Chapter 10 from:

Bacillus

Cellular and Molecular Biology (Third edition)

Edited by Peter L. Graumann

ISBN: 978-1-910190-57-9 (paperback)
ISBN: 978-1-910190-58-6 (ebook)

Caister Academic Press https://www.caister.com/bacillus2017

The Cell Wall of *Bacillus subtilis*

10

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https://doi.org/10.21775/9781910190579-10

Abstract

The cell wall of *Bacillus subtilis* is a rigid structure on the outside of the cell that forms the first barrier between the bacterium and the environment, and at the same time maintains cell shape and withstands the pressure generated by the cell's turgor. In this chapter, the chemical composition of peptidoglycan, teichoic and teichuronic acids, the polymers that comprise the cell wall, and the biosynthetic pathways involved in their synthesis will be discussed, as well as the architecture of the cell wall. *B. subtilis* has been the first bacterium for which the role of an actin-like cytoskeleton in cell shape determination and peptidoglycan synthesis was identified and for which the entire set of peptidoglycan synthesizing enzymes has been localized. The role of the cytoskeleton in shape generation and maintenance will be discussed and results from other model organisms will be compared to what is known for *B. subtilis.* Finally, outstanding questions in the field of cell wall synthesis will be discussed.

Introduction

The cell wall is a critical structural component of each bacterial cell, except for those few bacteria that lack a cell wall (Mollicutes). It determines bacterial cell shape and bears the stress generated by the intracellular pressure, called turgor. The integrity of the cell wall is of critical importance to cell viability. In both Gram-positive and Gram-negative bacteria, the scaffold of the cell wall consists of the cross-linked polymer peptidoglycan (PG). In Gram-negative bacteria the cell wall lies in the periplasmic space, between the inner and the outer membrane of the cell, and consists of only 1–3 layers of PG. Gram-positive bacteria, like *Bacillus subtilis*, lack an outer membrane and so the cell wall constitutes the contact area with the external milieu (Fig. 10.1A). The Gram-positive cell wall contains 10 to 30 layers of PG, as well as covalently linked teichoic and teichuronic acid polymers and attached proteins. For a long time, Gram-positive bacteria were thought not to contain a region comparable to the periplasmic space in Gram-negative bacteria, because ultrastructural studies on the Gram-positive envelope showed the cell wall in close apposition to the cytoplasmic membrane. Matias and Beveridge have revealed the existence of a periplasmic space in both *B. subtilis* and *Staphylococcus aureus*, using cryo-electron microscopy on frozen-hydrated bacteria (Fig. 10.1; Matias and Beveridge, 2005, 2006). The existence of such a space would provide Gram-positives with the opportunity to move enzymes and solutes within a confined region, but without these having to be in direct contact with either the plasma membrane or the highly negatively charged polymers in the cell wall (Matias and Beveridge, 2005). Fractionation studies also provide evidence for the existence of a functional homologue of a periplasmic space in *B. subtilis* (Merchante *et al.*, 1995). Similar techniques have also been used to identify novel bacterial structures, such as an outer membrane in the Gram-positive Mycobacteria (Hoffmann *et al.*, 2008; Zuber *et al.*, 2008).

The discovery of an actin-like cytoskeleton in *B. subtilis* (Jones *et al.*, 2001) and its role in synthesis of the cell wall (Daniel and Errington, 2003) have sparked a renewed effort to understand cell wall

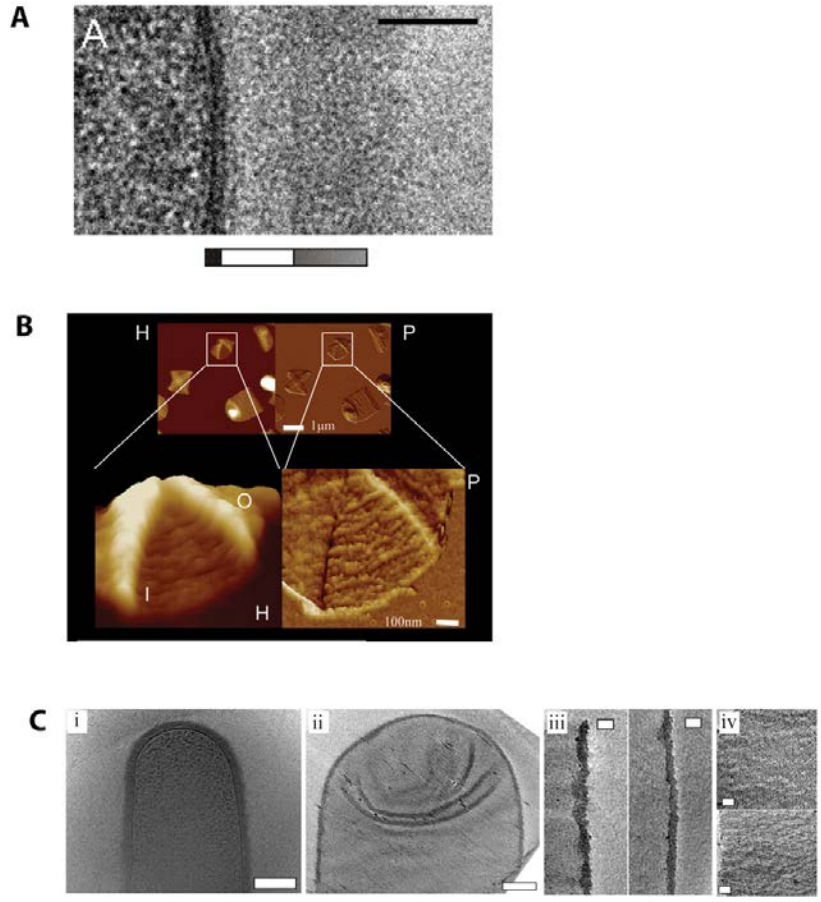

Figure 10.1 Cell wall architecture studied by various microscopy techniques (A) High magnification images of cell walls from frozen hydrated cells of *Bacillus subtilis*. The bars below the images indicate the different structures observed: black: cytoplasmic membrane; white: the Inner Wall Zone (IWZ), the Gram-positive equivalent of the periplasm; grey: the Outer Wall Zone containing the bacterial cell wall. Scale bar: 50 nm. Reprinted, with permission from ASM, from Matias and Beveridge (2005). (B) PG architecture of *B. subtilis.* AFM height (H) and phase (P) images of purified PG sacculi from broken *B. subtilis* cells. In the enlarged portions a cabling pattern is visible on the inside (I) surface of the sacculi, not on the outside (O). Scale bar: 1 μm. Reprinted, with permission, from (Hayhurst *et al*., 2008). (C) PG studied by cryo-tomography reveals that PG density and texture is homogenous in cross-sections of both intact cells and purified sacculi. (i) Tomographic slice through a *B. subtilis ΔponA* mutant (a mutant that is thinner than wild-type *B. subtilis* and thus amenable to ECT). Scale bar: 200 nm. (ii) Tomographic slice through an isolated wild-type *B. subtilis* sacculus. Scale bar: 250 nm. (iii) Two representative tomographic cross-sections across the wall of isolated *B. subtilis* sacculi perpendicular to the viewing plane reveal a globally straight sacculus side-wall with local variations in thickness. In both tomographic slices the sacculus interior is to the left. (iv) Two representative top-down slices through tomograms parallel to the plane of the sacculus illustrating surface textures (red arrows) previously interpreted to be the surfaces of coiled cables composed of helical coils of peptidoglycan. In both tomography slices the long axis of the cell runs vertically. Scale bars: 50 nm. Reprinted, with permission, from Beeby *et al*. (2013).

growth and shape determination in *Bacillus* as well as in other bacteria. Fluorescence microscopy techniques have made it possible to study the localization of enzymes involved in cell wall synthesis in growing cells, as well as to look at localization of newly incorporated PG in live cells (see Scheffers and Pinho, 2005). More recently, the development of fluorescent D-amino acid analogues (FDAAs) and click chemistry has made it possible to track cell wall synthesis (Kuru *et al.*, 2012) and furthermore, to visualize cell walls in organisms such as *Chlamydia* and *Planctomycetes* that for a long time were thought to be lacking a cell wall (Jeske *et al.*, 2015; Liechti *et al.*, 2014; Pilhofer *et al.*, 2013; van

Teeseling *et al.*, 2015). Electron cryotomography (ECT), pioneered by Grant Jensen and co-workers, has enabled us, for the first time, to see bacterial cytoskeletal elements *in situ* without any additional labelling technique (see Knowles *et al.*, 2009), and atomic force microscopy (AFM) has been successfully used to study cell wall architecture in *B. subtilis* (Hayhurst *et al.*, 2008) and several other organisms (below).

In this chapter, the chemical composition, architecture and synthesis of the cell wall of *B. subtilis* will be discussed. We will address how new findings have deepened our understanding of bacterial cell wall synthesis, but simultaneously have uncovered discrepancies in classical models of PG synthesis and have raised many new questions about the way bacteria grow.

Cell wall structure and composition

The two major structural components of the Gram-positive cell wall are peptidoglycan and anionic polymers that are covalently attached to PG or that are linked to the cytoplasmic membrane via acyl chain membrane anchors. Fractionation studies have revealed that about 9.8% of the total protein content of *B. subtilis* cells consists of periplasmic/wall associated proteins (Merchante *et al.*, 1995), and a further proteomic analysis identified 11 protein that are bound to the cell wall (Antelmann *et al.*, 2002), such as the wall associated protein A (WapA) that functions in intercellular competition (Koskiniemi *et al.*, 2013), a wall associated protease (WprA) and several autolysins that are involved in wall turnover (discussed below). Not much is known about the role of these proteins in *B. subtilis*, and they will not be discussed in detail in this chapter. For a review on protein sorting to the cell wall of Gram-positives see Schneewind and Missiakas (2014).

Peptidoglycan

Peptidoglycan (PG), also called murein, is a polymer that consists of long glycan chains that are cross-linked via flexible peptide bridges to form a strong but elastic structure that protects the underlying protoplast from lysing due to the high internal osmotic pressure. The basic PG architecture is shared between all eubacteria that contain a cell wall (e.g. like all Mollicutes, *Mycoplasma* lack a cell wall). The glycan chains are built up of alternating, β-1,4-linked, N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) subunits. An average glycan chain length for *B. subtilis* of 54–96 disaccharide (DS) units as determined by Ward using differential sodium borohydride labelling has been cited as a textbook value for a long time (Ward, 1973). Separation of radiolabelled *B. subtilis* glycan strands by size exclusion chromatography revealed that glycan strands display a wide mass distribution with the largest strands having a mass of > 250 kDa, corresponding to at least 500 DS units (Hayhurst *et al.*, 2008). Further inspection of the glycan strands by AFM revealed strand lengths of up to 5000 nm, corresponding to 5000 DS units. Again, a wide length distribution was found with an average length of 1300 nm (1300 DS units). Interestingly, when *S. aureus* glycan strands were analysed using similar methods, no such long strands were reported, suggesting that *S. aureus* PG strands are short as reported earlier (Boneca *et al.*, 2000) So, it appears that *B. subtilis* contains glycan strands of extreme lengths, which may be the result of polymerization of shorter glycan strands into one long chain rather than of continuous synthesis of one such strand. Glycan strand length is controlled by various systems that were recently identified in *S. aureus* and *Escherichia coli*. *S. aureus* uses extracellular *N*-acetylglucosaminidases, notably, SagA, to control glycan strand length and cell wall stiffness (Wheeler *et al.*, 2015). In *E. coli*, a membrane-bound endolytic transglycosylase MltG functions as a terminator of glycan strand elongation (Yunck *et al.*, 2016). These results warrant a re-evaluation of the length of glycan strands, and their control, in several other organisms as it has fundamental implications for PG architecture.

Between different bacterial species, there is considerable variation in the composition of stem peptides that are linked to the carboxyl group of MurNAc (the landmark overview is Schleifer and Kandler, 1972). The stem peptides are synthesized as penta-peptide chains, containing L- and D-amino acids, and one dibasic amino acid, usually *meso*-diamoinopimelic acid (*m*-A_2pm). In *B. subtilis*, the stem peptide composition is L-$Ala_{(1)}$-D-$Glu_{(2)}$-*m*-$A_2pm_{(3)}$-D-$Ala_{(4)}$-D-$Ala_{(5)}$, with L-$Ala_{(1)}$ attached to the MurNac (Foster and Popham, 2002; Warth and Strominger, 1971) (Fig. 10.2A). The peptide

cross-bridge is formed by the action of a transpeptidase (see below) that links D-Ala$_{(4)}$ from one stem peptide to the free amino group of *m*-A$_2$pm$_{(3)}$ from another stem peptide.

