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1 **Regulation of bacterial cell wall growth**

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14 **Running title:** Regulation of peptidoglycan growth

15

16 **Keywords:** Peptidoglycan; Penicillin-binding protein; divisome; elongasome; protein
17 phosphorylation.

18

19

20 **Abstract**

21 During growth and propagation a bacterial cell enlarges and subsequently divides its peptidoglycan
22 (PG) sacculus, a continuous mesh-like layer that encases the cell membrane to confer mechanical
23 strength and morphological robustness. The mechanism of sacculus growth, how it is regulated, and
24 how it is coordinated with other cellular processes is poorly understood. In this article we will discuss
25 briefly the current knowledge of how cell wall synthesis is regulated, on multiple levels, from both
26 sides of the cytoplasmic membrane. According to the current knowledge, cytosolic scaffolding
27 proteins connect PG synthases with cytoskeletal elements, and protein phosphorylation regulates cell
28 wall growth in Gram-positive species. PG-active enzymes engage in multiple protein-protein
29 interactions within PG synthesis multi-enzyme complexes, and some of the interactions modulate
30 activities. PG synthesis is also regulated by central metabolism, and by PG maturation through the
31 action of PG hydrolytic enzymes. Only now are we beginning to appreciate how these multiple levels
32 of regulating PG synthesis enable the cell to propagate robustly with a defined cell shape under
33 different and variable growth conditions.

34

35

36 Introduction

37 During growth and propagation a bacterium must regulate macromolecular synthesis in order to
38 replicate precisely. One of the largest macromolecules of the cell is the $>10^9$ Da peptidoglycan (PG)
39 sacculus, an essential, continuous mesh-like layer that encases the cell membrane and which is needed
40 to confer osmotic stability and morphological robustness [1]. The PG sacculus achieves these
41 structural feats through a deceptively simple composition of glycan chains of alternating *N*-
42 acetylglucosamine and *N*-acetylmuramic acid residues that are connected by short peptides
43 containing both L- and D-amino acids [2] (Figure 1). This basic arrangement is widely conserved
44 across most bacterial species, however, variation in the chemistry of this basic unit, particularly
45 variations in the residues of the peptide stem and secondary modifications in the glycan chains, allow
46 for great diversity in fine structure and architecture [1, 3]. In the well-studied Gram-negative model
47 bacterium *Escherichia coli* the newly made peptide has the sequence L-Ala-D-*iso*Glu-*meso*-Dap-D-
48 Ala-D-Ala (*meso*-Dap, *meso*-diaminopimelic acid). Many Gram-positive species have D-*iso*Gln
49 (generated by amidation of D-*iso*Glu) at position 2, L-Lys instead of *meso*-Dap at position 3, and a
50 peptide branch of 1-7 L-amino acids (or glycine) attached to the sidechain of the L-Lys (Figure 1).
51 The terminal D-Ala-D-Ala motif is highly conserved as this is a crucial substrate for peptide cross-
52 linking enzymes. In Gram-negative bacteria the PG sacculus is integrated within a complex cell
53 envelope between the cytoplasmic and outer membranes, and is mostly a single layer 3 – 6 nm thick.
54 By contrast, the cell wall of Gram-positive species is thicker - 10 – 40 nm - and contains secondary
55 polymers such as teichoic acids and capsular polysaccharides [4, 5] (Figure 1). In addition, many
56 proteins with various functions are covalently anchored to the stem peptides by sortase enzymes [6].
57 Some Gram-negative species covalently attach an abundant OM-anchored lipoprotein, Lpp (Braun's
58 lipoprotein), to the *meso*-Dap residue of the stem peptide thus tightly connecting the PG and OM [7].
59 Bacteria also possess an array of peptidoglycan-binding proteins, some of which play roles in the
60 process of sacculus growth.

61 The mechanisms by which bacteria enlarge and divide the PG sacculus during cell growth and
62 division are poorly understood. According to a current model, PG growth is facilitated by dynamic
63 multi-protein complexes containing PG synthases and hydrolases and cell morphogenesis proteins
64 [8]. These complexes are positioned and/or are controlled by cytoskeletal elements to form large cell
65 morphogenesis complexes, the elongasome for cell growth (in rod-shaped bacteria) and the divisome
66 for cell division [9]. This model is supported by the phenotypes of mutant strains, the sub-cellular
67 localization of key proteins and the existence of a large number of protein-protein interactions [9-11].
68 However, the exact composition of these complexes, and how they function in the cell, is not known
69 and we presumably do not yet know all of the proteins involved.

70 Bacteria typically possess several PG synthases capable of polymerising the PG precursor
71 lipid II (undecaprenol pyrophosphate disaccharide pentapeptide) and cross-linking the stem peptides.
72 Penicillin-binding proteins (PBPs), so named because they are the primary target of the β -lactam
73 antibiotics, are the major PG synthases [12]. They exhibit a modular architecture with distinct
74 domains for catalysis and for interactions/regulation. Class A PBPs have domains for both glycan
75 strand polymerisation (glycosyltransferase, GTase) and peptide cross-linking (transpeptidase, TPase)
76 activities. *E. coli* possesses three class A PBPs; PBP1A, PBP1B and PBP1C, and though PBP1C is
77 dispensable for growth the loss of both PBP1A and PBP1B is lethal [13]. This situation is mirrored
78 across many bacterial species, such that they need at least one class A PBP for growth [12]. However,
79 some Gram-positive bacteria, *B. subtilis* and *E. faecalis*, can grow upon deletion of all class A PBP
80 genes [14, 15] and it was recently proposed that the integral membrane protein RodA and other SEDS
81 proteins, which are capable of flipping lipid II *in vitro* [16], have a GTase activity [17]. Class B PBPs
82 have a TPase domain and a non-catalytic domain that might function as 'pedestal' for the positioning
83 of the catalytic domain away from the cell membrane, but the pedestal could also participate in
84 protein-protein interactions [18]. Finally, there are monofunctional GTases, such as MgtA in *E. coli*,
85 with currently unknown cellular function. All PG synthases are anchored to the cytoplasmic

86 membrane by a single transmembrane region near their N-terminus, and they possess a cytoplasmic
87 tail ranging in size from 4 amino acids to more than 100.

