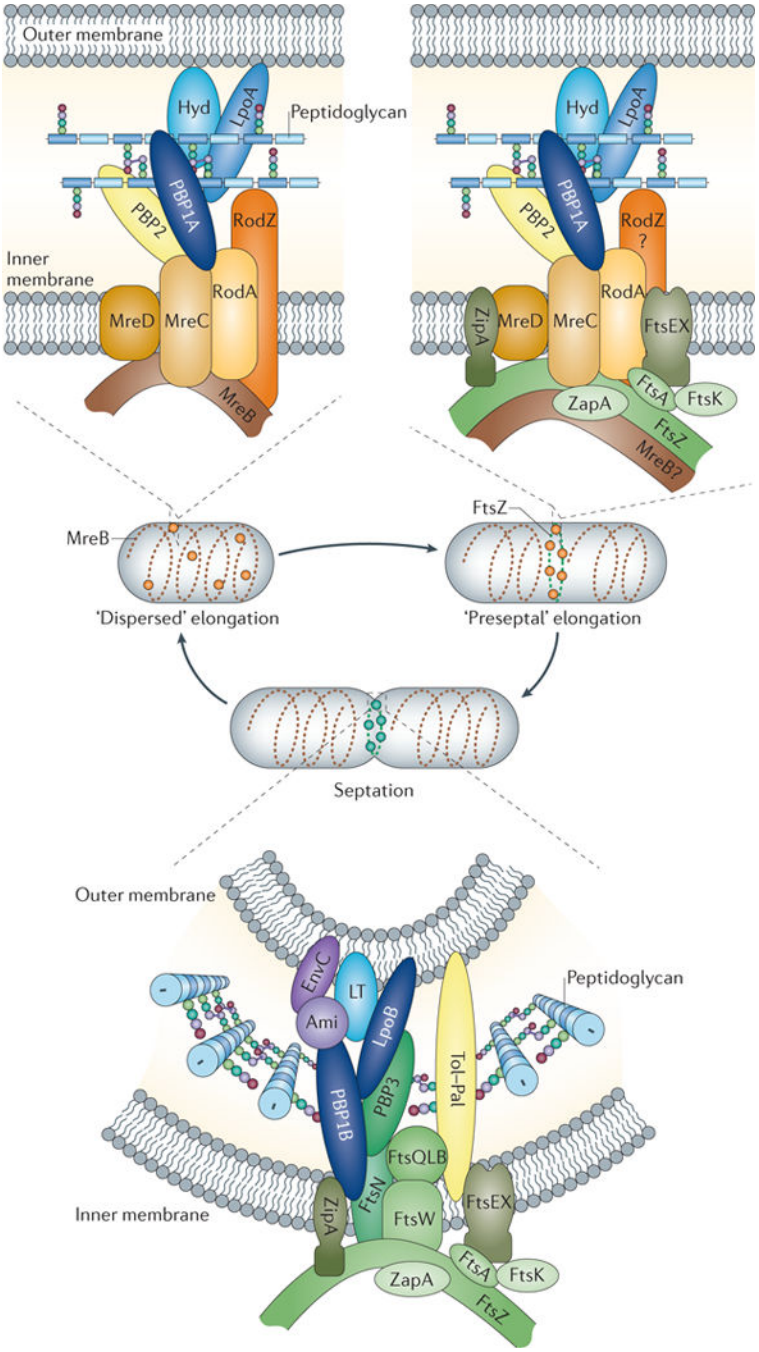

Figure 2. Different peptidoglycan synthesis complexes are active at different stages of the *Escherichia coli* cell cycle

As shown in the upper left panel, MreB and associated membrane proteins control or position the peptidoglycan synthases penicillin-binding protein 1A (PBP1A) and PBP2, as well as still-unknown hydrolases (Hyd), during the 'dispersed' mode of elongation. As illustrated in the upper right panel, FtsZ and other early cell division proteins control the elongation-specific peptidoglycan synthesis complex during a 'preseptal' mode of elongation. It is not known whether MreB and associated proteins participate in preseptal elongation. Finally, as depicted in the lower panel, the cell division complex contains essential, inner membrane-localized cell division proteins, the peptidoglycan synthases PBP1B and PBP3, and amidase enzymes (Ami) with their activators, as well as proteins of the Tol–Pal complex for constriction of the outer membrane. Activity of the PBPs is regulated in part by outer membrane-anchored lipoproteins such as LpoA and LpoB. LT, lytic transglycosylase.

