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Chemical tools to characterize peptidoglycan synthases

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Abstract

The peptidoglycan cell wall is a unique macromolecular structure in bacteria that defines their shape and confers protection from the surrounding environment. Decades of research has focused on understanding the peptidoglycan synthesis pathway and exploiting its essentiality for antibiotic development. Recently, a new class of peptidoglycan polymerases known as the SEDS (shape, elongation, division and sporulation) proteins was identified; these polytopic membrane proteins function together with the better-known penicillin-binding proteins (PBPs) to build the cell wall. In this review, we will highlight recent developments in chemical tools and methods to label the bacterial cell wall and discuss how these developments are leading to a better understanding of peptidoglycan synthases and their cellular roles.

INTRODUCTION

The bacterial cell envelope contains an intricate set of polymers and molecules that have evolved to promote survival and fitness in a hostile environment. Although components of the cell envelope are often present in only a subset of species, the peptidoglycan cell wall is a defining feature of prokaryotes that is present with few exceptions (e.g. *Mycoplasma*). The chemical steps of the peptidoglycan synthesis pathway were largely worked out by the end of the 1960s (Figure 1).^{1,2} In the first phase of synthesis, which is carried out by soluble enzymes in the cytoplasm, UDP-N-acetylglucosamine (UDP-GlcNAc) is converted to UDP-N-acetylmuramic acid (UDP-MurNAc)-pentapeptide via a multistep process. The basic structure of the stem pentapeptide is L-Ala-D- α Glu-*m*DAP (*meso*-2,6-diaminopimelate)/L-Lys-D-Ala-D-Ala in most species. The membrane protein MraY then catalyzes a pyrophosphate exchange reaction to couple phospho-MurNAc-pentapeptide to a polyprenyl phosphate in the membrane, and the resulting species, Lipid I, is converted by the glycosyltransferase MurG to Lipid II. Additional transformations that modify the stem pentapeptide, such as the amidation of D- α Glu at the second position and the addition of an

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CONFLICTS OF INTEREST STATEMENT

Nothing declared.

interpeptide bridge at the third position, occur in some organisms. The complete peptidoglycan Lipid II precursor is then translocated to the extracytoplasmic side of the membrane where it is polymerized to form nascent glycan chains that are crosslinked to form cell wall peptidoglycan. Crosslinking is catalyzed by transpeptidases that are the targets of the β -lactam antibiotics; because these enzymes were discovered based on their ability to bind penicillin, they are called penicillin-binding proteins, or PBPs. A variety of tailoring reactions, including attachment of wall teichoic acids and acetylation of the sugar backbone, can occur to diversify peptidoglycan in different organisms.³

Although the conserved chemical steps of biosynthesis have long been known, the past decade has witnessed major findings with respect to the enzymes that catalyze these steps. In part, these findings have been enabled by advances in tools to study peptidoglycan biosynthesis *in vitro* and in cells. This perspective will describe the recent discovery of a new family of peptidoglycan polymerases and connect this discovery to enabling advances in chemical biology.

DISCOVERY AND CHARACTERIZATION OF SEDS FAMILY PEPTIDOGLYCAN POLYMERASES

The availability of labeled beta-lactams that served as affinity-based probes for protein profiling led to the early discovery that bacteria typically contain several different PBPs having different molecular sizes.^{4,5} One class of high molecular weight PBPs, the class A PBPs (aPBPs), was found to contain a polymerase activity in addition to a crosslinking activity.⁶ The polymerase activity was found in an N-terminal domain that, for a time, served as the paradigm for what was widely believed to be the only family of peptidoglycan glycosyltransferases (PGTs). This domain was subsequently found in monofunctional glycosyltransferases (MGTs), and these were assumed to function in concert with a PBP that could catalyze crosslinking.^{6,7} Although early evidence suggested that bacteria contain another, unrelated family of PGTs, this evidence was overlooked until Popham and coworkers reported in 2003 that all aPBPs can be deleted in *Bacillus subtilis*, which does not encode any MGTs.⁸ Similar observations were subsequently reported in *Enterococcus faecalis* and *Enterococcus faecium*.^{9,10} The requirement for aPBPs or MGTs in peptidoglycan synthesis was further questioned when peptidoglycan was detected in *Chlamydia trachomatis*, which was previously thought to lack a cell wall.¹¹ The *Chlamydiales* family does not encode any aPBPs or MGTs, implying that an unknown PGT was responsible for making peptidoglycan polymers.¹²

