Uncovering the activities, biological roles, and regulation of bacterial cell wall hydrolases and tailoring enzymes

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Bacteria account for 1000-fold more biomass than humans. They vary widely in shape and size. The morphological diversity of bacteria is due largely to the different peptidoglycan-based cell wall structures that encase bacterial cells. Although the basic structure of peptidoglycan is highly conserved, consisting of long glycan strands that are cross-linked by short peptide chains, the mature cell wall is chemically diverse. Peptidoglycan hydrolases and cell wall–tailoring enzymes that regulate glycan strand length, the degree of cross-linking, and the addition of other modifications to peptidoglycan are central in determining the final architecture of the bacterial cell wall. Historically, it has been difficult to biochemically characterize these enzymes that act on peptidoglycan because suitable peptidoglycan substrates were inaccessible. In this review, we discuss fundamental aspects of bacterial cell wall synthesis, describe the regulation and diverse biochemical and functional activities of peptidoglycan hydrolases, and highlight recently developed methods to make and label defined peptidoglycan substrates. We also review how access to these substrates has now enabled biochemical studies that deepen our understanding of how bacterial cell wall enzymes cooperate to build a mature cell wall. Such improved understanding is critical to the development of new antibiotics that disrupt cell wall biogenesis, a process essential to the survival of bacteria.

At least a million different species of bacteria inhabit the earth, and they are incredibly diverse with respect to size, shape, life cycle, and ecological niche (1, 2). Bacteria can be as small as 0.2 μm in diameter or larger than 300 μm across (3). They come in many shapes, including spheres, rods, spirals, crescents, and even stars (4–6). Some bacteria can adopt alternate shapes in response to changes in the environment or during different phases of growth. Although many factors contribute to establishing bacterial size and shape, the peptidoglycan cell wall is central (7).

Peptidoglycan is a polymer composed of linear carbohydrate chains held together by peptide cross-links (8). This polymer, which is present in nearly all bacteria, forms a single, enormous macromolecule that surrounds the cell with the glycan chains oriented roughly parallel to the cytoplasmic membrane (9, 10). Gram-negative bacteria have a thin peptidoglycan layer between the inner and outer membrane, whereas Gram-positive bacteria have a thick peptidoglycan cell wall containing many layers of glycan chains (Fig. 1*A*) (11). Whether thin or thick, the peptidoglycan cell wall is essential not only for bacterial morphology but also for bacterial survival. Indeed, the role of peptidoglycan in bacterial survival has made peptidoglycan biosynthesis a target for important classes of antibiotics, including the β-lactams (*e.g.* the penicillins, cephalosporins, and carbapenems) and the glycopeptides (*e.g.* vancomycin) (12). Despite the importance of the cell wall both as a therapeutic target and as the key determinant of bacterial morphology, we still know relatively little about how it is assembled and remodeled during bacterial growth, division, and development (13–15). Moreover, much of what we do know about peptidoglycan assembly and remodeling is based on mutant phenotypes because the development of biochemical tools to study these processes has lagged behind the development of genetic and cell biological tools. Interpreting the biological functions of cell wall enzymes based on mutant phenotypes alone is problematic because these enzymes can have distinct biochemical and functional activities even if the mutants produce similar terminal phenotypes (*e.g.* cell growth or division defects). With recent advances in obtaining defined substrates for cell wall biochemistry, we are moving toward a more complete understanding of peptidoglycan biogenesis (16). This knowledge will facilitate the development of new antibiotics that specifically target and cripple cell wall assembly to ultimately kill the bacteria.

Peptidoglycan is built from a lipid-linked disaccharide-peptide precursor called Lipid II, which is synthesized inside the cell in two stages (Fig. 1*B*) (17, 18). The first stage takes place in the cytoplasm, where soluble enzymes convert GlcNAc to *N*-acetylmuramic acid (MurNAc)[2]-pentapeptide (19). The pentapeptide varies between different bacterial species. The

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[2] The abbreviations used are: MurNAc, *N*-acetylmuramic acid; GT, glycosyltransferase; PBP, penicillin-binding protein; aPBP and bPBP, class A and B PBP, respectively; RBB, Remazol Brilliant Blue; WTA, wall teichoic acid; CPS, capsular polysaccharide; SEDS, shape, elongation, division, and sporulation; ColM, colicin M; PDB, Protein Data Bank; anhMurNAc, 1,6-anhydro-*N*-acetylmuramic acid.

most common structures are L-Ala-D-isoGlu-mesoDAP-D-Ala-D-Ala in Gram-negative organisms and L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ala in Gram-positive organisms. In the next stage, MurNAc-pentapeptide is coupled to a C_{55}-undecaprenyl phosphate carrier lipid that is anchored in the cytoplasmic membrane. This pyrophosphate exchange reaction produces Lipid I, which is converted to Lipid II by the transfer of GlcNAc from UDP-GlcNAc to the C4 hydroxyl of Lipid I. In some species, the stem pentapeptide of Lipid II is additionally tailored. In many Gram-positive organisms, isoGlu at the second position is converted to isoGln by amidation of the α-carboxylate, and a short branch is attached to the L-Lys side chain (12). For example, *Staphylococcus aureus* has a pentaglycine branch, *Streptococcus pneumoniae* has an L-Ser-L-Ala or L-Ala-L-Ala branch, *Enterococcus faecalis* has an L-Ala-L-Ala branch, and *Enterococcus faecium* has a D-Asp branch. Once fully assembled, Lipid II is exported by the flippase MurJ across the cell membrane for incorporation into the cell wall (20, 21).

Cell wall assembly begins with polymerization of Lipid II by glycosyltransferases (GTs). The GTs add new Lipid II units to the reducing end of a growing glycan chain, releasing the carrier lipid in the process to be recycled back inside the cell (Fig. 1*B*) (22, 23). The polymer chains are then cross-linked by transpeptidases to the existing peptidoglycan matrix. Cross-linking occurs in two steps, starting with formation of a covalent intermediate via transpeptidase attack of the terminal amide bond in a donor stem peptide (Fig. 1*C*). A new peptide cross-link is formed when the intermediate reacts with the nucleophilic amine of an adjacent acceptor stem peptide. The transpeptidases are the targets of penicillin and other β-lactam antibiotics, which is why they are referred to as penicillin-binding proteins (PBPs) (24). There are two distinct families of glycosyltransferases that make the peptidoglycan polymers. The best-characterized glycosyltransferases are exemplified by the N-terminal domains of class A penicillin-binding proteins (aPBPs) (25). For over 60 years, it was believed that these glycosyltransferase domains were the only machinery that could polymerize Lipid II. This changed in 2016 when RodA, an essential integral membrane component of the cell elongation machinery, was shown to have Lipid II polymerization activity (26). In 2019, FtsW, a related protein essential for cell division, was also found to polymerize Lipid II (27). These SEDS (shape, elongation, division, and sporulation) proteins are structurally and mechanistically distinct from the aPBPs, and they function in complex with monofunctional bPBP transpeptidases (27–29). It is remarkable that it took this long to identify an enzymatic function for such a highly conserved family of proteins. A major impediment to progress was the lack of sufficient substrates for reconstitution.

The peptidoglycan synthases have been the subject of intense research, but they alone cannot assemble a complete cell wall. Cell wall maturation requires installing tailoring modifications, controlling the degree of cross-linking, trimming glycan strands to their final lengths post-synthesis, and growth-phase remodeling (12, 30). Peptidoglycan hydrolases[3] play key roles in cell wall maturation (31–33). Many hydrolases that cleave different bonds in peptidoglycan have been identified and can be readily discovered by mining microbial genomes for characteristic hydrolase domains. However, we are only beginning to understand the many ways that these hydrolases act in cell wall assembly and remodeling. We have also begun to appreciate that understanding hydrolases requires identifying regulatory mechanisms that control their activities. Here we will use selected examples to highlight the activities, biological roles, and regulation of hydrolases. We will show how the ability to obtain defined substrates for biochemical study of hydrolases and other peptidoglycan-modifying enzymes has deepened our understanding of their biological functions.

Peptidoglycan hydrolases have diverse biochemical activities

For virtually every linkage in peptidoglycan, there is a hydrolase with the capacity to cleave it (31). The first peptidoglycan hydrolase was discovered in 1922 by Alexander Fleming, who isolated a bacteriolytic agent that he called "lysozyme" from the nasal mucus of a patient with rhinitis (34). Lysozyme belongs to a class of hydrolases known as the *N*-acetylmuramidases, which cleave the β-1,4-glycosidic linkage between MurNAc and GlcNAc. New classes of hydrolases have since been discovered.

