Trends in Microbiology

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Review

Elucidating Peptidoglycan Structure: An Analytical Toolset

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Peptidoglycan (PG) is a ubiquitous structural polysaccharide of the bacterial cell wall, essential in preserving cell integrity by withstanding turgor pressure. Any change that affects its biosynthesis or degradation will disturb cell viability, therefore PG is one of the main targets of antimicrobial drugs. Considering its major role in cell structure and integrity, the study of PG is of utmost relevance, with prospective ramifications to several disciplines such as microbiology, pharmacology, agriculture, and pathogenesis. Traditionally, high-performance liquid chromatography (HPLC) has been the workhorse of PG analysis. In recent years, technological and bioinformatic developments have upgraded this seminal technique, making analysis more sensitive and efficient than ever before. Here we describe a set of analytical tools for the study of PG structure (from composition to 3D architecture), identify the most recent trends, and discuss future challenges in the field.

Highlights

PG is a major bacterial cell wall component, crucial for cell integrity, and strongly related to antibiotic sensitivity.

Methods to analyze PG structure have evolved over time, and new and improved methods are currently replacing previous tedious and long procedures.

Current tools, ranging from chromatography–mass spectrometry methods to imaging techniques, allow characterizing PG from a structural and architectural perspective.

Peptidoglycan Structure and Diversity

Peptidoglycan (PG) (also known as *murein*, from Latin 'murus', wall) is a major component of the bacterial cell wall [1] (Figure 1). Present in both Gram-positive and Gram-negative bacteria [2], this polysaccharide forms a flexible, net-like structure that determines cell shape and provides mechanical strength and osmotic stability. Although the structure and architecture of PG has been studied for a long time (for reviews see [3–5]), its detailed 3D structure is still an open question [5,6].

PG is a heteropolymer of linear **glycan** (see Glossary) strands crosslinked by short peptide bridges (Figure 2). The glycan strands are formed by repeating units of the disaccharide β-1,4-linked *N*-acetylglucosamine–*N*-acetylmuramic acid (GlcNAc-MurNAc). In Gram-negative bacteria the glycan strands end with a 1,6-anhydro-MurNAc residue, which results from the action of lytic transglycosylases [7]. Chemical variation of the glycan strands – for example, *O*-**acetylation**, *O*-glycolylation, and de-*N*-acetylation of GlcNAc, MurNAc, or both – is one of the sources of PG diversity, and some of these modifications have been related to changes in antibiotic sensitivity [8]. Another source of diversity in PG structure is due to variation of the peptide moiety [4]. In fact, the amino acid composition of PG stem peptides has been characterized in many species and is the basis for the PG bacterial classification system proposed by Schleifer and Kandler [9] that is still used today. These PG stem peptides are formed by five amino acids with L- and D-configurations, attached to the lactyl groups of MurNAc by an amide linkage [3]. They are crosslinked to each other through the second amino group of the diamino acid present at positions two or three of the peptide chain (Figure 2). In Gram-negative bacteria, the stem peptide is a L-Ala–D-*i*Glu–*m*-DAP–D-Ala–D-Ala pentapeptide, where *i*Glu and *m*-DAP correspond to *iso*-glutamate and *meso*-diaminopimelic acid, respectively. This PG chemotype has been named A1γ and is rarely found in Gram-positive bacteria [10], where often the D-*i*Glu residue at position two is amidated, resulting in D-*i*Gln, and L-Lys replaces *m*-DAP at position three [11]. Aside from **amidation**, the formation of cyclic imides in the peptide stem has also

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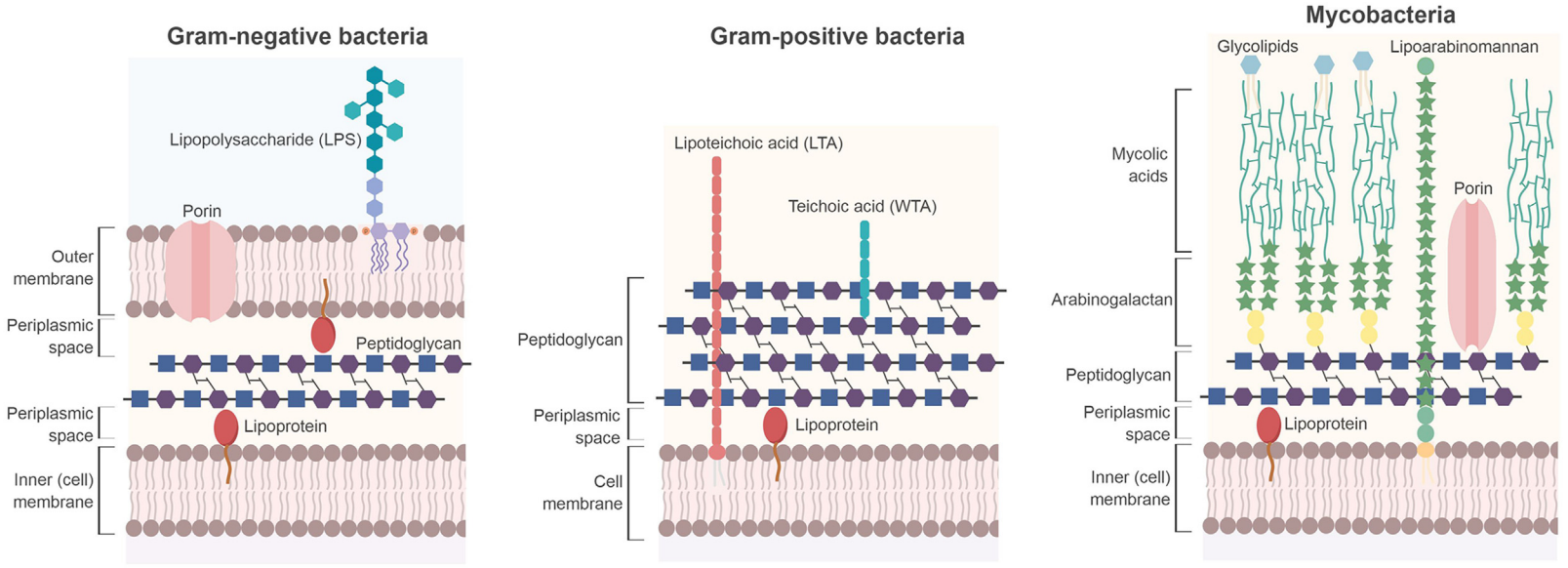

