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MINIREVIEW

An Update on Some Structural Aspects of the Mighty Miniwall

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Abstract

Peptidoglycan (PG), the mighty miniwall, is the main structural component of practically all bacterial cell envelopes and has been the subject of a wealth of research over the past 60 years, if only because its biosynthesis is the target of many antibiotics that have successfully been used in the treatment of bacterial infections. This review is mainly focused on the most recent achievements in research on the modification of PG glycan strands, which contribute to the resistance of bacteria to the host immune response to infection and to their own lytic enzymes, and on studies on the spatial organization of the macromolecule.

K e y w o r d s: glycan strands, *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac) modifications, peptidoglycan (PG), spatial organization

In 1884 Christian Gram devised a staining procedure that allowed classifying almost all bacteria to one of two groups, the Gram-positive and Gram-negative bacteria. The simple staining procedure is still widely used almost 130 years later, with practically all bacteria being classified to one group or the other. However, it was not until many years after Gram's invention that light was shed on the complexity of bacterial cell envelopes and the structures forming them. Studies of bacterial cell surfaces began in earnest in the 1950s and 1960s, following the isolation by Park and Johnston (1949) of what were later found to be cell wall PG precursors and the purification of bacterial cell walls by *e.g.* Salton (1952, 1957) and Work (1957). The term "microdermatology" was coined. It was found that all bacteria, except for some notable exceptions, *i.e.* mycoplasmas, *Planctomyces* and *Orientia tsutsugamushi* contain peptidoglycan (PG, syn. murein). PG has never been found in chlamydia either, although the bacteria have a functional pathway for meso-diaminopimelate, one of the unique structural building bricks of the macromolecule (Pavelka, 2007). More recently, an interesting new phylum of PG-free bacteria, the *Verrucomicrobia*, has been established (Yoon *et al.*, 2010).

There has been a resurgence in studies on PG in the past few years fired, amongst others, by the increased prevalence of antibiotic resistance among bacteria that cause life-threatening infections and the need to find new agents that inhibit bacterial cell-wall biosynthesis (Bugg *et al.*, 2011); the interaction of PG with innate immunity proteins (PG Recognition Proteins, PGRPs or PGLYRPs) that are conserved from insects to animals and the mechanisms that lead to bacterial cell death (Dziarski and Gupta, 2010; Kietzman and Tuomanen, 2011); last but not least, the role of D-amino acids, which are a universal component of PG, in nature (Cava *et al.*, 2011).

The main structure of PG involves linear glycan strands cross-linked by short peptides. "Normal", that is unaltered, glycan chains, have universally been found to be composed of alternating *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac) residues linked by β-1 → 4 bonds. The D-lactoyl group of each Mur*N*Ac residue is substituted by a peptide stem whose composition is most often L-Ala-γ-D-Glu-meso-A2pm (or L-Lys)-D-Ala-D-Ala in nascent PG, the last D-Ala residue being lost in the mature macromolecule. Cross-linking of the glycan strands generally occurs between the carboxyl group of D-Ala at position 4 and the amino group of the diamino acid at position 3, either directly or through a short peptide bridge. The unique chemical traits of PG thus include the presence of *N*-acetylmuramic acid, γ-bonded D-Glu, L-D (and even D-D) bonds and non-protein amino acids, *e.g.* 2,6-diaminopimelic acid (A2pm) (*e.g.* Cummins, 1956; Work, 1957, 1961, 1969; Rogers, 1974; Glauner, 1988; Höltje and Glauner, 1990; Markiewicz *et al.*, 1983; Markiewicz, 1993; Vollmer *et al.*, 2011). These structural features are basically retained in all bacteria, though many differences in the glycan strands, composition of the stem peptide and/or interpeptide bridge are known and are taken into account in the tri-digital

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PG classification system established by Schleifer and Kandler (1972). In some cases the differences may be quite extreme like, for example, the occurrence of mostly (75%) A2pm → A2pm (*i.e.* 3 → 3) crosslinks in *Clostridium difficile* PG (Peltier *et al.*, 2011)