Peptidoglycan hydrolases can be broadly classified as glycosidases or peptidases based on their chemical cleavage specificity (Fig. 2) (31, 35). In addition to *N*-acetylmuramidases, there are two other types of glycosidases. *N*-Acetylglucosaminidases cleave the β-1,4-glycosidic bond between GlcNAc and MurNAc. Lytic transglycosylases nonhydrolytically cleave the β-1,4-glycosidic bond between MurNAc and GlcNAc with the concomitant conversion of MurNAc into anhMurNAc (1,6-anhydro-*N*-acetylmuramic acid) via an intramolecular reaction. Peptidases, on the other hand, attack linkages within the stem peptide of peptidoglycan. *N*-Acetylmuramoyl-L-alanine amidases release stem peptides from the glycan backbone by hydrolyzing the amide bond between L-Ala at the first position and the lactyl group of MurNAc. DD-Carboxypeptidases are exolytic peptidases that convert stem pentapeptides into tetrapeptides by cleaving the terminal D-Ala–D-Ala bond. There are also LD-carboxypeptidases that further convert stem tetrapeptides into tripeptides by cleaving the L-D bond between mesoDAP or L-Lys at position 3 and D-Ala at position 4. The other amide linkages within the stem peptide, and within interpeptide bridges of cross-linked peptidoglycan, are processed by endopeptidases.

Functional diversity of peptidoglycan hydrolases

The biochemical diversity of peptidoglycan hydrolases translates to even greater functional diversity. Bacterial predators such as bacteriophages produce peptidoglycan hydrolases to pierce the cell wall of their hosts during host cell infection (36–38). *Bdellovibrio bacteriovorus* is another bacterial predator that weaponizes peptidoglycan-modifying enzymes to manipulate the host cell niche (39). To compete with other bacteria, *Pseudomonas aeruginosa* uses a type VI secretion system to deliver the peptidoglycan hydrolase effectors Tse1 and Tse3 into the periplasmic compartment of an enemy cell, where they chew the cell from within (40). As a first line of defense, we

[3] In this review, the term hydrolase refers to lytic enzymes that are hydrolases or lyases (*e.g.* lytic transglycosylases).

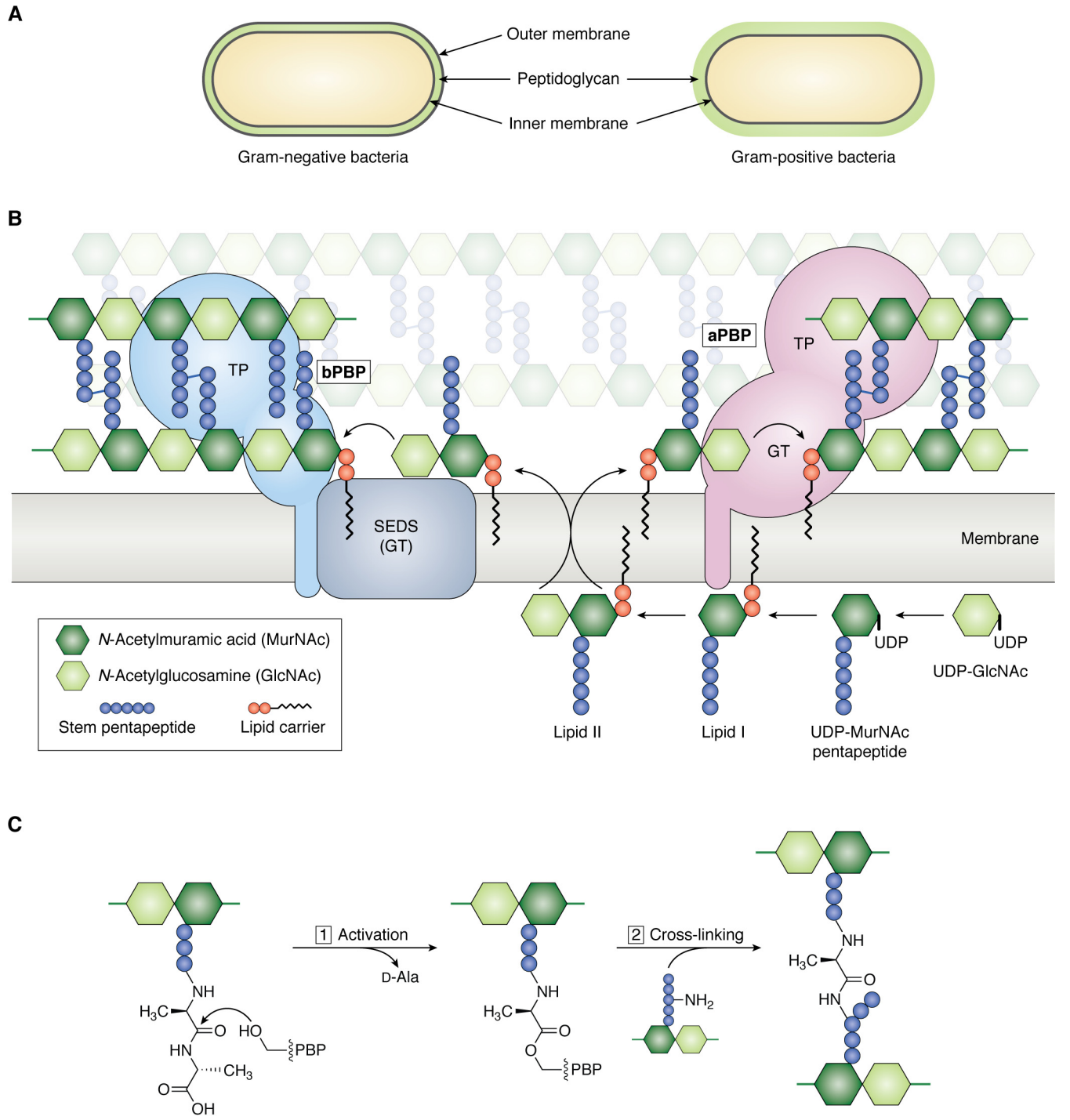


Figure 1. Overview of peptidoglycan assembly pathway. *A*, Gram-negative bacteria have an inner (*IM*) and outer (*OM*) cell membrane; a thin layer of peptidoglycan is sandwiched between the cell membranes. Gram-positive bacteria only have an inner cell membrane that is surrounded by a thick layer of peptidoglycan. *B*, Lipid II, the undecaprenyl pyrophosphate lipid-linked precursor for peptidoglycan synthesis, is assembled on the inner leaflet of the inner cell membrane. Once fully assembled, Lipid II is flipped to the outer leaflet of the inner cell membrane by a flippase. Peptidoglycan GTs polymerize Lipid II into glycan strands, which are cross-linked by transpeptidases (*TP*) into the existing matrix. There are two families of glycosyltransferases: the GT module of aPBPs and SEDS proteins. Class A PBPs are bifunctional, meaning they have polymerization and cross-linking activities. SEDS proteins cooperate with a partner bPBP, which only has transpeptidase activity. *C*, the active-site serine of PBP transpeptidases attacks the terminal D-Ala–D-Ala amide bond in a stem peptide (donor), forming an acyl-enzyme covalent intermediate and kicking out the terminal D-Ala. Resolution of the covalent intermediate occurs upon reaction with a nucleophilic amine from an incoming stem peptide (acceptor), producing a new peptide bond that links two glycan strands.

produce lysozyme in our mucus membranes to kill bacterial invaders (34). However, hydrolases are not simply lytic enzymes that destroy the cell wall. Their activities are harnessed to support cell growth, division, and differentiation, enabling bacteria to propagate and adapt to changing environmental conditions (Fig. 3).