Figure 1. Cell Wall Structure of Gram-Positive and Gram-Negative Bacteria. (Left) Gram-negative bacteria display a complex cell wall formed by two lipid bilayers (inner and outer membranes) which enclose the periplasmic space where peptidoglycan (PG) is located. In this case, PG forms a thin layer that surrounds the cell and it is tethered to both cell membranes by lipoproteins. The outer membrane is permeated by porins – proteins that cross the cell membrane and act as a pore, through which molecules can diffuse – and decorated with lipopolysaccharides (LPS). LPS, also referred to as endotoxin, is the major surface component of Gram-negative bacteria, contributing greatly to the structural integrity of the cell, and represents one of the microbial molecular signals responsible for activation of the host innate immune system. (Center) Gram-positive bacteria are surrounded by a single cell membrane and possess a much thicker layer of PG than Gram-negatives. Similarly, PG is attached to the cell membrane through a lipoprotein. Gram-positives also functionalize their cell wall with non-PG surface polymers. Teichoic acids and lipoteichoic acids are the main cell wall-bound polymers in Gram-positive bacteria and they play crucial roles in cell shape determination, regulation of cell division, pathogenesis, and antibiotic resistance. (Right) Acid-fast bacteria, such as mycobacteria, are a subset of Gram-positive bacteria with highly complex cell walls. While teichoic acids and lipoteichoic acids are not found in this group of organisms, their cell envelope is formed by a large macromolecular structure, termed the mycolylarabinogalactan-peptidoglycan (mAGP) complex. The mAGP complex is formed by characteristic long-chain mycolic acids, a highly branched arabinogalactan (AG) polysaccharide, and a crosslinked PG network. In addition, an outer membrane segment that contains solvent-extractable lipids, such as lipoarabinomannan (LAM) and other glycolipids, completes the cell wall. Monosaccharides shown in this figure follow the Symbol Nomenclature for Graphical Representation of Glycans (https://www.ncbi.nlm.nih.gov/glycans/snfg.html).

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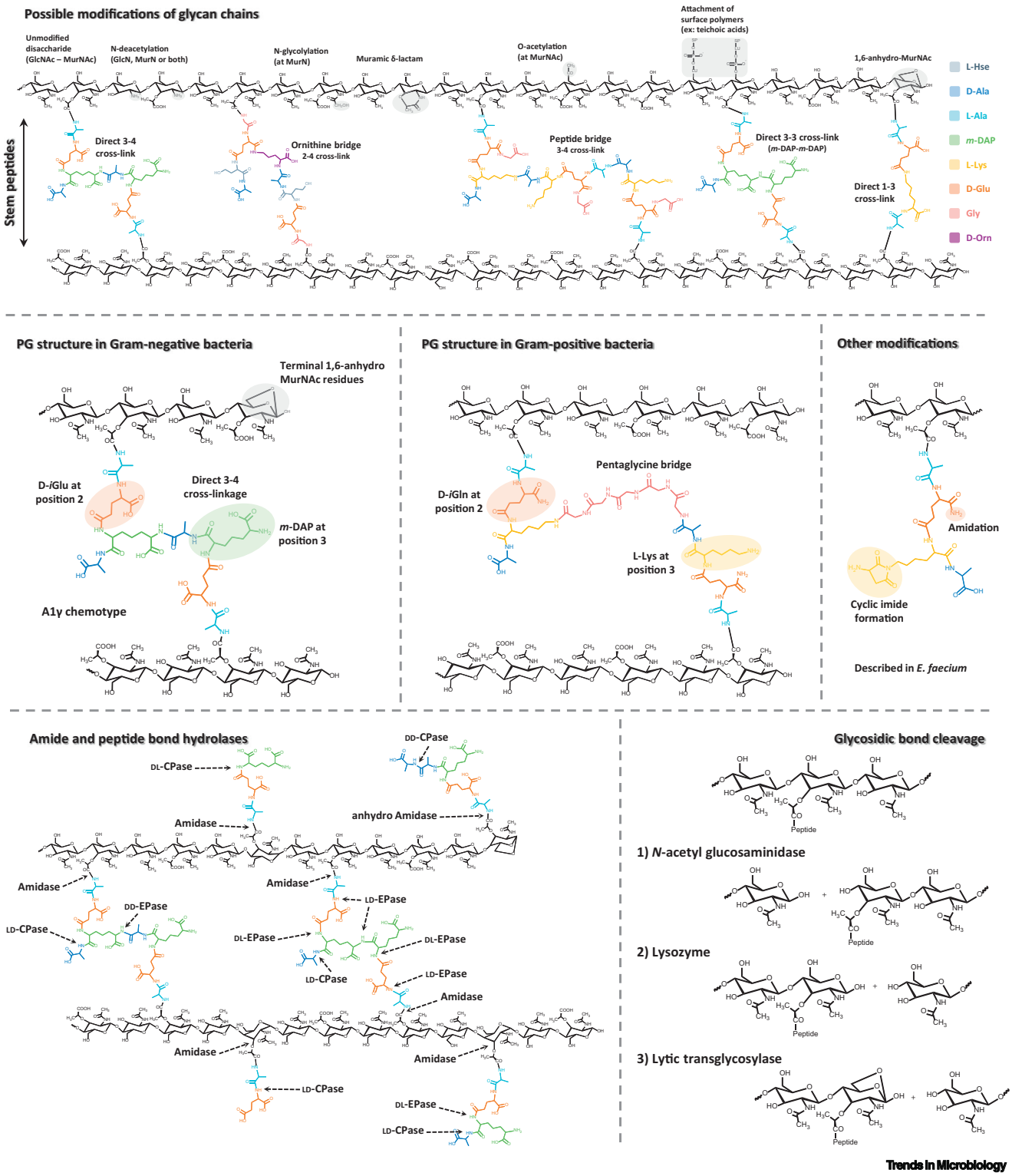

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been described [12] (Figure 2). The interpeptide bridges also vary depending on the species of bacteria, and define the type of PG [9]. In type A (3-4 crosslinkage), the crosslinking occurs between the side-chain amino group of the diamino acid in position three and the carboxyl group of the D-Ala residue on position four of the adjacent stem peptide. This is the most common type of crosslinkage and it can be direct (most Gram-negative bacteria) or by means of an interpeptide bridge (most Gram-positive bacteria) [4,13]. In type B (2-4 crosslinkage), which has been found only in coryneform bacteria, the α-carboxyl group of D-iGlu at position two of one peptide is connected, directly or indirectly, to the carboxyl of the D-Ala residue in position four of another peptide [4,13]. Interpeptide bridges in 2-4 crosslinkages must contain a diamino acid, such as L- or D-Lys, D-Orn, D-2,4-diaminobutyrate, to allow the formation of the peptide linkage to the adjacent peptide [13]. The diversity of interpeptide bridges, as well as the amino acid variation of stem peptides, have been thoroughly discussed elsewhere [4].