Variations in the structure of the glycan strands of PG have recently been elegantly reviewed by Vollmer (2008). A review by Davis and Weiser (2011) focuses specifically on the role of peptidoglycan modifications and their effects on the host immune response to infection. A unique modification of glycan strands is the presence of muramic δ-lactam, which occurs *e.g.* in the thick PG of *Bacillus* sp. and *Clostridium sporogenes* endospores. In *Bacillus subtilis* approximately every second Mur*N*Ac residue along the glycan strands is modified to muramic δ-lactam. The modification of Mur*N*Ac involves the action of two enzymes, the Mur*N*Ac deacetylase PdaA and the amidase CwlD. Studies with mutants lacking these enzymes have shown that intact endospores are formed but that the spores are not able to germinate since unmodified spore PG is not recognized by germination-specific hydrolases (Popham *et al.*, 1996, Atrih and Foster, 2001; Gilmore *et al.*, 2004). More recently, it has been demonstrated that in *Bacillus anthracis* germination is mediated by the action of germination-specific lytic enzymes (GSLEs), one of which is SleB. SleB functions independently as a lytic transglycosylase on both intact and fragmented cortex. Most of the muropeptide products that SleB generates are large and are potential substrates for other GSLEs present in the spore, such as a glucosaminidase that cleaves between N-acetylglucosamine and muramic-δ-lactam. SleB has two domains, the *N*-terminal domain is required for stable PG binding, while the C-terminal domain is the region of PG hydrolytic activity, which is dependent on cortex containing muramic-δ-lactam in order to carry out hydrolysis (Dowd *et al.*, 2008; Heffron *et al.*, 2011).

PdaA, mentioned above, is an example of several N-deacetylases found in different Gram-positive bacteria, which carry out the N-deacetylation of MurNAc or Glc*N*Ac (or both) in polymerized PG. N-deacetylation of Mur*N*Ac was found to protect bacterial cell walls from degradation by lysozyme, an important factor of the innate immune system (Araki *et al.*, 1971). These enzymes, which have a predicted extracytoplasmic location in the cell, have been thoroughly reviewed by Vollmer (2008). The N-deacetylase of *Streptococcus pneumoniae* (PgdA) has been shown to be a putative virulence factor (Vollmer and Tomasz, 2002). Deacetylation of PG increases the positive charge of the cell wall, possibly contributing to protection of the pathogens against the binding of cationic antimicrobial peptides of the host organism. Similar observations were made more recently by Popowska *et al.* (2009) who found that a *pgdA* mutant of *Listeria monocytogenes* was more prone to autolysis and was more susceptible to cationic antimicrobial peptides, and by Meyrand *et al.* (2007) for *Lactococcus lactis*. In other studies, a mutant strain of *L. monocytogenes* lacking PdaA activity induced a massive IFN-beta response in a TLR2 and Nod1-dependent manner and was rapidly destroyed within macrophage vacuoles (Boneca *et al.*, 2007; Corr and O'Neill, 2009) and in bone-marrow derived macrophages, *pgdA* mutants of *L. monocytogenes* demonstrated intracellular growth defects and increased induction of cytokine transcriptional responses that emanated from a phagosome and the cytosol (Rae *et al.*, 2011). In *Streptococcus suis* expression of the pgdA gene was increased upon interaction of the bacterium with neutrophils *in vitro* as well as *in vivo* in experimentally inoculated mice, suggesting that *S. suis* may enhance PG N-deacetylation under these conditions. Evaluation of the *pgdA* mutant in both the CD1 murine and the porcine models of infection revealed a significant contribution of the *pgdA* gene to the virulence traits of *S. suis* (Fittipaldi *et al.*, 2008). In an interesting study, it was found that neither PgdA inactivation nor PgdA overexpression in *Lactobacillus lactis* leading to different levels of PG deacetylation confers any advantage in the persistence of this bacterium in the gastrointestinal tract and its ability to enhance host immune responses (Watterlot *et al.*, 2010). Bacterial N-deacetylases have been considered to be exported enzymes but it has recently been reported that some of these enzymes may also be cytoplasmic, with a potential role in PG turnover and recycling (Popowska *et al.*, 2011, for a very good review on turnover and recycling, see Reith and Mayer, 2011). A similar N-deacetylase lacking a signal peptide for secretion into the periplasmic space has been found in *Helicobacter pylori* (Shaik *et al.*, 2011). The enzyme showed no *in vitro* activity on the typical polysaccharide substrates of peptidoglycan and results from crystallization and structure studies suggest that it binds a small molecule at the active site, even though the peptidoglycan of a HP0310 (syn. HpPgdA) knock-out mutant was characterized by higher degree of acetylation compared to the wild-type, along with increased susceptibility to lysozyme degradation.