Many hydrolases function to effect daughter cell separation. When bacteria divide, they form a partition called a septum that

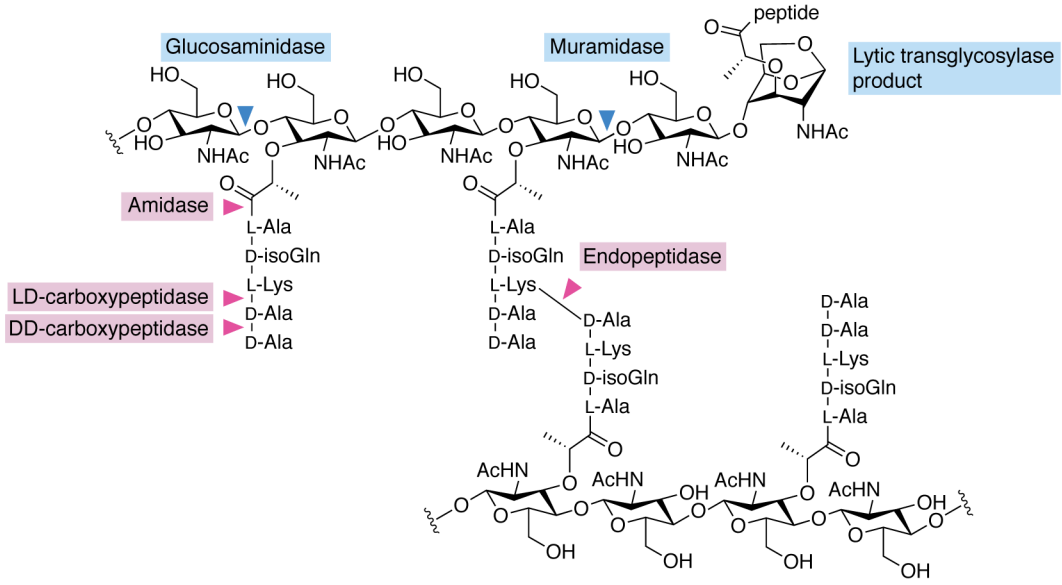


Figure 2. Cleavage sites of peptidoglycan hydrolases. Enzymes that hydrolyze peptidoglycan are broadly classified as glycosidases and peptidases, depending on where they cleave. Glycosidases (glucosaminidases, muramidases, and lytic transglycosylases) cleave within the glycan backbone. Peptidases (amidases, endopeptidases, LD-carboxypeptidases, and DD-carboxypeptidases) cleave peptide cross-links and within the stem peptides. The *carets* represent sites of hydrolysis.

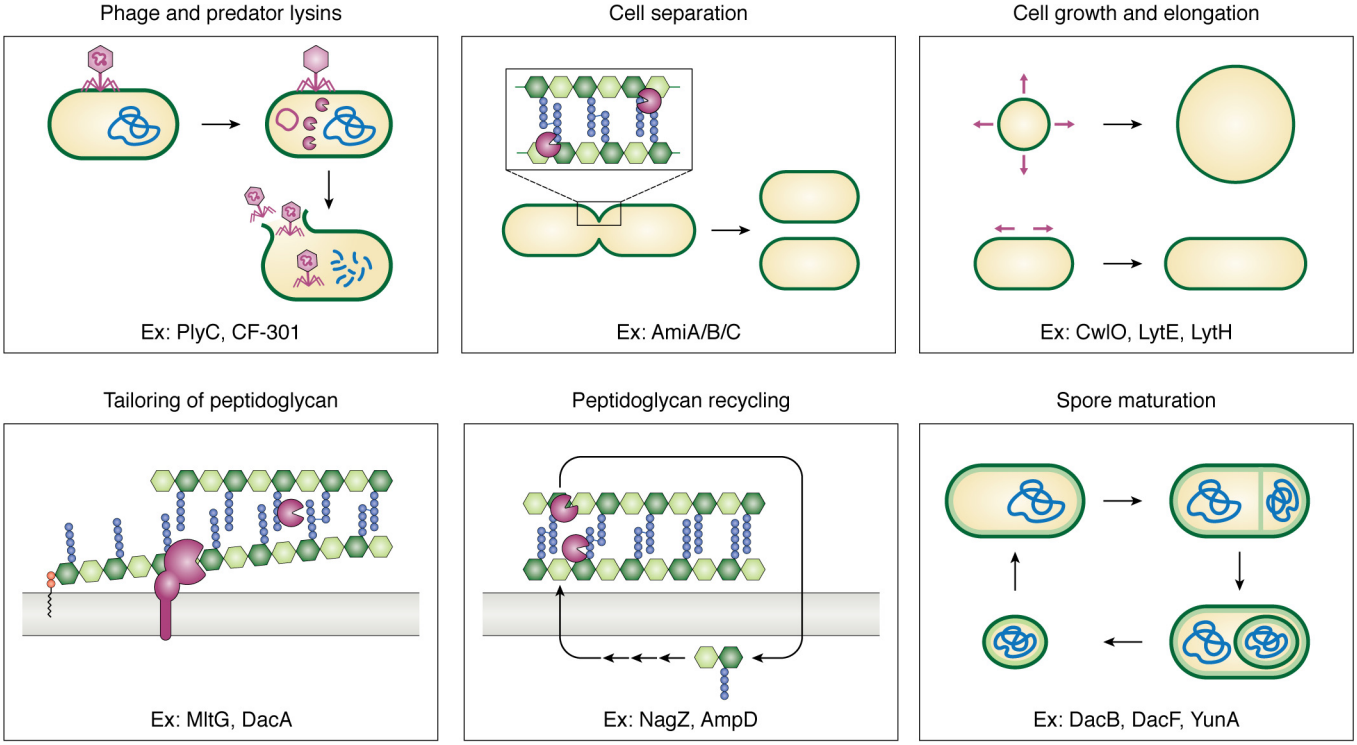


Figure 3. Functions of peptidoglycan hydrolases. Bacterial predators weaponize hydrolases to degrade the peptidoglycan cell wall of their hosts, leading to host cell lysis. But hydrolases are more than just lysins. Bacteria harness endogenous hydrolases to support fundamental cellular processes. Peptidoglycan hydrolases play important roles in bacterial cell growth, differentiation, and the separation of daughter cells that have divided. They also tailor the peptidoglycan cell wall, controlling the length of glycan strands and the degree of cross-linking. Bacteria that recycle components of the cell wall use hydrolases to break the peptidoglycan matrix into smaller pieces that are transported back into the cell.

is shared between daughter cells (41). Each daughter cell contains a membrane with a shared layer of peptidoglycan that must be split for separation to occur. This splitting is catalyzed by hydrolases. Bacterial mutants lacking splitting hydrolases fail to properly divide and form chains of unseparated cells. For example, an *Escherichia coli* triple deletion mutant lacking the amidases AmiA/B/C displays a filamentation phenotype in which cells that have divided remain stuck together (42).

Hydrolases are also needed for cell growth and development prior to cell division. As cells grow in size, hydrolases ensure that the peptidoglycan polymer surrounding the bacterium can stretch to accommodate cell expansion. These hydrolases break

peptidoglycan cross-links in the existing matrix to make space for insertion of new peptidoglycan (43). For example, in *Bacillus subtilis*, CwlO and LytE are endopeptidases that break peptidoglycan cross-links along the lateral cell wall to support cell elongation. Deletion of either *cwlO* or *lytE* produces shorter cells, and a double deletion mutant is nonviable (44).

Bacteria also deploy hydrolases to chemically modify the structure of peptidoglycan to control its physical properties. Peptidoglycan in different organisms varies considerably in the length of glycan strands and the degree of cross-linking; the identity of the sugar at the ends of glycan strands can also vary. These physical properties affect cell wall rigidity, which in turn can have significant functional consequences. A rigid or stretchy cell wall can be the difference between life or death for a bacterium that is subjected to unfavorable osmotic environments (45). In *E. coli*, the lytic transglycosylase MltG has been proposed to control strand length by cleaving within a glycan strand. In so doing, MltG produces an anhMurNAc sugar at the reducing end of one strand and a GlcNAc sugar at the nonreducing end of the other strand (46). In *S. aureus*, the *N*-acetylglucosaminidase SagB has also been proposed to control strand length by cleaving within a glycan strand. But in this case, SagB produces a GlcNAc sugar at the reducing end of one strand and a MurNAc sugar at the nonreducing end of the other strand (47). The nature of the termini of glycan strands may dictate whether the strands can be further processed by downstream cell wall-tailoring enzymes (48). Of the *S. aureus* glucosaminidases, SagB is believed to play the predominant role in controlling peptidoglycan strand length, which is a key determinant of cell wall stiffness (49). Another property that determines cell wall stiffness is the degree of peptidoglycan cross-linking (45). Carboxypeptidases that trim stem pentapeptides into tetrapeptides, which can only act as acceptor substrates in DD-transpeptidase reactions, are believed to control the extent of peptidoglycan cross-linking. Other types of hydrolases that act early in cell wall assembly to trim stem peptides may also regulate peptidoglycan cross-linking (31).

