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Recent progress in our understanding of peptidoglycan assembly in Firmicutes

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Abstract

Years of intense research have shown that the assembly of peptidoglycan, the extracellular mesh-like polymer surrounding the bacterial cell, is incredibly complex. It requires a suite of reactions catalyzed by dynamic macromolecular protein complexes whose localization and activity should be finely regulated in space and time. In this review, we focus on the main developments reported over the last 5 years for the assembly of peptidoglycan in Firmicutes, a bacterial phylum that comprises monoderm bacteria and that encompasses well studied bacterial models with different cell shapes and lifestyles.

Introduction

The bacterial cell wall consists mainly of a giant biopolymer named peptidoglycan. The latter surrounds the whole cell and is commonly described as a mesh-like structure consisting of glycan strands that are cross-linked by short peptides [1] (Figure 1). The assembly of this cage-like structure starts at the inner face of the cytoplasmic membrane by a series of reactions involving a set of well conserved proteins generating a lipid-linked disaccharide-pentapeptide precursor named lipid II. This precursor is then flipped across the membrane, polymerized and inserted into the existing peptidoglycan mesh. Polymerization involves both transglycosylation reactions to elongate the glycan strands and transpeptidation reactions that cross-link the pentapeptides of two adjacent glycan strands [1]. These reactions are performed by either the penicillin-binding proteins (PBPs), the L,D-transpeptidases (Ldt), and the SEDS (Shape, Elongation, Division and Sporulation) proteins. The peptidoglycan polymer is also continuously remodeled by a variety of hydrolases, cleaving either the glycan strand, the link between the sugar moieties and the peptide or inside the peptide itself, to promote the insertion of new precursors or to simply mediate cell separation after division [2] (Figure 2).

This general view of peptidoglycan synthesis derives mostly from studies on model organisms such as *Escherichia coli* or *Bacillus subtilis*, but does not reflect the great diversity encountered in bacteria with different morphology or physiology. The most prominent sources of variability are the composition of the peptide, the mode of pentapeptides cross-linking or the sites of peptidoglycan synthesis (polar, septal or lateral) [3] (Figure 1). In this review, we highlight some of the recent breakthroughs that have improved, and even changed, our understanding of some key aspects of peptidoglycan assembly in monoderm bacteria of the Firmicute phylum. Firmicutes are a well-studied group of Gram-positive bacteria and a number of important new discoveries regarding peptidoglycan synthesis have been published

during the last five years. To get more information about other bacterial models and the assembly of other compounds of the cell wall, we suggest the following reviews [4-6]. Furthermore, recent developments allowing the high through peptidoglycan analysis as well as advances in microscopy techniques allowing the investigation of spatial and temporal changes in peptidoglycan synthesis will be covered in this issue in a review by Sara B. Hernández and Felipe Cava [7].

Lipid II transport, polymerization and cross-linking

Once assembled at the inner face of the cytoplasmic membrane by the Mur proteins [1], the lipid II precursor must be translocated across the membrane in order to be incorporated in the peptidoglycan mesh. For many years, it was thought that the translocation step was carried out by the two SEDS proteins, FtsW and RodA, whereas polymerization and cross-linking were performed by two classes of PBPs : the bi-functional class A PBPs that display both a transpeptidase and a transglycosylase activity (aPBPs) and the mono-functional class B PBPs that display only a transpeptidase activity (bPBPs) [1]. This model has however been considerably modified based on the results of several seminal studies, revealing that MurJ mediates the translocation of lipid II [8] and that the two SEDS proteins, FtsW and RodA, possess an unexpected glycosyltransferase activity [9] [10] (Figure 1). Subsequently, it has been shown that the interaction of SEDS proteins with bPBPs was required to stimulate their respective activity [9]. Although initially demonstrated for *B. subtilis*, a series of evidence support that the same model is true for other Firmicutes such as *Staphylococcus aureus* in which the RodA/bPBP3 and FtsW/bPBP1 pairs mediate the sidewall and septal assembly of the peptidoglycan framework, respectively [11], *Streptococcus pneumoniae* in which FtsW works with bPBP2x in septal peptidoglycan assembly [12] or *Listeria monocytogenes* in which RodA and FtsW might work with the bPBP B1 and B2, respectively

[13]. Interestingly, *L. monocytogenes* possesses two FtsW and three RodA homologs that can compensate each other, at least partially, and whose expression levels are modulated upon treatment with antibiotics [13]. In addition to MurJ, a second unrelated protein, Amj, was shown to also flip the lipid II across the membrane in *B. subtilis* [14]. Amj forms a synthetic lethal pair with MurJ and its expression can be induced by cell wall stress. Together, these observations suggest that multiple SEDS and translocases might have redundant and/or specific role to support peptidoglycan synthesis during different environmental conditions/stresses.