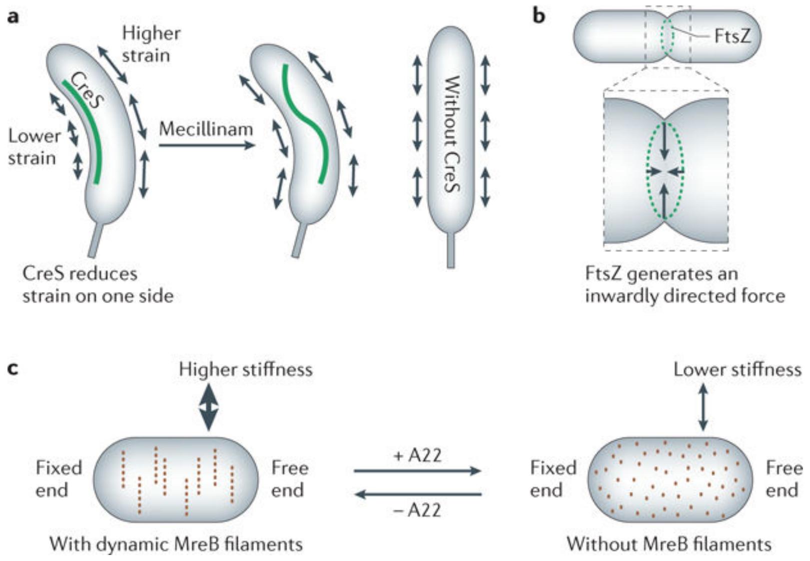

Figure 3. Force generation by cytoskeletal elements

a | Crescentin (CreS) reduces the strain at one side of the cell, causing *Caulobacter crescentusto* grow in a bent shape. Detachment of the CreS filament from the membrane (on addition of mecillinam) results in rapid loss of the filament's stretched form but does not cause an instant change in cell shape. Cells lacking CreS grow with a straight shape. **b** | FtsZ generates an inwardly directed constriction force in vesicle tubes and presumably also in the cell. **c** | Depolymerization of MreB filaments by addition of the drug A22 (*S*-(3,4-dichlorobenzyl)isothiourea) reduces the stiffness of *Escherichia coli* cells.

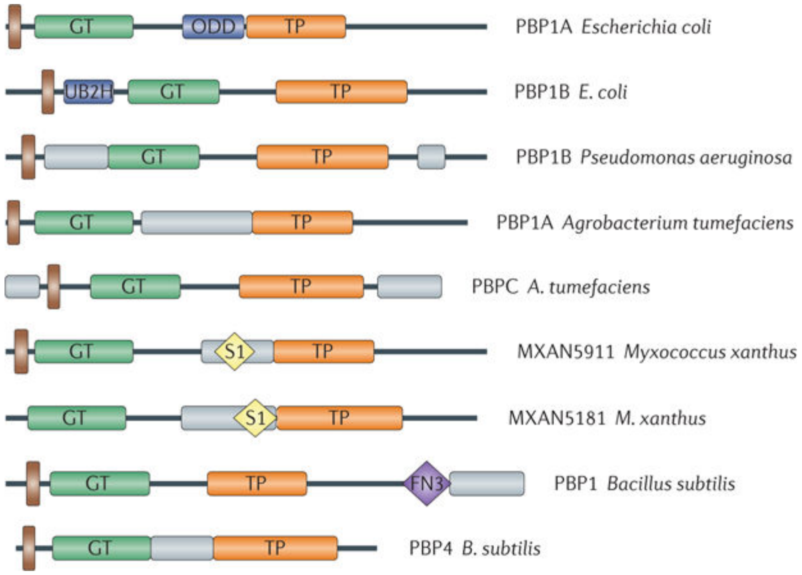

Figure 4. Species-specific non-catalytic regions in penicillin-binding proteins

Different class A penicillin-binding proteins (PBPs) in comparison with *Escherichia coli* PBP1A and PBP1B. Predicted or known transmembrane domains are shown in brown, newly evolved domains in *E. coli* PBP1A and PBP1B in dark blue and other species-specific regions with no function prediction in grey. Glycosyltransferase (GT) and transpeptidase (TP) domains are labelled, along with the fibronectin type 3 (FN3) domain and the ribosomal protein S1-like RNA-binding (S1) domains. The species-specific regions with no function prediction in the two Myxococcus *xanthus* proteins contain an S1 domain and are only conserved in *Stigmatella* and *Myxococcus* spp., whereas the analogous regions in the two *Bacillus subtilis* proteins consist of one that is unique in *B. subtilis* (the carboxy-terminal region in PBP1) and one that is more conserved among the bacilli (the domain in PBP4); the FN3 domain found in PBP1 is also conserved only in bacilli. ODD, outer-membrane PBP1A docking domain; UB2H, UvrB domain 2 homologue.