In most bacteria, PG is functionalized with proteins or other glycopolymers (Figure 1). Surface proteins (Gram-positives) and lipoproteins (Lpp) (Gram-negatives) are covalently linked to PG through the peptide stem [14]. Teichoic acids, the main PG-linked glycopolymer in Gram-positive bacteria, are attached to MurNAc residues through a phosphodiester bond and it has been estimated that every ninth MurNAc residue in *Bacillus subtilis* and *Staphylococcus aureus* PG is modified by one teichoic acid [15]. Other glycopolymers attached to PG through a phosphodiester bond include teichuronic acids, capsular polysaccharides [4] and, in the particular cases of corynebacteria, mycobacteria, and *Nocardia*, arabinogalactan [16].

While PG isolation strategies have not changed greatly, the methods used to study PG structure have significantly improved over time. Classical techniques [17] are still broadly used to purify PG from cell cultures and other cell wall components (Figure 3), although currently the process is more streamlined [18]. Historically, PG structure was studied in terms of amino acid and monosaccharide composition and susceptibility to PG-degrading enzymes [19]. Improved analytical techniques, such as cryotransmission electron microscopy (cryo-TEM), atomic force microscopy (AFM), high-performance liquid chromatography/ultra-performance liquid chromatography (HPLC/UPLC), and mass spectrometry (MS) have allowed identification and exploration of previously unknown features of PG structure as well as better characterization of its building blocks. In this review, we describe a set of analytical tools commonly used for the purification and structural analysis of PG that provide a comprehensive and multidisciplinary approach for the study of this polysaccharide.

Composition Analysis: Sample Composition and Purity at a Glance

Although the composition of PG is known and relatively conserved, composition analysis is a useful tool for determining sample purity and for aiding the differentiation of PG types. There are several ways of evaluating the glycosyl and amino acid composition of PG preparations. Typically, to release monosaccharides and amino acids, purified PG is hydrolyzed under harsh acidic conditions (6 N HCl) at high temperature (100–166°C) for several hours, and this is followed by different methods of analysis (Table 1).

Glossary

Acetylation: a chemical reaction that introduces an acetyl functional group into a chemical compound. Acetic anhydride is commonly used as an acetylating agent reacting with free hydroxyl groups.
Alditol: any of a class of acyclic polyols formally derived from an aldose by reduction of the carbonyl functional group.
Amidation: a chemical reaction where the formation of an amide group occurs.
Anomer: a type of geometric variation found at certain atoms in carbohydrate molecules. An *epimer* is a stereoisomer that differs in configuration at any single stereogenic center. An *anomer* is an epimer at the hemiacetal/acetal carbon in a cyclic saccharide, an atom called the 'anomeric carbon'. The anomeric carbon is the carbon derived from the carbonyl carbon (the ketone or aldehyde functional group) of the open-chain form of the carbohydrate molecule.
Chiral: a chiral molecule is distinguishable from its mirror image; that is, it has a non-superimposable mirror image. Chiral isomers are called enantiomers (or optical isomers) and are typically optically active. Common examples of chiral compounds are amino acids.
Derivatization: a procedure commonly used in chemistry which transforms a chemical compound into a product of similar chemical structure, called a derivative.
Glycan: a compound consisting of a large number of monosaccharides linked by glycosidic bonds. Glycan is a broad term that includes oligosaccharides and polysaccharides, but may also be used to refer to the carbohydrate portion of a glycoconjugate, such as a glycoprotein, glycolipid, or a glycosaminoglycan. Large-molecular-weight glycans can also be called glycopolymers.

Figure 2. Diversity and Regulation of Peptidoglycan Structure. (Top) Peptidoglycan (PG) structure. Glycan strands formed by repeating units of the β-1,4-linked disaccharide *N*-acetylglucosamine–*N*-acetylmuramic acid (GlcNAc-MurNAc) are interconnected by small peptides. Possible modifications of the glycan chains are highlighted. Several examples of peptide crosslinkage are also shown: direct 3-4 crosslink (*Escherichia coli*), 2-4 crosslink with a D-ornithine bridge (*Corynebacterium pointsettiae*), 3-4 crosslink with a peptide stem bridge (*Micrococcus luteus*), direct 3-3 crosslink (*E. coli*), and direct 1-3 crosslink (Acetobacteria). (Center) Typical PG structures of Gram-negative and Gram-positive bacteria. The pentaglycine interpeptide bridge is an example of a possible 3-4 crosslinkage in Gram-positives. Other possible modifications include amidation and the formation of cyclic imides in the peptide stem. (Bottom left) Hydrolysis of amide and peptide bonds in PG from *E. coli* or *Bacillus subtilis*. Amide bonds between the lactyl group of MurNAc and the L-alanine of the stem peptide are hydrolyzed by *N*-acetylmuramyl-L-alanine amidases (amidases). Some amidases (anhydro amidase) specifically cleave at 1,6-anhydroMurNAc residues, which are the hallmarks of PG turnover products in many species. Amide bonds in the peptides are cleaved by endopeptidases (DD-EPase, LD-EPase, DL-EPase). Carboxypeptidases (DD-CPase, LD-CPase, DL-CPase) hydrolyze peptide bonds to remove C terminal D- or L-amino acids. (Bottom right) Cleavage of glycosidic bonds in PG glycan chains by different enzymes. Note the formation of 1,6-anhydro-MurNAc as a result of the intramolecular transglycosylation reaction catalyzed by lytic transglycosylases. Abbreviations: LU, linkage unit; SP, surface polymer.

