During growth and propagation, a bacterial cell enlarges and subsequently divides its peptidoglycan (PG) sacculus, a continuous mesh-like layer that encases the cell membrane to confer mechanical strength and morphological robustness. The mechanism of sacculus growth, how it is regulated and how it is coordinated with other cellular processes is poorly understood. In this article, we will discuss briefly the current knowledge of how cell wall synthesis is regulated, on multiple levels, from both sides of the cytoplasmic membrane. According to the current knowledge, cytosolic scaffolding proteins connect PG synthases with cytoskeletal elements, and protein phosphorylation regulates cell wall growth in Gram-positive species. PG-active enzymes engage in multiple protein–protein interactions within PG synthesis multienzyme complexes, and some of the interactions modulate activities. PG synthesis is also regulated by central metabolism, and by PG maturation through the action of PG hydrolytic enzymes. Only now are we beginning to appreciate how these multiple levels of regulating PG synthesis enable the cell to propagate robustly with a defined cell shape under different and variable growth conditions.
D-Ala-D-Ala motif is highly conserved as this is a crucial substrate for peptide cross-linking enzymes. In Gram-negative bacteria, the PG sacculus is integrated within a complex cell envelope between the cytoplasmic and outer membranes (OM), and is mostly a single layer 3–6 nm thick. By contrast, the cell wall of Gram-positive species is thicker – 10–40 nm – and contains secondary polymers such as teichoic acids and capsular polysaccharides [4,5] (Fig. 1). In addition, many proteins with various functions are covalently anchored to the stem peptides by sortase enzymes [6]. Some Gram-negative species covalently attach an abundant OM-anchored lipoprotein, Lpp (Braun’s lipoprotein), to the meso-Dap residue of the stem peptide thus tightly connecting the PG and OM [7]. Bacteria also possess an array of peptidoglycan-binding proteins, some of which play roles in the process of sacculus growth.

The mechanisms by which bacteria enlarge and divide the PG sacculus during cell growth and division are poorly understood. According to a current model, PG growth is facilitated by dynamic multiprotein complexes containing PG synthases and hydrolases and cell morphogenesis proteins [8]. These complexes are positioned and/or are controlled by cytoskeletal elements to form large cell morphogenesis complexes, the elongasome for cell growth (in rod-shaped bacteria) and the divisome for cell division [9]. This model is supported by the phenotypes of mutant strains, the subcellular localisation of key proteins and the existence of a large number of protein-protein interactions [9–11]. However, the exact composition of these complexes and how they function in the cell are not known and we presumably do not yet know all of the proteins involved.

Bacteria typically possess several PG synthases capable of polymerising the PG precursor lipid II (undecaprenol pyrophosphate disaccharide pentapeptide) and cross-linking the stem peptides. Penicillin-binding proteins (PBPs), so named because they are the primary target of the β-lactam antibiotics, are the major PG synthases [12]. They exhibit a modular architecture with distinct domains for catalysis and for interactions/regulation. Class A PBPs have domains for both glycan strand polymerisation (glycosyltransferase, GTase) and peptide cross-linking (transpeptidase, TPase) activities. *E. coli* possesses three class A PBPs; PBP1A, PBP1B and PBP1C, and though PBP1C is dispensable for growth, the loss of both PBP1A and PBP1B is lethal [13]. This situation is mirrored across many bacterial species, such that they need at least one class A PBP for growth [12]. However, some Gram-positive bacteria, *Bacillus subtilis* and *Enterococcus faecalis*, can grow upon deletion of all class A PBP genes [14,15] and it was recently proposed that the integral membrane protein RodA and other SEDS proteins, which are capable of flipping lipid II in vitro [16], have a GTase activity [17]. Class B PBPs have a TPase domain and a noncatalytic domain that might function as ‘pedestal’ for the positioning of the catalytic domain away from the cell membrane, but the pedestal could also participate in protein-protein interactions [18]. Finally, there are monofunctional GTases, such as MtgA in *E. coli*, with currently unknown cellular function. All PG synthases are anchored to the cytoplasmic membrane by a single transmembrane region near their N terminus, and they possess a cytoplasmic tail ranging in size from four amino acids to more than 100.
In this article, we will discuss how cell wall synthesis is regulated from both sides of the cytoplasmic membrane. We will illustrate how PG synthases are connected to the bacterial cytoskeleton by cytosolic scaffolding proteins, and describe how protein phosphorylation regulates cell wall synthesis in Gram-positive species. We will discuss the role of protein–protein interactions within the elongasome and the divisome, and we will outline how PG maturation and central metabolism affect PG growth.