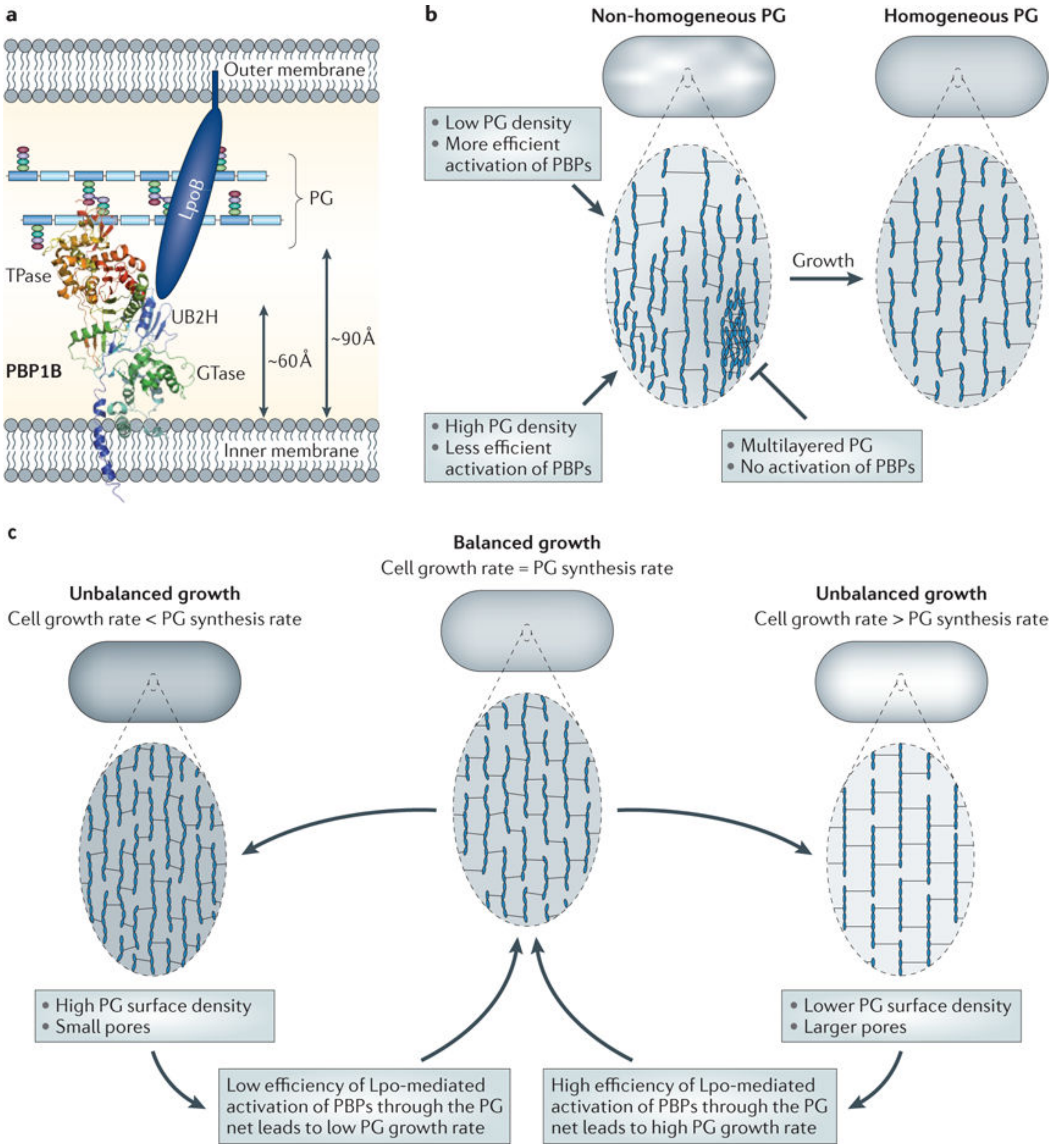

Figure 5. Regulation of peptidoglycan synthesis by outer-membrane proteins

a | Side view of the *Escherichia coli* cell envelope with the crystal structure of penicillin-binding protein 1B (PBP1B; Protein Data Bank accession 3FWM)[14] and the distances between the inner membrane, peptidoglycan (PG) and outer membrane drawn to scale. The glycosyltransferase (GTase) and transpeptidase (TPase) domains are shown. The structure of the activator protein for PBP1B, LpoB, is unknown. LpoB is anchored in the outer membrane and interacts with the PBP1B UB2H (UvrB domain 2 homologue) domain, which is situated between the inner membrane and the PG layer, not more than ~60 Å away from the inner membrane[14]. The distance from the inner membrane to the PG is ~90 Å[138]. **b** | A hypothetical self-repair mechanism to maintain a homogeneous peptidoglycan layer. The cell on the left has a non-homogeneous peptidoglycan layer consisting of large and small pores. Pore size-responsive activation of peptidoglycan synthase activity results in a more homogeneous peptidoglycan layer (on the right). **c** | A hypothetical homeostatic mechanism to balance the peptidoglycan growth rate with the overall cellular growth rate. When the peptidoglycan growth rate falls behind or exceeds that of overall cell growth, the peptidoglycan net stretches or relaxes, respectively. The resulting change in pore size alters the efficiency with which Lpo proteins can activate peptidoglycan synthases and therefore re-aligns the peptidoglycan growth rate with the overall cellular growth rate.

Table 1

Peptidoglycan synthesis enzymes and cell morphogenesis proteins in *Escherichia coli*

Function	**Activity or category**	**Proteins**[*]	**Relevant features and remarks**[‡]