88 In this article we will discuss how cell wall synthesis is regulated from both sides of the
89 cytoplasmic membrane. We will illustrate how PG synthases are connected to the bacterial
90 cytoskeleton by cytosolic scaffolding proteins, and describe how protein phosphorylation regulates
91 cell wall synthesis in Gram-positive species. We will discuss the role of protein-protein interactions
92 within the elongasome and the divisome, and we will outline how PG maturation and central
93 metabolism affects PG growth.

94

95 **The role of scaffolding proteins and the architecture of synthesis machinery**

96 Scaffolding proteins have a well-established role in numerous cellular processes in all cell types [19].
97 Scaffolds influence bacterial cell wall synthesis by controlling the spatial arrangement of PBPs and
98 PG hydrolases, ensuring that the PG meshwork remains intact as the cell wall is remodelled and
99 expanded during cycles of cell division and growth. In this regard, the dynamic filaments formed by
100 the actin homolog MreB have been proposed to have an essential function as a scaffold around which
101 the PBPs and PG hydrolases assemble [20, 21]. MreB (and orthologues Mbl and MreBH in *B. subtilis*)
102 is essential for the elongation of many rod-shaped bacteria. MreB moves along the lateral wall of the
103 cell depending on ongoing PG synthesis [22-24] and is connected to the cell wall synthesis machinery
104 via the cell morphogenic protein RodZ [25]. The N-terminal, cytosolic ~90 amino acids of RodZ that
105 interact with MreB [26] are separated from the C-terminal, ~170 residue extracellular domain by a
106 single trans-membrane region, but otherwise the biochemical properties of RodZ are unknown. While
107 these findings support a scaffolding role for MreB in controlling cell wall growth from inside the cell,
108 the movement of MreB is not required to maintain the rod-shape of *E. coli* under standard laboratory
109 conditions [25]. The extent to which MreB forms filaments within the cell, and whether such
110 filaments directly promote a long range ordered arrangement of other proteins, has been debated
111 extensively and the various models of MreB function have been discussed in depth recently [27]. The

112 scaffolding mechanism of regulation is also key during cell division, in which new cell poles are
113 created. A further cytoskeletal protein, the tubulin homologue FtsZ, controls the assembly of the
114 divisome from inside the cell [28]. The precise nature of the ring-like structure that FtsZ filaments
115 form (the Z-ring) with other division proteins is also subject to much debate, see recent reviews for a
116 discussion of the divisome interactions and stoichiometry [11, 29]. Recent structural and biochemical
117 studies have cast light on how two other conserved proteins, GpsB and EzrA, in Gram-positive
118 organisms may regulate cell wall synthesis by acting in a scaffolding mechanism in consort with the
119 cytoskeleton (Figure 2).

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

133 GpsB is a hexameric protein that associates with the cytoplasmic face of the cell membrane
134 [32]. High resolution crystal structures have been determined for the two domains of GpsB in
135 isolation [32], and these were used to build a low resolution model of the full length hexameric protein
136 based on small angle X-ray scattering (SAXS) data [34] (Figure 2). The hexamer is highly elongated,
137 with an overall wedge or fan shape. Binding sites for the bifunctional class A PBPs from *B. subtilis*

138 and *L. monocytogenes*, PBP1 and PBPA1 respectively, have been mapped by mutagenesis [32] and
139 are clustered together at one end of the hexamer, at the “thick” end of the wedge [34]. Intriguingly,
140 GpsB is phosphorylated *in vivo* at Thr75 in both *B. subtilis* [35, 36] and *S. agalactiae* [37] by the
141 respective kinases, PrkC and Stp1. Phosphomimetic mutations of Thr75 to Asp or Glu renders *B.*
142 *subtilis* GpsB non-functional [36] by an as yet undeciphered mechanism. Phosphomimetic mutations
143 do not alter the stability of the GpsB hexamer *in vitro* [34], but it is possible that phosphorylation
144 causes an alteration in the spatial arrangement of subunits to affect the arrangement of PBPs in the
145 membrane either at the septum during division (Figure 2) or in the lateral cell wall during growth.
146 Indeed phosphorylation of various proteins has emerged as one of the mechanisms by which cell wall
147 growth is controlled, which we discuss in more detail below.

148 The N-terminal domain of GpsB [32] is highly homologous (40% sequence identity, <1.0 Å
149 rmsd on superimposed C α atoms) to another scaffolding protein, DivIVA [38], which fulfils a crucial
150 role in cell division and sporulation by recruiting certain proteins to the cell poles. For example,
151 MinCD, an inhibitor of FtsZ polymerization [39], is recruited to the cell poles by DivIVA [40] to
152 ensure proper placement of the Z-ring at mid-cell during cell division in *B. subtilis*. GpsB and DivIVA
153 are both two-domain proteins in which homology is restricted to their N-terminal domains; both
154 proteins have C-terminal domains that are all α -helical, and drive higher-order homo-assemblies – a
155 hexamer for GpsB and a tetramer for DivIVA – that appear to be critical for proper function of both
156 proteins [32, 38]. The N-terminal domains of GpsB and DivIVA are responsible for driving the
157 interaction of the former with bifunctional PBPs in *Listeria monocytogenes* and *B. subtilis* [32] and
158 the latter with other cell morphogenesis proteins, including RodA in *Corynebacterium glutamicum*
159 [41] and MinJ in *B. subtilis* [42, 43], whereas the C-terminal domain of DivIVA is required to interact
160 with the kinetochore-like protein, RacA, during chromosome segregation in *B. subtilis* [43]). In
161 bacterial two-hybrid assays *Streptococcus pneumoniae* DivIVA interacts with itself and the cell
162 division proteins FtsZ, FtsA, ZapA, FtsK and FtsL, the PG hydrolase PcsB, and the chromosome

163 segregation protein Spo0J [44]. The same study did not detect an interaction of DivIVA with either
164 PBP2X or PBP1A.