Peptidoglycan recycling is another important function of hydrolases. As bacteria grow, they shed components of the cell wall. These turnover products are recycled for reincorporation into the cell wall (50). Finally, peptidoglycan hydrolases mediate transitions between cell states. In sporulating species, hydrolases promote mother cell lysis and spore germination (51, 52). In actinobacteria such as *Mycobacterium tuberculosis*, muralytic enzymes are involved in resuscitating cells from a dormant state (53, 54).

Peptidoglycan hydrolases therefore play essential and diverse functions across the life cycle of bacteria. What determines the function of any given hydrolase is a combination of intrinsic properties, including whether it recognizes uncross-linked peptidoglycan, cross-linked peptidoglycan, or peptidoglycan that is chemically modified in some other way and its regulation. When and where a hydrolase acts is crucial to its biological function, and hydrolases must be carefully controlled to avoid cell lysis. In the following section, we will summarize some regulatory mechanisms for cell wall hydrolases.

Regulation of peptidoglycan hydrolases

Several mechanisms exist to prevent hydrolases from compromising the structural integrity of the cell. One level of regulation is provided by transcriptional control over the expression of cell wall genes. In *B. subtilis*, the *lytE* and *cwlO* endopeptidase genes are regulated by the WalKR signal transduction pathway that governs cell wall homeostasis (55). In response to an unknown signal generated during cell wall metabolism, *lytE* and *cwlO* transcription is regulated to keep pace with growth (56). Protein localization provides another level of control over hydrolases. The activity of cell wall enzymes can be confined to specific sites in the cell. In *E. coli*, the amidases AmiB and AmiC are recruited to the site of division at midcell, whereas AmiA remains dispersed throughout the periplasm (57). Some hydrolases have tags that direct them to specific compartments of the cell. In *S. aureus*, the endopeptidase LytN contains a YSIRK signal that exports it to the cross-wall (septum) and a cell wall–binding domain that targets it to peptidoglycan so that it can split daughter cells (58). Proteolysis of a hydrolase can also contribute to its regulation. The steady-state levels of MepS, an *E. coli* endopeptidase that cleaves cross-links to enable cell wall expansion and consequently cell growth, depend on Prc protease-mediated degradation (59). Bacteria can also chemically modify their cell wall as a mechanism for regulating hydrolases that only act on certain substrates. For example, the peptidoglycan in many organisms is *O*-acetylated at the C6 hydroxyl group of MurNAc. This modification protects against cleavage by lytic enzymes, such as lysozyme (60), but is also used by some organisms to control the activity of endogenous hydrolases (61). The activity of lytic transglycosylases is especially sensitive to the acetylation state of peptidoglycan due to the nature of the reaction, which involves an intramolecular nucleophilic attack at C1 of MurNAc by the C6 hydroxyl group. Consequently, *O*-acetylation of the C6 hydroxyl group inhibits lytic transglycosylase activity because an unmodified group is required for the reaction. In *Lactobacillus plantarum*, high levels of MurNAc acetylation induce autolysis by a putative amidase. The peptidoglycan of *L. plantarum* is also *O*-acetylated at GlcNAc residues; this modification prevents cleavage by the glucosaminidase and major autolysin Acm2 (62). The mechanisms by which *O*-acetylation controls the activities of these endogenous hydrolases are unknown. Finally, it has recently become apparent that many cell wall enzymes are regulated by direct interaction with other proteins. Starting in the late 2000s, regulator proteins that directly interact with hydrolases to inhibit or activate their activities were discovered in rapid succession.

The first protein proposed to be a direct inhibitor of a cell wall hydrolase was IseA in *B. subtilis*. In 2008, Sekiguchi and co-workers (63) found that IseA inhibits the cell wall lytic activity of the endopeptidase LytF *in vitro*. Overexpression of IseA in cells phenocopied the cell-chaining defect of a Δ*lytF* mutant, consistent with a role for IseA in inhibiting LytF. A solution NMR structure of IseA suggested that an acidic loop in IseA reaches into and blocks the positively charged active site pocket of LytF (64). IseA is proposed to inhibit LytF in the transition from vegetative growth to stationary phase when less LytF activity is needed as cell growth slows. A direct inhibitor of a

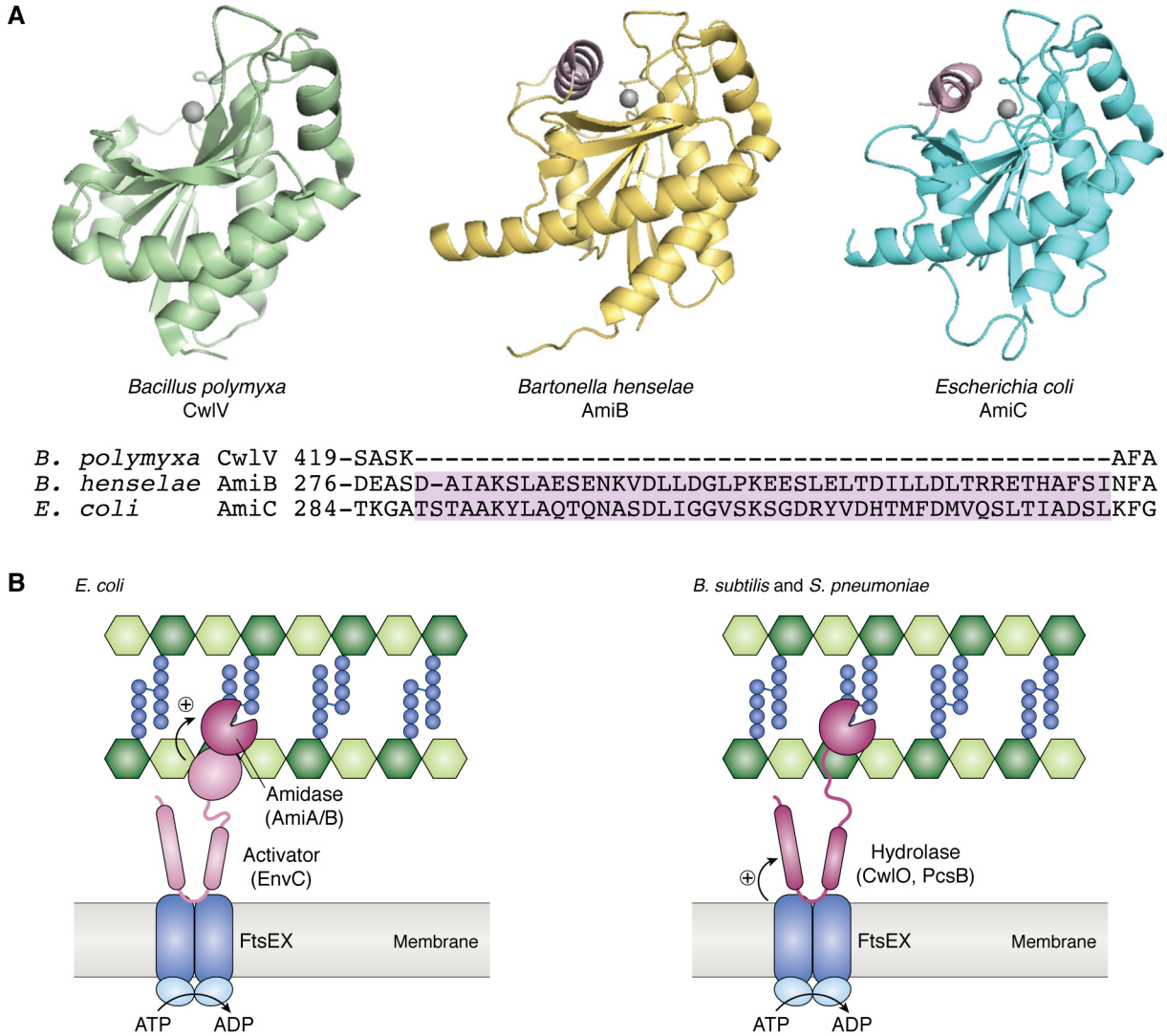


Figure 4. Direct regulation of peptidoglycan hydrolases. *A* (*top*), CwlV (PDB code 1JWQ) is an amidase that is active on its own. AmiB (PDB code 3NE8) and AmiC (PDB code 4BIN) are amidases that are directly activated by a regulator protein (69, 70). A helical domain, shown here in *pink*, sterically blocks the active site of AmiB and AmiC; displacement of this occluding helix activates the amidases. *Bottom*, a partial sequence alignment showing that the regulatory helix is present in AmiB and AmiC, but not CwlV. *B*, the *E. coli* amidases AmiA and AmiB are activated by direct interaction with EnvC, which itself is thought to be activated by FtsEX. In *B. subtilis* and *S. pneumoniae*, FtsEX is believed to regulate the hydrolases CwlO and PcsB, respectively. The ABC transporter FtsEX is presumed to harness ATP hydrolysis to adopt a conformation that is capable of activating the hydrolases.

glycosidase has also been reported. This inhibitor, Ivy, is produced by bacteria that do not *O*-acetylate their peptidoglycan to block the activity of lytic transglycosylases (65).