The first clue to the identity of this mystery family of PGTs was found in the work of Matsushashi and coworkers in 1986.¹³ This group reported that *E. coli* membranes overexpressing both RodA and PBP2 could make peptidoglycan, but this polymer was not made if only one of the proteins was overexpressed. PBP2 is a member of the class B PBPs (bPBPs), which only catalyze the transpeptidase reaction, and RodA is a core component of the Rod complex required for making side wall peptidoglycan.⁶ RodA belongs to the SEDS (for sporulation, elongation, division, and septation) family of polytopic membrane proteins, and a related protein, FtsW, is an essential component of the divisome that makes septal

peptidoglycan.^{14,15} These proteins contain ten transmembrane helices, and due to the challenges of isolating membrane protein complexes and the lack of tools to reconstitute peptidoglycan synthase activity, no further studies to investigate the polymerase activity of SEDS-bBPB complexes were reported for more than thirty years. Moreover, an alternative proposal that SEDS proteins function as Lipid II flippases diverted attention from other possible functions for these essential proteins.^{16,17} It is now widely held that MurJ, a membrane protein that belongs to the MOP (multidrug/oligo-saccharidyl-lipid/polysaccharide) transporter family, is the primary lipid II flippase.^{18–20}

A major breakthrough in the field was reported in 2016 when it was shown that purified *B. subtilis* RodA can polymerize synthetic Lipid II.²¹ Although the polymerase activity was weak, most likely because the bBPB partner was absent, genetic evidence was fully consistent with the role of RodA as a peptidoglycan polymerase.^{21–23} Homology and structural predictions identified similarities between SEDS proteins and O-antigen ligases, which catalyze the transfer of lipid-linked O-antigen to lipid A-core oligosaccharide to produce lipopolysaccharide in Gram-negative bacteria.^{21,24} A crystal structure of *Thermus thermophilus* RodA, solved using an innovative phasing strategy, revealed a conserved central cavity that was predicted to be a binding site for lipid-anchored substrates.²⁵ At this point, however, it was still unclear whether the PGT activity observed for *B. subtilis* RodA was shared by other SEDS proteins. It was also unclear what role, if any, the bBPBs that form complexes with SEDS proteins played in polymerase activity.^{26,27}

One major hurdle that for decades hindered the study of enzymes that make and modify peptidoglycan was limited access to peptidoglycan precursor. Lipid II is present in very low amounts in bacterial cells during normal growth owing to rapid turnover, and its complex structure containing an undecaprenyl (C55) pyrophosphate linked to a disaccharide-peptide moiety makes it challenging to synthesize (Figure 2a).^{2,28} Although chemical and enzymatic methods to prepare Lipid II were reported^{29–33}, these required substantial effort and obtaining sufficient amounts of substrate remained one of the bottlenecks in the field for many years, particularly because structural variation in the peptide unit among different bacterial species made it necessary to tailor chemical or enzymatic routes to study key reactions (Figure 2a).³ This problem has now been solved. Following the discovery of labeling reaction that enables detection of Lipid II in cell extracts, Qiao *et al.* found that Lipid II accumulates in large amounts in cells treated with antibiotics or other conditions that prevent its turnover (*e.g.*, inactivation of the Lipid II flippase).³⁴ Subsequently, a facile two-step extraction procedure was developed to isolate Lipid II and one can now obtain any Lipid II variant in substantial quantities overnight, allowing extensive exploration of peptidoglycan assembly and processing enzymes (Figure 2b).^{20,35,36}

Using the newly available lipid II substrates, it has now been shown that RodA and FtsW orthologs from several different organisms have peptidoglycan polymerase activity in complex with their cognate bBPBs.^{37,38} It is remarkable that protein complexes with a combined eleven transmembrane helices are functional in detergent. The bBPB is required for robust polymerase activity of the SEDS proteins, but the crosslinking activity does not depend on the presence of a SEDS protein, only on the availability of a polymer substrate.³⁸ The dependence of polymerase activity on the bBPB mirrors the initial observation from the