After the incorporation of disaccharide subunits with stem peptides in glycan strands, the stem peptide can be modified in several ways to yield mature PG. Depending on the strain and growth conditions, the cross-linking index of PG is between 29% and 33% of muramic acid residues (Atrih *et al.*, 1999). The terminal D-Ala residue on the peptide which had its D-Ala$_{(4)}$ cross-linked is removed during the transpeptidation reaction (see below), whereas the two terminal D-Ala residues on the other stem peptide are removed by the action of carboxypeptidase, either before or after the cross-linking reaction has taken place (see below). Stem peptides that have not been cross-linked are usually present as tri-peptides which are amidated on the free carboxylic group of the *m*-A$_2$pm (Atrih *et al.*, 1999). Depending on growth media, the stem peptides occasionally (max. 2.7%) have a glycine at position 5 (Atrih *et al.*, 1999). De-N-acetylation of the glucosamine has been found to occur in ~17% of the muropeptides, which results in incomplete digestion of the cell wall by lysozyme and may play a role in the regulation of autolysis of the cell wall (Atrih *et al.*, 1999). Some evidence that acetylation of PG is important for its regulation has been reported for *Bacillus anthracis*. Mutant cells

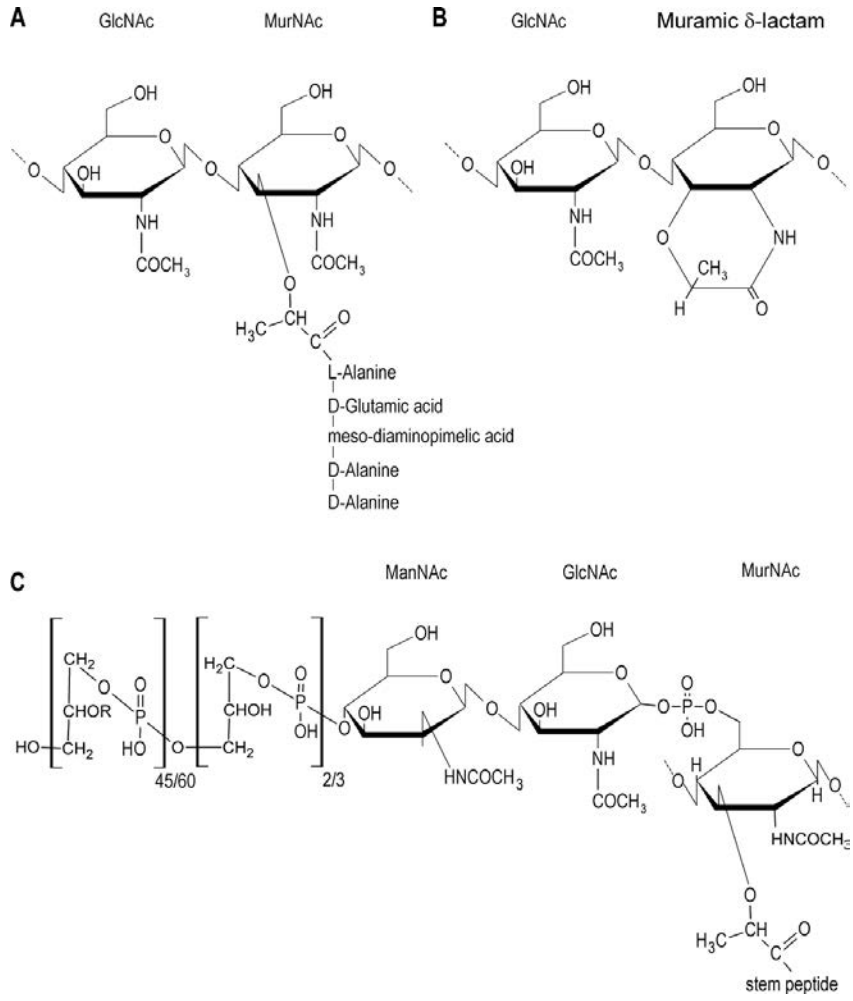

Figure 10.2 Structures of *B. subtilis* cell wall components. (A and B) The disaccharide subunits in peptidoglycan of the vegetative wall (A) and of the spore cortex with a muramic-δ-lactam (B). (C) The major wall teichoic acid, with its linkage to peptidoglycan via the MurNAc residue on the right hand side. R is either a D-alanine or glucose coupled to the C2 residues of poly(Gro-P).

carrying deletions of two peptidoglycan deacetylases, BA1961 and BA3979, grow as long twisted chains, with thickened PG at some spots at the division site and lateral wall (Balomenou *et al*., 2013).

Spore peptidoglycan

Upon nutrient starvation *B. subtilis* can switch from vegetative growth to the development of spores (discussed in detail in Chapter 11). The peptidoglycan of *B. subtilis* endospores is of a different composition than that of the vegetative cell. Spore PG consists of two layers, a thin inner layer that is closely apposed to the inner prespore membrane, and a thick outer layer, the cortex, that is close to the outer prespore membrane (for an extensive review, see Popham, 2002).

The inner layer is known as the primordial cell wall, or germ cell wall. The PG composition of the primordial wall is the same as that of the vegetative wall, and the primordial wall is not degraded during germination but forms the initial cell wall of the germinating spore. The cortex on the other hand is much thicker, contains a unique structure and is degraded during spore germination (Atrih *et al*., 1996, 1999; Popham and Setlow, 1996; Warth and Strominger, 1969, 1972). The stem peptides are removed from around 50% of the muramic acid residues and subsequently the MurNAc residues are converted to muramic-δ-lactam (see Fig. 10.2B). This results in a dramatically lower amount of possible cross-links. Additionally, around 24% of muramic acid residues have their stem peptides cleaved to single L-Ala residues precluding cross-linking. Thus, the cross-linking index for cortex PG is only 3%. The d-lactam in the cortex PG is part of the substrate recognition profile for lytic enzymes that are specific to germination, but does not play a role in dormancy and spore dehydration (Popham *et al*., 1996).

Peptidoglycan architecture

Our understanding of the architecture of the cell wall is still far from perfect, but in the past few years significant advances using advanced microscopical techniques have been made (see Vollmer and Seligman, 2010). In the absence of structural studies on discrete segments of bacterial PG, two models have been put forward in the literature for the architecture of PG. The first, also known as classical model, for PG architecture, states that the glycan strands run parallel to the plasma membrane and was first put forward by Weidel and Pelzer (Weidel and Pelzer, 1964). With the glycan strands parallel to the membrane and the stem peptides forming cross-bridges, PG is organized in several layers with the number of layers in the cell wall being different between Gram-negative and Gram-positive bacteria (Höltje, 1998; Vollmer and Höltje, 2004). An alternative model for PG architecture, the so-called scaffold model, was proposed by Dmitriev, Ehlers and co-workers. In the scaffold model, the glycan chains are in a perpendicular orientation to the membrane (with their ends pointing towards the membrane and to the outside) and form a sponge-like elastic matrix (see Dmitriev *et al*., 2005). Even though fundamentally different, the models are not mutually exclusive, nor do they exclude the possibility of other architectures (Young, 2006).

Meroueh *et al*. elucidated the 3D solution structure of a synthetic GlcNAc-MurNAc (-pentapeptide)-GlcNac-MurNAc(-pentapeptide) with NMR, providing the first glimpse of organization within a PG strand (Meroueh *et al*., 2006). The glycan backbone forms a right-handed helix with a periodicity of three disaccharide subunits, resulting in a threefold symmetry and a maximum of three neighbouring glycan strands that can be engaged in cross-links. It is not known whether these features can be extrapolated to model long glycan strands that are cross-linked, especially since PG is normally stretched by turgor pressure, which puts constraints on the spatial organization of PG.

Cryo-TEM revealed that the *B. subtilis* cell wall consists of an inner wall zone (IWZ; Fig 10.1A), the Gram-positive equivalent of the periplasm, and an outer wall zone (OWZ), containing the bacterial cell wall, with a thickness of about 33 nm (Matias and Beveridge, 2005) The discovery that *B. subtilis* glycan strands are extremely long (on average 1300 nm) makes it unlikely that *B. subtilis* PG is organized according to the scaffold model. Solid state NMR experiments on fully hydrated cell walls showed that the glycan strands are more rigid then the stem peptides, but that cross-linking of stem-peptides increases overall rigidity – thus the *S. aureus* cell wall with short glycan strands but an extremely high degree of cross-linking is more rigid than that of *B. subtilis* which has long glycan strands but not such a high degree of cross-linking (Kern *et al*., 2010).

AFM studies of gently broken cell walls revealed that the *B. subtilis* cell wall has a rough surface on the outside, but on the inside, where new PG is added to the wall, cables of about 50 nm in width were identified that run almost parallel to the short axis of the cell (Hayhurst *et al.*, 2008). Apparently helical cross-striations were observed along the cables with a periodicity of ~25 nm and the authors presented a model where glycan strands are bundled into a ~25 nm wide sheet that is coiled into a ~50-nm-wide helix (Fig. 10.1B). Interestingly, the glycan strand length was notably reduced, and the regular cabling feature on the inside of the CW lost, when CW material was isolated from a MreC mutant (Hayhurst *et al.*, 2008). A parallel organization for glycan strands was also found in *E. coli*, *C. crescentus* (Gan *et al.*, 2008), and *Lactococcus lactis* (Andre *et al.*, 2010). In *S. aureus*, nascent PG is laid down in concentric rings at the septum, again arguing for a parallel organization of glycan strands (Turner *et al.*, 2010). More recently, additional support for a parallel PG organization in *Bacillus* was provided by a combination of electron cryotomography and molecular dynamics simulations (Fig 10.1C) (Beeby *et al.*, 2013). Beeby *et al.* (2013) found that peptidoglycan strands are arranged as circumferential furrows and not as coiled cables. Moreover, peptide cross-links are placed parallel to the long axis of the cell as denatured sacculi in which peptide cross-links are broken increase in length but not in width.

Anionic polymers

Wall teichoic acids (WTA) and lipoteichoic acid (LTA) constitute up to 60% of the dry weight of the cell wall in *B. subtilis* and provide an overall negative charge to the cell wall (for an extensive review see Neuhaus and Baddiley, 2003). Both WTA and LTA are important as cells that cannot produce either LTA or WTA show morphological aberrations and can only be grown under certain conditions, whereas the absence of both is lethal (Swoboda *et al.*). LTA and WTA have several functions: (i) they can act as a reservoir for mono- and divalent cations, and cation binding in turn regulates porosity of the cell wall; (ii) their presence regulates the activity of autolysins; (iii) they can act as a scaffold for the anchoring of cell-surface proteins; (iv) WTAs function as the receptors for phage binding; and (v) their distribution is important for the regulation of cell division (Rahman *et al.*, 2009; Swoboda *et al.*, 2010). When grown under phosphate limiting conditions, teichuronic instead of teichoic acids are used, as teichuronic acid is free of phosphate. However, not all teichoic acid is replaced by teichuronic acid (Bhavsar *et al.*, 2004). WTA is covalently attached to the C6 of a MurNAc residue in the cell wall via its 'linkage unit': 1,3-glycerol-phosphate $(Gro\text{-}P)_{[2\ or\ 3]}$-N-acetyl-mannose (ManNAc)-β1,4-GlcNac-phosphate. Coupled to the linkage unit is a chain of poly(Gro-P) that can have either D-ala or glucose coupled to the C2, with chain lengths varying from 45 to 60 residues (Neuhaus and Baddiley, 2003). The composition of the chain varies between *Bacillus* species. A minor form of WTA comprises a polymer chain of N-acetylgalactosamine (GalNAc) and glucose-phosphate instead of poly-(Gro-P). Teichuronic acid consists of a chain of repeating glucuronic acid-N-GalNAc disaccharide residues (19–21 as determined for B. subtilis W23, Wright and Heckels, 1975), coupled to the cell wall via a phospho-diester bond similar to teichoic acid. LTA consists of a chain of poly(Gro-P) which contains D-Ala, glucose, or N-acetylglucosamine coupled to C2 in 40–60% of the units. LTA is anchored to the cytoplasmic membrane via a lipid anchor composed of a gentibiosyl-diacylglycerol, which is linked to the poly(Gro-P) via a glucose disaccharide. Nothing is known about the architecture of the anionic polymers in Gram-positives: they could be arranged either parallel or perpendicular to the cytoplasmic membrane, although the perpendicular orientation is favoured in discussions and figures on the topic. It has been established though that WTA and teichuronic acid are incorporated close to the membrane and move through the wall following the 'inside-to-outside' growth mechanism also proposed for PG (see Neuhaus and Baddiley, 2003).

Cell wall synthesis

All cell wall components are synthesized as precursors in the cytoplasm, which then need to be flipped across the cytoplasmic membrane to be incorporated into the cell wall. Interestingly, precursors for PG, WTA and teichuronic acid all use undecaprenyl-phosphate as carrier lipid. Synthesis of the cell wall can be subdivided in three stages: (1) synthesis of the cytoplasmic precursor and linkage

to the carrier lipid; (2) flipping across the membrane; (3) incorporation of the precursor into the cell wall. These stages will be discussed individually for the different wall components. PG and anionic polymer biosynthesis has been described in several reviews and book chapters (Neuhaus and Baddiley, 2003; Rogers *et al.*, 1980; van Heijenoort, 2001; Vollmer and Bertsche, 2008), and specifically for *B. subtilis* by Foster and Popham (2002) and Bhavsar and Brown (2006). Therefore, in this chapter the chemical reactions involved in PG synthesis will only be discussed briefly.

PG synthesis stage 1 – synthesis of Lipid II

The first dedicated step in PG precursor synthesis is the conversion of UDP-GlcNac to UDP-MurNac. A schematic outline of the steps in PG precursor synthesis and the proteins involved is shown in Fig. 10.3. Many of the proteins have been assigned based on sequence similarity to *E. coli* proteins, for which the function has been demonstrated (see Foster and Popham, 2002). The genes for *murE*, *mraY*, *murD*, *murG*, and *murB* are all present in one operon, whereas *murA (or murAA)*, *murZ (or murAB)* and *murC* lie on different places on the chromosome. MurA and MurZ are highly similar, can catalyse the same reaction and are possibly redundant, as a second *murA* copy is only present in low G+C Gram-positive bacteria. MurB is essential and the genetic organization of *murB* in the *dcw* gene-cluster is necessary for efficient growth and sporulation (Real and Henriques, 2006). MurC, D, E and F are all ATP-dependent amino acid ligases and have conserved ATP and amino acid binding motifs and common kinetic mechanisms (see El Zoeiby *et al.*, 2003). D-Ala is generated from L-Ala by the action of an alanine racemase (Diven *et al.*, 1964), encoded by the *dal* (or *alr*) gene (Ferrari *et al.*, 1985). D-Ala can function as a precursor for D-Glu, which can be generated by the action of a D-Alanine aminotransferase (*dat* or *yheM*) (Noback *et al.*, 1998), but D-Glu can also be generated by a Glu racemase of which *B. subtilis* has two, RacE and YrpC (Ashiuchi *et al.*, 1999; Fotheringham *et al.*, 1998). The cytoplasmic part of the precursor synthesis pathway is reviewed in (Barreteau *et al.*, 2008; Manat *et al.*, 2014).

Subsequently, at the cytoplasmic membrane, the monosaccharide-pentapeptide is coupled to a lipid and the second sugar is added (see Bouhss *et al.*, 2008). MraY catalyses the transfer of the phospho-MurNAc-pentapeptide moiety to the membrane acceptor undecaprenyl phosphate (bactoprenol), giving MurNAc-(pentapeptide)-pyrophosphoryl-undecaprenol (or lipid I). Then, UDP-GlcNAc is linked via a β-(1,4)-linkage to lipid I, yielding GlcNAc-b-(1,4)-MurNAc-(pentapeptide)-pyrophosphoryl-undecaprenol (or Lipid II). The coupling of the disaccharide precursor to a lipid molecule is required to facilitate the translocation of a hydrophilic substrate from one aqueous environment to another through the hydrophobic membrane. MraY and MurG have been found to interact with each other and cytoskeletal proteins MreB, MreD and FtsZ that are involved in positioning the PG synthesis machinery in *E. coli* and *C. crescentus* (Aaron *et al.*, 2007; Mohammadi *et al.*, 2007; White *et al.*, 2010).