As bPBPs work in concert with SEDS proteins to build the peptidoglycan framework, the function of aPBPs had to be revised. A first study has proposed that aPBPs could work outside and independently of the SEDS/bPBP complexes [15]. This view was recently strengthened in *S. pneumoniae* in which evidence was provided that aPBPs are required for the repair and/or maturation of the primary peptidoglycan framework independently of the SEDS/bPBP pairs FtsW/bPBP2x and RodA/bPBP2b [16]. A similar function is also proposed for aPBPs in diderm bacteria like *E. coli* [17]. Altogether, those recent findings support a new model with a division of labor between the SEDS/bPBPs and the aPBPs: while the SEDS/bPBPs complexes are essential for the synthesis of the nascent peptidoglycan, aPBPs would have complementary functions in the maintenance and maturation of the existing framework (Figure 1). The wide diversity in the number of aPBPs with an apparent redundancy among the Firmicutes suggests the existence of a whole repertoire of ancillary functions for aPBPs that have yet to be determined. Understanding how aPBPs operate and how their activity is regulated will be discussed in more detail in the same issue in a review by Manuel Pazos and Waldemar Vollmer [18], and will also be an exciting area of research for the coming years.

Modes of peptidoglycan synthesis

To maintain cell-wall homeostasis, the insertion of new material into the existing peptidoglycan mesh must be regulated in space and time. Different modes of peptidoglycan assembly (polar, lateral, septal) have been identified and characterized by the association between a scaffolding protein and a multiprotein complex responsible for the synthesis and the insertion of new material. In *B. subtilis*, a multiprotein complex called the elongasome which is scaffolded by the actin-like MreB protein, allows lateral insertion of peptidoglycan for cell elongation whereas another complex called the divisome which is scaffolded by the tubulin-like protein FtsZ, assembles the septal peptidoglycan [19]. The contribution of the scaffolding proteins MreB and FtsZ in the peptidoglycan synthesis is still actively debated, but over the last few years numerous insights have considerably improved our understanding of their dynamic and their function during the lateral and septal modes of peptidoglycan synthesis.

Initially thought to form a helical filament distributed along the length of cell, it is now clear that MreB forms small assemblies that move around the rod circumference and promote peptidoglycan synthesis in the radial direction in *B. subtilis* and *E. coli*. Thanks to the emergence of new labelling methods for nascent peptidoglycan and the use of super-resolution imaging, it has also become clear that this circumferential rotation is powered by the active peptidoglycan assembly (reviewed in [20]). While the molecular details regarding the organization of these MreB assemblies are still debated [21], it has become evident that the density of rotating MreB assemblies relies on RodA/bPBP2a but not on the activity of aPBPs [22]. In addition, there is growing evidence that the orientation and/or the motion of MreB filaments depend on the membrane curvature and fluidity, and very interestingly, on the nutrient availability and the growth rate suggesting an intimate link between the cell elongation and the central metabolism [23] [24] [25]. Resource sharing between central

metabolism and cell envelope synthesis will be covered in more detail in this issue in a review by Ankita J. Sachla and John D. Helmann [26].

FtsZ was initially thought to form a continuous structure named the Z-ring at the division septa. However, the advent of super-resolution imaging has also drastically changed our conception of the organization of the Z-ring and revealed that FtsZ forms discontinuous patches/filaments that also move circumferentially around the cell division site [27, 28]. Unlike MreB, the apparent circumferential movement of FtsZ was shown to be independent of cell wall synthesis and rather due to a treadmilling mechanism in which one end of the polymer grows and the other shrinks. While FtsZ treadmilling could guide the progressive insertion of nascent peptidoglycan during cell division, the functional link between FtsZ and peptidoglycan assembly remains more enigmatic and seems to be strain-specific. For instance, it has been shown that the treadmilling of FtsZ filaments drive the motion of bPBP2b in *B. subtilis* whereas the rotation of the bPBP2x:FtsW complex depends on peptidoglycan synthesis and not on FtsZ treadmilling in *S. pneumoniae* [12, 27]. In *S. aureus*, FtsZ treadmilling is proposed to serve for the initial invagination of the membrane while peptidoglycan synthesis occurs at the cell periphery. Upon recruitment of the protein complex DivIB/DivIC/FtsL and the protein MurJ, peptidoglycan assembly is reoriented at the division site and would provide the constriction force independently of FtsZ treadmilling [29]. Further studies will likely tell us whether these observations reflect the specificity of each species in regards to their cell cycle and/or in response to the growth conditions.