Compared with direct inhibitors, there are more examples of direct activators of peptidoglycan hydrolases. The best-characterized activators are EnvC and NlpD, which control the cell separation amidases AmiA/B/C (66–68). EnvC and NlpD resemble endopeptidases that cleave peptidoglycan cross-links, but the residues required for catalytic activity are missing, which suggested that they have a different function. Genetic studies provided clues to their function. An *E. coli* Δ*envC* Δ*nlpD* mutant phenocopies a Δ*amiABC* triple deletion mutant, forming chains of unseparated cells (66). This result implied that EnvC and NlpD act in the same pathways as the amidases. Subsequent work showed that EnvC specifically activates AmiA and AmiB, whereas NlpD activates AmiC (67). Hydrolysis of a crude bacterial cell wall preparation took several hours when the amidases alone were added. However, in the presence of the appropriate activator protein, hydrolysis was rapid. Structural studies later hinted at a mechanism of activation. Crystal structures of AmiB and AmiC showed that their active sites are occluded by an α-helix (Fig. 4*A*) (69, 70). Amidases such as *Bacillus polymyxa* CwlV that are active on their own lack this α-helix (69). Moreover, removal of the α-helix sequence from AmiC rendered AmiC highly active even in the absence of its cognate activator NlpD. These data suggested that the amidases switch from an inactive to active conformation when the occluding α-helix is displaced through an interaction with the activator. How EnvC and NlpD physically interact with the amidases to lead to this proposed displacement of the α-helix is unclear (71).

Until recently, the only other factor proposed to directly activate hydrolases was the widely conserved FtsEX transmembrane complex. FtsEX is an ABC transporter in which FtsE forms the ATP-binding component and FtsX forms the transmembrane channel (72). Rather than acting as a transporter,

FtsEX is thought to harness ATP hydrolysis to drive a conformational change in the transmembrane domain that somehow induces hydrolase activity. Two examples of hydrolases regulated by FtsEX are the *S. pneumoniae* hydrolase PcsB and the elongation-specific endopeptidase CwlO in *B. subtilis* (Fig. 4*B*) (73–75). Depletion of pneumococcal FtsEX phenocopies the cell division defects of a PcsB-depletion mutant (73). For *B. subtilis*, deletion of *ftsEX* produces shorter cells similar to deletion of *cwlO* (74, 75). Co-immunoprecipitation and bacterial two-hybrid assays further supported a direct interaction between FtsEX and the hydrolases. Recent structural and biochemical studies have proposed the following model for how the FtsEX complex regulates these hydrolases (76–78). In addition to a catalytic domain, both PcsB and CwlO have a coiled-coil domain, which appears to interact with two extracellular loops of FtsEX. The coiled-coil domain forms a V-shaped cleft that can lock around the catalytic domain like molecular tweezers. Interaction with FtsEX somehow releases the catalytic domain from the cleft to activate the hydrolase. This proposed mechanism for regulation by FtsEX has not been directly demonstrated through reconstitution.

Since the discovery of EnvC, NlpD, and FtsEX, new direct regulators of peptidoglycan hydrolases have been found in other organisms (79, 80). All but one are homologs of EnvC, NlpD, and FtsEX. The exception is a recently discovered *S. aureus* polytopic membrane protein called ActH, which activates an amidase, LytH, that controls *S. aureus* cell size (81). The LytH-ActH complex will be described below as the first example of how defined substrates can be used to characterize peptidoglycan hydrolase activity and to discover a new regulator protein. Given the diversity of hydrolases, we expect that other types of direct regulators with distinct structures and mechanisms remain to be identified.

Previous methods to characterize peptidoglycan hydrolases

Peptidoglycan hydrolases have traditionally been characterized using assays that involve bacterial sacculi, which are obtained by boiling bacteria in SDS and deproteinizing. Sacculi are highly heterogeneous and heavily cross-linked. A common assay to detect hydrolase activity is the zymogram (82, 83). Here, purified hydrolases are resolved on SDS-polyacrylamide gels containing sacculi in the gel matrix. Following separation, the gel is incubated in renaturation buffer to promote protein refolding and, as needed, subsequently stained with methylene blue to detect intact sacculi. Methylene blue staining is required to detect Gram-negative, but not Gram-positive, sacculi. Clear bands in the stained gel are interpreted as zones of hydrolase activity leading to cleaved sacculi. Zymography was for a long time the standard technique to assay hydrolase activity despite several drawbacks. One drawback is that charge-based exclusion of methylene blue leads to false positive results (84). As one example, *E. coli* EnvC was initially reported to have hydrolase activity by zymogram, but subsequent studies found that the signal was an artifact (67, 85). Another drawback is that zymograms cannot detect hydrolases that require an activator. The zymogram assay is also poorly suited for hydrolases that cannot spontaneously refold.

An alternative method to monitor peptidoglycan hydrolysis makes use of the dye Remazol Brilliant Blue (RBB), which under basic conditions forms a vinylsulfone species that can react with peptidoglycan (86). Isolated sacculi labeled with RBB are treated with a purified hydrolase, and reactions are terminated and centrifuged to pellet the undigested sacculi. The amount of dye released into the supernatant reports on hydrolytic activity. One drawback is that hydrolases that cannot act on cross-linked substrate would appear inactive in this dye release assay. Moreover, the RBB labeling is heterogeneous and, like other tailoring modifications, may interfere with the activities of some hydrolases. This assay also provides no information on the chemical cleavage specificity and product species. More recently, sacculi hydrolysis has been combined with LC-MS analysis to characterize the muropeptide cleavage products (87). But even with LC-MS analysis, the substrate preference of a hydrolase can only be indirectly deduced based on detectable changes in muropeptide species. The deficiencies in these assays underscore the need to obtain defined peptidoglycan substrates.