Teichoic/teichuronic acid synthesis stage 1

As with PG synthesis, the synthesis of the precursors of anionic polymers starts with UDP-linked N-acetylated sugars, glucosamine for teichoic acid and galactosamine for teichuronic acid (Fig. 10.3). In *B. subtilis* 168, the genes involved in WTA synthesis are *tagABDEFGHO* and *gtaBmnaA* (reviewed in Neuhaus and Baddiley, 2003). In the case of teichoic acid synthesis, the lipid-linkage reaction precedes the synthesis of the linkage unit and the elongation of the poly-(Gro-P) chain. This reaction is catalysed by TagO. Work from Eric Brown's group has shown that, unlike previously thought, WTA synthesis is not essential. However, the synthesis pathway can only be disrupted when *tagO* is deleted, either because otherwise undecaprenol that is also required for PG synthesis is sequestered, or because the accumulation of toxic WTA synthesis intermediates kills the cell (D'Elia *et al.*, 2006a; D'Elia *et al.*, 2006b). UDP-ManNAc, the product of an epimerization reaction of UDP-GlcNac catalysed by MnaA, is linked to undecaprenol-PP-GlcNac by TagA (Ginsberg *et al.*, 2006; Zhang *et al.*, 2006). TagD functions as a CTP-glycerol-3-phosphate cytidyltransferase to provide CDP-Gro and GtaB functions as a UTP-glucose-6-phosphate uridyltransferase to provide UDP-glucose. TagB functions as the 'Tag primase' that adds the first glycerol-phosphate residues to

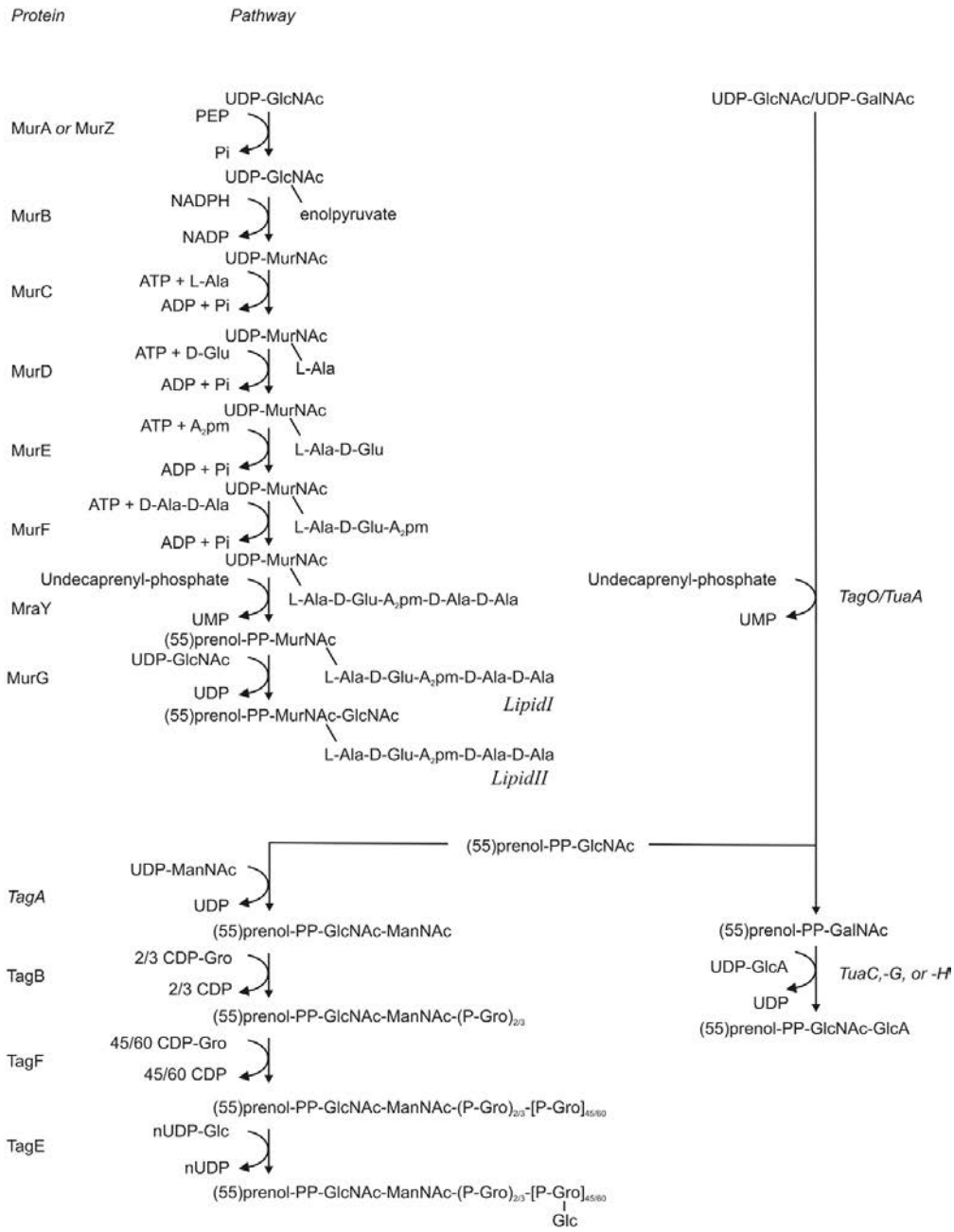

Figure 10.3 Synthesis of precursors of peptidoglycan, teichoic acid and teichuronic acid. Proteins denoted in italics have been predicted to be involved in the synthesis steps indicated. (55)prenol is undecaprenol. For more information see the text.

undecaprenol-PP-GlcNAc-ManNAc, the disaccharide linkage unit (Ginsberg *et al.*, 2006). Elongation of the glycerol-phosphate chain is mediated by TagF (Pereira *et al.*, 2008) and glucosylation by TagE, the only non-essential gene product in the *tag* operons (Pooley *et al.*, 1991). Coupling of D-Ala to the C2 on poly-(Gro-P) is mediated by the genes in the *dltABCDE* operon. The lipid-linked precursor is then ready to be translocated across the cytoplasmic membrane. The genetics of LTA biosynthesis are still enigmatic. The *dltABCDE* operon plays a

role in D-alanylation of LTA and the *ypfP* gene functions to couple the gentiobiosyl to the lipid anchor.

Teichuronic acid synthesis is mediated by the products of the *tuaABCDEFGH* operon. This operon is transcribed upon phosphate limitation (Soldo *et al.*, 1999). The reactions involved have been characterized for *B. subtilis* strain W23, and interestingly, synthesis of one repeating unit coupled to a lipid anchor takes place in the cytoplasm, after which the precursor is flipped and the repeating unit is added to a growing chain on a lipid anchor in the periplasm, before coupling of the chain to PG. WTA in *B. subtilis* W23 contains poly(ribitol-5-P) instead of poly(glycerol-3-P) and its is synthesized by a set of *tar* genes. *TarO*, *-A*, *-B* and *-F* are homologous to their *tag* equivalents, although TarF acts only as a primase to add one glycerol-3-P to the linkage unit, after which TarK primes this product by adding one ribitol-5-P and TarL acts as the poly(ribitol-5-P) polymerase (see Swoboda *et al.*, 2010). In LTA synthesis, the poly(glycerol-3-P) chain is synthesized on the outside of the cell – but the glycolipid anchor diglucosyl-diacylglycerol is synthesized at the cytoplasmic side of the membrane through coupling of UDP-Glc and diacylglycerol by UgtP (Jorasch *et al.*, 1998).

Peptidoglycan synthesis stage 2 – translocation of Lipid II

Several candidate proteins have been proposed to function as specific translocase or flippase for Lipid II, and various translocases have been identified over the last decade (reviewed in Ruiz, 2016; Scheffers and Tol, 2015). Experimental evidence for translocation activity has now been provided for three different protein families: the FtsW/RodA family (Mohammadi *et al.*, 2011, 2014), MurJ (Ruiz, 2008; Sham *et al.*, 2014) and Amj (Meeske *et al.*, 2015).

FtsW and RodA homologues were originally proposed as candidate translocases. RodA and FtsW are members of the SEDS (shape, elongation, division and sporulation) family and homologues have been found in many bacteria that contain a cell wall, but not in the wall-less *Mycoplasma genitalium* or the archaeon *Methanococcus janasschii* (Henriques *et al.*, 1998). RodA and FtsW are integral membrane proteins (generally predicted to have ten membrane spanning α-helices), which is in accordance with the suggestion that they may channel the lipid precursors to their cognate PBPs (Ehlert and Holtje, 1996; Ishino and Matsuhashi, 1981; Ishino *et al.*, 1986). Depletion of RodA in *B. subtilis* leads to the conversion from rod-shaped to spherical cells, which implicates RodA in growth of the lateral cell wall (Henriques *et al.*, 1998), and *E. coli rodA* (Ts) mutants have also been found to grow as spheres (Begg and Donachie, 1985). In various bacteria, *rodA* and *ftsW* are organized in operons with a cognate *pbp*, *e.g.* in *E. coli rodA/pbpA* and *ftsW/ftsI* (Ishino *et al.*, 1989; Matsuzawa *et al.*, 1989). In *B. subtilis*, *rodA* and *ftsW* are not found in operons with *pbp* genes, but a third homologue, *spoVE*, is part of the *mur* operon that also contains the upstream transpeptidase *spoVD* (Daniel *et al.*, 1994; Henriques *et al.*, 1992). During sporulation *spoVE* and *spoVD* are transcribed from σ^E dependent promoters, and both proteins function in PG synthesis during engulfment and the formation of the spore cortex (Daniel *et al.*, 1994; Henriques *et al.*, 1992; Meyer *et al.*, 2010). SpoVD and SpoVE physically interact and SpoVD localization is dependent on SpoVE, whereas SpoVE is protected from proteolysis by SpoVD (Fay *et al.*, 2010). The Breukink lab reconstituted FtsW/RodA mediated Lipid II translocation in an *in vitro* assay. First, a fluorescently labelled Lipid II analogue was developed, by linking the fluorophore NBD (7-nitro-2,1,3-benzoxadiazol-4-yl) to a lysine residue on position 3 in the stem peptide (van Dam *et al.*, 2007). NBD-Lipid II fluorescence can be quenched by dithionite or by specific antibodies. This allows the detection of a flipping reaction to the outside of a vesicle or liposome through reduction of total fluorescence in the presence of quencher. Using this assay, it was shown that NBD-Lipid II does not flip spontaneously across artificial membranes, but can flip across the membrane in *E. coli* membrane vesicles in an ATP-and pmf- (proton motive force) independent manner. This means that the flipping reaction was protein mediated, but excluded ABC-type transporters or other energy-dependent transport mechanisms (van Dam *et al.*, 2007). Breukink and co-authors continued by studying NBD-Lipid II flipping in membrane vesicles isolated from cells that over-expressed FtsW, or were depleted for FtsW, and found that the amount of NBD-Lipid II translocated depended on the amount of FtsW present in the vesicles (Mohammadi *et al.*, 2011). This flippase activity could

also be detected when purified FtsW was reconstituted into proteoliposomes and was specific for FtsW as several control proteins, including the other putative translocase, MurJ (below), did not show flipping activity (Mohammadi *et al.*, 2011). Recently, the same group showed that the FtsW transmembrane domain 4 (TM4) is important for translocation of Lipid II. The charged amino acids in TM4, Arg145 and Lys153, may be responsible for the interaction with Lipid II as mutation of these residues abolished FstW activity (Mohammadi *et al.*, 2014). To test FtsW pore size, Lipid II analogues attached to a rigid spherical molecule were used to test FtsW pore size. An analogue of 2464.21 Da was unable to be translocated, while a smaller analogue of 2190.13 Da was successfully translocated suggesting the size of the analogue is a limiting factor and thus that FtsW translocates Lipid II via pore mechanism with limited size (Mohammadi *et al.*, 2014).

A second class of candidate translocases is formed by the MurJ family of proteins. MurJ is a 14 TM member of the MOP (multidrug/oligosaccaridyl-lipid/polysaccharide) exporter superfamily (Hvorup *et al.*, 2003). Other MOP exporters transport other undecaprenol-linked precursors such as the O-antigen flippase Wzx in *Pseudomans aeruginosa* (Burrows and Lam, 1999). MurJ was identified in a bioinformatics search for genes that are specific for bacteria that have a cell wall, is essential in *E. coli*, and when depleted leads to cell shape defects (Inoue *et al.*, 2008; Ruiz, 2008). The Ruiz, Kahne and Bernhardt laboratories developed an assay to test the Lipid II flipping ability of *E. coli* MurJ *in vivo* based on the activity of the toxin colicin M (ColM) (Sham *et al.*, 2014). ColM cleaves periplasmic Lipid II producing PP-disaccharide-pentapeptide, which is further cleaved by carboxypeptidase to tetrapeptide, whereas Lipid II that has remained in the inner membrane leaflet will not be cleaved as ColM cannot cross the inner membrane. Thus, ColM mediated production of PP-disaccharide-tetrapeptide is a measure for Lipid II flippase activity, whereas Lipid II accumulation in the membrane is a measure for blocked flippase. Use of a single-Cys MurJ mutant (MurJA29C) that can be inactivated by the addition of MTSES (2-sulfonatoethyl methanethioulfonate) allowed the monitoring of Lipid II translocation in the presence and absence of active MurJ. Modification of MurJA29C also abolished PG synthesis *in vivo*, whereas unmodified MurJA29C allowed growth. Interestingly, whereas MurJ was not active in an *in vitro* assay (above), cells depleted for FtsW/RodA still displayed detectable flippase activity in the *in vivo* ColM assay (Sham *et al.*, 2014).