Precursor synthesis	Transferase and dehydrogenase, respectively	MurA, MurB	• Synthesis of UDP-MurNAc from UDP-GlcNAc
	Amino acid ligases	MurC, MurD, MurE, MurF, Ddl	• Cytoplasmic steps leading to the UDP-MurNAc pentapeptide
	Racemases	Alr, DadX, MurI	• Synthesis of D-Ala or D-Glu from L-Ala or L-Glu, respectively
	GTases	MraY, MurG	• Inner membrane-localized steps of lipid II synthesis from the UDP-MurNAc pentapeptide
Peptidoglycan synthesis	GTases and DD-TPases (class A PBPs)	PBP1A	• Major peptidoglycan synthase, mainly involved in cell elongation • Anchored in the inner membrane • Interacts with LpoA
		PBP1B	• Major peptidoglycan synthase, mainly involved in cell division • Anchored in the inner membrane • Dimerizes and interacts with PBP3, FtsN, MipA and LpoB • Crystal structure available
		PBP1C	• Cellular role unknown • Anchored in the inner membrane
	DD-TPases (class B PBPs)	PBP2	• Essential for cell elongation • Dependent on MreB filament for localization • Anchored in the inner membrane
		PBP3	• Essential for cell division • Part of the divisome • Anchored in the inner membrane • Interacts with PBP1B, MtgA, FtsQLB, FtsW and FtsN
	GTase	MtgA	• Localizes to the division site • Interacts with PBP3, FtsW and FtsN • Anchored in the inner membrane
Regulation of peptidoglycan synthesis	Activators of peptidoglycan synthase	LpoA, LpoB	• Regulate PBP1A (LpoA) and PBP1B (LpoB) TPase activity • Outer-membrane lipoproteins
Formation of 3–3 crosslinks	LD-TPases	YnhG,YcbB	• Form the minor type of β-lactam-insensitive peptide crosslinks, the function of which is unknown
Cell envelope stability and the creation of a firm connection between	Structural protein	Lpp (Braun's lipoprotein)	• Outer-membrane lipoprotein