Matsuhashi laboratory that peptidoglycan synthesis in *E. coli* membranes required that both RodA and the bPBP be overexpressed.¹³ Evidence indicates that the transmembrane helix of the cognate bPBP for each SEDS protein plays a crucial role in complex formation.^{24,25,38} Using state-of-the-art microscopy, it has been found that SEDS proteins and bPBPs form complexes in cells.^{39,40} Taken together, recent work has established these SEDS-bPBP complexes as peptidoglycan synthases. At this point, almost nothing is known about the polymerase mechanism except that divalent cations are required for activity, unlike for aPBP-mediated peptidoglycan polymerization.³⁸

CHEMICAL PROBES FOR LABELING PEPTIDOGLYCAN

A full understanding of peptidoglycan synthesis requires not only tools that enable *in vitro* reconstitution, but methods to monitor peptidoglycan synthesis in cells. Seminal studies have demonstrated that noncanonical D-amino acids can be incorporated into peptidoglycan to modulate peptidoglycan structure and vancomycin resistance. While vancomycin resistance in *Enterococcus* was shown to be mediated by VanA, a ligase responsible for replacing D-Ala-D-Ala with D-Ala-D-lactate at the stem peptide terminus, the noncanonical D-amino acid incorporation seen in a wide range of species has been attributed to transpeptidase activities of PBPs and L,D-transpeptidases (Figure 3a).^{41–44} PBP-mediated D-amino acid incorporation has been confirmed *in vitro* for aPBPs and a family of low molecular weight PBPs that act as transpeptidases rather than carboxypeptidases.^{36,45} These low molecular weight PBPs can catalyze exchange of unnatural D-amino acids, including biotin-D-lysine (BDL), into Lipid II, unlike the aPBPs that only act on polymeric peptidoglycan. The ability to incorporate BDL is useful for *in vitro* detection of peptidoglycan (Figure 3b).

To detect active peptidoglycan synthesis in cells, a number of fluorescent D-amino acid (FDAA) probes have been developed (Figure 3b).⁴⁶ By combining these probes with methods to fluorescently label proteins, it is possible to investigate the relationship between protein localization and peptidoglycan synthesis in cells.⁴⁷ Recent studies have investigated the relationship between septal peptidoglycan synthesis and treadmilling of FtsZ, a tubulin homolog required for bacterial cell division.^{40,48,49} FDAAs can be tailored for each species to increase incorporation efficiency. In *B. subtilis* and some other organisms better peptidoglycan labeling has been observed for FDAAs in which the acid is converted to a carboxamide, perhaps because the stem peptides modified with carboxamide probes are poorer substrates for transpeptidase or hydrolysis activities that could cleave other probes.^{50,51} One drawback of FDAAs is that it is necessary to wash out excess probe before imaging. To circumvent this issue, Hsu *et al.* recently reported the design and synthesis of rotor-fluorogenic D-amino acids, which only fluoresce upon peptidoglycan incorporation and do not require any washing prior to imaging, thus providing better temporal resolution.⁵²

In addition to stem peptide modifications, strategies to label the sugar backbone of peptidoglycan have been explored. Incorporation of MurNAc derivatives containing a bioorthogonal handle into peptidoglycan has been demonstrated in live cells, and it has been shown that the modified cell wall can be labeled with an appropriate fluorophore (Figure 3c).⁵³ Another strategy to label the MurNAc residues takes advantage of PatB, a

peptidoglycan O-acetyltransferase with promiscuous properties, to install a bioorthogonal handle at the 6-OH position of MurNAc.⁵⁴ These studies have established that peptidoglycan disaccharide units are targets for installation of chemical probes and that labeling the glycan backbone can have advantages over stem peptide modification for studying the peptidoglycan architecture. Finally, fluorescent substrate-binding antibiotics that bind Lipid II and nascent peptidoglycan are also useful probes because they report on sites of new polymer synthesis.^{55,56}

CONCLUSION

Despite being a subject of intense research for decades, the bacterial cell wall still contains many surprises. Here we have highlighted recent innovative methods in chemical biology that have made it possible to study peptidoglycan biosynthesis both in vitro and in cells. Although we focused on the recent discovery of a new family of peptidoglycan polymerases, the methods mentioned here more generally make it possible to link cell biology, genetics, and biochemistry to arrive at a comprehensive understanding of the role of cell wall assembly and modification enzymes in bacterial physiology.