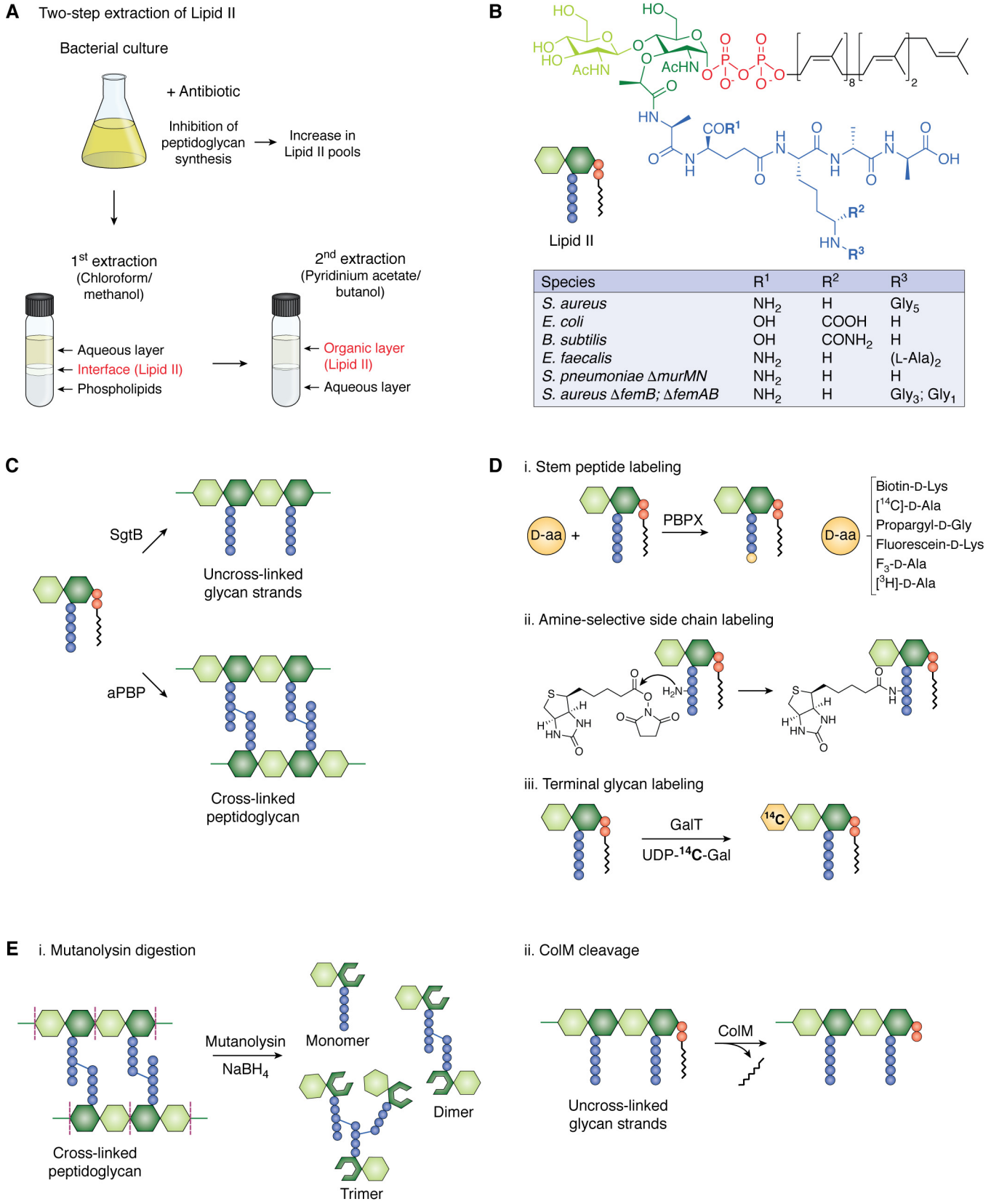
In vitro assembly of peptidoglycan enables characterization of cell wall hydrolases and tailoring enzymes

To study peptidoglycan hydrolases or tailoring enzymes ideally requires defined substrates and methods to detect reaction. This has been a challenge for several reasons. First, the peptidoglycan precursor, Lipid II, historically has been very challenging to obtain in practical quantities for biochemistry (88). Second, methods to build peptidoglycan substrates from Lipid II and label them for detection were lacking.

Starting in the late 1990s, several methods to obtain Lipid II were developed (89–94). The first methods involved chemical synthesis, but enzymatic methods were subsequently developed. These methods only provided access to Lipid II for research groups with the appropriate expertise, and even then, considerable effort was required. Moreover, each synthetic route was designed to make a particular Lipid II molecule. Painstaking modifications were required to acquire other variants. Because Lipid II structures vary and cross-linked peptidoglycan can only be made from variants having the correct stem peptide, the lack of a ready, general solution to obtain substrates hampered progress. This obstacle to progress has now been largely overcome.

The first finding that led to a breakthrough was that Lipid II can be accumulated in cells. Steady-state levels of the cell wall precursor Lipid II are normally low, but levels of undecaprenyl-phosphate, the carrier lipid on which Lipid II is built, are estimated at $1–3 \times 10^5$ molecules/cell (17, 18, 95). Therefore, cells have the capacity to make substantially more Lipid II. Antibiotics that block Lipid II utilization (*e.g.* moenomycin or vancomycin) were shown to cause a rapid (10–20 min), substantial increase in Lipid II pools (96, 97). The next challenge was to identify a strategy to isolate Lipid II. Because it has a C_{55} lipid chain, Lipid II is difficult to separate from other cellular lipids. However, it was found that Lipid II can be isolated at reasonable purity from large-scale bacterial cultures using a two-step extraction (Fig. 5*A*) (98). Lipid II accumulation and extraction

have been successfully adapted to isolate Lipid II variants from diverse species, including *S. aureus*, *B. subtilis*, *E. coli*, *S. pneumoniae*, *E. faecalis*, and *Mycobacterium smegmatis* (Fig. 5*B*) (98–101). Lipid II has also been isolated from mutant strains of some of these species, expanding the repertoire of Lipid II structural variants. In *S. aureus*, the Fem proteins sequentially



install five glycines to the side chain of L-Lys in the stem peptide: FemX adds the first glycine, FemA adds the second and third glycines, and FemB adds the final two glycines. Lipid II-monoglycine and Lipid II-triglycine have been prepared from *S. aureus* Δ*femAB* and Δ*femB* mutants, respectively (99). Similarly, an *S. pneumoniae* Δ*murMN* mutant provides access to Lipid II with unmodified lysine at the third position (100). With sufficient quantities of different Lipid II in hand, previously inaccessible peptidoglycan substrates can now be easily obtained.

Methods have been developed to build both uncross-linked and cross-linked peptidoglycan from Lipid II and to label the substrates for detection. To make uncross-linked glycan strands, Lipid II is polymerized with a monofunctional glycosyltransferase, such as *S. aureus* SgtB (Fig. 5*C*) (102). As this enzyme does not recognize the stem peptide, it can be used to polymerize diverse Lipid II variants. Glycan strand length can be controlled to some extent using glycosyltransferase mutants with processivity defects. Cross-linked peptidoglycan is made using an appropriate PBP that recognizes the stem peptide (98, 99, 103). Peptidoglycan substrates synthesized *in vitro* can be detected by incorporating a chemical probe within the glycan backbone or stem peptide (Fig. 5*D*). One approach for stem peptide labeling uses PBPX, a functionally unique transpeptidase from *E. faecalis* that does not cross-link peptidoglycan, to catalyze D-amino acid exchange (100). This reaction leads to an exchange of the terminal D-Ala on stem peptides for D-amino acids bearing a detectable tag. Alternatively, stem peptides can be labeled at the side-chain primary amine of the third amino acid with an amine-reactive probe (104, 105). These two strategies work for labeling Lipid II prior to polymerization or the stem peptides of preassembled glycan strands. Making labeled cross-linked peptidoglycan is also relatively straightforward. By incubating a D-amino acid probe with Lipid II and an appropriate PBP that both cross-links peptidoglycan and performs D-amino acid exchange, the PBP incorporates the probe as it synthesizes peptidoglycan (98). These labeling methods make it possible to detect reaction of peptidoglycan substrates in downstream enzymatic assays. In cases where stem peptide labeling interferes with enzymes that distinguish different stem peptides, a probe can instead be incorporated within the glycan backbone. One simple strategy uses the bovine β-1,4-galactosyltransferase GalT to attach a [^{14}C]galactose radiolabel onto the C4 hydroxyl of GlcNAc at the nonreducing end of glycan strands (106).

In addition to labeling the substrates for detection, analytical methods allow for structural characterization of the peptidoglycan products (Fig. 5*E*). High-resolution MS is typically used for characterization because it can detect low amounts of material. For analysis of cross-linked peptidoglycan, material is first digested with mutanolysin, an *N*-acetylmuramidase that cleaves between MurNAc and GlcNAc units (98). Because cleavage results in anomeric mixtures of muropeptides with different retention times, the mutanolysin digests are reduced with sodium borohydride prior to LC-MS analysis. For uncross-linked peptidoglycan, degradation methods that remove the lipid from the reducing end of the polymers are required for good ionization. One method uses acid hydrolysis to remove the lipid but can produce mixtures of glycan mono- and diphosphates (98). An enzymatic method makes use of the protein toxin colicin M (ColM) from *E. coli*, which was originally shown to cleave Lipid II and yield a 1-pyrophospho-MurNAc-(peptide)-GlcNAc product (107). It has recently been shown that ColM can also cleave the lipid tail from uncross-linked glycan strands, generating glycan diphosphate products (23).

The ability to make labeled, defined peptidoglycan substrates now enables a systematic characterization of hydrolase activity (108). In the next section, we will highlight recent studies that have used defined substrates to characterize peptidoglycan hydrolases and cell wall–tailoring enzymes.

Connecting cell wall biochemistry with biological function

Some cell wall hydrolases act on cross-linked peptidoglycan, whereas others can only cleave uncross-linked substrates. Still others may cleave only substrates having particular stem peptides and tailoring modifications. Proving the biochemical activities of these enzymes is needed to uncover their functions in cells. The trend toward building substrates *in vitro* for biochemical reconstitution has started and already yielded new insights into cell wall biogenesis.