Regulation of peptidoglycan hydrolases

To promote the insertion of new precursors into the existing peptidoglycan or to simply mediate cell separation after division, the peptidoglycan polymer needs to be continuously remodeled by a variety of proteins with hydrolytic functions (Figure 2). These

hydrolases also serve other functions, like cell wall recycling or signaling in the host, that are detailed in [30, 31]. For cell division and morphogenesis, these hydrolases include glycosidases that cleave the glycosidic bonds in the glycan strand or amidases and peptidases that cleave the amide bond either between the glycan strand and the first amino acid of the stem peptide or within the stem peptide itself, respectively [2] (Figure 2). As observed for the synthesis machineries, the action of hydrolases must be regulated to maintain cell-wall homeostasis. While many of them are biochemically and structurally well characterized, their regulation remains largely unknown.

Over the last few years, the conserved transmembrane FtsEX complex has emerged as a major regulator of different hydrolases, notably in *S. pneumoniae* and *B. subtilis*. In *S. pneumoniae*, the cell division endopeptidase PcsB is autoinhibited and activated by an ATP-driven conformational change of the FtsEX complex [32] [33]. In *B. subtilis*, a similar observation was made for the endopeptidase CwlO that also requires FtsEX for activation [34]. CwlO however degrades peptidoglycan at the lateral cell wall allowing cell elongation and two new co-factors, SweC and SweD, are required for the FtsEX-CwlO complex to function outside the divisome [35]. In *E. coli*, FtsEX is able to interact with several cell division proteins including FtsZ and FtsA [36]. Based on these findings, it has been proposed that FtsEX could act as a mechano-transmitter coupling cell division with the activation of the endopeptidases required for cell division and morphogenesis. Recently, a new amidase, LytH, contributing to the coordination between cell expansion and cell division was discovered in *S. aureus* [37]. Although independent of FtsEX, LytH is also controlled by a new co-factor, named ActH, to be fully active. Last, the glucosaminidase SagB was recently shown to work in complex with the integral membrane protein SpdC to release the growing glycan strand from the membrane. Indeed, SpdC would bind the lipid carrier and would orient the SagB active site for efficient clivage [38].

These few examples illustrate that we likely only know the tip of the iceberg regarding the regulation of the peptidoglycan hydrolase activities. Interesting directions for future research will be the identification of additional co-factors of cell-wall hydrolases and to investigate the mechanisms by which these hydrolytic enzymes are regulated.

New factors and regulatory mechanisms

An impressive array of studies has led to the characterization of new factors involved in peptidoglycan assembly. Notable examples are the *S. pneumoniae* CozE protein, which directs the activity of the aPBP PBP1a to specific cell wall areas [39]. It has also been proposed that CozE also interacts with MreC and MreD to promote cell elongation. Interestingly, a CozE homolog, named CozEb, was then identified both in *S. pneumoniae* and *S. aureus* [40] [41]. The two proteins interact together and harbor some overlapping functions required for the maintenance of the cell shape. Another factor that is crucial for pneumococcal cell morphogenesis is the RNA-binding protein EloR (also named KhpA) [42] [43]. Inactivation of EloR suppresses the requirement of the essential bPBP PBP2b and SEDS RodA and generates shorter cells indicating that EloR is involved in cell elongation. Interestingly, EloR is phosphorylated by the serine-threonine kinase StkP [42], the key regulator of the pneumococcal cell elongation and division [44]. In absence of EloR phosphorylation, cells length is reduced as for $\Delta eloR$ cells suggesting that EloR is likely inactive. By contrast, the increase in EloR phosphorylation is tolerated only in the presence of suppressor mutations that reduce the functionality of the cell elongation proteins MreC or RodZ [42]. Altogether, it is thus proposed that EloR phosphorylation would stimulate the elongosome activity. EloR phosphorylation echoes another study that has identified a second regulator, specific of the aPBP PBP2a in *S. pneumoniae* [45]. The phosphorylation of this protein, named MacP, by StkP is also required for promoting PBP2a function.