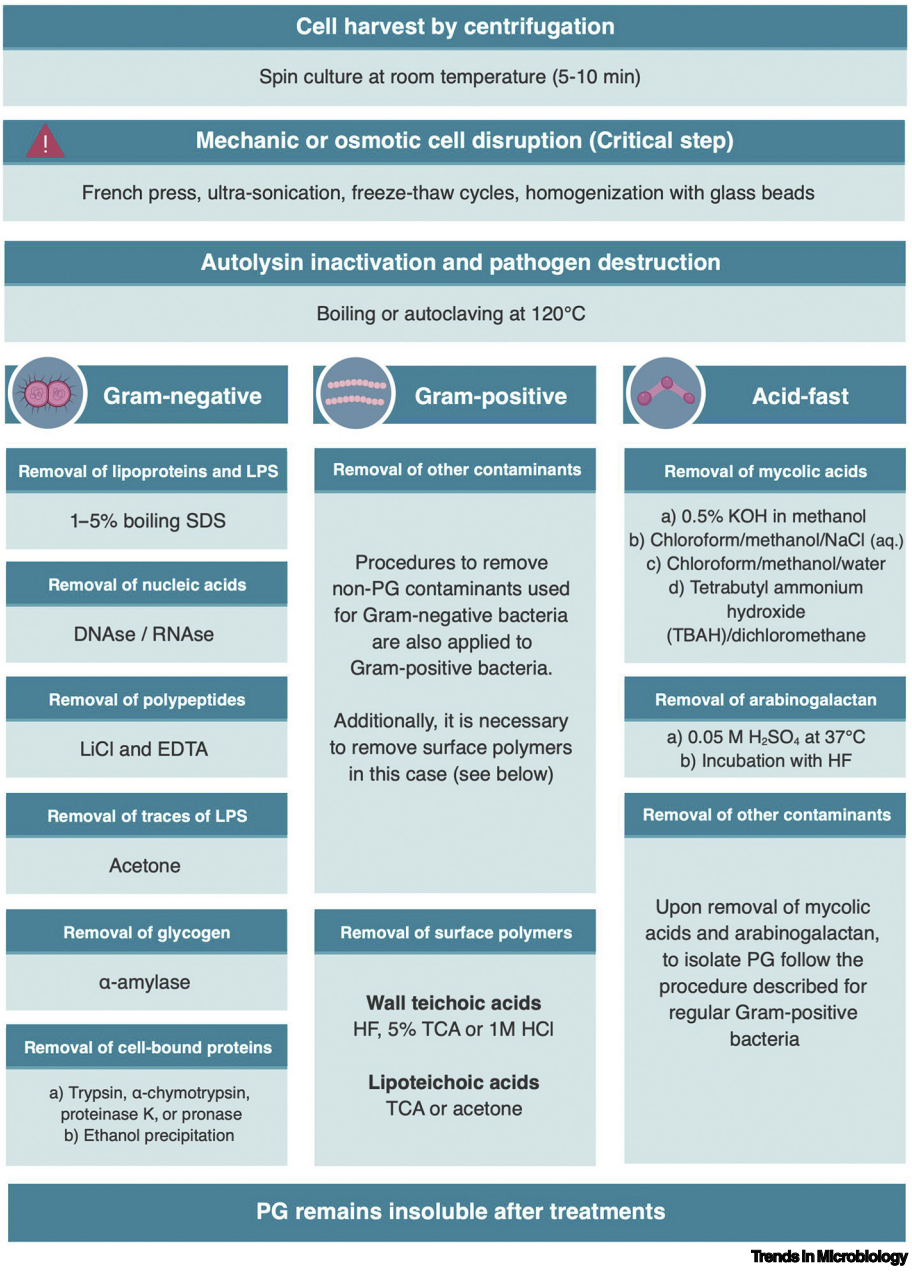

Figure 3. General Protocol for Peptidoglycan (PG) Isolation from Gram-Negative, Gram-Positive, and Acid-Fast Cells. Steps described at the top are common to all types of bacteria. The general protocol used for Gram-negative cells (left) involves differential removal of non-PG components of the cell wall and can also be used for Gram-positive bacteria, with added steps to remove surface polymers (center). In the case of acid-fast bacteria (right), non-PG components of the mAGP complex must be removed before PG isolation. Upon removal of mycolic acids and arabinogalactan, the same protocol used for PG isolation from Gram-positive cells can be applied. In all cases, PG is collected as the insoluble material after all treatments described. EDTA, ethylenediaminetetraacetic acid; HF, hydrofluoric acid; mAGP, mycolylarabinogalactan-peptidoglycan; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

Glycoside: a molecule in which a sugar is bound to another functional group via a glycosidic bond.
Methanolysis: a chemical reaction used to hydrolyze polysaccharides and proteins under acidic conditions and simultaneously derivatize them with a methyl substituent group.
Mutarotation: the change in the optical rotation because of the change in the equilibrium between two anomers. Cyclic sugars (such as monosaccharides) show mutarotation as α and β anomeric forms interconvert.
Silylation: a derivatization procedure in which, typically, a trimethylsilyl group is attached to a certain molecule.
Stereoisomers: molecules that have the same molecular formula and sequence of bonded atoms, but differ in the 3D orientations of their atoms in space.
Transglycosylation: the transfer of a sugar residue from one glycoside to another.