165 Despite the close similarities in structure and sequence of the N-terminal domains of GpsB
166 and DivIVA, their respective interactions may be specific to each protein. The divergence in function
167 between the two proteins is starting to emerge from the recent high resolution structural analyses of
168 both proteins [32, 38], coupled to functional analyses of sequence conservation hotspots. For instance,
169 the residues critical for the interaction of GpsB with PBPs (Y27, D33, D37, I40 in *L. monocytogenes*
170 GpsB) are considerably better conserved in GpsB proteins in comparison to DivIVA orthologues
171 [32].

172 A notable characteristic of DivIVA is a preferential interaction with negatively curved
173 membranes [45], a crucial factor driving its localization to the cell poles and the cell division septum.
174 The binding of DivIVA to concave membranes is believed to be driven by the complementarity
175 between a concave membrane and the elongated “dumbbell” shape of the DivIVA tetramer [46], in
176 which there are membrane binding sites at both ends of the dumbbell [38]. The subcellular
177 localization of GpsB is also dynamic in rod-shaped bacteria; after division GpsB transitions from the
178 cell poles to the lateral wall of the cell and back again to mid-cell for the next cell division [33, 47].
179 Whether this re-localization of GpsB to the division site is also driven by a preference for negatively
180 curved membranes is unknown. The architecture of the GpsB hexamer [34], though very different
181 from that of the DivIVA tetramer [38], is reminiscent of the hexameric chemotaxis receptor TlpA
182 [48], which displays a similar subcellular localization pattern as GpsB. GpsB and TlpA hexamers are
183 both formed from a trimer of dimers architecture [32, 34, 48]; in the case of GpsB the N-terminal
184 domain is dimeric and further oligomerization to the hexamer is driven by interactions between C-
185 terminal domains at the thin end of the wedge shape [34]. In TlpA, sensory N-terminal domains are
186 dimeric and hexamerisation occurs by the association of the C-terminal regions of three TlpA dimers
187 [48]. In both instances, the membrane-associating sites are clustered at the thick end of the hexameric
188 wedges. The comparison between the chemotaxis receptor TlpA and cell division regulator GpsB is

189 important because the shape, flexibility and multimeric state of the TlpA hexamer has been found to
190 have a crucial effect upon its subcellular localization [48], and it is also apparent that GpsB variants
191 that no longer form hexamers have a null phenotype in *L. monocytogenes* [32], indicating that
192 maintenance of a hexamer with a defined shape is critical to function for both proteins.

193 EzrA is one of the first proteins to be recruited to mid-cell during cell division [47] and has
194 been shown to interact with PBPs in bacterial two hybrid assays [33], as well as with a number of
195 other cell division proteins including FtsA, FtsZ and GpsB [33]. In *B. subtilis* *eZR*A deletions impact
196 the assembly of the Z-ring, causing the appearance of extra Z-rings at the cell poles and at mid-cell
197 [33]. In *S. aureus*, an *eZR*A deletion does not lead to such a pronounced extra Z-ring phenotype but
198 nevertheless still results in the mislocalization of FtsZ [49].

199 A direct interaction between EzrA and FtsZ is supported by *in vitro* assays using different approaches
200 [50-55]. EzrA may therefore act as an adapter, linking the PBPs to the cytoskeletal protein FtsZ, the
201 central scaffold around which the components of the cell division machinery assemble.

202 In addition to regulating the assembly of the Z-ring, EzrA influences cell wall synthesis. In *B.*
203 *subtilis* an *eZR*A null mutant has a similar growth phenotype as a deletion of *ponA*, which encodes
204 PBP1; cells are elongated and have a thinner cell wall [33, 56]. In these *eZR*A null strains the
205 localization of PBP1 is perturbed because the recruitment of PBP1 to division sites at mid-cell is
206 impaired. In *S. aureus*, an *eZR*A deletion results in enlarged cells with unusual patterns of PG insertion
207 [49], an observation again consistent with perturbed PBP localization. Others have reported that the
208 *eZR*A deletion in *S. aureus* [57] and *L. monocytogenes* [58] is lethal.

209 EzrA is an integral membrane protein with a single transmembrane helix followed by an
210 approximately 540 amino acid elongated cytoplasmic domain, with a fold that is intriguing in its
211 resemblance to the eukaryotic spectrin-type proteins [51, 59]. As in spectrin-like proteins [60], the
212 EzrA cytoplasmic domain contains multiple copies of a three helical bundle repeat unit connected in
213 a head-to-tail fashion [51]. The arrangement of helices in the repeat units from EzrA and eukaryotic
214 spectrins superimpose very closely but the connectivity between the helices differs in the two cases

215 [51]. The difference in connectivity explains why the intriguing relationship between EzrA and
216 spectrins was not previously detected on the basis of sequence homology alone.

217 Spectrins have an established role in eukaryotic cells as adaptor proteins linking the actin
218 cytoskeleton to the membrane [61]. A similar role can be envisaged for EzrA as a linker between the
219 FtsZ cytoskeleton and the membrane [51]. The unusual horseshoe shape of the EzrA cytoplasmic
220 domain raises the possibility that it could in fact act as a clamp attaching FtsZ filaments to the
221 membrane, and/or as a divider to separate FtsZ protofilaments prior to their bundling [51]. In the
222 simplest model for the association of EzrA with the membrane, the cytoplasmic domain of EzrA
223 forms a bridge over the membrane surface under which there is sufficient space to accommodate FtsZ
224 protofilaments [51] (Figure 2). Such a model could explain how EzrA negatively regulates Z-ring
225 assembly. The trapping of protofilaments under the bridge will impact lateral interactions between
226 FtsZ filaments, which should in turn modulate the structure of the Z-ring. Although the ultrastructure
227 of the Z-ring has – like several other aspects of bacterial cell division – been a controversial topic,
228 recent evidence supports the important role played by the lateral interactions between FtsZ filaments
229 [28, 62].