The role of scaffolding proteins and the architecture of synthesis machinery

Scaffolding proteins have a well-established role in numerous cellular processes in all cell types [19]. Scaffolds influence bacterial cell wall synthesis by controlling the spatial arrangement of PBPs and PG hydrolases, ensuring that the PG meshwork remains intact as the cell wall is remodelled and expanded during cycles of cell division and growth. In this regard, the dynamic filaments formed by the actin homologue MreB have been proposed to have an essential function as a scaffold around which the PBPs and PG hydrolases assemble [20,21]. MreB (and orthologues Mbl and MreBH in B. subtilis) is essential for the elongation of many rod-shaped bacteria. MreB moves along the lateral wall of the cell depending on ongoing PG synthesis [22–24] and is connected to the cell wall synthesis machinery via the cell morphogenic protein RodZ [25]. The N-terminal, cytosolic ~90 amino acids of RodZ that interact with MreB [26] are separated from the C-terminal, ~170 residue extracellular domain by a single transmembrane region, but otherwise the biochemical properties of RodZ are unknown. While these findings support a scaffolding role for MreB in controlling cell wall growth from inside the cell, the movement of MreB is not required to maintain the rod-shape of E. coli under standard laboratory conditions [25]. The extent to which MreB forms filaments within the cell and whether such filaments directly promote a long-range ordered arrangement of other proteins have been debated extensively and the various models of MreB function have been discussed in depth recently [27]. The scaffolding mechanism of regulation is also key during cell division, in which new cell poles are created. A further cytoskeletal protein, the tubulin homologue FtsZ, controls the assembly of the divisome from inside the cell [28]. The precise nature of the ring-like structure that FtsZ filaments form (the Z-ring) with other division proteins is also subject to much debate, see recent reviews for a discussion of the divisome interactions and stoichiometry [11,29]. Recent structural and biochemical studies have cast light on how two other conserved proteins, GpsB and EzrA, in Gram-positive organisms may regulate cell wall synthesis by acting in a scaffolding mechanism in consort with the cytoskeleton (Fig. 2).

The role of GpsB in cell wall synthesis in Gram-positive bacteria is apparent from the pronounced elongated growth phenotypes upon gpsB deletion in Streptococcus pneumoniae and Listeria monocytogenes [30–32]. Furthermore, gpsB mutants are more sensitive to lysis [31] and in the case of L. monocytogenes, also to β-lactams [32], consistent with a weakened cell wall in the gpsB mutant. The S. pneumoniae gpsB null mutant has a defect in the closure of the cell division septum, producing multiple, unconstricted Z-rings along the length of the elongated cells [30,31]. By comparison, a gpsB mutant of B. subtilis is largely unaffected under normal conditions but shows increased sensitivity to high-salt conditions [33]. However, cells become elongated and prone to lysis when gpsB and ezrA are simultaneously deleted in B. subtilis [33], which might be caused by mislocalisation of PBP1, the major bifunctional PBP at the cell division septum. The mechanism underlying the synergistic effect of a gpsB ezrA double mutant is unknown, but considering that both proteins interact with PBP1, and with each other, the effect might be caused by alterations in the assembly of protein complexes.

![Fig. 2. Schematic model of the association of EzrA (dark and light blue) and GpsB (orange/grey) with the membrane and possible arrangements for interaction partners in the divisome, including FtsZ filaments (magenta), FtsA filaments (red) and three representative bifunctional PBP molecules. The GpsB structure represented is the molecular envelope calculated by SAXS (grey) with the crystal structures of the individual GpsB domains (orange) docked inside it. EzrA is represented in the antiparallel dimer form with the component subunits coloured blue and light blue. In the model of a bifunctional PBP, the transmembrane region is coloured grey, the GTase domain coloured green, the TPase domain coloured blue.](image-url)
GpsB is a hexameric protein that associates with the cytoplasmic face of the cell membrane [32]. High-resolution crystal structures have been determined for the two domains of GpsB in isolation [32], and these were used to build a low-resolution model of the full-length hexameric protein based on small-angle X-ray scattering (SAXS) data [34] (Fig. 2). The hexamer is highly elongated, with an overall wedge or fan shape. Binding sites for the bifunctional class A PBPs from *B. subtilis* and *L. monocytogenes*, PBPA1 and PBPA2 respectively, have been mapped by mutagenesis [32] and are clustered together at one end of the hexamer, at the ‘thick’ end of the wedge [34]. Intriguingly, GpsB is phosphorylated in vivo at Thr75 in both *B. subtilis* [35,36] and *Streptococcus agalactiae* [37] by the respective kinases, PrkC and Stp1. Phosphomimetic mutations of Thr75 to Asp or Glu renders *B. subtilis* GpsB nonfunctional [36] by an as yet undeciphered mechanism. Phosphomimetic mutations do not alter the stability of the GpsB hexamer in vitro [34], but it is possible that phosphorylation causes an alteration in the spatial arrangement of subunits to affect the arrangement of PBPs in the membrane either at the septum during division (Fig. 2) or in the lateral cell wall during growth. Indeed phosphorylation of various proteins has emerged as one of the mechanisms by which cell wall growth is controlled, which we discuss in more detail below.