O-acetylation of Mur*N*Ac, similarly to N-deacetylation, is typically associated with bacterial resistance to lysozyme PG from degradation by lysozyme as well as by endogenous autolytic enzymes, *e.g.* the lytic transglycosylases. As a protective modification it is more ubiquitous than *N*-deacetylation and has been found to occur in many different bacterial species, both Gram-positive and Gram-negative (Vollmer, 2008). An additional acetyl group is linked to the C6-OH of Mur*N*Ac to form a 2,6-*N*,*O*-diacetylo muramic acid residue. The ester bond of *O*-linked acetate is significantly weaker than the amide bond of *N*-linked acetate.

Two types of unrelated *O*-acetyltransferases have been described, corresponding to different mechanisms of peptidoglycan *O*-acetylation (Clarke *et al.*, 2000; Bera *et al.*, 2005, Crisostomo *et al.*, 2006). The first mechanism involves a single protein (an OatA-type enzyme) which performs both the transport of acetate across the membrane and its transfer onto the peptidoglycan. The second mechanism involves two proteins, one for acetate transport across the membrane and the other for catalyzing its transfer to Mur*N*Ac. The acetate transport genes of this system are unknown. There are several candidate genes for these *O*-acetyltransferases (named Pat). A new peptidoglycan *O*-acetyltransferase has been found in *E. coli*. The enzyme, named PatB, *O*-acetylates peptidoglycan within the periplasm. This activity was found to be dependent upon a second protein, PatA, which functions to translocate acetate from the cytoplasm to the periplasm, demonstrating that the *O*-acetylation of peptidoglycan in Neisseria gonorrhoeae, and other Gram-negative bacteria, requires a two component system (Moynihan and Clarke, 2010).

The *O*-acetyltransferase reaction is reversed by peptidoglycan *O*-acetyl esterase activity (Weadge *et al.*, 2005; Vollmer, 2008). In *Bacillus anthracis*, in contrast to other bacteria, *O*-acetylation of peptidoglycan is combined with *N*-deacetylation to confer resistance of cells to lysozyme and is conferred by two unrelated *O*-acetyltransferases. Activity of the Pat *O*-acetyl-transferases is also required for the separation of the daughter cells following bacterial division and for anchoring of one of the major S-layer proteins (Laaberki *et al.*, 2011).

Until very recently it was thought that only the Mur*N*Ac residues in the PG polymer can be *O*-acetylated. However, the presence of *O*-acetylation on *N*-acetylglucosamine (Glc*N*Ac) in *Lactobacillus plantarum* PG has just been reported (Bernard *et al.*, 2011). Detailed structural characterization of acetylated muropeptides released from *L. plantarum* PG revealed that both Mur*N*Ac and Glc*N*Ac are *O*-acetylated in this species. These two PG modifications are carried out by two dedicated *O*-acetyltransferases, OatA and OatB, respectively. Analysis of the resistance of mutant strains to cell wall hydrolysis demonstrated that Glc*N*Ac *O*-acetylation inhibits the activity of the major *L. plantarum* autolysin, *N*-acetylglucosaminidase Acm2. In this bacterial species, inactivation of *oatA*, encoding Mur*N*Ac *O*-acetyltransferase, resulted in marked sensitivity to lysozyme. Moreover, Mur*N*Ac over-*O*-acetylation was shown to activate autolysis through the putative *N*-acetylmuramoyl-l-alanine amidase LytH enzyme. In *L. plantarum*, two different *O*-acetyltransferases seem to play original and antagonistic roles in modulating the activity of endogenous autolysins.