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

239

240 **Regulation through protein phosphorylation**

241 Bacteria often use phosphorylation/dephosphorylation cascades to sense and to respond to external
242 signals, such as nutrients, oxygen, light and osmotic pressure, to adapt to changes in their
243 environment. In prokaryotes this adaptation depends mainly upon two-component signal transduction
244 (TCS) systems that allow communication between the cell envelope and the cytoplasm based on the
245 transient phosphorylation of a response regulator by a membrane anchored histidine kinase [64]. In
246 *B. subtilis*, the WalRK TCS system senses cell wall growth by binding wall teichoic acid precursors
247 [65], whereas other TCS systems sense, for example, compromised membrane integrity (LiaRS) [66]
248 or secretion stress (CssRS) [67]; the activating molecular cues for many TCS systems remain
249 unknown.

250 Serine/threonine protein kinases (STPKs) and their cognate Ser-P/Thr-P phosphatases
251 represent another major mechanism of transmembrane signalling and were thought for many years to
252 be specific to eukaryotic cellular processes. However, since the early 1990s many eukaryotic-type
253 STPKs have been identified in bacterial genomes (including a broad spectrum of pathogens [68, 69]),
254 where they regulate various cellular functions, including biofilm formation, stress responses,
255 sporulation, metabolic processes, pathogenicity, and cell wall synthesis and cell division through
256 phosphorylating key proteins on Ser/Thr residues to elicit specific downstream effects [70].

257 *Mycobacterium tuberculosis* has 11 eukaryotic-type STPKs [71, 72]. The genes of two of
258 these, *pknA* and *pknB*, are part of an operon encoding cell wall synthesis and cell shape control genes
259 [73], and are mainly expressed during exponential growth; *pknA* and *pknB* are essential under
260 laboratory growth conditions [73-75]. The overexpression of both kinases slows cell growth and
261 changes cellular morphology, while the partial depletion of both genes results in elongated cells [73].
262 The essential mycobacterial protein Wag31, a homolog of DivIVA, was identified as a substrate of
263 PknA and PknB *in vivo* [73]. The phosphorylation of Wag31 may trigger the remodelling of bacterial
264 morphology, but the molecular mechanism by which phosphorylation of Wag31 is transduced to
265 changes in cellular structure is unknown. Furthermore, PknA was subsequently shown to
266 phosphorylate FtsZ and to reduce septum formation by affecting the GTP-dependent polymerization

267 of FtsZ [76] but the phosphorylation site(s) on FtsZ are not known and it remains to be determined
268 how phosphorylation affects GTP binding and/or hydrolysis.

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

279 Several other PknB substrates that belong to different functional categories were identified
280 [81], implying that PknB controls multiple steps in cell envelope biogenesis. PknB modulates the
281 acetyltransferase activity of GlmU by phosphorylating threonine residues in its C-terminal domain;
282 GlmU is a bifunctional enzyme that synthesises UDP-*N*-acetylglucosamine, a critical precursor for
283 the synthesis of PG and other cell surface polymers [82]. Another PknB substrate is MurJ (a.k.a.
284 MviN) [83] which has been proposed to be the essential flippase for the PG precursor lipid II [84].
285 PknB may also regulate PG polymerization by phosphorylating Thr34 of the cytoplasmic tail of a
286 class A PBP called PonA1 [85, 86], but it is currently not known how phosphorylation of the
287 cytoplasmic tail of PonA1 affects the GT and/or the TP activity that are housed on the other side of
288 the cytoplasmic membrane.

289 Another substrate of PknB, CwlM, coordinates PG synthesis in a nutrient-dependent fashion
290 [87]. CwlM is homologous to PG amidases but unlike these seems to be inactive and localize to the
291 cytoplasm. Phosphorylated CwlM activates the UDP-*N*-acetylglucosamine 1-
292 carboxyvinyltransferase MurA, the first enzyme in the PG precursor pathway [87]. According to this

293 model CwlM is unphosphorylated in nutrient-depleted cells, which reduces the stimulation of MurA
294 resulting in decreased PG precursor synthesis and increased tolerance to many antibiotics [87].

295 *Streptococcus pneumoniae* contains one STPK (StkP) that forms a functional pair with its
296 corresponding cytoplasmic phosphatase PhpP [88]. StkP is involved in the regulation of virulence,
297 competence, stress resistance and biofilm formation [89, 90]. StkP and PhpP localize to the sites of
298 PG synthesis and both proteins delocalize in the presence of cell-wall targeting antibiotics and in non-
299 dividing cells [91]. StkP/PhpP have presumed roles in coordinating cell wall growth, since an *stkP*
300 mutant and a PhpP overexpression strain in the unencapsulated *S. pneumoniae* strain Rx1 and in the
301 encapsulated strain D39 background grew as elongated cells with mostly unconstricted division rings
302 [91]. By contrast, a StkP overexpression strain and a PhpP depletion strain were rounder and smaller
303 than wildtype *S. pneumoniae* [91]. However, a different study showed that the *stkP* deletion strain
304 and a strain expressing truncated StkP that lacks the kinase domain, produced round and chaining
305 cells [92], phenotypes that were not observed in previous studies of the *stkP* mutant [93]; this
306 discrepancy may be explained because of the different genetic backgrounds or growth conditions
307 used, or because of the presence of suppressor mutations [94].