The N-terminal domain of GpsB [32] is highly homologous (40% sequence identity, <1.0 Å rmsd on superimposed Cx atoms) to another scaffolding protein, DivIVA [38], which fulfils a crucial role in cell division and sporulation by recruiting certain proteins to the cell poles. For example, MinCD, an inhibitor of FtsZ polymerisation [39], is recruited to the cell poles by DivIVA [40] to ensure proper placement of the Z-ring at mid-cell during cell division in *B. subtilis*. GpsB and DivIVA are both two-domain proteins in which homology is restricted to their N-terminal domains; both proteins have C-terminal domains that are all α-helical, and drive high-order homo-assemblies – a hexamer for GpsB and a tetramer for DivIVA – that appear to be critical for proper function of both proteins [32,38]. The N-terminal domains of GpsB and DivIVA are responsible for driving the interaction of the former with bifunctional PBPs in *L. monocytogenes* and *B. subtilis* [32] and the latter with other cell morphogenesis proteins, including RodA in *Corynebacterium glutamicum* [41] and MinJ in *B. subtilis* [42,43], whereas the C-terminal domain of DivIVA is required to interact with the kinetochore-like protein, RacA, during chromosome segregation in *B. subtilis* [43]). In bacterial two-hybrid assays, *S. pneumoniae* DivIVA interacts with itself and the cell division proteins FtsZ, FtsA, ZapA, FtsK and FtsL, the PG hydrolase PcsB, and the chromosome segregation protein Spo0J [44]. The same study did not detect an interaction of DivIVA with either PBP2x or PBP1a.

Despite the close similarities in structure and sequence of the N-terminal domains of GpsB and DivIVA, their respective interactions may be specific to each protein. The divergence in function between the two proteins is starting to emerge from the recent high-resolution structural analyses of both proteins [32,38], coupled to functional analyses of sequence conservation hotspots. For instance, the residues critical for the interaction of GpsB with PBPs (Y27, D33, 284 in *L. monocytogenes* GpsB) are considerably better conserved in GpsB proteins in comparison to DivIVA orthologues [32].

A notable characteristic of DivIVA is a preferential interaction with negatively curved membranes [45], a crucial factor driving its localisation to the cell poles and the cell division septum. The binding of DivIVA to concave membranes is believed to be driven by the complementarity between a concave membrane and the elongated ‘dumbbell’ shape of the DivIVA tetramer [46], in which there are membrane-binding sites at both ends of the dumbbell [38]. The subcellular localisation of GpsB is also dynamic in rod-shaped bacteria; after division, GpsB transitions from the cell poles to the lateral wall of the cell and back again to mid-cell for the next cell division [33,47]. Whether this relocalisation of GpsB to the division site is also driven by a preference for negatively curved membranes is unknown. The architecture of the GpsB hexamer [34], though very different from that of the DivIVA tetramer [38], is reminiscent of the hexameric chemotaxis receptor TlpA [48], which displays a similar subcellular localisation pattern as GpsB. GpsB and TlpA hexamers are both formed from a trimer of dimers architecture [32,34,48]; in the case of GpsB, the N-terminal domain is dimeric and further oligomerisation to the hexamer is driven by interactions between C-terminal domains at the thin end of the wedge shape [34]. In TlpA, sensory N-terminal domains are dimeric and hexamerisation occurs by the association of the C-terminal regions of three TlpA dimers [48]. In both instances, the membrane-associating sites are clustered at the thick end of the hexameric wedges. The comparison between the chemotaxis receptor TlpA and cell division regulator GpsB is important because the shape, flexibility and multimeric state of the TlpA hexamer has been found to have a crucial effect upon its subcellular localisation [48], and it is also apparent that GpsB variants that no longer form hexamers have...
a null phenotype in *L. monocytogenes* [32], indicating that maintenance of a hexamer with a defined shape is critical to function for both proteins. EzrA is one of the first proteins to be recruited to mid-cell during cell division [47] and has been shown to interact with PBPs in bacterial two-hybrid assays [33], as well as with a number of other cell division proteins including FtsA, FtsZ and GpsB [33]. In *B. subtilis*, *ezrA* deletions impact the assembly of the Z-ring, causing the appearance of extra Z-rings at the cell poles and at mid-cell [33]. In *Staphylococcus aureus*, an *ezrA* deletion does not lead to such a pronounced extra Z-ring phenotype but nevertheless still results in the mislocalisation of FtsZ [49].

A direct interaction between EzrA and FtsZ is supported by *in vitro* assays using different approaches [50–55]. EzrA may therefore act as an adapter, linking the PBPs to the cytoskeletal protein FtsZ, the central scaffold around which the components of the cell division machinery assemble.

In addition to regulating the assembly of the Z-ring, EzrA influences cell wall synthesis. In *B. subtilis*, an *ezrA* null mutant has a similar growth phenotype as a deletion of *ponA*, which encodes PBP1; cells are elongated and have a thinner cell wall [33,56]. In these *ezrA* null strains, the localisation of PBP1 is perturbed because the recruitment of PBP1 to division sites at mid-cell is impaired. In *S. aureus*, an *ezrA* deletion results in enlarged cells with unusual patterns of PG insertion [49], an observation again consistent with perturbed PBP localisation. Others have reported that the *ezrA* deletion in *S. aureus* [57] and *L. monocytogenes* [58] is lethal.