Another kind of modification of muramic acid occurs in the PG of most genera of the order *Actinomycetales* that contain mycolic acids, *e.g. Mycobacterium* sp. In these bacteria muramic acid is *N*-glycolylated and not *N*-acetylated, and this modification is introduced during the synthesis of UDP-linked PG precursors, specifically into the last cytoplasmic precursor, UDP-Mur*N*Ac-pentapeptide. The *N*-glycolylated form arises through the action of an *N*-acetyl muramic acid hydroxylase (NamH) (Raymond *et al.*, 2005) present only in the *Actinomycetales*. The importance of glycolylation has been elusive, with a hypothesis proposed based on studies with *M. smegmatis namH* mutants that it may protect PG from the action of lysozyme. However, recent findings (Coulombe *et al.*, 2009) identify N-glycolyl MDP (Peptidoglycan-derived Muramyl Dipeptide) as more stimulatory than *N*-acetyl MDP at eliciting NOD2-mediated immune responses in the context of both an intact bacterium and as a pure compound, consistent with early observations attributing exceptional immunogenic activity to the mycobacterial cell wall. Disruption of *namH* in *M. smegmatis* nulled NOD2-mediated TNF secretion, which could be restored upon gene complementation. In mouse macrophages, *N*-glycolyl MDP was more potent than *N*-acetyl MDP at activating RIP2, nuclear factor kappaB and proinflammatory cytokine secretion. Finally, *N*-glycolyl MDP was found to be more efficacious than *N*-acetyl MDP at inducing ovalbumin-specific T cell immunity in a model of adjuvancy (Coulombe *et al.*, 2009; Davis and Weiser, 2011).

A different modification of the glycan strands that can be found in the PG of many Gram-negative bacteria, but also in some Gram-positive ones is the presence of a 1,6-anhydroMur*N*Ac residue at the end of the chain. These are formed by the action of lytic transglycosylases (LTs), that have the same bond specificity as lysozyme (Höltje *et al.*, 1975). These ubiquitous enzymes are classified to one of four distinct families, based on sequence similarities and identified consensus motifs (Blackburn and Clarke, 2001). The importance of these enzymes is reflected in the fact that the bacterium *Escherichia coli* has six of them, representing different families and subfamilies. LTs can be viewed as space-making enzymes. They cleave glycosydic bonds within the PG sacculus to allow for a number of different processes to occur. They play an important a critical role in the expansion of the sacculus and consequent cell growth by creating sites for the insertion of PG precursors (Höltje, 1998). They are also required for PG turnover and recycling. In concert with amidases LTs function to split the septum, thereby permitting the separation of dividing cells (Heidrich *et al.*, 2002). LTs have also been suggested to contribute to pathogenesis (reviewed in Cloud-Hansen *et al.*, 2006). An important question has always been how large structures, *e.g.* protein complexes, penetrate the PG layer (Scheurwater

and Clarke, 2008). LTs have always been implicated in these processes. This topic has been very well reviewed by Scheurwater and Burrows (2011).

Glycan strands of PG are also modified *via* the attachment of many different types of compounds and polymers to muramic acid, usually *via* a phosphodiester bond. The most notable of these are the teichoic and teichuronic acids as well as the arabinogalactans. Very rarely, structures are attached to Glc*N*Ac, as in *Streptococcus agalactiae*. This topic is vast and well beyond the scope of this review.