With respect to SEDS-bPBP complexes, their primary function has been uncovered, but many outstanding questions remain to be answered. As noted, the biochemical mechanisms of these complexes are not understood. Moreover, how these complexes coordinate with aPBPs to build peptidoglycan in cells has only recently begun to be examined.^{22,57} Perhaps the most important question from the standpoint of significance is whether SEDS proteins are “druggable” antibiotic targets. SEDS proteins are found in all bacteria that have a cell wall and could thus be broad-spectrum targets like the PBPs. Moreover, their active sites are accessible from the extracytoplasmic side, which may allow targeting by compounds that cannot cross the cytoplasmic membrane. Errington and coworkers have recently described a partially purified set of natural products that appear to have an inhibitory effect on *B. subtilis* RodA, but the structures of these molecules have not been reported and inhibition has not been confirmed in a pure assay.²³ Nevertheless, this report is promising and gives reason for optimism.

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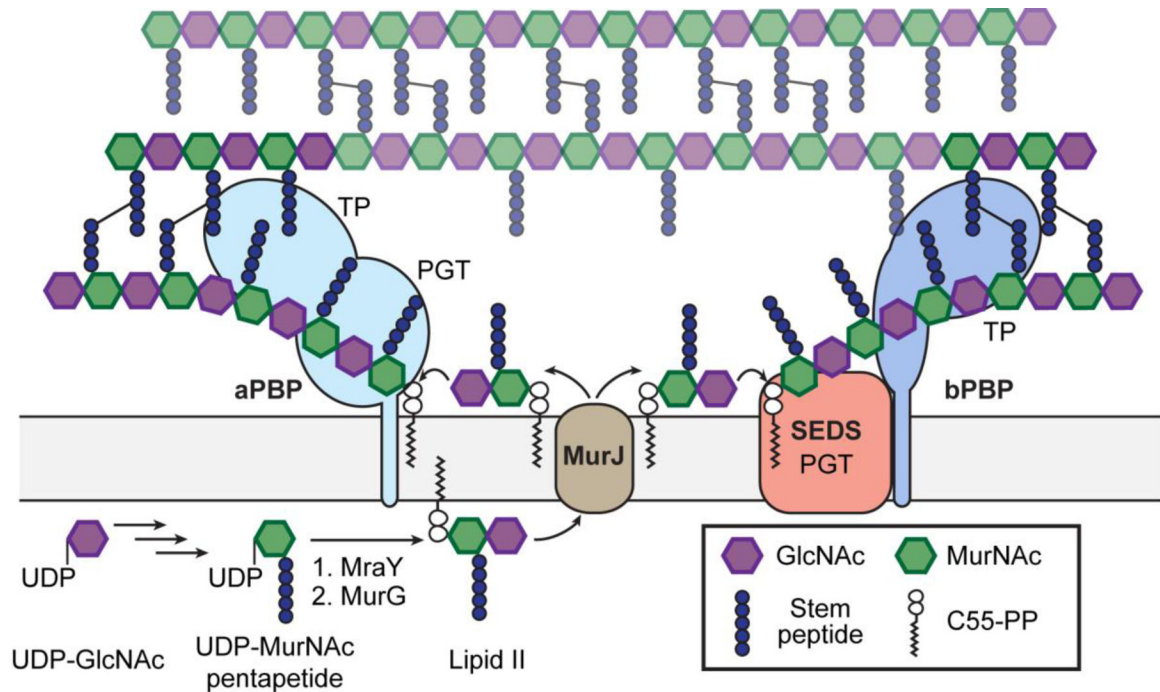
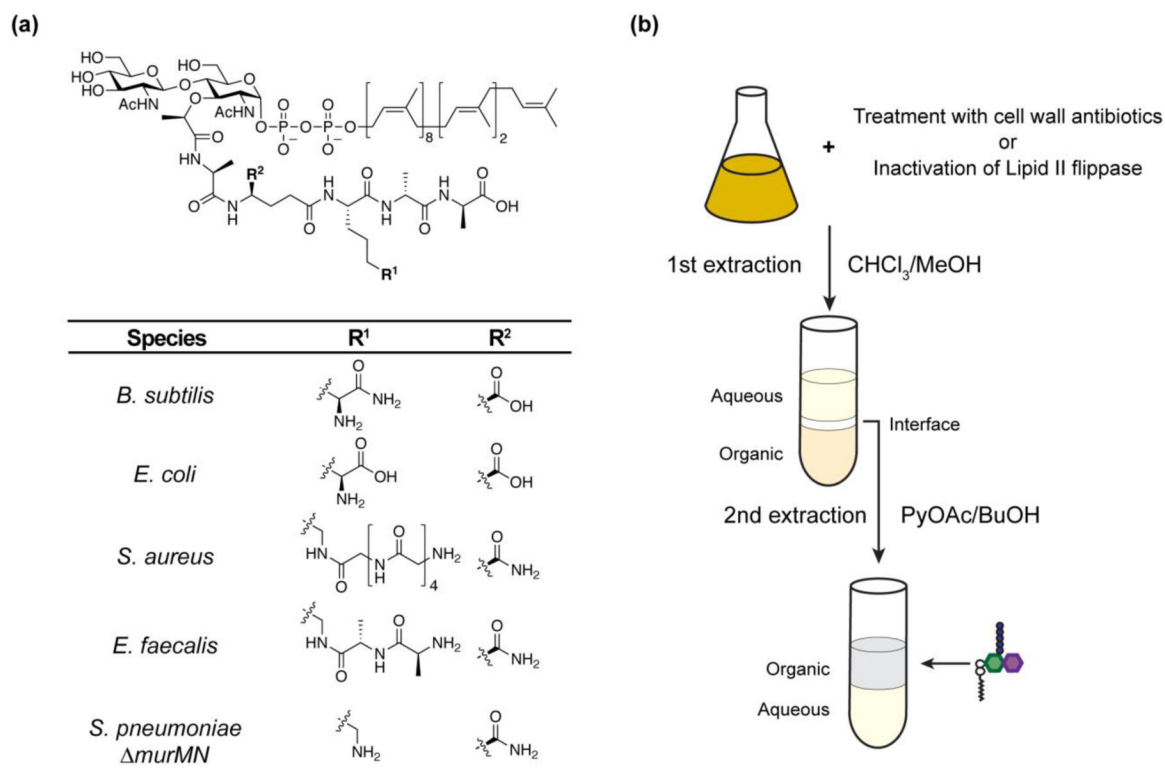
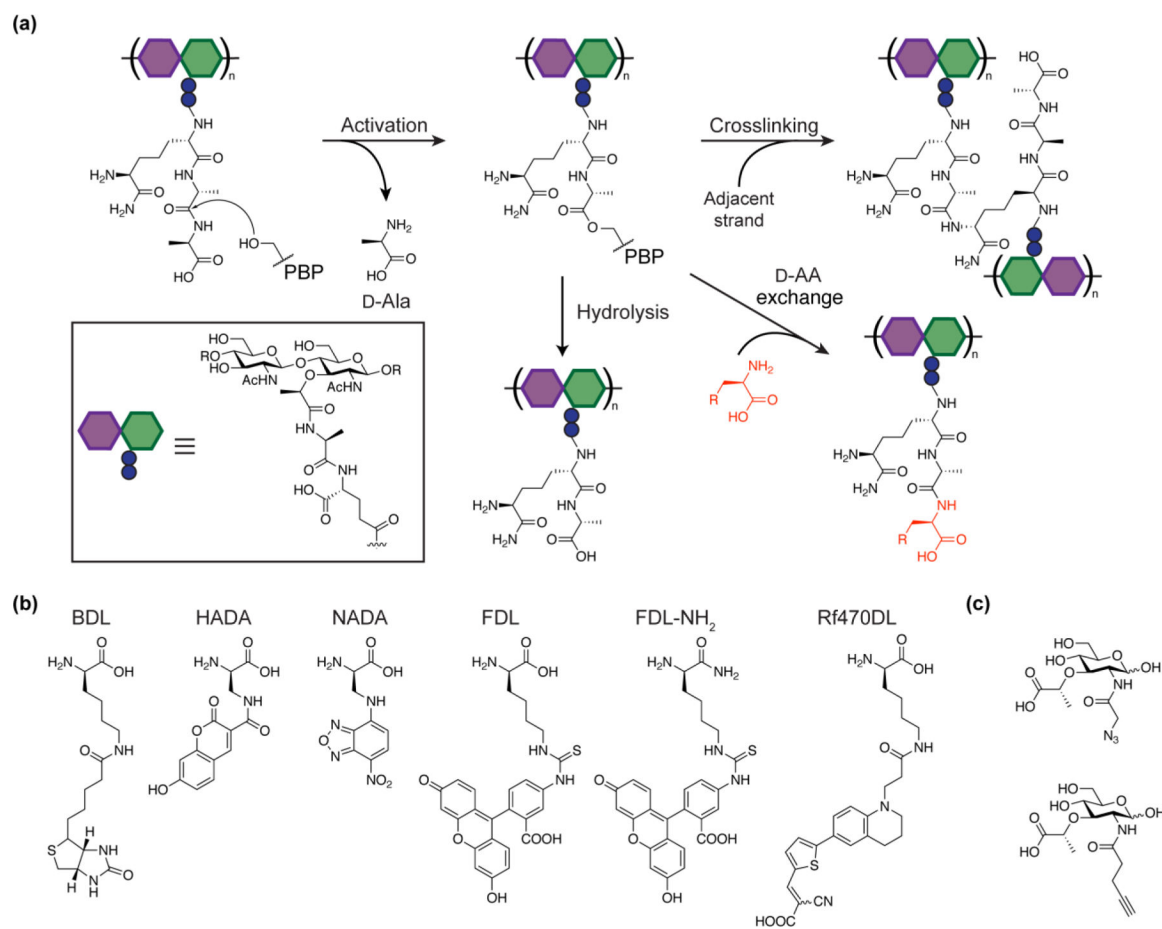