EzrA is an integral membrane protein with a single transmembrane helix followed by an ~540 amino acid elongated cytoplasmic domain, with a fold that is intriguing in its resemblance to the eukaryotic spectrin-type proteins [51,59]. As in spectrin-like proteins [60], the EzrA cytoplasmic domain contains multiple copies of a three helical bundle repeat unit connected in a head-to-tail fashion [51]. The arrangement of helices in the repeat units from EzrA and eukaryotic spectrins superimpose very closely but the connectivity between the helices differs in the two cases [51]. The difference in connectivity explains why the intriguing relationship between EzrA and spectrins was not previously detected on the basis of sequence homology alone.

Spectrins have an established role in eukaryotic cells as adaptor proteins linking the actin cytoskeleton to the membrane [61]. A similar role can be envisaged for EzrA as a linker between the FtsZ cytoskeleton and the membrane [51]. The unusual horseshoe shape of the EzrA cytoplasmic domain raises the possibility that it could in fact act as a clamp attaching FtsZ filaments to the membrane, and/or as a divider to separate FtsZ protofilaments prior to their bundling [51]. In the simplest model for the association of EzrA with the membrane, the cytoplasmic domain of EzrA forms a bridge over the membrane surface under which there is sufficient space to accommodate FtsZ filaments [51] (Fig. 2). Such a model could explain how EzrA negatively regulates Z-ring assembly. The trapping of protofilaments under the bridge will impact lateral interactions between FtsZ filaments, which should in turn modulate the structure of the Z-ring. Although the ultrastructure of the Z-ring has – like several other aspects of bacterial cell division – been a controversial topic, recent evidence supports the important role played by the lateral interactions between FtsZ filaments [28,62].

A final aspect of EzrA pertinent to a potential scaffolding role is its oligomerisation *in vivo*. A characteristic of spectrin proteins, notably alpha-actinin, is the formation of an antiparallel dimer in which two separate chains of spectrin repeats align in an antiparallel fashion [63]. A similar arrangement is observed in the crystal packing of the structure of the EzrA cytoplasmic domain [51] and the formation of such a dimer is compatible with the simplest model for the membrane-associated EzrA protein. Further biochemical and structural studies will be required to clarify precisely how EzrA assembles with partner proteins in the cellular environment. Nonetheless, the structure of the EzrA cytoplasmic domain arguably points to a key architectural role within the machinery responsible for cell wall synthesis and cell division.

**Regulation through protein phosphorylation**

Bacteria often use phosphorylation/dephosphorylation cascades to sense and to respond to external signals, such as nutrients, oxygen, light and osmotic pressure, to adapt to changes in their environment. In prokaryotes, this adaptation depends mainly upon two-component signal transduction systems (TCS) that allow communication between the cell envelope and the cytoplasm based on the transient phosphorylation of a response regulator by a membrane-anchored histidine kinase [64]. In *B. subtilis*, the WalRK TCS system senses cell wall growth by binding wall teichoic acid precursors [65], whereas other TCS systems sense, for example, compromised membrane integrity (LiaRS) [66] or secretion stress (CssRS) [67]; the activating molecular cues for many TCS systems remain unknown.
Serine/threonine protein kinases (STPKs) and their cognate Ser-P/Thr-P phosphatases represent another major mechanism of transmembrane signalling and were thought for many years to be specific to eukaryotic cellular processes. However, since the early 1990s, many eukaryotic-type STPKs have been identified in bacterial genomes (including a broad spectrum of pathogens [68,69]), where they regulate various cellular functions, including biofilm formation, stress responses, sporulation, metabolic processes, pathogenicity, and cell wall synthesis and cell division through phosphorylating key proteins on Ser/Thr residues to elicit specific downstream effects [70].

Mycobacterium tuberculosis has 11 eukaryotic-type STPKs [71,72]. The genes of two of these, pknA and pknB, are part of an operon encoding cell wall synthesis and cell shape control genes [73], and are mainly expressed during exponential growth; pknA and pknB are essential under laboratory growth conditions [73–75]. The overexpression of both kinases slows cell growth and changes cellular morphology, while the partial depletion of both genes results in elongated cells [73]. The essential mycobacterial protein Wag31, a homologue of DivIVA, was identified as a substate of PknA and PknB in vivo [73]. The phosphorylation of Wag31 may trigger the remodelling of bacterial morphology, but the molecular mechanism by which phosphorylation of Wag31 is transduced to changes in cellular structure is unknown. Furthermore, PknA was subsequently shown to phosphorylate FtsZ and to reduce septum formation by affecting the guanosine triphosphate (GTP)-dependent polymerisation of FtsZ [76] but the phosphorylation site(s) on FtsZ are not known and it remains to be determined how phosphorylation affects GTP binding and/or hydrolysis.

PknB is the only mycobacterial STPK that belongs to a distinct and ultraconserved subfamily of STPKs restricted to Gram-positive bacteria that contain several PBP and serine/threonine kinase-associated (PASTA) domains in their extracellular region [77]. PknB comprises a conserved N-terminal kinase domain, a transmembrane region and four PASTA domains in the surface-exposed C-terminal region [77,78]. These PASTA domains appear to bind muropeptide PG fragments depending on the identity of the amino acids at positions two and three of the peptide [79]. These PG fragments may be produced locally at mid-cell and the cell poles by PG turnover and may recruit PknB to cell wall growth sites, where it has a regulatory function [79]. Overexpression of the extracellular PASTA domains of PknB leads to elongated cells, a delay in the regrowth from stationary phase and to an increased sensitivity to β-lactam antibiotics [80].