Amino Acid Composition

The most traditional methods for amino acid composition analysis include the use of amino acid analyzers or thin layer chromatography (TLC) separation. Amino acid analyzers are widely used

Table 1. Methods Available for Determining Sample Composition

	Technique/Instrument	Refs
Amino acids	Amino acid analyzer Based on ion-exchange chromatography and ninhydrin labeling for UV detection.	[22–26]
	Waters PicoTag system Based on precolumn derivatization with Edman's reagent (phenyl isothiocyanate) followed by HPLC-UV separation.	[27–30]
	Derivatization For GC-MS analysis: Reagents such as tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MSBSTFA), hexamethyldisilazane (HMDS), bis-(trimethylsilyl)-trifluoroacetamide (BSTFA), trifluoroacetic acid (TFAA), or heptafluorobutyric anhydride (HFBA) are frequently used prior to GC-MS analysis. Alternatively, EZ: faast GC-MS kits, which combine solid-phase extraction (SPE) with derivatization of both the carboxylic acid and amino groups can be used. For HPLC analysis: Ortho-phthaldialdehyde (OPA) derivatization can be used as pre- or post-column detection reagent for amino acid analysis by HPLC. Derivatives can be detected and quantified using fluorescence detection. To determine **stereoisomer** configuration: Amino acids are derivatized with L-FDAA (Nα-(2,4-dinitro-5-fluoro-phenyl)-L-alanineamide) followed by liquid chromatography–mass spectrometry (LC-MS) analysis or using GC-MS **chiral** columns.	[13,31–39] (J.K. Loraine, PhD thesis, University of Leicester, 2013)
Monosaccharides	Alditol acetates **Alditol** acetates are one of the most classical derivatization methods for glycosyl composition analysis. After hydrolysis, polysaccharides are reduced and acetylated to produce alditol acetates, which can then be separated and analyzed by GC-MS. These derivatives are widely used for identification of neutral as well as amino sugar residues, producing a single peak for each derivatized monosaccharide and allowing sample storage for long periods of time. Thus, this method has been broadly applied to PG analysis for a long time, mostly with the purpose of identifying PG-specific MurNAc residues.	[35,40–45]
	Trimethylsilyl (TMS) methyl glycosides Another common derivatization method used for glycosyl composition analysis is **silylation** after **methanolysis** and re-*N*-acetylation. This results in the formation of TMS methyl **glycosides**, which are then analyzed by GC-MS. This method has the advantage of allowing identification of neutral, amino, and acidic sugar residues.	[40,46]
	High-pH anion-exchange chromatography (HPAEC) This highly sensitive technique separates mono and oligosaccharides under alkaline conditions, and it is frequently coupled with pulsed amperometric detection (PAD). In contrast to GC-MS methods, this procedure does not require chemical derivatization after acidic depolymerization of PG.	[47,48]

but, despite being a reliable option, the need for specific instrumentation limits their widespread application. On the other hand, TLC separation is restricted to qualitative analysis. Resolution can be improved using 2D-TLC; however, diamino acids fundamental for crosslinking may not

be identified by this technique [13]. Alternatively, PG-derived amino acids can be derivatized with different reagents prior to gas chromatography–mass spectrometry (GC-MS) or HPLC analysis (Table 1). **Derivatization** improves analytical performance and chromatography, thus allowing analysis of a compound that may not be suited for a specific technique in its native form.

Monosaccharide Composition

Glycosyl composition analysis is an important first step in the structural characterization of complex carbohydrates, and it provides information not only on the type and amount of each monosaccharide present but also on the overall percentage of total carbohydrate in the sample, and thus is a good indicator of sample purity. Some of the procedures used for amino acid analysis are also applicable for monosaccharide composition. For instance, heptafluorobutyric anhydride (HFBA) derivatization can also detect monosaccharide residues [20], including GlcNAc and MurNAc [20,21]. Nevertheless, there are other methods more specific for glycosyl residue analysis that can be used to determine the monosaccharide composition of a PG sample (Table 1).

Autolysins: Breaking Down PG for Analysis

PG is a high-molecular-weight polymer, thus it is insoluble unless partially digested or hydrolyzed. Therefore, most methods used for PG structural characterization require prior solubilization of PG samples, which can be achieved by digestion with PG hydrolases. There are several different classes of PG hydrolase [49], defined based on their specificity for PG covalent bonds (Figure 2). These enzymes, called autolysins, are part of the enzymatic machinery that regulates PG recycling in bacteria [50–52].

Amidases, Endopeptidases, and Carboxypeptidases

N-acetylmuramoyl-L-alanine amidases (amidases) hydrolyze the amide bond between MurNAc and L-Ala, separating the glycan chain from the peptides, and releasing *stem peptides*. This family of enzymes is one of the most commonly used to hydrolyze PG samples [53–58]. One example is the amidase domain from *S. aureus* Atl autolysin, a bifunctional enzyme also containing an *N*-acetyl-glucosaminidase domain (see 'Glycosidases' below) which is frequently used in PG characterization studies [59–61]. Another example of a widely used amidase is LytA from pneumococcus [54,57,62]. Cleavage under alkaline conditions [63–65] can be used as an alternative to the use of enzymes to obtain lactoyl peptides, which correspond to stem peptides containing the lactoyl group of MurNAc.

Endo- and carboxypeptidases hydrolyze the LD- and DD-bonds in PG stem peptides. These enzymes are referred to as DD-peptidases if they cleave the bond between D-amino acids, and are referred to as DL- or LD-peptidases if they cleave the bond between D- and L- amino acids [52]. A commonly used example of these enzymes is lysostaphin, a glycyl-glycyl endopeptidase cleaving the pentaglycine bridges of *S. aureus*, and thus broadly used to mimic the effect of penicillin or identify the type of peptide cross-link [60,66–68].

Glycosidases (*N*-Acetyl-Glucosaminidases, *N*-Acetyl-Muramidases)

These enzymes cleave the β-1→4 glycosidic bonds of PG glycan strands, releasing glycopeptides containing at least one GlcNAc-MurNAc disaccharide unit still attached to its corresponding peptide chain, structures also called muropeptides.

N-acetyl-glucosaminidases hydrolyze glycosidic bonds between GlcNAc and other monosaccharides. This implies that these enzymes can act on PG, but also on other substrates such as chitin, chitosan, or *N*-glycans [49,69]. An example of this family of enzymes is NagZ [70,71], which is frequently used in the study of β-lactam antibiotic resistance [38,72,73].

N-acetyl-muramidases, often called simply muramidases, cleave the glycosidic bonds between MurNAc and GlcNAc residues. Two types of muramidase can be distinguished, depending on substrate specificity and how the glycosidic bond is cleaved: lysozymes and lytic transglycosylases (LTs).