Even though the structure of PG and the various species-specific and function-determined modifications of its structural elements have been thoroughly investigated, there are still numerous unresolved fundamental questions regarding the architecture of the peptidoglycan, *i.e.* the orientation of the glycan strands and stem peptides in relation to the surface and axes of a cell (Vollmer and Seligman, 2010). Various models for the spatial organization of peptidoglycan have been proposed over the years and currently two opposing models are considered: a model in which the glycan strands run parallel to the cytoplasmic membrane (the "classical" or "classical" model, *e.g.* Höltje, 1998; Pink *et al.*, 2000) recently supported by experimental data by Gan *et al.* (2008) and Hayhurst *et al.* (2008) and the opposing "scaffold" model in which the glycan strands are oriented perpendicularly to the membrane (Dmitriev *et al.*, 2003, 2004, 2005; Meroueh *et al.*, 2006). Unraveling the architectural issues is compounded by the differences of the thickness of PG in Gram-negative versus Gram-positive cells, which is approximately 5–10 times thicker in the latter compared to the former and the fact that structure may be affected by *i.e.* growth conditions, gene activity (*e.g.* Höltje and Glauner, 1990; Vollmer *et al.*, 2008; Korsak *et al.*, 2005, 2010; Cava *et al.*, 2011), antibiotic production or resistance (Sieradzki and Markiewicz, 2004; Schaberle *et al.*, 2011) and many other factors. Moreover, recent studies using cryo-transmission electron microscopy (cryo-EM) have conclusively demonstrated the existence of the equivalent of the Gram-negative periplasmic space in at least *B. subtilis* and *Staphylococcus aureus* (Matias and Beveridge, 2005; 2006), which also complicates interpretation in spatial structure studies. The technique reveals in Gram-positive bacteria two different cell wall layers: an inner wall zone (IWZ) of low-electron density, whose main component is lipoteichoic acid (Matias and Beveridge, 2008), and a high-electron density outer wall zone (OWZ). In the "layered" model the glycan strands are believed to run parallel to the plasma membrane, arranged as hoops or helices around the short axis of the cell, resulting in a woven fabric-like structure (Verwer *et al.*, 1978; Vollmer and Höltje, 2004; Vollmer *et al.*, 2008). A recent study by Gan *et al.* (2008), in which frozen-hydrated sacculi from *E. coli* and *Caulobacter* crescentus were examined by electron cryotomography, confirmed the layered model, showing that in the Gram-negative PG sacculus a single layer of glycan strands lie parallel to the cell surface, roughly perpendicular to the long axis of the cell, encircling the cell in a disorganized hoop-like fashion. Their data also precluded the scaffold model. However, assuming that Gram-negative bacteria do have a single layer of PG, then how can one explain the difference in the thickness of *E. coli* PG versus *Pseudomonas aeruginosa* PG (approximately 2 : 1, respectively, for either dry or hydrated isolated sacculi (Vollmer and Seligman, 2010)? The organization of PG in ovococcoidal mutant *Lactobacillus lactis* cells lacking cell wall exopolysaccharides was studied using AFM (Atomic Force Microscopy) topographic and recognition imaging. Topographic images showed periodic ridges on the mutant surface that always ran parallel to the short cell axis. Recognition imaging demonstrated that these ridges consisted of peptidoglycan. The results are consistent with a PG organization in the plane perpendicular to the long axis of the cell (Andre *et al.*, 2010). It would thus seem that the 3-D architecture of PG in both Gram-negative and Gram-positive cells is of the layered type. However, observations of isolated *Bacillus subtilis* PG using AFM show that, at least in this species, spatial organization is more complex (Hayhurst *et al.*, 2008). This may be related to the existence of the IWZ in *B. subtilis* (Matias and Beveridge, 2005; Zuber *et al.*, 2006) and the finding that the glycan strands of the bacterium are longer 50 times longer than previously calculated. The model of Hayhurst *et al.* (2008) proposes that during biosynthesis small numbers of glycan strands are polymerized and cross-linked to build a peptidoglycan "rope", which is coiled into a helix to form inner surface cable structures. The nascent helix (cable) is inserted into the cell wall by cross-links between two existing cables and the overlying cable interface cleaved by autolysins known to be essential for cell growth. As part of cable maturation, the structure may become stabilized by inter/intra glycan strand cross-links. The model also predicts that the cell wall is likely only one intact cable thick with partially hydrolyzed cables also present externally. Solid-state NMR data obtained for *Staphylococcus aureus* PG, which contains an interpeptide pentaglycyl bridge, show that the spatial arrangement of the polymer in staphylococci may be even more complex. Partial characterization of the structure was achieved by measuring spin diffusion from $^{(13)}$C labels in pentaglycyl cross-linking segments to natural-abundance $^{(13)}$C in the surrounding intact cell walls. The measurements were performed using a version of Centerband-Only Detection of EXchange (CODEX). The CODEX spin diffusion rates established that the pentaglycyl bridge of one peptidoglycan repeat unit of *S. aureus* is within 5 angstroms of the glycan chain of another repeat unit, which

shows surprising proximity compared to earlier theoretical considerations and was interpreted in terms of a model for the peptidoglycan lattice in which all peptide stems in a plane perpendicular to the glycan main chain are parallel to one another (Sharif *et al.*, 2009).

This minireview reflects the most recent achievements in research on peptidoglycan, with focus on modifications of the glycan chains and the spatial organization of the polymer.

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