B. subtilis homologues of MurJ are not essential for growth (Fay and Dworkin, 2009), even more, a deletion of all 10 *B. subtilis* MOP homologues does not have important effects of growth and morphology (Meeske *et al.*, 2015). This observation led to the suggestion that an additional flippase may be present. A synthetic lethal screen in a strain in which four the MOP members most homologous to *E. coli* MurJ (YtgP, YabM, SpoVD and YkvU) were deleted, identified Amj (Alternate to MurJ) as a putative flippase. The role of Amj as a flippase was confirmed in *E. coli* – Amj could compensate for the loss of MurJ and was functional in the *in vivo* ColM flippase assay (Meeske *et al.*, 2015). Interestingly, it was impossible to make a double deletion of *amj* and *ytgP*, the closest homologue to *E. coli* MurJ, indicating that YtgP is the *B. subtilis* MurJ homologue (Meeske *et al.*, 2015).

The identification of these three families of Lipid II flippases over the past years means tremendous progress. The question how Lipid II is precisely translocated by these various flippases remains to be resolved and may require crystal structures of the proteins with bound Lipid II.

The PG precursor that is translocated does not necessarily have to contain a penta-peptide side chain. MraY has been shown to accept substrates containing di-, tri-, tetra- and modified pentapeptide side chains, which can be coupled to a undecaprenyl-phosphate (Price and Momany, 2005). Some bacteria are capable of translocating incomplete PG precursors. In *S. aureus* a conditional *murF* mutant has been constructed by placing the *murF* gene under control of an inducible promoter. Suboptimal concentrations of inducer block the addition of the final two D-Ala residues to the stem peptide, which leads to the accumulation of UDP-linked muramyl tripeptides in the cytoplasm. However, these muramyl-tripeptides can still be incorporated into the cell wall (Sobral *et al.*, 2006).

Anionic polymer synthesis stage 2 – translocation

Translocation of teichoic acid precursors is probably mediated by TagGH. Both *tagG* and *tagH* are essential genes encoding a two-component ABC transporter. Limited expression of these genes results in cells with aberrant cell walls containing reduced amounts of both the major and minor components of WTA (Lazarevic and Karamata, 1995). Translocation of the repeating unit of teichuronic acid is thought to be mediated by TuaB, a protein with 11 or 12 predicted transmembrane helices that is homologous to the Wzx proteins described above (Soldo *et al.*, 1999). The glycolipid anchor for LTA synthesis is flipped to the outside of the cell by LtaA (Grundling and Schneewind, 2007a).

Peptidoglycan synthesis stage 3 – incorporation of precursors into peptidoglycan

The third and final stage of PG biosynthesis takes place at the outer side of the cytoplasmic membrane and involves the polymerization of the translocated disaccharide-peptide units and their incorporation into the growing PG. The periplasmic space poses a topological problem in PG synthesis: the lipid-linked precursor and the majority of the proteins catalysing PG synthesis are embedded in the membrane, whereas the PG to which the precursor has to be attached is at a distance of some 22 nm. There is quite some flexibility between the TGase and TPase domains in Class A PBPs that could account for this bridging, especially if one imagines that a new glycan strand is being assembled at the membrane, and the threaded into the cell wall at a slight distance from the membrane. Dmitriev and coworkers have proposed that the membrane bulges to bring the cell wall and the sites of PG synthesis (Dmitriev *et al.*, 2005). It still has to be resolved whether such membrane bulging occurs.

Incorporation of PG-precursors into PG is mediated mainly through the action of the so-called penicillin-binding proteins (PBPs), which catalyse the transglycosylation and transpeptidation reactions responsible for the formation of the glycosidic and peptide bonds of the PG. In the transglycosylation reaction, the glycan chain is elongated by the formation of a glycosidic bond between Lipid II and the lipid-linked PG strand. An elegant *in vitro* study from Nguyen-Distèche and co-workers, using *E. coli* PBP1b, showed that in this reaction the reducing end of MurNac on the growing glycan chain acts as a donor and the C-4 carbon of GlcNac moiety of Lipid II acts as an acceptor (Fraipont *et al.*, 2006), as was previously concluded for cell wall growth in *B. licheniformis* (Ward and Perkins, 1973). As a result, undecaprenyl-phosphate will be released from the donor (and flipped across the membrane to act again as a substrate for MraY), and the growing glycan chain will remain attached to the membrane through the lipid anchor at its new reducing end. Termination of elongation of peptidoglycan strands is performed by LTs (lytic transglycosylases). In *E. coli*, MltG has been identify recently as a terminase (Yunck *et al.*, 2016). MltG is the first LT reported to localize at the inner membrane and interacts with PBP1b. In the absence of MltG, the amount of anhydromuropeptides, which are a characteristic feature of the caps of glycan strands, was reduced, a direct measure of glycan strand length confirmed that the overall length of glycan strands was increased (Yunck *et al.*, 2016).

In the transpeptidation reaction, the terminal D-Ala-D-Ala of one stem peptide is bound to the active site of the enzyme through binding of the penultimate D-Ala to the catalytic Serine in the protein, concomitant with the release of the terminal D-Ala. Subsequently, the stem-peptide is coupled to the dibasic A_2pm that functions as an acceptor on another stem-peptide. The acceptor peptide does not have to be a pentapeptide: for example, tri- and tetra-peptide acceptors can also be used by *E. coli* PBP1b (Bertsche *et al.*, 2005). However, the specificity for acceptor substrates may be more stringent for other transpeptidases (see below). Terminal D-Ala residues are removed from acceptor stem peptides by the D,D-carboxypeptidase activity of some PBPs. This reaction can also take place before the stem-peptide has been cross-linked to another peptide, thus presenting a level of control for acceptor substrate specificity. Finally, PBPs with D,D-endopeptidase activity can cleave cross-links in order to allow the PG mesh-work to expand.

During spore PG synthesis, around 50% of the muramic acid residues are converted to muramic-δ-lactam by the concerted efforts of the CwlD and PdaA proteins. CwlD acts as an amidase and removes the stem peptide from muramic acid, after which PdaA acts as a de-acetylase and generates the lactam ring (Gilmore *et al.*, 2004). This

process must be tightly regulated as every second muramic acid in the PG strand is converted to muramic-δ-lactam and the reactions have to occur before the stem peptide is involved in a transpeptidation reaction. Nothing is known about regulatory factors for CwlD or for PdaA. PdaA is expressed in the prespore only (Fukushima *et al.*, 2002) whereas CwlD is expressed both in the prespore and in the mother cell, and mother cell expression alone is enough to produce spores with normal muramic-δ-lactam levels (Gilmore *et al.*, 2004; Sekiguchi *et al.*, 1995). Interestingly, both proteins have to cross the membrane from different compartments and act in the intermembrane space between the inner- and outer prespore membrane where they generate a highly ordered muramic-δ-lactam distribution.

Anioic polymer synthesis stage 3

It is not known how teichoic and teichuronic acid are coupled to PG. Chain formation during teichuronic acid biosynthesis occurs in the periplasm and is thought to be mediated by TuaE, which is a membrane protein homologous to a polymerase involved in O-antigen synthesis (Soldo *et al.*, 1999).

B. subtilis contains four paralogues of LtaS, the enzyme that can synthesize the poly(glycerol-3-P) backbone on the glycolipid anchor. Of these homologues only $LtaS_{BS}$ is capable of replacing the single LtaS of *S. aureus* (Grundling and Schneewind, 2007b). Recent work, in which LTA synthesis activity was reconstituted *in vitro*, has shown that of the four LtaS paralogues, YvgJ acts as a primase adding the first glycerolphosphate onto the glycolipid anchor, with $LtaS_{BS}$ acting as the housekeeping LTA synthase and YqgS as a sporulation-specific LTA synthase and YfnI as the LTA synthase active during stress. YfnI can also act as a primase and its expression is controlled by σM, hence its suggested role as a LTA synthase active during stress (Schirner *et al.*, 2009; Wörmann *et al.*, 2011).

Penicillin-binding proteins in *Bacillus subtilis*: activity and expression

Penicillin binding proteins (PBPs) belong to the family of acyl serine transferases, which comprises high molecular weight (HMW; > 60 kDa) PBPs (catalysing transglycosylation and transpeptidation reactions), low molecular weight (LMW; < 60 kDa) PBPs (catalysing carboxypeptidase and endopeptidase reactions) and β-lactamases (which cleave β-lactam rings and thereby mediate resistance to penicillin and analogous antibiotics) (Ghuysen, 1991). An overview of *B. subtilis* PBPs is given in Table 10.1. The functional redundancy between PBPs from all classes has made it difficult to assign specific functions to individual PBPs in *B. subtilis* and most of our current understanding of PBP functions in *B. subtilis* is the result of extensive genetic studies performed by Popham, Setlow and their co-workers.

HMW PBPs are further subdivided into two classes, A and B, on the basis of their primary structure and the catalytic activity of the N-terminal domain (Goffin and Ghuysen, 1998). All HMW PBPs are anchored to the cytoplasmic membrane via a transmembrane helix.

Class A PBPs have an N-terminal domain with transglycosylase activity and a C-terminal domain with transpeptidase activity, which makes them capable of both glycan strand elongation and formation of cross-links between glycan strands. Therefore, these proteins are also known as bifunctional PBPs. *B. subtilis* contains four genes encoding Class A PBPs, but the *ponA* gene gives rise to both PBP1a and PBP1b which are different due to C-terminal processing of the protein (Popham and Setlow, 1995). Class A PBPs are the only genes with an identified transglycosylation activity in *B. subtilis*, yet a mutant strain in which all four Class A *pbp* genes have been deleted is still capable of PG synthesis, although the strain grows much slower and displays some abnormalities in its cell wall (McPherson and Popham, 2003). *B. subtilis* lacks the so-called monofunctional glycosyltransferases (Mgt) that have been found in several other bacteria (e.g. Di Berardino *et al.*, 1996), so it remains to be identified which other protein(s) are capable of performing the transglycosylation reaction. PBP1 performs a non-essential function in cell division, as mutants exhibit slower growth and form slightly elongated cells (Popham and Setlow, 1995). Recently, Claessen *et al.* showed that PBP1 also plays a role in pole maturation and cell elongation, and shuttles between the division site and lateral wall (Claessen *et al.*, 2008). PBP-2c and -2d are involved in sporulation, as cells lacking both of these PBPs are incapable of forming viable spores

Table 10.1 An overview of penicillin-binding proteins from *B. subtilis*

Class	Gene	Protein	Function/expression	Localization (method)[a]
A	*ponA* (Popham and Setlow, 1995)	PBP1a/b	TG/TPase[b] involved in cell division and diameter control in elongation (Claessen *et al*., 2008), veg[c] (Popham and Setlow, 1995)	Septal (IF, GFP) (Pedersen *et al*., 1999; Scheffers *et al*., 2004), distinct foci and bands at cell periphery (Claessen *et al*., 2008)
	pbpD (Popham and Setlow, 1994)	PBP4	Not known, veg (Popham and Setlow, 1994)	Distributed along membrane with distinct spots at periphery (GFP) (Scheffers *et al*., 2004)
	pbpF (Popham and Setlow, 1993b)	PBP2c	Synthesis of spore PG (McPherson *et al*., 2001), veg, late stages of spo (Popham and Setlow, 1993b)	Distributed along membrane, redistributed to prespore during sporulation (GFP) (Scheffers *et al*., 2004; Scheffers, 2005)
	pbpG (Pedersen *et al*., 2000)	PBP2d	Synthesis of spore PG (McPherson *et al*., 2001), spo (Pedersen *et al*., 2000)	Distributed along membrane (GFP) (Scheffers *et al*., 2004), redistributed to prespore during sporulation (Scheffers, 2005)
B	*pbpA* (Murray *et al*., 1997)	PBP2a	Synthesis of lateral wall (Wei *et al*., 2003), veg (Murray *et al*., 1997)	Evenly distributed along the membrane (GFP) (Scheffers *et al*., 2004); depends on Lipid II (Lages *et al*., 2013)
	pbpH (Wei *et al*., 2003)	PbpH	Synthesis of lateral wall veg (Wei *et al*., 2003)	Evenly distributed along the membrane (GFP) (Scheffers *et al*., 2004); depends on Lipid II (Lages *et al*., 2013)
	pbpB (Yanouri *et al*., 1993)	PBP2b	Cell division specific TPase (Daniel *et al*., 2000), veg, spo (Yanouri *et al*., 1993)	Septal (IF, GFP) (Daniel *et al*., 2000; Scheffers *et al*., 2004)
	pbpC (Murray *et al*., 1996)	PBP3	Not known, veg, low expression during spo (MURRAY *et al*., 1996)	Distinct foci and bands at cell periphery (GFP) (Scheffers *et al*., 2004)
	spoVD (Daniel *et al*., 1994)	SpoVD	Synthesis of spore PG, spo (Daniel *et al*., 1994)	outer prespore membrane (GFP) (Fay *et al*., 2010)
	pbpI (Wei *et al*., 2004)	PBP4b	Not known, spo (Wei *et al*., 2004)	evenly distributed along the membrane (GFP) (Scheffers *et al*., 2004)
Low MW CPase	*dacA* (Todd *et al*., 1986)	PBP5	Major D,D-carboxypeptidase (Lawrence and Strominger, 1970)	distributed along membrane with distinct spots at periphery (GFP) (Scheffers *et al*., 2004)
	dacB (Buchanan and Ling, 1992)	PBP5*	Control of peptide cross-linking in spore PG (Popham *et al*., 1999), spo (Buchanan and Ling, 1992)	not known
	dacC (Pedersen *et al*., 1998)	PBP4a	not known, late stationary phase (Pedersen *et al*., 1998)	Distinct foci and bands at cell periphery (GFP) (Scheffers *et al*., 2004)
	dacF (Wu *et al*., 1992)	DacF	Control of peptide cross-linking in spore PG (Popham *et al*., 1999), spo (Wu *et al*., 1992)	Not known
Low-MW EPase	*PbpE* (Popham and Setlow, 1993a)	PBP4*	Not known, spo (Popham and Setlow, 1993a)	Distinct foci and bands at cell periphery (GFP) (Scheffers, 2005)
	PbpX	PbpX	Not known, veg (Scheffers, 2005)	Septal, spiral outgrowth to both asymmetrical septa during sporulation (Scheffers *et al*., 2004; Scheffers, 2005)

[a]IF: Immunofluorescence; GFP: fluorescence of a GFP-fusion.
[b]TGase: transglycosylase; TPase: transpeptidase; CPase: carboxypeptidase; EPase: endopeptidase.
[c]For the expression or transcription factor dependency of most *pbp* genes has been determined and is indicated; veg: expression during vegetative growth; spo: expression during sporulation. Reprinted, with permission from ASM, from (Scheffers and Pinho, 2005).