Function	**Activity or category**	**Proteins**[*]	**Relevant features and remarks**[‡]
peptidoglycan and the outer membrane			• The bound form is covalently attached to peptidoglycan • The free form forms trimers and is embedded in the outer membrane
	LD-TPases	ErfK, YbiS, YcfS	• Attachment of Lpp to peptidoglycan
Regulation of peptidoglycan structure	DD-CPases (class C PBPs)	PBP5, PBP4B, PBP6, PBP6B	• Proposed regulatory role in peptidoglycan synthesis by removal of excess pentapeptide donors in newly made peptidoglycan
Peptidoglycan hydrolysis (autolysis)	DD-EPases	PBP4, PBP7	• Septum cleavage (PBP7) • Biofilm formation (PBP7) • Also has DD-CPase activity (PBP4)
	DD-and LD-EPase	MepA	• LAS family metallopeptidase
	LTs	Slt70, MltA, MltB, MltC, MltD, MltE (also known as EmtA), MltF	• Major autolysins • Interact with PBP7 (Slt70) or PBP1B via MipA (MltA) • Septum cleavage (Slt70, MltA, MltB, MltC, MltD) • Outer membrane-anchored lipoproteins (Mlt proteins)
	Amidases	AmiA, AmiB, AmiC, AmiD	• Septum cleavage (AmiA, AmiB and AmiC)
Regulation of peptidoglycan hydrolysis	Activators of amidases	EnvC, NlpD, YgeR, YebA	• Have a LytM peptidoglycan-binding domain • Activators of AmiA and AmiB (EnvC) or AmiC (NlpD) • The roles of YgeR and YebA are unknown
	Inhibitor of LTs	Ivy	• Inhibitor of MltB
Cell elongation	Cytoskeletal structure, ATPase, GTPase	MreB	• Actin structural homologue • Forms a cytoplasmic, membrane-attached helix or patches
	MreB-associated proteins	MreC, MreD, RodZ, RodA, PBP2	• MreB-associated and inner membrane-associated proteins (MreC, MreD and RodZ) • Lipid II flippase (RodA)
Cell division	Cytoskeletal structure, GTPase	FtsZ	• Tubulin structural homologue • Forms a dynamic cytoplasmic ring structure at midcell
	‘Early’ association with the Z ring	FtsA, ZipA, ZapA, ZapB, ZapC, FtsE, FtsX, FtsK	• Stabilization and membrane-attachment of FtsZ polymers (FtsA, ZipA, ZapA, ZapB, ZapC) • Recruitment of proteins and DNA transport (FtsK)
	‘Late’ association with the Z ring	FtsQ, FtsL, FtsB, FtsW, FtsN, PBP3, DamX, DedD, RlpA	• Interactions with peptidoglycan synthases PBP3 (FtsQLB, FtsW and FtsN) and PBP1B (PBP3 and FtsN) • Lipid II flippase (FtsW) • Peptidoglycan binding (FtsN, DamX, DedD and RlpA)

Function	Activity or category	Proteins[*]	Relevant features and remarks[‡]
	Outer-membrane invagination	TolQ, TolR, TolA, TolB, Pal	• Form an envelope-spanning complex for outer-membrane invagination during septation • Peptidoglycan binding (Pal)

Alr, Ala racemase, biosynthetic; CPase, carboxypeptidase; DadX, Ala racemase, catabolic; Ddl, D-Ala–D-Ala ligase; EPase, endopeptidase; GlcNAc, *N*-acetlyglucosamine; GTase, glycosyltransferase; Ivy, inhibitor of vertebrate lysozyme; LT, lytic transglycosylase; Mlt, membrane-bound lytic murein transglycosylase; MraY, UDP-MurNAc-pentapeptide phosphotransferase; MurA, UDP-GlcNAc enolpyruvyl transferase; MurB, UDP-MurNAc dehydrogenase; MurC, UDP-MurNAc–L-Ala ligase; MurD, UDP-MurNAc-L-Ala–D-Glu ligase; MurE, UDP-MurNAc-L-Ala-D-Glu–*meso*-diaminopimelic acid ligase; MurF, UDP-MurNAc-tripeptide–D-alanyl-D-Ala ligase; MurG, UDP-GlcNAc-undecaprenoyl-pyrophosphoryl-MurNAc-pentapeptide transferase; MurI, Glu racemase; MurNAc, *N*-acetylmuramic acid; PBP, penicillin-binding protein; TPase, transpeptidase.

*Proteins were assigned to one category, although many of them would fit into more than one category. The DD-TPases (class B PBPs) were added to peptidoglycan synthesis and to cell elongation (PBP2) or cell division (PBP3) to illustrate their specific functions in the cell cycle. ‡References are given in the main text.