As one example, Lee *et al.* (48) reconstituted the activities of all known lytic transglycosylases from *P. aeruginosa* using defined peptidoglycan substrates. They showed that the lytic transglycosylases differ in substrate recognition. Some required an anhMurNAc terminus at the +2 position (*i.e.* two sugars toward the reducing end) from the cleavage site. One lytic transglycosylase, RlpA, preferred cleaving glycan strands lacking stem peptides (109). RlpA must therefore work in tandem

Figure 5. Making and labeling peptidoglycan substrates to characterize cell wall enzymes. *A*, accumulation and extraction of Lipid II. Bacterial cultures are treated with an antibiotic that inhibits peptidoglycan synthesis to accumulate Lipid II in cells. The cells are spun down and resuspended in chloroform/methanol for the first extraction, which produces three layers. Lipid II is enriched in a thick interface fraction, whereas the majority of cellular phospholipids partition to the organic layer. A second extraction results in partitioning of Lipid II into the organic phase and UDP-MurNAc-pentapeptide, which is also present in the interface layer, into the aqueous phase. *B*, the two-step extraction allows isolation of Lipid II variants from the indicated species. *C*, uncross-linked glycan strands are synthesized from Lipid II using the monofunctional glycosyltransferase SgtB, which recognizes Lipid II variants with different stem peptides. To make cross-linked peptidoglycan, an appropriate bifunctional aPBP that recognizes the stem peptide is used. The aPBP polymerizes Lipid II and cross-links glycan strands. *D*, a chemical probe can be incorporated within the glycan backbone or stem peptide of peptidoglycan substrates to visualize and assess reaction of the substrates. *i*, one strategy to label the stem peptide of Lipid II or glycan strands makes use of the transpeptidase PBPX, which exchanges the terminal D-Ala in the stem peptide for a D-amino acid bearing a detectable tag. *ii*, another stem peptide labeling strategy selectively couples an amine-reactive probe to the side-chain primary amine at position 3 of the stem peptide. *iii*, a strategy to label the sugar backbone uses GalT to attach a [^{14}C]galactose radiolabel at the nonreducing end of glycan strands. *E*, methods to digest peptidoglycan products for structural characterization by LC-MS. *i*, mutanolysin digestion and $NaBH_4$ reduction of cross-linked peptidoglycan produce muropeptide species. *ii*, ColM treatment of uncross-linked glycan strands produces delipidated glycan diphosphate products.

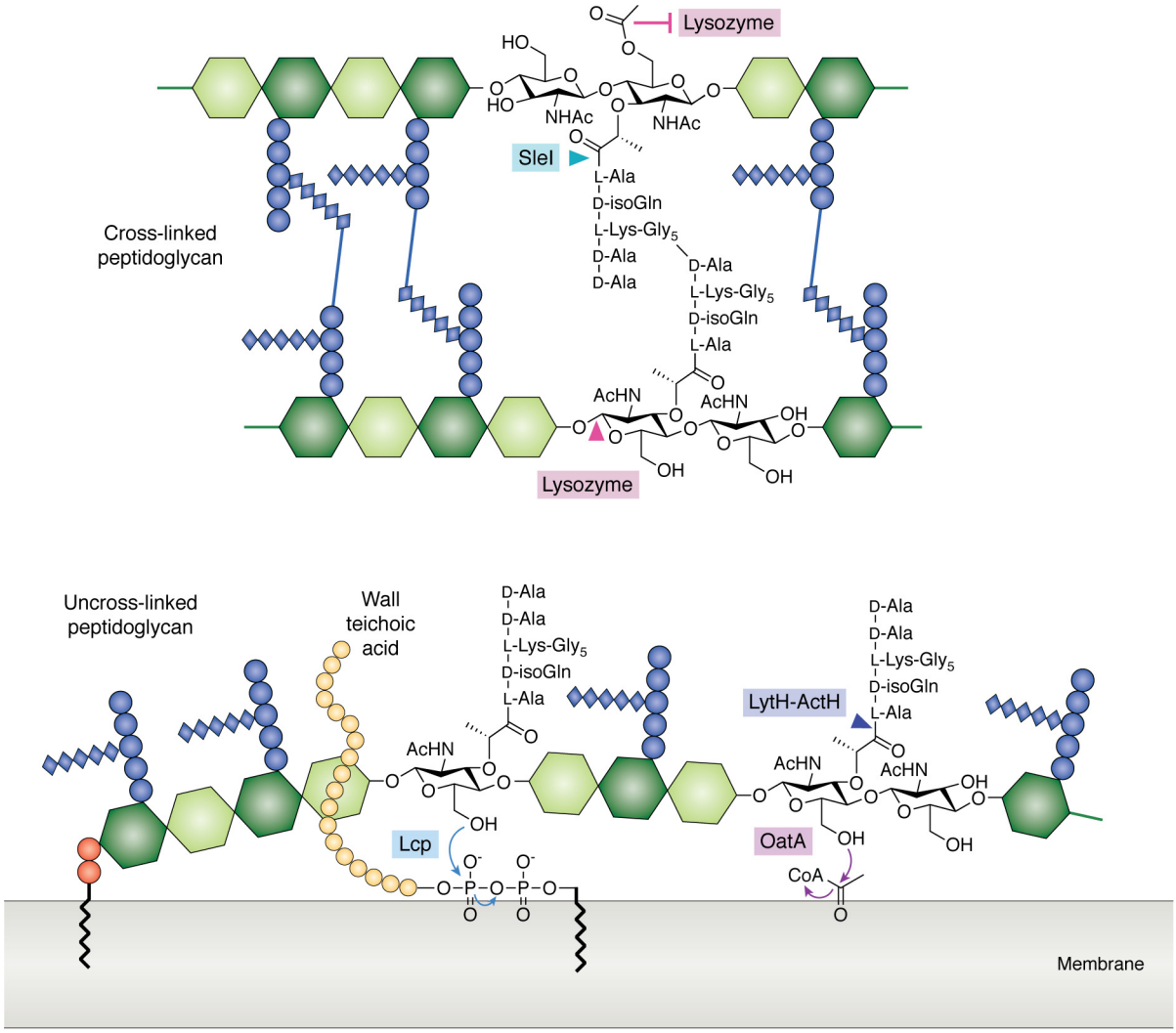


Figure 6. Peptidoglycan hydrolases and tailoring enzymes have distinct substrate preferences. In *S. aureus*, the LCP wall teichoic acid ligases and the LytH-ActH amidase-activator complex are membrane-anchored proteins that only act on uncross-linked peptidoglycan substrates. The *S. aureus O*-acetyltransferase OatA is also a membrane-anchored protein and may act preferentially on uncross-linked peptidoglycan. By contrast, *S. aureus* Sle1 is an amidase that acts on cross-linked peptidoglycan. Another example of substrate selectivity is provided by lysozyme, which cleaves unacetylated, but not *O*-acetylated, backbones. Substrate selectivity can offer insights into the functions of these enzymes, as those enzymes that act at an earlier stage of peptidoglycan synthesis may show a preference for nascent peptidoglycan substrates.

with amidases in cells. Only some of these reconstituted lytic transglycosylases have been functionally characterized in cells. Understanding which substrates are recognized will help elucidate the biological functions of the remaining glycosidases.

A second example relates to a recent study that used synthetic peptidoglycan fragments to define the binding mechanism of the endopeptidase lysostaphin (110). Produced by *Staphylococcus simulans* biovar *staphylolyticus*, lysostaphin shows highly specific and potent lytic activity against other staphylococci (111). Lysostaphin has an SH3b cell wall–binding domain that recognizes the pentaglycine cross-bridges present exclusively in staphylococcal peptidoglycan, positioning the enzyme to cleave the cross-bridges. Within the SH3b domain resides a shallow groove that appears to sterically select for pentaglycine (112). Just this year, Gonzalez-Delgado *et al.* (110) discovered that the SH3b domain drives not only the specificity, but also high-affinity binding, of lysostaphin toward staphylococcal peptidoglycan. A co-crystal structure of the SH3b domain with a tetrapeptide-pentaglycine ligand revealed two separate binding sites that are on opposite sides of each SH3b monomer. Surprisingly, the ligand was found to simultaneously bind two SH3b monomers: the pentaglycine branch sits within one pocket of the first SH3b monomer, and the tetrapeptide stem protrudes into the opposite pocket on a second SH3b monomer. The authors proposed that this two-site binding mechanism enables lysostaphin to cluster on staphylococcal peptidoglycan, leading to the observed potency in lytic activity. Therefore, juxtaposition of substrate-binding sites serves as another way to regulate peptidoglycan hydrolase activity.