Several other PknB substrates that belong to different functional categories were identified [81], implying that PknB controls multiple steps in cell envelope biogenesis. PknB modulates the acetyltransferase activity of GlmU by phosphorylating threonine residues in its C-terminal domain; GlmU is a bifunctional enzyme that synthesises uridine diphosphate (UDP)-N-acetylglucosamine, a critical precursor for the synthesis of PG and other cell surface polymers [82]. Another PknB substrate is MurJ (a.k.a. MviN) [83] which has been proposed to be the essential flippase for the PG precursor lipid II [84]. PknB may also regulate PG polymerisation by phosphorylating Thr34 of the cytoplasmic tail of a class A PBP called PonA [85,86], but it is currently not known how phosphorylation of the cytoplasmic tail of PonA affects the GT and/or the TP activity that are housed on the other side of the cytoplasmic membrane.

Another substrate of PknB, CwlM, coordinates PG synthesis in a nutrient-dependent fashion [87]. CwlM is homologous to PG amidases but unlike these seems to be inactive and localise to the cytoplasm. Phosphorylated CwlM activates the UDP-N-acetylg glucosamine 1-carboxyvinyltransferase MurA, the first enzyme in the PG precursor pathway [87]. According to this model, CwlM is unphosphorylated in nutrient-depleted cells, which reduces the stimulation of MurA resulting in decreased PG precursor synthesis and increased tolerance to many antibiotics [87].

Streptococcus pneumoniae contains one STPK (StkP) that forms a functional pair with its corresponding cytoplasmic phosphatase PhpP [88]. StkP is involved in the regulation of virulence, competence, stress resistance and biofilm formation [89,90]. StkP and PhpP localise to the sites of PG synthesis and both proteins delocalise in the presence of cell wall-targeting antibiotics and in nondividing cells [91]. StkP/PhpP have presumed roles in coordinating cell wall growth, as an stkP mutant and a PhpP overexpression strain in the unencapsulated S. pneumoniae strain Rx1 and in the encapsulated strain D39 background grew as elongated cells with mostly unconstricted division rings [91]. By contrast, a StkP overexpression strain and a PhpP depletion strain were rounder and smaller than wild-type S. pneumoniae [91]. However, a different study showed that the stkP deletion strain and a strain expressing truncated StkP that lacks the kinase domain, produced round and chaining cells [92], phenotypes that were not observed in previous studies of the stkP mutant [93]; this discrepancy may be explained because of the different genetic backgrounds...
or growth conditions used, or because of the presence of suppressor mutations [94].

The four extracellular PASTA domains of StkP bind β-lactam antibiotics as well as native and synthetic PG [95], and are required for proper localisation of StkP at mid-cell with the FtsZ ring [91,96]. StkP is presumably recruited to cell division sites by the interaction of its PASTA domains with nascent, un-crosslinked PG chains [91,92]; the binding of the nascent PG to the PASTA domains activates the kinase function of StkP towards its substrate(s) [93].

StkP regulates cell division and PG synthesis enzymes in order to maintain the characteristic ellipsoid cell shape of *S. pneumoniae* during growth and division. StkP phosphorylates several cell division proteins, including the cytoskeletal elements FtsZ [96] and FtsA [91], the cell wall precursor enzymes UDP-N-acetylglucosamine mutase GlmM [88], and the cell cycle regulators LocZ/MapZ [98,99] and DivIVA [91,92]. The nonessential cell division protein LocZ/MapZ localises to future cell division sites before FtsZ to mark the new cell wall growth zone for Z-ring assembly [98,99]. DivIVA is specifically phosphorylated at Thr201 [92]: *S. pneumoniae* R800 cells expressing the nonphosphorylatable DivIVA (T201A) variant had an elongated cell shape with a polar bulge and aberrant spatial organisation of nascent PG synthesis [92]. However, in sharp contrast, *S. pneumoniae* Rx1 and D39 strains expressing DivIVA (T201A) did not display cell shape defects [91], and because of these contradictory results, the importance of the phosphorylation of DivIVA remains unclear.

*Staphylococcus aureus* has one eukaryotic-like STPK (Stk1 or PknB) containing three extracellular PASTA domains and a cognate, cotranscribed cytoplasmic phosphatase Stp1, both of which play major roles in regulating virulence. An *stk1* null strain was more resistant than wild-type to fosfomycin, which inhibits MurA, and against Triton X-100-induced lysis [100], and more sensitive to the cell wall-targeting antibiotics cephalosporin and carbapenem [101]. The *stk1/stp1* double mutant had defects in cell division and septum formation, producing cells with irregular sizes, bulging and multiple and incomplete septa [101]. These results suggest a link between Stk1/Stp1 activity and cell division.

Altogether these findings show the diversity of STPKs and their cognate phosphatases in Gram-positive species and their potential for regulating cell wall synthesis, though the precise regulatory mechanisms remain to be determined for most cases.