(McPherson *et al.*, 2001). Both PBPs are expressed in the prespore during sporulation, and in the double mutant, PG is synthesized that has an altered composition and does not completely surround the prespore, suggesting that these PBPs play a role in the synthesis of the germ cell wall that serves as a template for synthesis of cortex PG (McPherson *et al.*, 2001). PBP4 is probably involved in synthesis of the vegetative cell wall, but a *pbp4* deletion has no obvious phenotype (Popham and Setlow, 1994), although it recently was proposed as the main PBP involved in the incorporation of unnatural D-amino acids (Fura *et al.*, 2015).

B. subtilis contains six genes encoding Class B PBPs, which, like class A PBPs, contain a C-terminal domain with transpeptidase activity. The N-terminal domain of Class B PBPs has an unknown, non-catalytic function. The best studied protein in this class is the essential *E. coli* PBP3, which functions in cell-division (see Errington *et al.*, 2003). Here, the N-terminal domain is important for protein folding and stability (Goffin *et al.*, 1996) and for the recruitment of other cell division proteins (Wissel and Weiss, 2004). The crystal structure of the Class B PBP2x from *Streptococcus pneumoniae* showed that the N-terminal domain resembles a sugar tong, but structural homologues have not been found in the databases (Parès *et al.*, 1996) so the function of this domain is still enigmatic. It has been proposed that this domain plays a role as a morphogenetic determinant, as some of the Class B PBPs have a specific role in cell wall synthesis during either division or elongation (Goffin and Ghuysen, 1998). PBP2b, the homologue of *E. coli* PBP3, is the only essential PBP in *B. subtilis*, functions in cell division and is expressed during vegetative growth and sporulation (Yanouri *et al.*, 1993). Modelling of the interaction between DivIB and PBP2b suggests that it is not the N-terminal domain, but rather the TPase domain of PBP2b that contacts DivIB, the protein that recruits PBP2b to the septum (Rowland *et al.*, 2010). PBP2a and PbpH are expressed during vegetative growth and play redundant roles in cell wall growth during elongation: a double mutant of these PBPs is not viable and depletion of one in the absence of the other leads to swelling of the cells and eventually to lysis (Wei *et al.*, 2003). SpoVD is expressed during sporulation (Daniel *et al.*, 1994) and is responsible for PG synthesis during engulfment and synthesis of the spore cortex PG (Bukowska-Faniband and Hederstedt, 2013). The remaining two PBPs, PBP3 and PBP4b, are expressed during vegetative growth and sporulation, respectively, and have unknown functions (Murray *et al.*, 1996; Wei *et al.*, 2004).

The low-MW PBPs can be subdivided in two classes: carboxypeptidases, of which *B. subtilis* has four, and endopeptidases, of which *B. subtilis* has two. PBP5 is the major D,D-carboxypeptidase, and in a PBP5 deletion strain, the terminal D-Ala residues are not removed from pentapeptide side chains that either were not cross-linked or functioned as acceptors during transpeptidation (Atrih *et al.*, 1999), which is also true for the fluorescent D-amino acid analogue HADA, which is incorporated in position 5 of the stem peptide and a substrate for PBP5 (Kuru *et al.*, 2012) (Fig. 10.4B). PBP5* has been shown to function as a D,D-carboxypeptidase during sporulation (Buchanan and Ling, 1992; Todd *et al.*, 1985). Together with DacF, PBP5* regulates the degree of cross-linking in spore cortex PG (Popham *et al.*, 1999). The final carboxypeptidase, PBP4a, is expressed during late stationary phase (Pedersen *et al.*, 1998) and is capable of catalysing peptidation reactions on mDAP both with and without an amidated N-carboxylic acid (Nemmara *et al.*, 2013). Classically, D,D-carboxypeptidases are thought to play a role in PG maturation, cleaving off terminal D-Ala residues from stem peptides after transpeptidation. However, it is also possible that these proteins control the length of the stem peptides that function as substrate for transpeptidation, thereby controlling substrate availability for Class B PBPs with different morphogenetic properties (see below). The endopeptidases in *B. subtilis* were assigned on the basis of their homology to the known *E. coli* endopeptidase PBP4 (Korat *et al.*, 1991), but both PBP4*, which is expressed during sporulation, and PbpX, which is expressed during vegetative growth, can be lost through deletion of the genes without any phenotypic effects (Popham and Setlow, 1993b; Scheffers, 2005).

Structure of PBPs

The transpeptidase domains of both Class A and B HMW PBPs contain conserved motifs that constitute the unique signature of all penicillin interacting proteins: SXXK, with the active site serine, (S/Y) XN and (K/H) (T/S)G. These motifs are always present in the same order with similar spacing in the

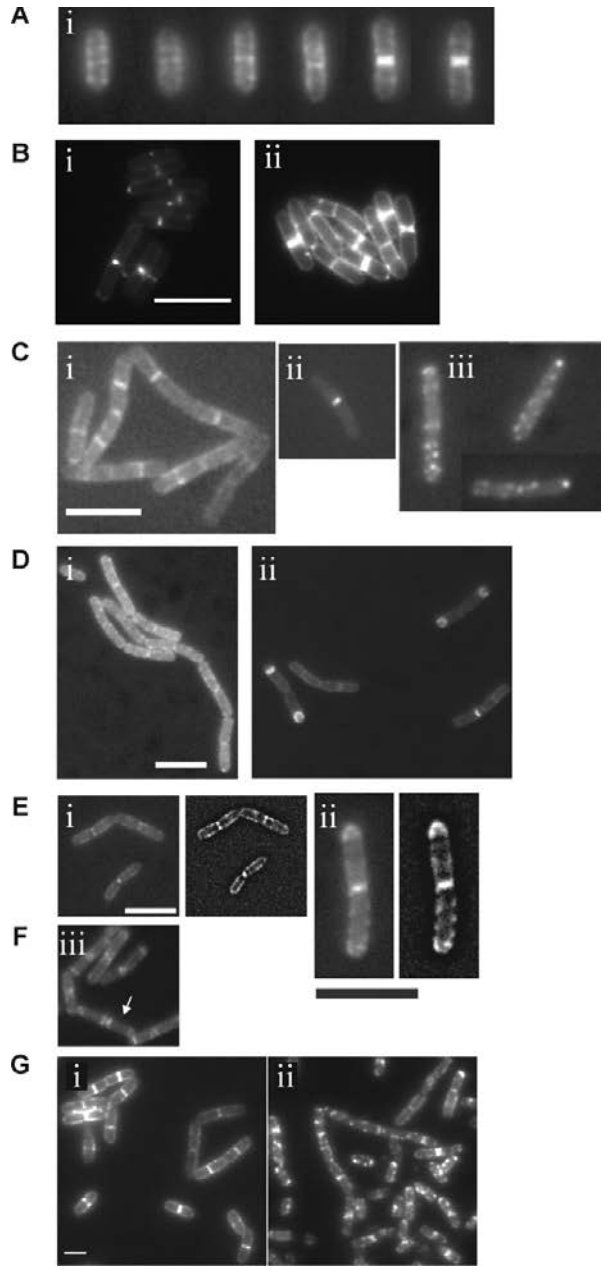

Figure 10.4 Localization of PG precursor insertion and PBPs in *B. subtilis.* (A) Van-FL staining of nascent PG during various stages in the cell cycle in a wild-type strain (adapted, with permission from Elsevier, from Daniel and Errington, 2003). (B) HADA staining of *B. subtilis* wild type (i) and *B. subtilis* Δ*dacA* (ii) (DMA, unpublished). (C) Representative patterns for PBP distribution: (i) disperse, shown is GFP-PBP2a; (ii) septal, shown is GFP-PBP1; (iii) spotty, shown is GFP-PBP3 (adapted, with permission from Blackwell Publishing, from Scheffers *et al*., 2004). (D) Redistribution of GFP-PBP2d during sporulation: (i) disperse localization of GFP-PBP2d during vegetative growth; (ii) GFP-PBP2d localization to the prespore membrane, 2 h after resuspension in sporulation medium (adapted, with permission from SGM, from Scheffers, 2005). (E) Redistribution of GFP-PbpX during sporulation. (i, ii) GFP-PbpX localization changes from a septal to membrane localization (i) and appears to spiral out (ii, note difference in magnification) to both asymmetrical division sites (iii). Right hand panels in (i) and (ii) show the images after deconvolution (adapted, with permission from SGM, from Scheffers, 2005). (G) PP- nisin delocalizes Lipid II and specific PBPs. GFP-PbpH localization in untreated cells (i) and after treatmeant with PP-nisin (ii) (adapted with permission from Blackwell Publishing, from Lages *et al*., 2013). Scale bars: 5 μm (A–F) 2 μm (G).

primary protein structure, forming the active site in the tertiary structure of the domain (Ghuysen, 1991; Goffin and Ghuysen, 1998; Massova and Mobashery, 1998).

The crystal structures of several high- and low-molecular-weight PBPs from various organisms have been determined in the last few years (for an overview see Mattei *et al.*, 2010; Sauvage and Terrak, 2016). TP domains are highly similar, with a central, mixed, β-sheet surrounded by α-helices. The structure of *E. coli* PBP6 in complex with a MurNAc-pentapeptide substrate revealed that the D-Ala-D-Ala part of the peptide is positioned within the active side cleft with the rest of the substrate accessible to the solvent, leaving the third stem peptide residue free to participate in a TP reaction (Chen *et al.*, 2009). The crystal structure of a soluble form of *S. pneumoniae* PBP1b revealed a conformational change upon ligand binding (Macheboeuf *et al.*, 2005). The active site of the transpeptidase domain of PBP1b was found to exist in an 'open' and 'closed' conformation, and the open conformation was dependent on the presence of ligand, whereas the closed conformation showed blocked substrate accessibility. The difference between the structures suggests that PBPs may be activated by the availability of transpeptidation substrate, as the 'open' conformation could only be obtained by soaking crystals containing the 'closed' form of PBP1b with a stem peptide analogue (Macheboeuf *et al.*, 2005). Similar local flexibility has been confirmed in a number of other structures, and is linked to the development of resistance to β-lactam antibiotics (see Mattei *et al.*, 2010). Insight into TG domain structure has come from the structures of 2 Class A PBPs and two isolated TG domains, which display a lysozyme-like fold (Heaslet *et al.*, 2009; Lovering *et al.*, 2007; Sung *et al.*, 2009; Yuan *et al.*, 2007). Next to the TG and TP domains *E. coli* PBP1b contains an additional domain, UvrB domain 2 homologue (UB2H), which is required for the interaction of PBP1b with the lytic transglycosylase MltA and the lipoprotein LpoB that activates PBP1b (Markovski *et al.*, 2016; Sung *et al.*, 2009).

Cell wall turnover

The cell wall is subject to continuous turnover, with PG being hydrolysed and synthesized at the same time. Autolysins, proteins that hydrolyse PG, play a role in various processes in *B. subtilis*, such as PG maturation, separation of the cell wall at the septum during division, motility, competence development, spore development, germination and protein secretion (for reviews see Smith *et al.*, 2000; Vollmer *et al.*, 2008). Hydrolysis activity must be tightly controlled to allow insertion of a PG strand in the meshwork without disrupting the structural integrity of the PG (especially in Gram-negative organisms because the PG is only 1–3 layers thick). Not much is known about the turnover of anionic polymers, but evidently, the PG linked WTA and teichuronic acid will be released from the cell wall when PG is hydrolysed.

Genome analysis revealed the presence of 35 definite or predicted autolysins in *B. subtilis* that cluster in 11 different protein families (Smith *et al.*, 2000). These proteins hydrolyse all the different bonds in PG: glucosaminidases LytD and LytG hydrolyse the bond between GlcNAc and MurNAc; muramidases (enterococcal muramidase family) and lytic transglycosylases (Slt70 family, germination specific lytic enzyme family) hydrolyse the bond between MurNAc and GlcNac; amidases (LytC family, XlyA family) cleave the bond between MurNac and L-Ala$_{(1)}$ on the stem-peptide and D,L-endopeptidases (families I and II) and L,D-endopeptidase cleave D,L and L,D peptide bonds in the stem peptides and cross-links. Two additional protein families were identified. Firstly, proteins homologous to Lysostaphin, an endopeptidase that cleaves pentaglycine cross-bridges occurring in *Staphylococcus* species but not in *Bacillus*, suggesting that these proteins may be secreted by *Bacillus* as antibiotics against staphylococci. Secondly, proteins homologous to LrgB, a putative autolysin from *S. aureus* with no identified function.

During vegetative growth, about 95% of the autolyic activity is mediated by the amidase LytC and the glucosaminidases LytD and LytG. Inactivation of these autolysins, in various combinations with the D,L-endopeptidases LytE and LytF, and YwbG (LrgB family) results in formation of chains of cells indicating a role in cell separation (Blackman *et al.*, 1998; Ishikawa *et al.*, 1998; Margot *et al.*, 1998, 1999; Ohnishi *et al.*, 1999). LytG functions as an exoglucosaminidase that removes GlcNAc residues from glycan strands, resulting in glycan strands with MurNac at their non-reducing termini (Atrih *et al.*, 1999; Horsburgh *et al.*, 2003b).

Interestingly, overproduction of the predicted endopeptidase PBP4* also causes chain formation (Zellmeier *et al.*, 2003). Expression of LytC, -D and –F as well as of genes for flagellar motility and chemotaxis are under control of the transcription factor σ^D (Helmann and P, 2002) and inactivation of LytC and LytD causes diminished swarming motility (Blackman *et al.*, 1998; Margot *et al.*, 1994; Rashid *et al.*, 1995), suggesting that autolysins play an as yet unidentified role in motility.