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

313 StkP regulates cell division and PG synthesis enzymes in order to maintain the characteristic
314 ellipsoid cell shape of *S. pneumoniae* during growth and division. StkP phosphorylates several cell
315 division proteins, including the cytoskeletal elements FtsZ [96] and FtsA [91], the cell wall precursor
316 enzymes UDP-*N*-acetylmuramate-L alanine ligase MurC [97] and phosphoglucosamine mutase
317 GlmM [88], and the cell cycle regulators LocZ/MapZ [98, 99] and DivIVA [91, 92]. The non-essential
318 cell division protein LocZ/MapZ localizes to future cell division sites before FtsZ to mark the new

319 cell wall growth zone for Z-ring assembly [98, 99]. DivIVA is specifically phosphorylated at Thr201
320 [92]: *S. pneumoniae* R800 cells expressing the non-phosphorylatable DivIVA(T201A) variant had an
321 elongated cell shape with a polar bulge and aberrant spatial organization of nascent PG synthesis [92].
322 However, in sharp contrast, *S. pneumoniae* Rx1 and D39 strains expressing DivIVA(T201A) did not
323 display cell shape defects [91] and, because of these contradictory results, the importance of the
324 phosphorylation of DivIVA remains unclear.

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

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

336

337 **Regulation through protein-protein interactions**

338 Since the multi-enzyme complex hypothesis for PG sacculus growth was proposed by Höltje [102],
339 many direct interactions between PG enzymes and between PG enzymes and cell morphogenesis
340 proteins have been identified. We shall not list all the interactions in detail here as these have been
341 reviewed comprehensively elsewhere recently [9-11]. The importance of protein-protein interactions
342 for structural/scaffolding purposes has been discussed above. However, within multi-protein
343 complexes there are likely interactions that impact on the activities of PG synthases and hydrolases.
344 The divisome in *E. coli* can be broken down, in gross terms, to early and late subsets based on

345 localisation hierarchy and timing of arrival at mid-cell [103]. The early proteins, including FtsZ and
346 associated proteins such as FtsA and ZipA, are responsible for initiating Z-ring assembly and its
347 stabilization at the membrane at midcell for subsequent recruitment of the later proteins consisting of
348 PG synthases and their interacting proteins such as FtsQLB, PBP3/1B, FtsW and FtsN, and proteins
349 involved in coordinating OM constriction with PG synthesis (the Tol-Pal machinery) in Gram-
350 negatives. The interaction network between the divisome proteins is extensive, including many
351 interactions between the various late and early proteins (reviewed in [11]). A key example is between
352 FtsA, FtsN and FtsQLB, which are all essential for division in *E. coli*. FtsA is an actin-like
353 cytoskeletal protein and crucial in Z-ring formation, it associates with the inner leaflet of the
354 cytoplasmic membrane and contributes to anchoring of FtsZ [104]. FtsN is an integral bitopic
355 membrane protein with the majority of the protein present in the periplasm, including a C-terminal
356 PG binding domain. FtsQLB are integral membrane proteins whose multiple interactions with early
357 and late divisome proteins place them within the core of the divisome [105]. The cytosolic portion of
358 FtsN interacts with FtsA, and a periplasmic portion with FtsQLB [106]. Thus the arrival of FtsN is
359 thought to simultaneously signal to the early and late components to initiate cell division [106].
360 Consistent with this role, FtsN is also directly involved in the control of PG synthesis during division
361 (detailed below). There is still much to understand about the mechanisms of divisome function, but
362 it seems clear that the complex works through more than simply scaffolding the cell wall synthesis
363 machinery, with multiple signals transduced through protein-protein interactions, making the
364 divisome highly dynamic. These mechanisms also likely occur within the elongasome complex,
365 though this is less well understood because it is relatively under-studied.

366 PG synthase activity in the *E. coli* divisome is provided by PBP1B, the crystal structure of
367 which contains, in addition to the conserved glycosyltransferase and transpeptidase domains, a small,
368 non-catalytic domain, called UB2H (due to structural similarity to domain 2 of UvrB) [107]. The
369 UB2H domain acts as a docking site for an activator of PBP1B, the OM-anchored lipoprotein LpoB
370 [108]. LpoB is absolutely required for the functioning of PBP1B in the cell [108, 109]. The other

371 major class A synthase, PBP1A requires a different OM lipoprotein, LpoA, for its function in the cell
372 [108, 109]. LpoA docks to a predicted, cognate non-catalytic domain, called ODD (outer membrane
373 docking domain) in PBP1A [108]. The fact that both cytoplasmic membrane-anchored major class A
374 PBPs of *E. coli* are regulated from outside the PG sacculus by OM-anchored lipoproteins supports
375 the hypothesis that the γ -proteobacteria possess a homeostatic mechanism for regulating the PG
376 synthesis rate that responds to the status of the pores in the PG sacculus [9]. If the rate of cell growth
377 is greater than the rate of sacculus growth the increasing turgor stretches the sacculus, opening the
378 pores and potentially facilitates increased PBP activation. Both Lpo proteins span the periplasm to
379 interact specifically with the non-catalytic domains in their cognate PBP and to stimulate PG
380 synthesis activities [108, 110, 111]. As LpoB interacts only with the UB2H domain and not the
381 catalytic domains [110], the interaction must induce conformational changes in PBP1B that stimulate
382 the enzyme [110, 112]. The identification of LpoB-bypass mutants with amino acid substitutions in
383 PBP1B supports this hypothesis; these substitutions cluster in the interface between the UB2H, GT
384 and TP domains and in the GT domain itself [113]. The cluster of bypass substitution mutants in the
385 interfaces suggests an activation signal is transduced through PBP1B from the LpoB binding site on
386 the UB2H domain, but the precise PBP activation mechanism remains to be determined.