The contribution of serine-threonine-kinases in peptidoglycan assembly is not restricted to *S. pneumoniae* and it has been detected in many Firmicutes, including *Clostridium difficile* [46], *B. subtilis* [47], *Enterococcus faecalis* [48], *L. monocytogenes* [49] or *S. aureus* [50]. Quantitative phosphoproteomic studies also suggest that many more proteins involved in peptidoglycan assembly are likely regulated by phosphorylation and protein-kinases [51]. In line with this claim, the recently identified transglycosylase MltG was found to be phosphorylated in *S. pneumoniae* [51]. MltG was proposed to contribute to pneumococcal cell elongation as its mutation suppresses the requirement of PBP2b, RodA and other proteins from the elongasome [52]. Similarly, the glucosaminidase LytB, that is required for final separation of daughter cell, is controlled by the serine-threonine-kinase StkP [53]. Last, a protein of unknown function was found phosphorylated in both *E. faecalis* (IreB) and *L. monocytogenes* (ReoM). While the function IreB remains unclear [54], ReoM was shown to activate the ClpCP-dependent proteolytic degradation of MurA that is essential for the synthesis of the lipid II [55].

Conclusion and perspectives

The key observations described in this review have changed our view about the biosynthesis of peptidoglycan. However, many other findings, like the knowledge gained about the cell division protein GpsB (reviewed in [56]) have also provided interesting observations and clarifications regarding some aspects of the peptidoglycan assembly. In addition, recent observations provide new directions of research. For instance, peptidoglycan homeostasis is also tightly interconnected with the cell metabolism ([57,58]), regulated by other protein post-translational modification like acetylation ([59]) and the production of second messengers like c-di-AMP ([60,61]) or still dependent on the biosynthesis of other cell wall compounds like teichoic acids ([62]). Together with the recent advances in

peptidoglycan imaging [63,64], all these observations promise further discoveries as exciting as those presented in this review for the next years.

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 - ** The authors applied atomic force microscopy to show that the peptidoglycan layer could be composed of two different zones: an outer-surface composed of loose and disordered thick glycan strands and a dense inner-surface with thinner glycan strands.

Figure Legends

Figure 1: Composition and diversity of the peptidoglycan polymer

Glycan strands are made of alternating β -1,4-linked N-acetylmuramic acid (MurNac) and N-acetyl-glucosamine (GlcNac) cross-linked by peptide links. The assembly of this structure starts at the inner face of the cytoplasmic membrane by a series of reactions involving a set of well conserved proteins (MurABCDEFGL and MraY) generating a lipid-linked disaccharide-pentapeptide precursor named lipid II. The composition of the stem peptide could vary between species. For instance, the third amino acid could be a meso-DAP (meso-diaminopimelic acid) in proteobacteria, bacilli and mycobacteria or a L-Lys in many Firmicutes. The fifth amino acid could be a D-Ala in most of Firmicutes or a D-Lac in *Lactobacillus plantarum* (a). The lipid II is anchored to the membrane with a undecaprenol phosphate (C55-P). Lipid II is then flipped across the cytoplasmic membrane by MurJ and is used as the substrate for the polymerization of the nascent peptidoglycan chains by glycosyltransferases (GT: SEDS protein & class A PBPs) and transpeptidases (TP: class A & B PBPs). Transpeptidation reactions occur between the third amino acid (meso-DAP or L-Lys) of the acceptor stem and either the fourth amino acid (b and c) (DD-transpeptidation: 3-4 link) or the third amino acid (g) (LD-transpeptidation: 3-3 link) of the donor stem to form a dimer. Cross-linking can occur with or between two dimers producing cross-linked trimers (c) and tetramers. In some species the transpeptidation reactions can occur between the third amino acid (meso-DAP or L-Lys) of the acceptor stem and a bridge structure which is attached to the L-Lys of the third position of the stem. The composition and the length of the bridge structure could vary between species. For instance, the bridge may contain one amino acid in *Enterococcus faecium* (d) (D-Asp or D-Asn), one or two amino acid(s) in *S.*

pneumoniae (e) (L-Ala and L-Ala or L-Ser) or up to 5 amino acids in *S. aureus* (f) (Gly or Ser). The MurNac and the GlcNac can be O-acetylated and N-deacetylated, respectively.

Figure 2: Hydrolases required for peptidoglycan remodeling

The peptidoglycan structure is continuously remodeled by a variety of proteins with hydrolytic functions. For instance, peptides are trimmed by either DD-carboxypeptidases or LD-carboxypeptidases which cleave the bond respectively between the fourth D-Ala and the fifth D-Ala (or D-Lac) amino acid and between the third meso-DAP (or L-Lys) and the fourth D-Ala amino acid of the stem peptide. Crosslinks are cleaved by DD-endopeptidases or LD-endopeptidases while the peptide is removed from the glycan chain by amidases. Glycosydases cleave the backbone of glycan and consist of N-acetylglucosaminidases which hydrolyses N-acetyl-D-glucosamine (GlcNAc) residues from adjacent sugar residues and N-acetylmuramidases which cleaves the β 1-4 glycosidic bond between N-Acetylmuramic acid (MurNAc) and GlcNAc.