D,L-Endopeptidases as LytF, LytE, and CwlO have a similar C-terminal sequence, but different N-terminal domains which determine their localization in the cell and function (Hashimoto *et al.*, 2012). While LytF is the principal endopeptidase involved in cell separation, LytE and CwlO are required during elongation. LytF localizes to the division site (Yamamoto *et al.*, 2003) and poles (Hashimoto *et al.*, 2012) and is mainly expressed during mid-exponential phase (Hashimoto *et al.*, 2012). LytE localizes to the septum and poles, but also at the lateral wall in a helix-like manner (Hashimoto *et al.*, 2012; Kasahara *et al.*, 2016). And finally, CwlO localizes to the sidewall and is expressed during early exponential phase (Hashimoto *et al.*, 2012). LytF localization is affected by the presence of WTA and LTA. LytF loses it septal localization when the teichoic acids are depleted (Kiriyama *et al.*, 2014; Yamamoto *et al.*, 2008), but also the presence of LTA and WTA regulates the expression of the LytF transcription factor σ^D.

LytE and *cwlO* knockouts are viable, but a double deletion of *lytE* and of *cwlO* is lethal indicating that the have similar function (Bisicchia *et al.*, 2007). Interestingly, LytE interacts with MreBH (Carballido-López *et al.*, 2006), while CwlO interacts with Mbl (Dominguez-Cuevas *et al.*, 2013) two of the three actin homologues in *B. subtilis.* LytE and CwlO expression is regulated by the WalRK two component signal transduction pathway. LytE expression is up-regulated under stress conditions like heat shock and high temperatures (Huang *et al.*, 2013; Salzberg *et al.*, 2013; Tseng *et al.*, 2011), and again the presence of LTA or WTA play a role as LytE transcription is enhanced in the absence of LTA and WTA (Kasahara *et al.*, 2016). CwlO is also controlled by WalRK but has highly instable transcript, so that the levels of CwlO will decrease quickly after WalRK deactivation, allowing a tight regulation of CwlO (Noone *et al.*, 2014). CwlO is also regulated at the protein level by the ABC transporter FstEX. FstEX has been related to cell division in *E. coli* (Yang *et al.*, 2011); however, in *B. subtilis* it is involved in cell elongation. FtsE mutants with a defect in binding or hydrolysis of ATP are lethal in a *lytE* knockout background, strongly suggesting that FtsEX activity, probably through an ATP-mediated conformational change, is required to activate CwlO (Meisner *et al.*, 2013).

Autolysins also play critical roles during spore formation and germination. The autolysins SpoIID and SpoIIP form a complex with the membrane protein SpoIIM that drives membrane migration during engulfment (Abanes-De Mello *et al.*, 2002; Chastanet and Losick, 2007). In addition, SpoIID and SpoIIP degradation activity at the septum allows the recruitment of SpoIIIAH and SpoIIQ to the sporulation septum (Rodrigues *et al.*, 2013). The amidase CwlD (see above, LytC family) is involved in the generation of muramic-δ-lactam, which is recognized by lytic enzymes that break down the spore cortex during germination (Atrih *et al.*, 1996; Popham *et al.*, 1996; Sekiguchi *et al.*, 1995). During maturation of the spore cortex, stem peptides of non-cross-linked muramic acid residues are generally cleaved to single L-Ala residues by the action of LytH, a proposed L,D-endopeptidase, which is homologous to lysostaphin (Horsburgh *et al.*, 2003a). Release of spores occurs through lysis of the mother cell, which involves LytC, CwlC and CwlH (Nugroho *et al.*, 1999; Smith and Foster, 1995). Spore germination requires the action of the partially redundant autolysins SleB and CwlJ (Chirakkal *et al.*, 2002).

Since autolysins can disrupt the integrity of the PG structure, and therefore are potentially lethal, their activity needs to be under tight control. Little is known about this aspect, but it has been suggested that the energy state of the cell, through the proton motive force (pmf), controls aulolysin activity. The cell wall of *B. subtilis* is protonated (and thus acidic) under respiring conditions (Calamita *et al.*, 2001) and dissipation of the pmf renders cells more sensitive to lysis (Jolliffe *et al.*, 1981, 1983; Kemper *et al.*, 1993). This suggests that when the acidity of the cell wall decreases, the activity of autolysins increases, resulting in cell lysis. Exciting new work based has provided evidence for activation of cell wall hydrolases by other cell wall binding or degrading proteins: in *E. coli*, cell wall

binding proteins EnvC and NlpD activate the amidases AmiA, -B, and -C at the cell division site and thus control cell separation (Uehara *et al.*, 2010), whereas in *B. subtilis* SpoIIP cleaves stem peptides and activates the lytic transglysolyase SpoIID to remove PG during prespore engulfment (Morlot *et al.*, 2010). The molecular details of these activation mechanisms are beginning to be elucidated with the help of crystal structures (Nocadello *et al.*, 2016).

Although turnover of cell wall material has been studied for some time in Gram-negatives, the observation of large amounts of PG fragments shed by Gram-positives combined with the notion that the thick cell wall is degraded on the outside and thus fragments are free to diffuse, has for a long time led people to think that Gram-positives do not recycle their cell wall fragments (Johnson *et al.*, 2013; Reith and Mayer, 2011). The Mayer group discovered a recycling pathway in *B. subtilis* that contains several genes that are homologous to Gram-negative recycling genes (Litzinger *et al.*, 2010). Muropeptides released by autolysins are further processed in the 'cell wall compartment' on the outside of the cell to GlcNAc and MurNAc by the activites of NagZ and AmiE, and transported into the cytoplasm by the phosphotransferase systems NagP and MurP. Then, in the cell, the recycling enzyme MurQ converst MurNAc-6P to GlcNAc-6P, which can be reused. The released peptide fragments are likely to be taken up by Oligopeptide transport systems (Johnson *et al.*, 2013; Reith and Mayer, 2011). Reusing peptidoglycan fragments makes sense, not only from an energetic point of view, but also in cases where, for example, pathogens or symbionts do not want to overstimulate the hosts' immune system by the release of large amounts of peptidoglycan fragments. In addition, peptidoglycan fragments are used by bacteria to sense damage to the cell walls and stimulate the expression of β-lactamases to counteract the activity of antibiotics (Johnson *et al.*, 2013).

Organization of cell wall synthesis in *Bacillus subtilis*

The use of fluorescence microscopy, AFM and ECT has allowed new studies on the insertion of material into the cell wall, the architecture of the cell wall, the visualization of cytoskeletal elements, as well as localization studies on the proteins involved in cell wall synthesis. *B. subtilis* has played a leading role in these studies: it was the first bacterium for which Errington and co-workers (i) identified an actin-like cytoskeleton (Jones *et al.*, 2001); (ii) obtained high resolution images of localized PG precursor insertion along the lateral wall (Daniel and Errington, 2003); and (iii) generated a comprehensive data-set on PBP localization (Scheffers *et al.*, 2004). Also, *B. subtilis* was one of the first bacteria for which the PG architecture was studied by AFM (Hayhurst *et al.*, 2008) and cryotomography (Beeby *et al.*, 2013).

Two modes of cell wall growth in rod-shaped bacteria

Cell wall synthesis in rod-shaped bacteria like *B. subtilis* is thought to occur in two modes: one associated with elongation of the cell, and one associated with cell division. This is in contrast to cell wall synthesis in spherical cocci, which takes place only at the division site, or in 'rugby-ball' shaped streptococci that synthesize the cell wall at the septum and the so-called 'equatorial rings' (Turner *et al.*, 2014). The concept of lateral wall growth vs. division wall growth originates from the observation that various mutations in genes associated with cell wall synthesis in *B. subtilis* and *E. coli* block either elongation of the cells (lateral growth) or cell division. An elongation block leads to cells that loose shape control and start to grow as spheres and eventually lyse. On the other hand, a division block leads to filamentation and eventually lysis. In a classic paper, Spratt described Class B transpeptidases from *E. coli* specific for elongation (PBP2) or for division (PBP3) (Spratt, 1975). Similarly, *B. subtilis* contains a division specific Class B PBP, PBP2b (Yanouri *et al.*, 1993), and two Class B PBPs, PBP2a and PbpH, involved in elongation (Wei *et al.*, 2003). PBP1 is a Class A PBP that shuttles between cell division and elongation (Claessen *et al.*, 2008). Defects in growth of the lateral wall are also observed in mutants of the Lipid II translocase RodA in both *E. coli* and *B. subtilis* (de Pedro *et al.*, 2001; Henriques *et al.*, 1998), whereas mutations in the division-specific Lipid II translocase FtsW result in filamentation (Boyle *et al.*, 1997). Cells with a deficiency in WTA synthesis grow as spheres,

underscoring the role of the anionic cell wall polymers in shape maintenance in Gram-positive cells (D'Elia *et al.*, 2006a; Pollack and Neuhaus, 1994; Pooley *et al.*, 1992).

Cell wall synthesis and turnover involve biochemical reactions that are catalysed by different enzymes. The observation that labelled PG precursors are incorporated close to the membrane in Gram-positives and are gradually displaced within the wall to the outside led Koch and Doyle to propose the 'inside-to-outside' model for cell wall growth (Koch and Doyle, 1985). In this model, new material is inserted close to the membrane where the PBPs are, and are displaced outward as a result of the incorporation of new material. On moving out, the PG is stretched and becomes stress bearing, but also more susceptible to the activity of autolysins. Continuous synthesis of PG close to the membrane prevents the cell wall from rupturing, according to what is called the 'make-before-break' principle (Koch, 2000). As an extension to this model the existence of multi-enzyme complexes involved in cell wall synthesis was proposed by Höltje, originally for *E. coli* (Höltje, 1996, 1998). The multi-enzyme complexes ensure the coordinated activity of incorporating new material into the wall without compromising cell wall integrity, which is extra critical in Gram-negative cells that contain only 1–3 layers of continuous PG (Höltje, 1998; Turner *et al.*, 2014; Typas *et al.*, 2012). These complexes would combine transglycosylase, transpeptidase and PG hydrolysis activities and could allow for controlled insertion of glycan strands into an existing PG meshwork while simultaneously removing old material.

The discovery of bacterial cytoskeletal proteins and their influence of cell shape (Margolin, 2009) strongly suggested that positioning and activity of the multi-enzyme complexes that synthesize the cell wall is controlled by the cytoskeleton (Fig. 10.5). FtsZ, the bacterial homologue of tubulin, positions all cell division proteins at midcell and is required for the correct synthesis of the division septum. The actin homologue MreB, of which *B. subtilis* contains three paralogues (MreB, Mbl and MreBH), is similarly responsible for positioning

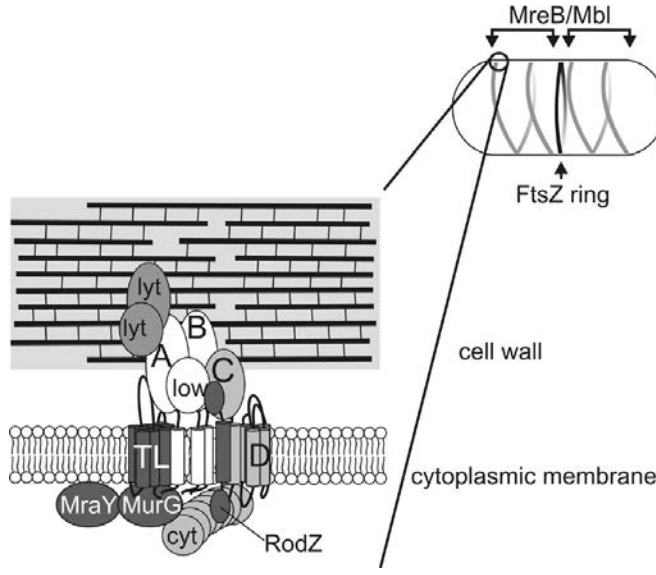

Figure 10.5 Putative multi-enzyme complexes functioning in cell wall synthesis in *Bacillus subtilis*. Depicted is a complex involved in growth of the lateral cell wall during elongation. The actin-like cytoskeletal protein MreB or Mbl (cyt) organizes the localization of MreC (C) and MreD (D, all light grey), possibly via the bridging protein RodZ (dark grey), and MurG (dark grey). MraY (also dark grey) interacts with MurG and together they are associated to a Lipid II translocase (TL, dark grey). PG synthesis requires at least a Class A HMW PBP, a Class B HMW PBP and a LMW carboxypeptidase (A, B, low, all in white). The Class B PBP (during *B. subtilis* elongation this could be either PBP2a or PbpH) is associated with MreC as has been reported for *C. crescentus*. Autolysins (lyt, grey) mediate cell wall turnover. During cell division, the cytoskeletal protein would be FtsZ that organizes other cell division proteins and cell division associated PBPs, such as PBP1 and PBP2b.

of the cell wall synthesis machinery during elongation. Localization and interaction studies in various bacteria have provided strong evidence for this hypothesis, even though it is not resolved whether both models are completely separate and whether simultaneous cell wall synthesis at both sites can occur.