**Regulation through protein–protein interactions**

Since the multienzyme complex hypothesis for PG sacculus growth was proposed by Hölting [102], many direct interactions between PG enzymes and between PG enzymes and cell morphogenesis proteins have been identified. We shall not list all the interactions in detail here as these have been reviewed comprehensively elsewhere recently [9–11]. The importance of protein–protein interactions for structural/scaffolding purposes has been discussed above. However, within multiprotein complexes, there are likely interactions that impact on the activities of PG synthases and hydrolases. The divisome in *E. coli* can be broken down, in gross terms, to early and late subsets based on localisation hierarchy and timing of arrival at mid-cell [103]. The early proteins, including FtsZ and associated proteins such as FtsA and ZipA, are responsible for initiating Z-ring assembly and its stabilisation at the membrane at mid-cell for subsequent recruitment of the later proteins consisting of PG synthases and their interacting proteins such as FtsQLB, PBP3/1B, FtsW and FtsN, and proteins involved in coordinating OM constriction with PG synthesis (the Tol-Pal machinery) in Gram-negatives. The interaction network between the divisome proteins is extensive, including many interactions between the various late and early proteins (reviewed in [11]). A key example is between FtsA, FtsN and FtsQLB, which are all essential for division in *E. coli*. FtsA is an actin-like cytoskeletal protein and crucial in Z-ring formation, it associates with the inner leaflet of the cytoplasmic membrane and contributes to anchoring of FtsZ [104]. FtsN is an integral bitopic membrane protein with the majority of the protein present in the periplasm, including a C-terminal PG-binding domain. FtsQLB are integral membrane proteins whose multiple interactions with early and late divisome proteins place them within the core of the divisome [105]. The cytosolic portion of FtsN interacts with FtsA, and a periplasmic portion with FtsQLB [106]. Thus, the arrival of FtsN is thought to simultaneously signal to the early and late components to initiate cell division [106]. Consistent with this role, FtsN is also directly involved in the control of PG synthesis during division (detailed below). There is still much to understand about the mechanisms of divisome function, but it seems clear that the complex works through more than simply scaffolding the cell wall synthesis machinery, with multiple signals transduced through protein–protein interactions, making the divisome highly dynamic. These mechanisms also likely occur...
within the elongasome complex, though this is less well understood because it is relatively understudied.

Peptidoglycan synthase activity in the *E. coli* division is provided by PBP1B, the crystal structure of which contains, in addition to the conserved GTase and TPase domains, a small, noncatalytic domain, called UB2H (due to structural similarity to domain 2 of UvrB) [107]. The UvrB domain 2 homologue domain (UB2H domain) acts as a docking site for an activator of PBP1B, the OM-anchored lipoprotein LpoB [108]. LpoB is absolutely required for the functioning of PBP1B in the cell [108,109]. The other major class A synthase, PBP1A, requires a different OM lipoprotein, LpoA, for its function in the cell [108,109]. LpoA docks to a predicted, cognate noncatalytic domain, called outer membrane docking domain (ODD) in PBP1A [108]. The fact that both cytoplasmic membrane-anchored major class A PBPs of *E. coli* are regulated from outside the PG sacculus by OM-anchored lipoproteins supports the hypothesis that the γ-proteobacteria possess a homeostatic mechanism for regulating the PG synthesis rate that responds to the status of the pores in the PG sacculus [9]. If the rate of cell growth is greater than the rate of sacculus growth, the increasing turgor stretches the sacculus, opening the pores and potentially facilitates increased PBP activation. Both Lpo proteins span the periplasm to interact specifically with the noncatalytic domains in their cognate PBP and to stimulate PG synthesis activities [108,110,111]. As LpoB interacts only with the UB2H domain and not the catalytic domains [110], the interaction must induce conformational changes in PBP1B that stimulate the enzyme [110,112].

The identification of LpoB-bypass mutants with amino acid substitutions in PBP1B supports this hypothesis; these substitutions cluster in the interface between the UB2H, GT and TP domains and in the GT domain itself [113]. The cluster of bypass substitution mutants in the interfaces suggests an activation signal is transmitted through PBP1B from the LpoB-binding site on the UB2H domain, but the precise PBP activation mechanism remains to be determined.

Other protein interaction partners have also been shown to have a direct effect on the PG synthesis activities of PBP1B and PBP1A from *E. coli* (reviewed in [10]). FtsN interacts with the membrane proximal portion of PBP1B and increases the rate of glycans strand synthesis synergistically with the effect of LpoB [10]. The Tol-Pal machinery, which ensures proper OM constriction during cell division, was recently shown to modulate the function of PBP1B-LpoB in the cell through direct interaction with the synthase and its regulator [114]. This interaction alters the cross-linking activity of PBP1B-LpoB in response to Tol-Pal function in the cell, possibly by interfering with the conformational change exerted by LpoB on PBP1B.