Lysozymes

These are endo-*N*-acetyl muramidases whose hydrolysis product contains a terminal reducing MurNAc residue (Figure 2). There are four different classes of lysozyme [hen egg-white lysozyme (HEWL), goose egg-white lysozyme (GEWL), bacteriophage T4 lysozyme (T4L), and *Chalaropsis* lysozyme], with well characterized structures and activities [49]. A particular enzyme in this family is cellosyl, a *Chalaropsis* enzyme which also exhibits a β-1,4-*N*,6-*O*-diacetylmuramidase activity and is able to degrade *O*-acetylated PG, which is present in the cell walls of *S. aureus* and other pathogens, and is typically resistant to other lysozymes [56,74]. Another example of a *Chalaropsis* lysozyme is the autolysin LytC from *Streptococcus pneumoniae* [75]. Similar to cellosyl in terms of activity, and with identical amino acid sequence [74,76], mutanolysin is a commercially available muramidase (unlike cellosyl) purified from *Streptomyces globisporus,* which is able to lyse *Listeria, Lactobacillus, Lactococcus*, and pathogens such as *S. aureus* and is probably the most broadly used muramidase in PG analysis [39,60,77]. Thus, muramidases with nonspecific activity, like mutanolysin and cellosyl, are preferable for most applications, although in some cases they have been used in combination with other lysozymes [63,64].

Lytic Transglycosylases (LTs)

These are exo-*N*-acetyl muramidases (that can also show endolytic activity [78]) whose reaction products undergo intramolecular **transglycosylation**, resulting in the formation of 1,6-anhydroMurNAc-containing disaccharide peptides, released from one of the ends of the glycan chain (Figure 2). The 1,6-anhydroMurNAc-muropeptides resulting from this reaction are then recycled in the cytoplasm and reused for *de novo* synthesis of new PG [79], and can contribute to bacterial virulence, while enabling defense mechanisms such as antibiotic resistance [7]. Furthermore, anhydromuropeptides can be released to the environment, acting as immunomodulatory effectors [80]. So far, six different families of LTs have been described, grouped according to their substrate preference and catalytic folds [7,81,82]. However, these enzymes are not commercially available and usually have to be purified in-house.

Structural Analysis: Understanding PG at the Molecular Level

Analysis of Stem Peptides/Muropeptides

Although low-resolution chromatographic methods like paper chromatography and gel permeation have been traditionally used to separate soluble PG fragments [19,83], ever since Glauner established the first HPLC approach to muropeptide separation in the late 1980s [84], HPLC indisputably became the preferred analytical method for separation and purification of stem peptides and muropeptides, as it provides comprehensive, high-resolution analysis of PG composition [46]. (Note: in some cases, muropeptides were separated using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) instead of HPLC [47]). Prior to HPLC separation, muropeptides are typically reduced with sodium borohydride to prevent peak doubling as a result of **mutarotation** of the reducing-end glycosyl residue of the muropeptide [48]. Typically, separation of muropeptide/stem peptide species is performed in reversed-phase C18 columns using sodium phosphate/methanol-buffered mobile phases, followed by UV detection (204–208 nm) [62,85–89]. This separation is usually long, taking up to 1–2 h, and the separated peaks are subsequently collected and desalted (in the same HPLC column used for separation or in smaller formats such as ZipTips) in preparation for MS analysis.

The preferred technique for offline MS analysis of purified muropeptides is matrix-assisted laser desorption ionization–time-of-flight–mass spectrometry (MALDI-TOF-MS) [with some reports of MALDI post source decay (MALDI-PSD)] mainly because it has an appropriate mass range for these analytes, requires small amounts of sample, and is relatively tolerant to salts (which may remain in the samples if buffer desalting is not complete) [28,46,86,90–93]. Alternatively, muropeptide or stem peptide HPLC peaks can be analyzed offline by quadrupole ion trap mass spectrometry (Q-Trap-MS) [94], or electrospray ionization–mass spectrometry (ESI-MS) [95,96].

Predictably, offline HPLC-MS analysis is long and tedious. More recently the development of UPLC methods, which can withstand much higher pressures than HPLC, has considerably improved resolution and speed of analysis. UPLC has other advantages such as low sample volumes (1–10 µl) and short run times (5–20 min) and, in fact, the number of references using UPLC for muropeptide analysis has increased considerably as these instruments become accessible to more laboratories [89,97–99]. However, notwithstanding the improvement in run time introduced by UPLC methods, when relying on UV detection, fractionation, desalting, and further offline MS analysis are still necessary steps to characterize muropeptides/stem peptides in terms of structure and molecular weight. This means that, overall, the total gain in analysis time just by switching from HPLC to UPLC is not significant. Indeed, most current quantification methods based on UV absorbance are still very long. Furthermore, and despite being the best option for quantification purposes, UV detection is heavily dependent on available standards (whether individual muropeptides or a reference chromatogram) for peak identification. Thus, the introduction of online UPLC-MS methods has proved to be a remarkable advance. By using MS-compatible mobile phases [formic acid or trifluoroacetic acid (TFA) in methanol or acetonitrile], these methods were able to reduce total sample preparation and analysis time to an impressive 24 h [48].

Despite the advantages of UPLC-MS methods, perhaps the biggest recent advancement in muropeptide analysis has been the use of online liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) methods [31,100]. Besides overcoming the time-consuming peak collection and desalting, online LC-MS/MS methods are associated with one major benefit: automated data analysis. Bern *et al.* [101] reported the first automated tandem MS data analysis strategy for systematic structural analysis of muropeptide samples. This approach allows one to search for modifications such as *O*-acetylation, deacetylation, amidation, formation of 1,6-anhydro-MurNAc ends, and even unanticipated modifications. This methodology not only allowed the identification and quantification of all previously reported PG monomers and dimers of *Clostridium difficile*, leaving disambiguation of 3-3 and 4-3 cross-linking as the only manual interpretation step, but it also detected some new structures not reported elsewhere.