Cell wall synthesis during division

Cell division starts with the assembly of the Z-ring, formed by polymers of the tubulin homologue FtsZ (Adams and Errington, 2009; Meier and Goley, 2014; Rowlett and Margolin, 2015). After assembly of the Z-ring, all other cell division proteins, including the division specific PBP2b localize to the division site and synthesis of the septum follows. In both *B. subtilis* and *E. coli*, the rate of PG synthesis during cell division is notably increased compared to synthesis during cell growth along the lateral wall (Cooper and Hsieh, 1988; Daniel and Errington, 2003; de Pedro *et al.*, 1997; Kuru *et al.*, 2012; Woldringh *et al.*, 1987). MurG, the last enzyme in the Lipid II synthesis pathway, localizes to the division site depending on FtsZ in both *E. coli* and *C. crescentus* (Aaron *et al.*, 2007; Mohammadi *et al.*, 2007). Several PBPs in *B. subtilis* show specific localization to the division site: PBPs 1, 2b and PbpX (Scheffers *et al.*, 2004). PBP2b is the cell division specific transpeptidase and the only essential PBP in *B. subtilis* – *pbpB* mutants form long filaments, the characteristic cell division mutant phenotype. PBP2b interacts with cell division DivIB as determined by a two-hybrid assay and a heterologous targeting assay (Daniel *et al.*, 2006; Robichon *et al.*, 2008; Rowland *et al.*, 2010). A PbpX-deficient strain has no observable phenotype (Scheffers, 2005). A role for PBP1 in cell division had been postulated on the basis of the phenotype of a PBP1 deficient strain, which grows at a reduced rate, has slightly elongated cells, less efficient spore formation (Popham and Setlow, 1996) as well as abnormal septal structures (Pedersen *et al.*, 1999). The sporulation deficiency in the PBP1 mutant is the cause of a defect in the formation of the asymmetrical sporulation septum. Also, the localization of GFP-PBP1 depends on the presence of other membrane-associated cell division proteins (all membrane bound cell division proteins in *B. subtilis* are interdependent in terms of localization, see Errington *et al.*, 2003), more specifically EzrA (Claessen *et al.*, 2008). Recently, it has become evident that PBP1 is also involved in lateral wall growth and shuttles its localization from the division site to the lateral wall, where it is bound by MreB (Kawai *et al.*, 2009b), through the interaction with GpsB (Claessen *et al.*, 2008). GpsB is a ~10 kDa membrane associated protein, conserved in Gram-positives. Furthermore, there is evidence that phosphorylation might be involved in the regulation of PBP1, GpsB and cell wall synthesis. PrkC belongs to a family of serine/threonine kinases that have been associated with regulation of cell wall synthesis and cell division (Manuse *et al.*, 2016). In *Bacillus*, PrkC interacts with GpsB, DivIVA and Ezra; however, only GpsB interacts with the active site of PrkC. PrkC and GpsB interact to control each other: on the one hand, PrkC phosphorylates GpsB at Thr75, while on the other hand PkrC auto-phosphorylation is stimulated by unphosphrylated GpsB, and inhibited by phosphorylated GpsB (Pompeo *et al.*, 2015). The link between Gpsb phosphorylation and PBP1 is not clear – both proteins interact *in vitro*, but the *in vitro* glycosyltransferase and transpeptidase activity of PBP1 are not affected by either GpsB or a phosphomimetic GpsBT57D/E mutant (Cleverley *et al.*, 2016; Rismondo *et al.*, 2016), suggesting that GpsB does not influence the enzymatic activity of PBP1. A possibility is that phosphorylation might be related with the shuttling of PBP1 from the division site to the lateral wall. Interestingly, another protein phosphorylated by PrkC has proven to have an effect in PBP1 localization. YvcK is a protein essential for growth under gluconeogenic condition (Gorke *et al.*, 2005). PrkC phosphorylates YvcK at Thr304 and PrpC dephosphorylates YvcK. In the absence of MreB, overproduction of YvcK restored PBP1 localization and the MreB phenotype. Furthermore, only the overproduction of a phosphomimetic version of Yvck (T304E) in the absence of MreB rescued the characteristic bulging phenotype, while the phosphoablative version (T304A) did not (Foulquier *et al.*, 2014).

It could very well be that PBP1 and PBP2b function together to make the septal wall but evidence for a specific interaction between these PBPs is lacking. The equivalent PBPs in *E. coli* (PBP1b and 3) have been shown to interact in two-hybrid and pull-down studies (Bertsche *et al.*, 2006), and FtsW, FtsN and the monofunctional glycosysltransferase

MgtA are part of this complex as well (Derouaux *et al.*, 2008; Muller *et al.*, 2007). A Native PAGE analysis of *E. coli* membranes also revealed a complex containing a number of cell division proteins including FtsZ, -Q, -L, -B, and -N, but no PBPs (Trip and Scheffers, 2015). A recent pull-down in *B. subtilis* with a Lipid II analogue containing a photocross-linker identified PBPs 1, 3, 4, 5 and X bound to Lipid II next to several other known cell wall synthesis and division proteins (EzrA, LytE, MurG, FtsX among others) (Sarkar *et al.*, 2016).

The housekeeping LTA synthase $LtaS_{BS}$ also localizes to the division site and *ltaS* mutants have a reduced diameter and aberrantly formed septa, suggesting that LTA may be specifically synthesized and localized at the cell division site (Schirner *et al.*, 2009).

The material that is synthesized at the division septum will form one of the two cell poles of each daughter cell after separation. These poles are called the 'new' poles, as opposed to the 'old' poles that are the poles from the mother cell. In *E. coli*, the cell wall at the poles is 'inert', i.e. no new material is inserted at these sites, and polar PG is not subject to degradation/turnover, in contrast to material in the lateral wall (Burman *et al.*, 1983; de Pedro *et al.*, 1997). Cell wall turnover at the poles in *B. subtilis* is very slow, but insertion of new material at the poles does occur (Graham and Beveridge, 1994; Mobley *et al.*, 1984). Whether this difference between *E. coli* and *B. subtilis* with respect to the insertion of material at the poles is a true difference between these organisms or the result of different experimental approaches is unclear. The cause for the (virtual) absence of cell wall turnover at the cell poles is not known but may be caused by a difference in structural PG composition at the pole compared to the lateral wall or the presence/absence of enzymes involved in PG turnover at the poles. The poles of rod-shaped cells constitute an area of restricted mobility for periplasmic proteins (Foley *et al.*, 1989) and for outer membrane proteins (de Pedro *et al.*, 2004; de Pedro *et al.*, 2003). Strikingly, an *E. coli* mutant for the major D,D-carboxypeptidase PBP5 displays a branching phenotype. The branches originate from areas of inert PG in the lateral wall that are thought to act as *de novo* poles from which the branches originate (de Pedro *et al.*, 2003; Gullbrand *et al.*, 1999).

Cell wall synthesis during elongation

Initial studies on the incorporation and turnover of cell wall material at the lateral wall used labelled PG precursors (mostly GlcNAc). Old wall material was found to co-segregate with DNA and new material was inserted in patches (Pooley *et al.*, 1978; Schlaeppi and Karamata, 1982; Schlaeppi *et al.*, 1985). Cell wall material is turned over rapidly at the cell cylinder but is retained at the cell poles, so that polar material is conserved for several generations, although some insertion of new material does occur at the poles (Mobley *et al.*, 1984). A study using phage SP50 as a marker for teichoic acid synthesis (SP50 binds specifically to TA containing bacteria) showed that TA becomes exposed at the lateral wall first and then migrates towards the poles, but that new material is incorporated along the entire wall, and thus also at the cell poles (Clarke-Sturman *et al.*, 1989). Teichoic acid is only accessible from the outside at discrete places along the cylinder, at division septa or at the junction between the cell pole and the cylinder, possibly indicating areas of high hydrolytic activity (Graham and Beveridge, 1994).

Daniel and Errington developed a new method to visualize cell wall synthesis in *B. subtilis* using a fluorescent derivative of vancomycin (Van-FL; see Fig. 10.4A and Daniel and Errington, 2003). The antibiotic vancomycin binds to the terminal D-Ala-D-Ala of PG precursors and thus blocks transpeptidation. In control experiments it was shown showed that Van-FL binds to externalized but unincorporated lipid-linked PG precursors and to the recently inserted lipid-linked subunit at the growing end of a glycan strand, and can therefore be used as a marker for nascent PG synthesis. Removal of the terminal D-Ala by the action of transpeptidases or carboxypeptidases precludes Van-FL binding, therefore older PG will not be labelled (Daniel and Errington, 2003). Van-FL does not penetrate the outer membrane of Gram-negative cells and can therefore only be used to label Gram-positives. Van-FL staining of growing *B. subtilis* cells showed prominent staining at an area around the division site, corresponding to areas of high PG synthesis activity observed with radio-labelled PG precursors. No staining was observed at the cell poles, showing that cell wall turnover at the poles is absent or very low. Although less bright, the staining pattern at the lateral wall could be resolved to follow a helical pattern. The helical pattern of PG

insertion was confirmed in an independent study by the Walker and Rudner groups, who developed fluorescent derivatives of vancomycin and ramoplanin, which binds to the reducing end of a nascent glycan chain as well as to Lipid II (Tiyanont *et al.*, 2006). The use of Van-FL in other Gram-positives revealed polar growth in hyphal growth of *Streptomyces coelicolor*. *Corynebacterium glutamicum*, which grows in a rod-like shape but lacks a homologue of MreB, grows both at the division site and at the poles (Daniel and Errington, 2003). These results illustrate the different growth modes of rod-shaped organisms that either contain or lack MreB-like proteins (see Carballido-López, 2006).

More recently, the ability of bacteria to incorporate D-amino acids different than D-ala into the stem peptide, has been used by several groups to developed fluorescent (FDAAs) and clickable analogues of D-amino acids (Kuru *et al.*, 2012; Kuru *et al.*, 2015; Shieh *et al.*, 2014; Siegrist *et al.*, 2013, 2015;) that can be visualized in real time by microscopy or further analysed by HPLC and mass spectrometry. In contrast to Van-FL labelling, FDAAs are incorporated into the stem peptide and can be used in Gram negatives without the need to permeabilize the outer membrane.

Interestingly, FDAAs are incorporated only at the fifth position in the stem peptide of *Bacillus*, while in other bacteria, such as *E. coli* they are incorporated at position 4 (Kuru *et al.*, 2012). The difference of the position at which FDAAs are incorporated into the stem peptides reflects the mechanism of how D-amino acids are incorporated into the cell wall. It has been reported that *E. coli* and *P. aeuruginosa* incorporate D-amino acids in the fourth position via an amino acid exchange reaction by L,D-transpeptidases (Ldts). In contrast, *B. subtilis*, *E. faecalis* and *S. aureus* incorporate D-amino acids in position 5. Incorporation in fifth position is performed by D-alanyl- D-alanine ligase (Ddl) which is part of Lipid II synthesis. Interestingly, when Ddl was inhibited with D-cycloserine in *B. subtilis* no effect was observed in the incorporation of some non-canonical D-amino acids suggesting a different mechanism (Cava *et al.*, 2011) for amino acids that are normally not substrates for Ddl.

B. subtilis has the D-carboxypeptidase PBP5 that removes the last D-ala of the stem peptide (Atrih *et al.*, 1999), and PBP5 also removes FDAAs that are incorporated at the fifth position reducing overall labelling (Kuru *et al.*, 2012). Recently, Fura and collaborators (Fura *et al.*, 2015) tested the ability of all *Bacillus* PBPs with transpeptidation activity to incorporate fluorescently labelled D-lysine analogues into the cell wall. Most of the PBPs showed a minor effect in labelling, however a PBP4 knockout had an significant decrease in labelling. Interestingly, signal at the division site was observed in all strains, which could indicate that FDAAs at the septum are incorporated via the Lipid II pathway rather than transpeptidation.

The helical patterns for PG insertion closely matched the helices formed by the bacterial actin homologue MreB. MreB proteins are discussed in detail in Chapter 8. Here, it is briefly summarized why the proteins are important for cell wall synthesis. *B. subtilis* contains three paralogues of MreB: *mreB*, which forms an operon with *mreC* and *mreD*, *mbl* and *mreBH*, and all three paralogues have a role in ensuring correct cell shape as mutants lose their rod shape (Abhayawardhane and Stewart, 1995; Defeu Soufo and Graumann, 2003; Jones *et al.*, 2001; Levin *et al.*, 1992; Varley and Stewart, 1992). The importance of *mreB* is underscored by the fact that it is essential in many bacteria, like *B. subtilis* (Formstone and Errington, 2005), *E. coli* (Kruse *et al.*, 2005), *C. crescentus* (Figge *et al.*, 2004), or *Rhodobacter sphaeroides* (Slovak *et al.*, 2005). Also, expression of *B. subtilis mreB* in *E. coli* leads to an altered cell morphology (Defeu Soufo and Graumann, 2010). Similarly, both *mreC* and *mreD* are essential in *B. subtilis* and in *E. coli*, and depletion of each results in spherical cells (Kruse *et al.*, 2005; Leaver and Errington, 2005; Lee and Stewart, 2003).

All MreB paralogues are part of a helical structures in *B. subtilis*, which undergo continuous assembly/disassembly, most likely through treadmilling (Carballido-López and Errington, 2003; Defeu Soufo and Graumann, 2004; Kim *et al.*, 2006). All three paralogues interact as shown by bimolecular fluorescence complementation (BiFC) and cross-linking although they are not always colocalized (Defeu Soufo and Graumann, 2006; Kawai *et al.*, 2009a). Single deletions of MreB paralogues are tolerated although in the case of *mreB* the medium has to be supplemented with high Mg^{2+} (Formstone and Errington, 2005), and the insertion of PG precursors along the lateral wall is not affected in single deletions (Kawai *et al.*,

2009a). Double mutants, except the *mbl mreBH* combination, are not viable (Defeu Soufo and Graumann, 2006), although a triple mutant can be constructed in the absence of the anti-sigma factor of σI (Schirner and Errington, 2009). When *mreB* is depleted in a *mbl* knockout background, cells show a strong growth defect and lose PG precursor insertion along the lateral wall, indicating that *mreB* and *mbl* have redundant functions and that either one is required for correct cell wall synthesis during elongation (Kawai *et al.*, 2009a). The role of MreBH in cell shape organization is slightly different. MreBH positions LytE, one of the cell wall hydrolases, in a helical pattern, which suggests that MreBH is involved in cell wall turnover rather than synthesis (Carballido-López *et al.*, 2006).

The obvious relation between MreB/Mbl and cell wall synthesis suggested that MreB/Mbl control cell wall synthesis during elongation by forming multi-enzyme complexes with cell wall synthesis proteins. MreB is a cytoplasmic protein, whereas PBPs have at best very short cytoplasmic tails, so the question was how connections between these proteins are made. MreC and MreD are membrane proteins that also form helical patterns and that are responsible for cell shape and PG-precursor insertion along the lateral wall, so they were obvious candidates to connect the MreB scaffold to cell wall synthesis proteins (Fig. 10.5; e.g. Daniel and Errington, 2003; Kruse *et al.*, 2005; Leaver and Errington, 2005). Other components of this multi-enzyme complex at the lateral wall would be a PG precursor translocase and several PBPs (such as the elongation specific PBPs-2a and -H) required for incorporation of PG precursors into glycan strands, as well as proteins involved in (lipo)teichoic acid synthesis. Very few PBPs actually localized in a helical pattern, nor was localization of PBPs affected in *mreB* or *mbl* mutants (Scheffers *et al.*, 2004), but increased resolution has shown that e.g. PBP1 does localize in a helical pattern (Claessen *et al.*, 2008) in a MreB dependent fashion (Kawai *et al.*, 2009b) and the redundancy of *mreB* and *mbl* explains why localization patterns were no affected in the absence of either MreB or Mbl.