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

395 There are other examples of conformational changes and structural dynamics that affect the
396 activity of PG synthesis enzymes. PBP2 of *S. aureus* was found to possess dynamic structural motifs

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

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

413

414 **The role of PG-binding proteins**

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

420 The SPOR domain of FtsN is not essential for cell division [128], but helps to accumulate the
421 protein at mid-cell depending on the activity of the PG synthase PBP3, the PG hydrolysis activities
422 of amidases, and the essential domain of FtsN itself, which is present in a surprisingly short

423 periplasmic region (35 aa) [106, 125]. FtsN binds long PG glycan strands released from sacculi by
424 amidases [128] and FtsN and the other SPOR domain proteins bind to septal regions of PG sacculi,
425 suggesting that they recognize 'denuded' glycan strands generated by amidases, which are transiently
426 available during the constriction process [127, 128]. The structures of the SPOR domains of FtsN and
427 DamX from *E. coli* and of the sporulation protein CwlC from *B. subtilis* were solved using NMR
428 spectroscopy [129-131], and crucial amino acids for PG-binding were identified [132]. SPOR
429 domains share a low amino acid sequence identity, but they have a similar core structure with a
430 ribonucleoprotein (RNP) fold composed of a $\beta\alpha\beta\beta\alpha\beta$ secondary structure [132].

431 Many proteins bind to PG by virtue of specific PG-binding domains. One example is the
432 abundant PG-binding domain LysM which is, for example, present in the *E. coli* PG hydrolases MltD
433 [133] and MepM (YebA), the PG amidase regulator NlpD [134], and the LD-transpeptidases YnhG
434 and YcfS [135]. How PG-binding affects the function of these proteins is not known in most cases,
435 but PG hydrolases often require a PG-binding domain for activity [136]. In addition, there are
436 numerous and often abundant proteins that bind to the PG sacculus. Some of these abundant proteins
437 stabilize the Gram-negative cell envelope, such as the outer-membrane anchored lipoproteins Lpp
438 and Pal [137, 138], and the integral outer membrane beta-barrel protein OmpA [138]. Pal interacts
439 with the Tol system, which constricts the outer membrane during cell division and affects PG
440 synthesis by modulating the function of PBP1B-LpoB (see above). However, in many cases we
441 simply don't know the effects of PG-interacting proteins on PG-synthesizing and hydrolysing
442 enzymes.

443

444 **Regulation of PG growth by 'redundant' DD-carboxypeptidases**

445 DD-Carboxypeptidases (DD-CPases) trim pentapeptides in newly made PG to tetrapeptides by
446 removing the terminal D-alanine. The seven DD-CPases of *E. coli* are all dispensable for the survival
447 of the cell and, with the exception of PBP5, their absence does not affect cell growth or morphology.
448 Mutants lacking PBP5 or PBP5 together with other DD-CPases, contain an increased level of

449 peptapeptides in the PG and have irregular cell shapes with kinks, bends or even branches [139, 140].
450 Pentapeptides can act as donor substrates for the TPase reaction performed by the PBPs and, hence,
451 DD-CPases can modulate PG synthesis by removing donor substrates for PBPs, producing the
452 resultant mature, tetrapeptide-rich PG [141]. Presumably, an excess of pentapeptides in the sacculus
453 (as in DD-CPase mutants) causes mal-functioning of PBPs that utilize donor peptides from the
454 sacculus instead of those present in the nascent PG, resulting in uncontrolled transpeptidase activity
455 and cell shape defects.

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

465

466 **Coordination with central metabolism**

467 Coordinating cell growth and division to ensure that daughter cells have sufficient internal space for
468 their cytoplasmic and genetic materials is a fundamental problem for all cells to solve [143-145]. It
469 has long been known that bacteria in differing nutrient environments, poor or rich, have different
470 sizes, with those grown in the former being smaller [143-145]. Given that the PG sacculus dictates
471 morphology it follows that bacteria must ensure the correct amount of new cell wall is synthesised
472 each generation, to accommodate such variations in size. A homeostatic, and rather indirect
473 mechanism that *E. coli* and other γ -proteobacteria might employ, is that cell wall synthesis is activated
474 if the PG pore size increases because of stretching caused by the cytoplasmic growth rate being greater

475 than the rate of wall growth [9]. However, this is unlikely to be the only mechanism of linking cell
476 wall and cytoplasmic growth, and of course cannot apply to Gram-positive species.

477 In *B. subtilis* the glucosyltransferase UgtP was identified as a metabolic sensor responsible
478 for increasing cell size under nutrient rich conditions by sensing UDP-glucose [146]. UgtP interacts
479 with FtsZ to inhibit Z-ring formation and delay cell division when UDP-glucose levels are high,
480 giving the cell longer to increase in size per generation [147]. An analogous protein was found in *E.*
481 *coli*, OpgH, which also inhibits Z-ring formation depending upon the availability of UDP-glucose
482 [148]. Both systems act to indirectly coordinate central metabolism with cell wall synthesis during
483 division, explaining why cells are longer but not how they are wider in nutrient rich growth
484 conditions. Whether there are regulators which coordinate lateral wall growth with metabolism
485 remains to be resolved. A putative candidate, YvcK, is required for rod-shaped growth of *B. subtilis*
486 under gluconeogenic conditions through a currently unknown mechanism, but one that is associated
487 with the functions of MreB and/or the major PG synthase PBP1 [149, 150]. YvcK is conserved in
488 spherical bacteria and is essential in *S. aureus* [9, 151]; the homologue YbhK is also found in *E. coli*
489 [150]. It remains to be seen whether YvcK/YbhK plays a similar role in these different organisms.

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

494

495 **Concluding remarks**

496 We are just beginning to understand the many ways bacteria regulate cell wall growth (Figure 3).
497 Though we have gained insight into some mechanisms, including roles of key proteins in scaffolding,
498 PG binding and maturation, and the effects of protein-protein interactions, more components of the
499 regulation of peptidoglycan growth are likely to be discovered even in well-studied model bacteria.
500 Given the extensive nature of the interaction network amongst cytoskeletal, cell morphogenesis

501 proteins and PG synthases, hydrolases and their respective regulators we anticipate a wealth of new
502 mechanistic insights to come to light in the coming years. Advances in the genetic and biochemical
503 tools such as high throughput mutagenesis and genetic screening technologies, *in vitro* activity assays,
504 and advanced structure determination technologies will greatly aid these studies.