Another notable development is the introduction of chemometric tools for bioinformatic analysis of large PG datasets. While the advances in chromatography/mass spectrometry techniques have enabled high-throughput screenings of muropeptide profiles, they have also generated large amounts of data, which can be difficult to analyze manually. Chemometrics uses advanced statistical methods such as principal component analysis (PCA) and constraint randomized non-negative factor analysis (CRNNFA) for faster and efficient analysis of large data sets resulting from analytical instruments such as UPLC and nuclear magnetic resonance (NMR) [98,102,103]. Recently, these approaches have been applied to large-scale comparison of UPLC chromatograms and used for sample classification and outlier identification. Using these methods, large numbers of samples can be grouped according to their PG composition, allowing the exploration of PG chemical diversity in an unprecedented way. Furthermore, chemometric tools have proven to be useful in the elucidation of novel PG structures conserved in Acetobacteria, such as amidation at the α-(L)-carboxyl of mesodiaminopimelic acid and the presence of muropeptides crosslinked by (1 – 3) L-Ala-D-(meso)-diaminopimelate crosslinks [98]. The main shortcoming of

these methods is the current nature of the datasets, usually UPLC-UV chromatograms, which are associated with the previously discussed limitations of UV detection.

In a less frequently used alternative procedure to HPLC/UPLC, muropeptides were size-fractionated by size-exclusion chromatography (SEC), labeled with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and further separated electrophoretically, using fluorophore-assisted carbohydrate electrophoresis (FACE) with UV detection. The bands collected from FACE gels can then be analyzed by MALDI-MS [104].

In some cases, whether as a complement of MS analysis of isolated muropeptides [29,31,98] or as a stand-alone method of analysis for cell wall or purified PG samples [62,105–108], 2D-NMR and solid-state NMR have been used to determine the identity of muropeptide species or elucidate the 3D structure of bacterial PG. Information obtainable by 2D-NMR includes monosaccharide composition, **anomeric** configurations, linkage positions, branch points, monosaccharide sequences, noncarbohydrate substituents, as well as conformational information. Furthermore, NMR has the advantage of being nondestructive to the sample, allowing coupling with other techniques, making it a powerful tool for comprehensive structural analysis. In fact, provided that there is enough sample, 2D-NMR spectroscopy is probably the single most powerful technique for the elucidation of precise carbohydrate structures. However, NMR analysis is hindered by the insolubility of PG. Analysis of intact PG requires the use of solid-state NMR, which not only is not a commonly accessible technique, requiring specialized facilities and instrumentation, it is also insensitive, thus requiring large amounts of sample or long analysis times. In turn, analysis of solubilized PG, in the form of muropeptides, is hampered by the fact that the sample is a mixture of molecules, which significantly complicates data analysis. Indeed, solution-state NMR is best used in combination with HPLC/UPLC separation of muropeptides, much like MS, but with the added disadvantage of low sensitivity. Nevertheless, it is worth mentioning the use of rotational-echo double-resonance (REDOR) NMR in the characterization of the tertiary structure of PG from different pathogens. REDOR NMR is a solid-state NMR experiment that can provide angstrom-resolution distance constraints necessary for structural characterization, while allowing analysis of the chemical composition of PG in cell walls or intact whole cells [109]. By growing cells in defined media containing combinations of ^{13}C- and ^{15}N-specific labels in D- and L-alanine and L-lysine (in the presence of an alanine racemase inhibitor), this approach allows unambiguous assignments of amino acid positions and linkages in the peptide stem and crosslinks. For example, REDOR NMR has been used in the elucidation of the PG lattice assembly for FemA mutants of *S. aureus*, which is consistent with a tightly packed, hybrid architecture containing both parallel and perpendicular peptide stems in a repeating structural motif [110], contrasting with the parallel-stem architecture of wild-type *S. aureus* [111]. Interestingly, applying the same technique to whole cells of *Enterococcus faecalis*, based on the connectivity of L-alanyl carbonyl-carbon bridge labels to D-[^{13}C]alanyl and L-[ε-^{15}N]lysyl stem labels, Yang *et al.* [112] were able to show that the PG of *E. faecalis* has the same hybrid short-bridge architecture as the FemA mutant of *S. aureus*.

Glycan Strand Length

The bulk properties of PG are a consequence of its nanoscale structure. Therefore, characteristics like glycan strand length and crosslinking degree are reflective of the overall 3D structure of PG [113] and are often used as a metric to compare PG from different species [5]. The length of glycan strands can be determined by different methods. Older protocols include quantification of the fraction of reducing hexosaminosyl residues after chemical reduction [114,115] or quantification of radioactive galactosamine residues enzymatically attached to the GlcNAc end [116]. A more recent approach consists in estimating the length of the glycan strands from the fraction of 1,6-anhydro muropeptides, which indicates the end of glycan strands [46,84]. For example, Desmarais *et al.* [97] calculated the average glycan strand length according to: average glycan

strand length = 100 muropeptides/sum of (% molar fraction of all anhydro peaks). However, this can only be applied to Gram-negative species, where 1,6-anhydro MurNAc residues are present [4].

As an alternative, glycan strands can be purified by digesting PG with an amidase that cleaves off the crosslinking stem peptides [62,117], followed by further purification in ion-exchange columns and size separation using HPLC for strands up to 30 disaccharides in length [4,19], and SEC for strands up to 200 disaccharides in length [118–121]. The main shortcoming of this approach is the difficulty in obtaining commercially available amidases to release the glycan strands from the stem peptides, implying that, as mentioned previously, often these enzymes have to be purified in-house [46]. Notably, upon purification following the protocol described above, AFM was applied to the study of *B. subtilis* glycan strands. This study was particularly relevant because it suggested for the first time that a glycan strand could be longer than a bacterial cell and that glycan strands must be 'wrapped' in some fashion in order to fit onto the cell surface [119]. Studies like this have elucidated fundamental aspects of PG architecture. For instance, while it was thought that *Escherichia coli* possessed a PG consisting of a single layer with relatively short glycan chains running mainly perpendicular to the long axis of the cell [10], recent studies have proven otherwise. Using a combination of AFM and SEC, Turner *et al.* [122] have shown that glycan chains from *E. coli* in its normal rod shape are long and circumferentially oriented but become short and disordered in cells chemically or genetically induced to display a spheroid form. Furthermore, this study also revealed glycan chains up to 200 nm long, much longer than initially thought.