Evidence for the existence of multi-enzyme complexes came from two-hybrid studies and pull-down experiments. MreC is a dimeric protein with a single TM and a large periplasmic domain (van den Ent *et al.*, 2006). Two-hybrid studies revealed that MreC interacts with all Class A and B PBPs (van den Ent *et al.*, 2006) and with proteins involved in WTA synthesis, that also localize in a helical pattern (Formstone *et al.*, 2008). Similarly, MreB was found to interact with several HMW PBPs in two-hybrid studies (Kawai *et al.*, 2009b). In a pull-down experiment using his-tagged MreB as bait, MreC and various fluorescently labelled PBPs (1, 2A, 2C and 4) could be copurified with MreB (Kawai *et al.*, 2009b), and labelled PBPs (1, 2a and 4) were also copurified with his-tagged Mbl (Kawai *et al.*, 2009b). This does not exclude the presence of other PBPs, as some PBPs are more abundant and/or more efficiently labelled in cell extracts – therefore several PBPs present in the complex may be missed in these experiments. Also, the pull-down experiments do not show that there is a direct interaction between PBPs and MreB/Mbl – there may be a bridging function for MreC. However, the two-hybrid and localization data do suggest that at least PBP1 and MreB interact directly (Kawai *et al.*, 2009b). Recently, another conserved protein that interacts directly with MreB was simultaneously discovered in *B. subtilis*, *E. coli* and *C. crescentus* (Alyahya *et al.*, 2009; Bendezu *et al.*, 2009; Shiomi *et al.*, 2008). RodZ is a bitopic membrane protein with a cytoplasmic domain that adopts a helix–turn–helix structure that interacts with MreB (van den Ent *et al.*, 2010). RodZ and MreB localization is interdependent and inactivation of RodZ leads to shape defects. The bitopic structure of RodZ, with a cytoplasmic MreB binding domain and a large periplasmic/extracellular domain, make it another candidate for communicating structural information from MreB to a cell wall synthesis machinery, although the mislocalization of MreB in the absence of RodZ suggests that this communication is not a one-way process. More recently, it has been shown that MreB is important for RodZ stability and localization. Pull-down and Bacterial two-hybrid studies revealed interactions of RodZ with MreB, MreBH and Mbl, and with MreD. Surprisingly, a strain expressing only the cytosolic part of RodZ is rod shaped only when grown in the absence of Mg^{2+} and not with Mg^{2+}, which normally stabilizes rod-shape in cells with defective cell wall synthesis (Muchova *et al.*, 2013).

More evidence for the possible existence of a complex comprising MreBCD and PBPs comes from studies in other organisms. *E. coli* MreC

interacts with both MreB and MreD (Kruse *et al.*, 2005). Immunoprecipitation of MraY showed interactions with MurG and MreB (Mohammadi *et al.*, 2007). Earlier affinity studies using *E. coli* proteins involved in cell wall synthesis identified protein–protein interactions between bifunctional transpeptidase-transglycosylases PBPs, monofunctional transpeptidases, lytic transglycosylases, DD-endopeptidases and structural proteins (Romeis and Holtje, 1994; Vollmer *et al.*, 1999; von Rechenberg *et al.*, 1996). In *C. crescentus*, MreB localizes in a helical pattern that condenses into a ring at the cell division site, potentially to aid PG synthesis at midcell (Aaron *et al.*, 2007; Figge *et al.*, 2004; Gitai *et al.*, 2004). PBP2 as well as several other PBPs were retained by MreC coupled to Sepharose, again suggesting a direct role for MreC in PBP localization (Divakaruni *et al.*, 2005). Recently, an extensive two-hybrid analysis combined with localization studies showed that the cell wall synthetic complex contains MreB, MreD, MraY, MurB, MurC, MurE, MurF, MurG, RodA and RodZ (White *et al.*, 2010). MreB and MreD localize interdependently in *C. crescentus* (White *et al.*, 2010), whereas MreC and MreB localize independently (Divakaruni *et al.*, 2005). Combined, these data make a strong case for a function of MreD in the localization of (some) elongation-specific PBPs, and for MreB playing a scaffolding role. Also, MreB does not only play a role in cell shape determination, but also in other processes like chromosome segregation (for an overview see Gitai *et al.*, 2005), which complicates the interpretation of mutant phenotypes.

Does MreB position PBPs directly? MreB dynamics require active PG synthesis in *B. subtilis* and *E. coli* (Dominguez-Escobar *et al.*, 2011; Garner *et al.*, 2011; van Teeffelen *et al.*, 2011). In *B. subtilis*, PBP2A and PbpH, the PBPs essential for elongation in *B. subtilis* are drivers for MreB dynamics (Dominguez-Escobar *et al.*, 2011; Garner *et al.*, 2011). These observations led to the suggestion that MreB(-like) polymers function by restricting the diffusion of PG synthesis complexes within the membrane rather than by actively positioning PBPs along a scaffold (Dominguez-Escobar *et al.*, 2011; Garner *et al.*, 2011). The MreB scaffold model was recently tested by analysis of the localization of PBP2A and PbpH in cells were MreB was delocalized by membrane depolarization, or Lipid II was delocalized by addition of the Lipid II clustering peptide nisin. The outcome of this test was that both PBPs, that are essential for MreB dynamics, localize to Lipid II, and do not delocalize when MreB is displaced (Lages *et al.*, 2013), which supports the notion that MreB does not actively position PBPs.

Cell wall synthesis during sporulation

At the onset of sporulation, the two copies of the chromosome are deposited in a DNA structure that stretches the length of the cell, called the axial filament, and two polar division sites are formed, one of which develops into the sporulation septum. Asymmetrical division triggers a developmental programme that is governed by mother cell and prespore specific transcription factors (see Chapter 11).

At the start of sporulation, a PBP of unknown function, PbpX, spirals out from the medial division site to both asymmetrical division sites during the switch from medial to asymmetrical cell division at the start of sporulation (Fig. 10.4E; Scheffers, 2005). This spiralling resembles patterns described for FtsZ, FtsA and EzrA (Ben-Yehuda and Losick, 2002), and PbpX localizes to both asymmetrical division sites, whereas the division specific PBPs 2B and 1 only localize to the one site that has committed to form the sporulation septum (Scheffers, 2005). The function of PbpX is unclear, as a *pbpX* deletion strain does not show cell division or shape defects in vegetative growth or in sporulation (Scheffers, 2005).

PBP-1 and -2b, which are both required for efficient formation of the asymmetrical division septum once the polar division site has been selected, localize to the polar division site (Daniel *et al.*, 2000; Scheffers *et al.*, 2004). Once the polar septum has been formed a process called engulfment starts, where the mother cell membrane migrates around the prespore and fuses at the cell pole to enclose the prespore within the mother cell. There is a special role for cell wall turnover and synthesis during this process. At the start of engulfment, the proteins SpoIIM, SpoIID and SpoIIP assemble into a complex, anchored in the mother cell membrane, at the middle of the septum (Abanes-De Mello *et al.*, 2002). After this localization, degradation of cell wall material at the septum is initiated to allow membrane movement and engulfment. SpoIIM is a membrane protein of unknown (scaffolding) function (Abanes-De Mello

et al., 2002; Chastanet and Losick, 2007). SpoIIP is an amidase and endopeptidase that removes cross-links and stempeptides from the septal PG. This allows SpoIID to become active. SpoIID is a lytic transglycosylase that acts on glycan strands without associated peptides. So SpoIID needs SpoIIP to become active, but in a unknown fashion, SpoIID also stimulates SpoIIP activity. The result of these combined activities, which were beautifully demonstrated by Rudner and colleagues (Morlot *et al.*, 2010), is a PG-degrading complex that uses cell wall degradation to drive membrane migration around the prespore. However, not only PG degradation is required for membrane migration during engulfment. Ramoplanin-FL staining of sites of PG synthesis during engulfment showed that there is active PG synthesis at the leading edge of the engulfing membrane. Moreover, membrane migration is blocked when PG synthesis is blocked (Fay *et al.*, 2010). This PG synthesis required the activity of SpoVD, a TPase that is essential for cortex synthesis (Daniel *et al.*, 1994) and that localizes to the outer prespore membrane dependent on its putative cognate Lipid II translocase SpoVE (Fay *et al.*, 2010). This leads to a model where degradation of PG that surrounds the entire cell functions as a track for membrane migration, which in turn is aided by the synthesis of new PG (the primordial cell wall) between the engulfing membrane (that will become the outer prespore membrane) and the inner prespore membrane (Meyer *et al.*, 2010).

Once engulfment is complete, the spore cortex is synthesized between the outer and inner prespore membranes. This process requires SpoVD (above). Early studies on PBP profiles of sporulating *B. subtilis* indicated that PBPs 3, 4* and 5* are involved in sporulation (Sowell and Buchanan, 1983; Todd *et al.*, 1983). Transcriptional profiling and studies on mutant strains have now identified several other PBPs involved in sporulation. Two Class A PBPs, 2c and 2d play redundant roles in sporulation (McPherson *et al.*, 2001). PBPs 2c and 2d are under control of forespore specific transcription factors, with PBP2d being activated by both σ^F (early factor) and σ^G (late factor) and PBP2c being activated by σ^G. PBP2c is also present in the mother cell as it is also under the control of the vegetative transcription factor σ^A (McPherson *et al.*, 2001; Popham and Setlow, 1993b). Interestingly, a GFP-fusion to PBP2c is stable in the mothercell, whereas a GFP-fusion to PBP2d is not (Scheffers, 2005). Both GFP-PBP2c and 2d redistribute from the peripheral wall to the sporulation septum when expressed under control of P_{xyl} (Fig. 10.4D). From the septum, these proteins follow the mother cell membrane during engulfment (see Chapter 11) and finally localize to the outer prespore membrane. This localization pattern was also observed when either GFP-PBP2c or 2d was expressed in the absence of PBP2d or 2c, respectively, indicating that these proteins do not depend on each other for their localization (Scheffers, 2005). Even though PBP2c is not essential, it would be interesting to see whether it also aides in the membrane migration during engulfment, as SpoVD can only catalyse TP reactions. Other PBPs that have been found to act during sporulation, such as PBP4b (Eichenberger *et al.*, 2003; Wei *et al.*, 2004), PBP3 and PBP4* (above) did not show a sporulation-associated change in their localization patterns. The carboxypeptidases PBP5* and DacF, that regulate the degree of cross-linking of the spore PG (Popham *et al.*, 1995, 1999; 1995), have not been characterized in terms of their localization.

Life without a wall – conundrums and open questions

As we have seen, the shape of a *Bacillus* cell is tightly regulated and the cell wall is normally considered to be a semi-rigid structure. A striking example of adaptation of bacterial growth and division in the absence of a cell wall was given by Leaver *et al.* who were able to reproducible produce L-forms (Leaver *et al.*, 2009). L-forms are bacteria that do not have a cell wall. L-forms are generated by prolonged growth of bacteria under conditions were PG synthesis is blocked – however resulting L-forms are very often unstable and whether or not they were completely devoid of PG has been a matter of discussion (Allan, 1991; Joseleau-Petit *et al.*, 2007). Leaver *et al.* isolated stable L-forms by growing a strain in which the *murE* operon was shut down on a osmotically protected, high Mg^{2+} medium that contained high concentrations of penicillin. Interestingly, the stable L-forms obtained contained a point mutation in the active site of *ispA*, a protein involved in the isoprenoid synthesis pathway which leads to the formation of several essential lipids

including bactoprenol that is required for Lipid II and TA precursor synthesis (Leaver *et al.*, 2009). Next to *ispA*, mutations in *walR* or *sepF* also facilitate the transition from rod to L-forms (Dominguez-Cuevas *et al.*, 2012). L-forms are spherical and have a much larger diameter than normal rod-shaped cells and propagate without FtsZ (Leaver *et al.*, 2009). Division of L-forms is dependent on membrane composition and fluidity, as L-forms deficient in branched-chain membrane synthesis are not able to perform scission and separate (Mercier *et al.*, 2012). Overproduction of fatty acids which generates an excess of membrane facilitates L-form division by increasing the cell surface to volume ratio, leading to cell shape deformation and division of the cell (Mercier *et al.*, 2013). A similar proliferation mechanism, based on simple biophysical principles, has been reported in L-forms of *S. aureus* and *E. coli*, t suggesting that this proliferation mechanism could also have been used by primitive cells (Mercier *et al.*, 2014). As expected, *B. subtilis* L-forms are resistant to antibiotics that inhibit the bactoprenol cycle or peptidoglycan synthesis, but hypersensitive to antibiotics that impact on membrane stability (Tol *et al.*, 2015; Wolf *et al.*, 2012).

Concluding remarks

The study of cell wall synthesis has received a tremendous boost in the last few years because of the development of fluorescent microscopy techniques that are applicable to bacteria, and the discovery of an actin-like cytoskeleton that plays a role in cell shape determination. *Bacillus subtilis* has been at the forefront of these developments, and will probably continue to be since it is one of the most extensively characterized bacteria in terms of mutant studies, the availability of localization data, and the ease with which new reporter strains and deletions can be generated. From the work in *B. subtilis* and other bacteria we now know that the cytoskeletal proteins FtsZ and MreB play a role the in organization of the cell wall by organizing PBPs and other proteins into multi-enzyme complexes to ensure correct PG synthesis. Few data exist on the exact composition of these complexes and elucidation of the architecture of these complexes, and in fact of the architecture of the cell wall itself is one of the main challenges for the future.

Acknowledgements

We would like to thank Valério Matias, Richard Daniel, Simon Foster, David Popham, Morgan Beeby and the late Terry Beveridge for the supply of, or the permission to adapt, figures. We would like to apologize to authors whose original work has not been cited due to space limitations. This work was supported by a VIDI grant (864.09.010) from the Netherlands Organization for Scientific Research (NWO).

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