505

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509

510

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940

941 **Figure legends**

942 **Figure 1.** Cartoon representations of the Gram-positive and –negative peptidoglycan. (A) The basic
943 subunit of peptidoglycan; *N*-acetylglucosamine and *N*-acetylmuramic acid sugar residues, with the
944 peptide stem. Residues of the peptide stem are labelled according to the sequences found in model
945 Gram-negative / -positive species. (B) A simplified representation of the peptidoglycan sacculus
946 meshwork, made up of polymerised glycan strands linked by the peptides. This is a small sample of
947 the much larger sacculus, with continuation in all directions indicated by the ... motif. (C) Simplified
948 view of the cell envelope/surface of Gram-negative and –positive rod-shaped bacteria. The blue box
949 on the right of the panel is an enlargement of the indicated area on the whole cell to the left.
950 Peptidoglycan-associated proteins and other surface polymers are omitted.

951

952 **Figure 2.** Schematic model of the association of EzrA (dark and light blue) and GpsB (orange/grey)
953 with the membrane and possible arrangements for interaction partners in the divisome, including FtsZ
954 filaments (magenta), FtsA filaments (red) and three representative bifunctional PBP molecules. The
955 GpsB structure represented is the molecular envelope calculated by SAXS (grey) with the crystal
956 structures of the individual GpsB domains (orange) docked inside it. EzrA is represented in the
957 antiparallel dimer form with the component subunits coloured blue and light blue. In the model of a
958 bifunctional PBP; the transmembrane region is coloured grey, the GTase domain coloured pink, the
959 TPase domain coloured blue.

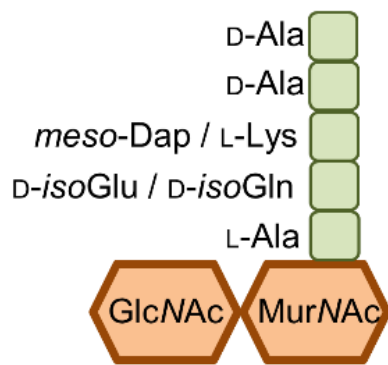
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961 **Figure 3.** A general representation of the regulatory mechanisms of cell wall growth. (A) Scaffolding;
962 cytoskeletal elements and their accessory proteins act to provide a scaffold on which the cell wall
963 synthesis machineries can assemble within the cytoplasmic membrane (CM). (B) Phosphorylation; a
964 Ser/Thr protein kinase (STPK) is depicted phosphorylating a target protein, the resultant form then
965 goes on to effect cell wall growth either directly through its own enzymatic activity or through
966 subsequent regulation of another enzyme (stimulating conversion of the substrate X to the product Y

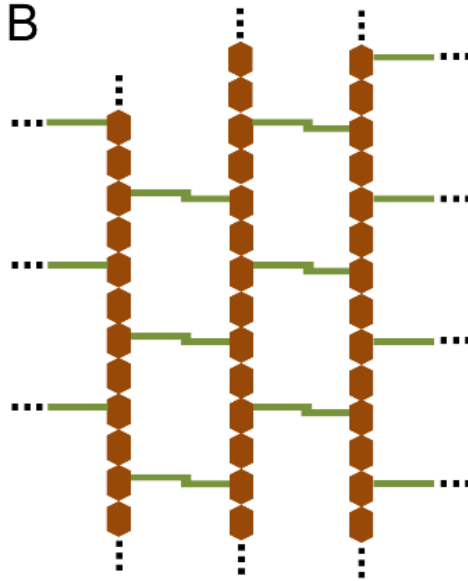
967 in this example). (C) Protein-protein interaction; in addition to the structural importance of
968 interactions in the scaffolding mechanism, interactions between proteins within the cell wall synthesis
969 machineries can lead to transduction of signals to initiate activity, such as the signal to begin
970 constriction delivered through the division machinery by FtsN binding to FtsQLB and FtsA. In
971 addition, PG synthetic and hydrolytic enzymes are controlled through the induction of conformational
972 changes upon binding of a regulator, presumably in a similar or complementary way to the
973 mechanisms depicted in panel D. (D) Structural dynamics; PG synthesis and hydrolysis enzymes
974 adopt different conformations through changes in dynamic structural elements they possess, which
975 impacts enzymatic activity. The example shown here depicts changes in the GTase domain of *S.*
976 *aureus* PBP2 enabling catalysis, and the inter-domain flexibility in this class A PBP for optimal
977 coupling of the GT and TP activities. (E) Carboxypeptidases; CPase enzymes in the periplasm act to
978 trim peptides from the peptide stems. In this top-down view example, DD-CPases trim the terminal
979 D-Ala residue (5th position) in nascent PG, thus removing this source of TPase donor substrates and
980 regulating the potential for new cross-linking. (F) Central metabolism; proteins have been found that
981 sense available nutrients and transduce this information to exert an effect on cell wall biosynthesis,
982 such as the effect of UgtP on the timing of cell division at high levels of UDP-glucose.