There are other examples of conformational changes and structural dynamics that affect the activity of PG synthesis enzymes. PBP2 of *S. aureus* was found to possess dynamic structural motifs in its GTase domain, proximal to the essential catalytic Glu114 residue [115]. These motifs (named I and II) are highly conserved across GTase domains in PG synthases and are thought to induce local unfolding, or at least structural flexibility, to assist catalysis [115]. Furthermore, it was noted that artificial removal of certain disordered structural loops of PBP2 increased interdomain flexibility between the TP and GT domains [115]. Though the authors note that the structural changes were artificially introduced, their extensive structural evidence supported the hypothesis that these motifs act to restrict the PBP's conformation until substrate binding [115–117]. Given that both GT and TP activities of bifunctional PBPs are coupled [118–120], it is likely that the relative orientation of the two domains within a synthase molecule could impact function. Whether these specific structural motifs in *S. aureus* PBP2 are subject to inputs from regulators through interaction, and whether this observation applies more broadly to other bifunctional PBPs remains to be determined.

Finally, some PG hydrolases have also been shown to be regulated through conformational changes induced by the binding of a regulator. For example, *E. coli* AmiB, one of the hydrolases important for daughter cell separation during division, exists in an inactive conformation until complexed by its regulator, EnvC [121] to prevent unregulated PG hydrolysis. Further examples of regulated hydrolases are reviewed in [11,122].

The role of PG-binding proteins

Most of the cell division proteins localise at mid-cell through interacting and binding to other proteins of the septal ring [123], but some cell division and sporulation proteins have so-called sporulation-related domains ( Pfam05036 (SPOR) and are recruited to the septum by binding to PG [124–126]. *E. coli* has four SPOR domain proteins, including the essential cell division protein FtsN (see above), DamX, DedD and the lytic transglycosylase RlpA, which all localise to mid-cell [127].

The SPOR domain of FtsN is not essential for cell division [128], but helps to accumulate the protein at mid-cell depending on the activity of the PG synthase
PBP3, the PG hydrolysis activities of amidases, and the essential domain of FtsN itself, which is present in a surprisingly short periplasmic region (35 aa) [106,125]. FtsN binds long PG glycan strands released from sacculi by amidases [128] and FtsN and the other SPOR domain proteins bind to septal regions of PG sacculi, suggesting that they recognise ‘denuded’ glycan strands generated by amidases, which are transiently available during the constriction process [127,128]. The structures of the SPOR domains of FtsN and DamX from *E. coli* and of the sporulation protein CwlC from *B. subtilis* were solved using NMR spectroscopy [129–131], and crucial amino acids for PG binding were identified [132]. SPOR domains share a low amino acid sequence identity, but they have a similar core structure with a ribonucleoprotein (RNP) fold composed of a ββββββ secondary structure [132].

Many proteins bind to PG by virtue of specific PG-binding domains. One example is the abundant PG-binding domain LysM which is, for example, present in the *E. coli* PG hydrolases MltD [133] and MepM (YebA), the PG amidase regulator NlpD [134], and the LD-TPase s YnhG and YcfS [135]. How PG binding affects the function of these proteins is not known in most cases, but PG hydrolases often require a PG-binding domain for activity [136]. In addition, there are numerous and often abundant proteins that bind to the PG sacculus. Some of these abundant proteins stabilise the Gram-negative cell envelope, such as the outer membrane-anchored lipoproteins Lpp and Pal [137,138], and the integral OM beta-barrel protein OmpA [138]. Pal interacts with the Tol system, which constricts the OM during cell division and affects PG synthesis by modulating the function of PBP1B-LpoB (see above). However, in many cases, we simply do not know the effects of PG-interacting proteins on PG-synthesising and -hydrolysing enzymes.

### Regulation of PG growth by ‘redundant’ DD-carboxypeptidases

DD-Carboxypeptidases (DD-CPases) trim pentapeptides in newly made PG to tetrapeptides by removing the terminal α-alanine. The seven DD-CPases of *E. coli* are all dispensable for the survival of the cell and, with the exception of PBP5, their absence does not affect cell growth or morphology. Mutants lacking PBP5 or PBP5 together with other DD-CPases, contain an increased level of peptidoglycans in the PG and have irregular cell shapes with kinks, bends or even branches [139,140]. Pentapeptides can act as donor substrates for the TPase reaction performed by the PBPs, and hence, DD-CPases can modulate PG synthesis by removing donor substrates for PBPs, producing the resultant mature, tetrapeptide-rich PG [141]. Presumably, an excess of pentapeptides in the sacculus (as in DD-CPase mutants) causes malfunctioning of PBPs that utilise donor peptides from the sacculus instead of those present in the nascent PG, resulting in uncontrolled TPase activity and cell shape defects.

*Escherichia coli* is able to grow in various different environments that affect the composition and features of the periplasm, where PG synthesis (or regulation thereof) takes place. Therefore, these processes must be robust in order to work properly under a range of different pH values, temperatures and osmolalities. A recent study revealed a specialised function of the DD-CPase PBP6b in *E. coli*, which is required at acidic pH to trim pentapeptides and maintain cell shape [142]. PBP6b is expressed predominantly at acidic pH and the enzyme is more active and more stable at lower pH values. Hence, *E. coli* appears to maintain sets of apparently redundant PG hydrolases with the same substrate specificity but with different activity ranges that together cover all physiological conditions, to ensure effective growth and adaptation to environmental changes [142].