Another example is provided by the recently discovered *S. aureus* amidase-activator complex, LytH-ActH (Fig. 6) (81). LytH is a membrane-anchored amidase that was shown to remove stem peptides exclusively from uncross-linked glycan strands. This substrate preference suggested that LytH acts early in cell division because amidases that effect cell separation would need to cleave cross-linked peptidoglycan (113, 114).

Accordingly, deletion of *lytH* led to defects in placement of nascent division sites as opposed to the final cell separation defects observed for mutants of amidases that separate daughter cells after cell division is complete. LytH trims stem peptides to control the density of peptidoglycan assembly sites at a given subcellular location. In doing so, LytH spatially regulates peptidoglycan synthesis to ensure cell growth is coordinated with cell division. Importantly, LytH was active only in the presence of a polytopic membrane protein partner, ActH, that was identified by co-immunoprecipitation. ActH is structurally distinct from EnvC, NlpD, and FtsEX and resembles a rhomboid serine protease, a family of proteins that is widespread in bacteria but whose biological roles have remained largely mysterious (115, 116). As such, ActH represents a new class of regulators that directly activate a partner cell wall hydrolase. Defined peptidoglycan substrates were instrumental in characterizing the LytH-ActH complex, as the complex only cleaves uncross-linked peptidoglycan. This work exemplifies how establishing the substrate preference of an enzyme is crucial in determining its biological function. How ActH activates LytH remains to be established.

Access to defined substrates has advanced studies of other cell wall enzymes besides hydrolases that act on peptidoglycan (Fig. 6). In some species, the peptidoglycan is decorated with additional chemical modifications that are important for bacterial physiology (12). *O*-Acetylation of the MurNAc C6 hydroxyl and *N*-deacetylation of the GlcNAc C2 acetyl are two examples (117, 118). Both of these modifications protect pathogens that colonize a host from host-derived lysozyme. Other examples of tailoring modifications are the covalent attachment of glycopolymers, including wall teichoic acid (WTA), teichuronic acid, and capsular polysaccharide (CPS), to the glycan backbone of peptidoglycan (30, 119). These polymers are important for pathogenesis, contributing to immune evasion and host colonization. Other functions of these polymers include scaffolding cell wall proteins, conferring cell morphology, and protecting cells against antibiotics. A detailed characterization of the kinetics and substrate preferences of these cell wall–tailoring enzymes will help establish their biological roles. Once enzymatic activity has been confirmed *in vitro*, homologs can also be identified and rapidly characterized in other organisms.

Recent work has shown that peptidoglycan-tailoring enzymes exhibit clear substrate preferences, which influence when they act during cell wall assembly. Differences in substrate preferences between homologs may also reveal distinct biological roles. One class of enzymes for which substrate preferences have been studied are the LCP (LytR-CpsA-Psr) proteins that couple both WTA and CPS glycopolymers to peptidoglycan. For a long time, the identity of the coupling enzymes was not known. Genetic studies in 2011 and 2014 suggested that the LCP proteins could be the WTA and CPS ligases, but reconstitution to demonstrate ligase activity was not done because appropriate substrates were unavailable (120, 121). Recently, these activities were confirmed biochemically, when it was also found that the LCP proteins have strict substrate preferences. *S. aureus* LcpB, a prototypical LCP enzyme, only attaches WTA glycopolymers to uncross-linked peptidoglycan oligomers that are at least 4 sugars long (122). Neither Lipid II nor cross-linked peptidoglycan were substrates (123). Similarly, evidence suggests that *S. aureus* LcpC transfers CPS at an early stage of peptidoglycan synthesis as well (124). In this case, LcpC appears to couple CPS to Lipid II, although the products were not characterized. Structural studies have revealed a possible mechanism for substrate selection by the LCP proteins based on steric exclusion (123). The LCP protein has an extended narrow groove that likely excludes cross-linked substrates. Similar to LCP proteins, *O*-acetylation enzymes that install an acetyl group on the C6 hydroxyl of MurNAc also discriminate between different peptidoglycan substrates. The Gram-negative *O*-acetyltransferase PatB can only acetylate substrates that are at least 3 sugars in length (125, 126). The *S. aureus O*-acetyltransferase OatA prefers to acetylate uncross-linked glycan strands containing intact stem pentapeptides as opposed to those with trimmed peptides (127). Whether OatA can transfer acetyl groups to cross-linked peptidoglycan was not tested.

These mechanistic studies of LCP proteins and *O*-acetyltransferases have important biological implications for ordering the steps in cell wall assembly. In the final stages of cell wall assembly, several biosynthetic pathways converge to produce the mature cell wall (30). However, the sequence of cell wall assembly has been unclear. After Lipid II is transported across the inner cell membrane, it is polymerized into glycan strands that are cross-linked into the peptidoglycan matrix. In most cases, it is not known when tailoring modifications are made to peptidoglycan during its assembly. In principle, modifications may be added to Lipid II, uncross-linked polymers, and/or cross-linked polymers. One way to address the order of cell wall assembly is through *in vitro* reconstitution using a range of peptidoglycan intermediates. This type of logic has been commonly used in the field of natural product biosynthesis, where elucidating the order of assembly often relies on testing the ability of a biosynthetic enzyme to convert a pathway intermediate to a product that can be used in the subsequent step. In *S. aureus*, WTA glycopolymers can only be transferred to nascent (uncross-linked) peptidoglycan strands, and it has been shown that these WTA-modified polymers can then be cross-linked (123). Indeed, it has even been proposed that WTAs regulate the localization of the PBP4 transpeptidase to control peptidoglycan cross-linking (128). The clear preference of WTA ligases for modifying nascent peptidoglycan is consistent with their membrane-anchored location. *S. aureus* OatA is also anchored in the membrane and may also display a preference for uncross-linked peptidoglycan, although detailed studies of its substrate preferences that could help elucidate when it acts have not been done (127). The studies described here exemplify how using defined substrates that mimic peptidoglycan intermediates can provide temporal information on cell wall assembly. Moreover, access to peptidoglycan species containing post-synthetic modifications expands the ensemble of substrates available to assess cell wall hydrolase activity.

Conclusions and outlook

Nearly all bacteria are encased in layers of peptidoglycan, which is a central determinant of cell morphology (4–7). The basic structure of peptidoglycan is a simple carbohydrate back-

bone with peptide cross-links that are relatively conserved across bacterial species (8). Yet there is a vast diversity in the structure of the mature cell wall, owing to tailoring modifications and the trimming of peptidoglycan by hydrolases (12, 30). Peptidoglycan hydrolases are essential for cell wall biogenesis (31–33). However, due to their destructive potential, these hydrolases must be regulated to prevent cell wall damage. Elegant studies from the past decade established that bacteria encode regulator proteins that directly interact with hydrolases to control their activity. Yet only a few types of direct regulators have been identified despite the diversity of hydrolases. In part, the search for new regulators has been hindered by a lack of suitable peptidoglycan substrates to evaluate hydrolase activity. This problem has now been solved with new approaches to rapidly access large quantities of Lipid II variants from different bacterial species. Using Lipid II as a building block, uncross-linked and cross-linked peptidoglycan can be assembled *in vitro* and labeled for detection. Studies using these defined substrates have uncovered a new class of direct regulators of peptidoglycan hydrolases and provided mechanistic insights into other aspects of cell wall biogenesis.

Looking forward, there are many open questions that these new tools can help answer. A robust cell wall depends on the ordered convergence of multiple biosynthetic pathways. Many of the enzymes involved in cell wall assembly have been identified or can be computationally predicted, but we have yet to fully understand their biological functions. Determining when, where, and on what these enzymes act in cells is necessary to elucidate their functions. In the past decade, new microscopy techniques to track cell wall proteins have been developed that will provide valuable spatiotemporal information (129). The tools we outlined here to biochemically characterize cell wall enzymes will complement future imaging studies by reporting on substrate specificity. Finally, the ability to obtain defined substrates will also be useful for developing sensitive, high-throughput assays to monitor cell wall enzyme kinetics and screen for inhibitors. For peptidoglycan hydrolases, there are a few reported activity assays, but all use crude cell wall substrates. These assays can now be revisited with the tools we have described in hand, facilitating the identification of new antibiotics that disrupt the activity of enzymes essential to the assembly of a complete cell wall.

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