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A

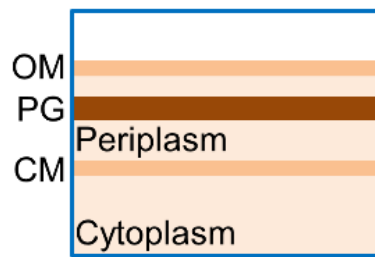
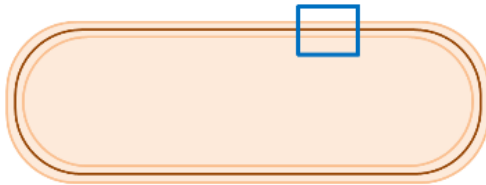


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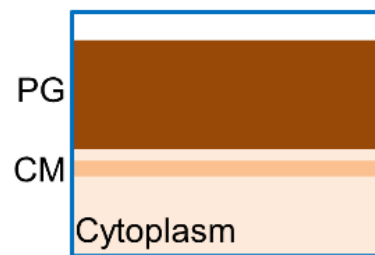
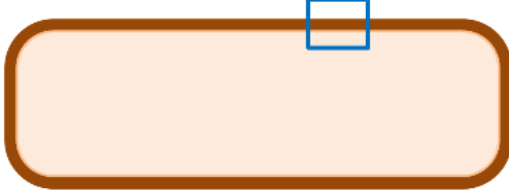


C

Gram-negative

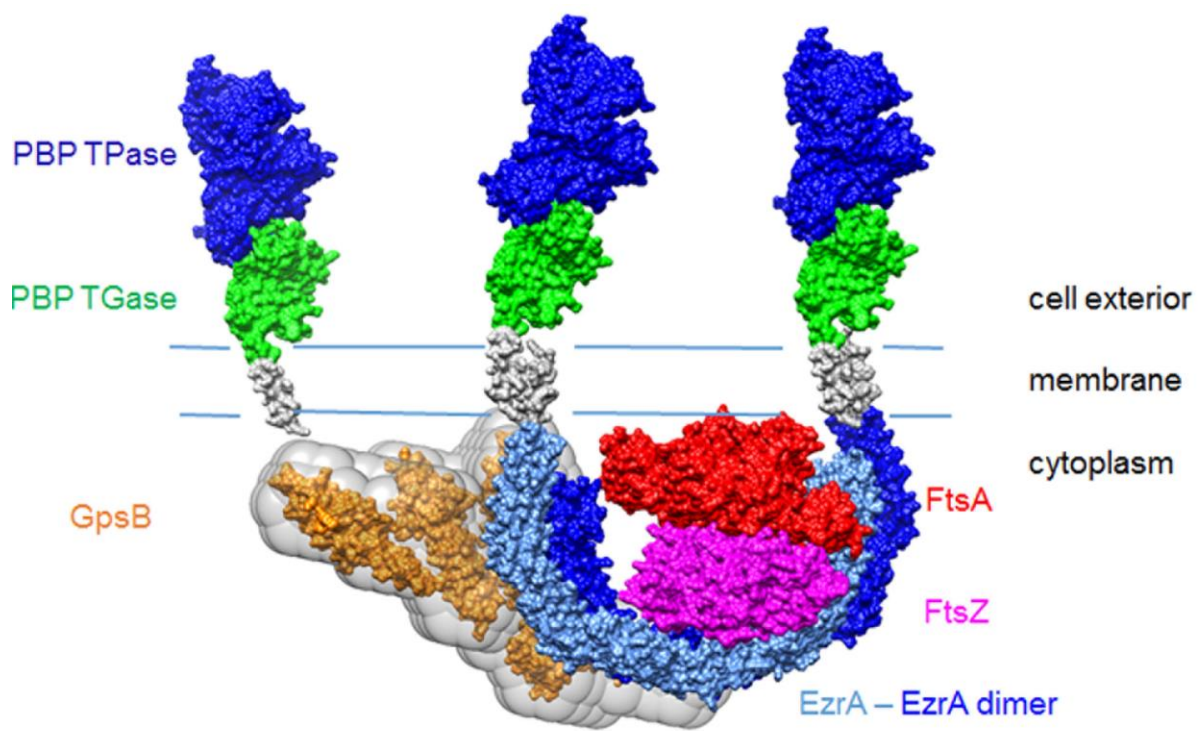


Gram-positive



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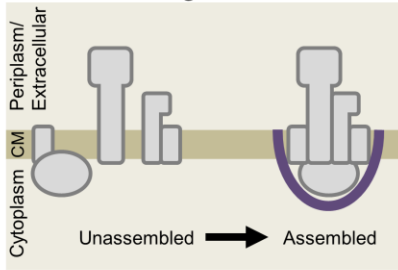
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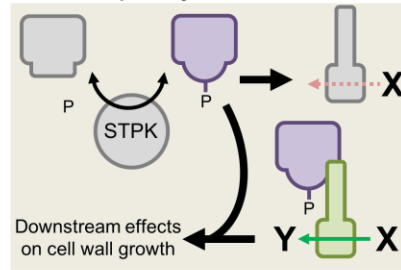
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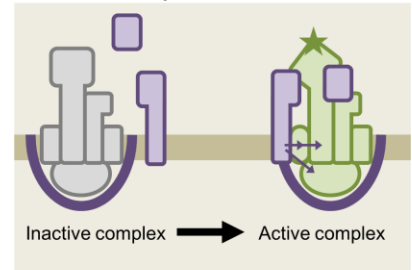
A Scaffolding



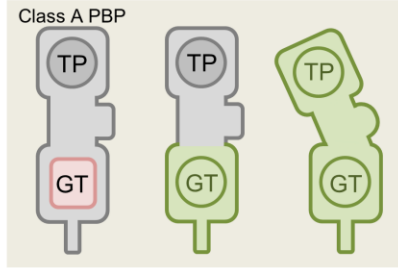
B Phosphorylation



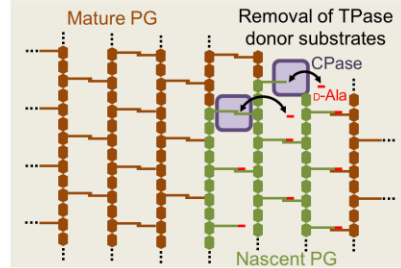
C Protein-protein interaction



D Structural dynamics



E Carboxypeptidases



F Central metabolism