### Coordination with central metabolism

Coordinating cell growth and division to ensure that daughter cells have sufficient internal space for their cytoplasmic and genetic materials is a fundamental problem for all cells to solve [143–145]. It has long been known that bacteria in differing nutrient environments, poor or rich, have different sizes, with those grown in the former being smaller [143–145]. Given that the PG sacculus dictates morphology, it follows that bacteria must ensure the correct amount of new cell wall is synthesised each generation, to accommodate such variations in size. A homeostatic, and rather indirect mechanism that *E. coli* and other γ-proteobacteria might employ, is that cell wall synthesis is activated if the PG pore size increases because of stretching caused by the cytoplasmic growth rate being greater than the rate of wall growth [9]. However, this is unlikely to be the only mechanism of linking cell wall and cytoplasmic growth, and of course cannot apply to Gram-positive species.

In *B. subtilis*, the glucosyltransferase UgpP was identified as a metabolic sensor responsible for increasing cell size under nutrient-rich conditions by sensing UDP-glucose [146]. UgpP interacts with FtsZ to inhibit Z-ring formation and delay cell division when UDP-glucose levels are high, giving the cell longer to...
increase in size per generation [147]. An analogous protein was found in *E. coli*, OpgH, which also inhibits Z-ring formation depending upon the availability of UDP-glucose [148]. Both systems act to indirectly coordinate central metabolism with cell wall synthesis during division, explaining why cells are longer but not how they are wider in nutrient-rich growth conditions. Whether there are regulators which coordinate lateral wall growth with metabolism remains to be resolved. A putative candidate, YvcK, is required for rod-shaped growth of *B. subtilis* under gluconeogenic conditions through a currently unknown mechanism, but one that is associated with the functions of MreB and/or the major PG synthase PBP1 [149,150]. YvcK is conserved in spherical bacteria and is essential in *S. aureus* [9,151]; the homologue YbhK is also found in *E. coli* [150]. It remains to be seen whether YvcK/YbhK plays a similar role in these different organisms.

It thus seems that bacteria possess mechanisms to coordinate cell wall synthesis and central metabolism during growth to ensure the appropriate amount of new wall is produced during the cell cycle. We anticipate that this emerging topic will provide interesting new insights in the coming years as the players and their cellular roles are identified.

**Concluding remarks**

We are just beginning to understand the many ways bacteria regulate cell wall growth (Fig. 3). Though we have gained insight into some mechanisms, including roles of key proteins in scaffolding, PG binding and maturation, and the effects of protein–protein interactions, more components of the regulation of PG growth are likely to be discovered even in well-studied model bacteria. Given the extensive nature of the

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**Fig. 3.** A general representation of the regulatory mechanisms of cell wall growth. (A) Scaffolding; cytoskeletal elements and their accessory proteins act to provide a scaffold on which the cell wall synthesis machineries can assemble within the cytoplasmic membrane (CM). (B) Phosphorylation; a Ser/Thr protein kinase (STPK) is depicted phosphorylating a target protein, the resultant form then goes on to affect cell wall growth either directly through its own enzymatic activity or through subsequent regulation of another enzyme (stimulating conversion of the substrate X to the product Y in this example). (C) Protein–protein interaction; in addition to the structural importance of interactions in the scaffolding mechanism, interactions between proteins within the cell wall synthesis machineries can lead to transduction of signals to initiate activity, such as the signal to begin constriction delivered through the division machinery by FtsN binding to FtsQ/LB and FtsA. In addition, synthetic and hydrolytic PG enzymes are controlled through the induction of conformational changes upon binding of a regulator, presumably in a similar or complementary way to the mechanisms depicted in panel D. (D) Structural dynamics; PG synthesis and hydrolysis enzymes adopt different conformations through changes in dynamic structural elements they possess, which impacts enzymatic activity. The example shown here depicts changes in the GTase domain of *Staphylococcus aureus* PBP2 enabling catalysis, and the interdomain flexibility in this class A PBP for optimal coupling of the GT and TP activities. (E) Carboxypeptidases; CPase enzymes in the periplasm act to trim peptides from the peptide stems. In this top-down view example, DD-CPases trim the terminal d-Ala residue (5th position) in nascent PG, thus removing this source of TPase donor substrates and regulating the potential for new cross-linking. (F) Central metabolism; proteins have been found that sense available nutrients and transduce this information to exert an effect on cell wall biosynthesis, such as the effect of UgpP in *B. subtilis* on the timing of cell division at high levels of UDP-glucose.
interaction network among cytoskeletal, cell morphogenesis proteins and PG synthases, hydrolases and their respective regulators, we anticipate a wealth of new mechanistic insights to come to light in the coming years. Advances in the genetic and biochemical tools such as high-throughput mutagenesis and genetic screening technologies, in vitro activity assays, and advanced structure determination technologies will greatly aid these studies.

Acknowledgements

This work was supported by the Wellcome Trust (101824/Z/13/Z to WV) and the UK Biotechnology and Biological Sciences Research Council (BB/M001180/1 to RJL).

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