Degree of Crosslinking

The extent of PG crosslinking (crosslinking indices), as basically every single PG trait, varies for a specific species with growth phase [29] and culturing conditions, but also among bacterial species and strains [6]. This parameter provides an indirect measure for the density and resistance of PG [123] and it can also be related with antibiotic resistance [124]. Typically, the degree of peptide crosslinking is determined from a ratio based on the overall abundance of monomers and multimers [46,84,123]. For instance, Desmarais *et al.* [97] calculated the degree of crosslinking as: % crosslinking = % molar fraction dimers + 2× (% molar fraction trimers) + 3× (% molar fraction tetramers), where the multipliers are used to account for the number of crosslinks per oligomer (e.g., a trimer contains two crosslinks and therefore is multiplied by two). Other authors [30,87] calculated the crosslinking index using the classical formula proposed by Glauner [84], where the degree of crosslinkage is defined as the percentage of crossbridges of a specified type relative to the total number of disaccharide peptide subunits of this type.

Much like glycan chain length, the degree of crosslinking has been used to evaluate PG diversity among bacteria. It has been estimated that this parameter can vary from ca. 20% in *E. coli* to over 93% in *S. aureus* [114], which reflects the differences in cell wall architecture between Gram-negative and Gram-positive bacteria.

Additionally, the degree of crosslinking, together with other parameters, like glycan chain length, has been used to define glycopeptide antibiotic binding sites in *Enterococcus faecium* [107]. Using LC-MS/MS, the authors defined crosslinking as: (dimers + 2× trimer + 3× tetramers)/(monomers + 2× dimers + 3× trimers + 4× tetramers); and the obtained value (≈40%) was comparable with that directly measured by solid-state NMR (47%).

Percent of O-Acetylation

O-Acetylation of PG occurs specifically at the C-6 hydroxyl group of MurNAc and GlcNAc residues, forming a 2,6-*N,O*-diacetyl derivative [8,125]. This modification, which is both strain

specific and dependent on culture age, confers resistance to lysozyme, explaining why it has been identified in many Gram-positive as well as Gram-negative species [8]. Furthermore, it is generally accepted that *O*-acetylation of PG serves to control autolysin activity [126].

Because *O*-acetylation happens through an ester bond rather than an amide bond, *O*-acetyl groups can easily be removed under mild alkaline or acidic conditions [4]. Therefore, to evaluate the degree of *O*-acetylation a common strategy involves releasing acetate groups (from intact PG or muropeptides) using a mild alkaline treatment, followed by quantification of the released acetic acid by HPLC [127] or using assay kits [128,129]. The extent of *O*-acetylation can then be expressed as a percentage of the MurNAc content.

Imaging of PG Structure

Finally, imaging techniques such as cryo-TEM and AFM have become crucial tools in the study of PG 3D structure and architecture [130–139]. Electron microscopy (EM) has long been applied to the visualization of biomolecules and it originally revealed that isolated sacculi maintained the morphology of the bacterial cell [140]. Later, EM became useful in the study of PG biosynthesis, allowing visualization of where and when new PG is inserted into existing PG, something that was attained after the development of staining methods that distinguished new from old PG using microscopy [141]. However, EM sample preparation involves cell fixation and staining, which may disrupt the structure of PG, causing cell shrinking, and, in turn, lead to inaccurate measurements of cell size [4,142]. More recently, this pitfall has been overcome with the advent of cryo-TEM, which allows the observation of specimens without fixation or staining. By preserving samples in the frozen-hydrated state using liquid nitrogen, cryo-TEM preserves the components and morphology of cells, capturing the natural state of cell wall organization [143]. Thus, cryo-TEM captures a snapshot of a living cell at a single moment in time [144]. Moreover, a specialized cryo-TEM application, cryoelectron tomography (CET), allows visualizing biomolecules, such as PG, in three dimensions, with high resolution, by tilting the specimen relative to the incident electron beam [145–147]. Using CET, Gan *et al.* [135] were able to determine that the glycan strands were oriented parallel to the surface of PG and roughly perpendicular to the long axis of the cell, clarifying the long debate between the 'layered' and 'scaffold' models for PG architecture [5,148]. AFM has some advantages over TEM, namely the fact that samples can be analyzed in water or buffer, and that it can be used to measure the rigidity and elasticity of specimens, which can be particularly useful in the study of PG surface properties [130]. Notably, AFM has been used to evaluate the degree of crosslinking in *S. aureus* [149]. For a very thorough review on these and other methods for visualization of PG, the reader is referred to Hsu *et al.* [143].

Concluding Remarks and Future Perspectives

Over the past decades, a lot of effort has been put into the improvement of methodologies for PG analysis. The choice of protocols for analysis of a particular sample relies obviously on the available instrumentation. However, some strategies provide more information than others, and the final goal of the project should dictate which protocol is most suitable. In terms of PG isolation, classical protocols are still commonly applied and these have not changed much since their development. A typical isolation strategy for Gram-positive PG involves extraction of cell pellets with boiling 5% sodium dodecyl sulfate (SDS), followed by pronase digestion, and treatment with hydrofluoric acid (HF). Depending on the nature of the sample, these steps can be modified according to Figure 3. We recommend performing composition analysis at the end of the purification procedure to determine sample purity. As described in Table 1, there are several methods that can be used for composition analysis, although GC-MS analysis of HFBA derivatives is probably the most effective for PG samples since it can detect both amino acids and monosaccharides. The presence of non-PG amino acids in the GC-MS chromatogram is a good indication that the sample is not pure.

Outstanding Questions

How can we upgrade old methods and optimize protocols for faster PG purification?

It is possible to solubilize PG, without the use of hydrolases, to allow for its analysis by solution-state NMR?

Will advances in MS software tools be able to turn LC-MS/MS data analysis into a fully automated process?

Will new tools help to elucidate currently unknown features of PG structure?

When aiming for structural analysis of PG, the most comprehensive strategy involves the enzymatic production of muropeptides, preferentially using mutanolysin, and their subsequent analysis by LC-MS/MS methods. Modern instrumentation offers the possibility of creating data-dependent fragmentation strategies, combining higher-energy collisional dissociation (HCD) and electron-transfer dissociation (ETD) fragmentation, where diagnostic oxonium ions (glycan-derived low-molecular-weight ions) formed by HCD can trigger ETD fragmentation, thus providing highly specific full sequence information. With current tools, PG can be, structurally, fully characterized. Nevertheless, there is still room for improvement in terms of protocols and techniques (see Outstanding Questions). The combination of analytical methods (chromatography, mass spectrometry, and spectroscopy) with imaging techniques allows in-depth study of this fascinating molecule whose biological roles we have known for so long, and yet, keeps surprising us.

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