Figure 1: Overview of peptidoglycan synthesis.

Peptidoglycan synthesis is initiated in the cytoplasm, where UDP-GlcNAc is converted to UDP-MurNAc pentapeptide, which is then converted by MraY and MurG at the cytoplasmic membrane to the peptidoglycan precursor Lipid II. After being translocated to the extracytoplasmic side, Lipid II is polymerized by peptidoglycan glycosyltransferases (PGTs) and crosslinked into the existing peptidoglycan by transpeptidases (TPs). Peptidoglycan polymerization is catalyzed by the bifunctional class A PBPs (aPBPs) or the SEDS proteins, the latter of which function together with the monofunctional class B PBPs (bPBPs).

**Figure 2:**

(a) Structure of Lipid II. Lipid II variants that have been obtained using the method described in (b) are listed.^{35,36} *S. pneumoniae* possesses a *murMN* operon which encodes ligases that attach L-Ser-L-Ala or L-Ala-L-Ala to the R¹ position. Lipid II extracted from *S. pneumoniae murMN* strain lacks this modification. **(b) Isolation of Lipid II from bacterial cells.** Accumulation of Lipid II is accomplished by treatment with vancomycin/moenomycin or conditional inactivation of the Lipid II flippase. After chloroform/methanol extraction, a white interface layer enriched with Lipid II and UDP-MurNAc-pentapeptide is observed. A second pyridinium acetate/1-butanol extraction from this interface separates Lipid II from other water-soluble molecules.

**Figure 3:****(a) Schematic of PBP transpeptidase and carboxypeptidase reactions in *B. subtilis*.**

After the formation of the acyl-enzyme intermediate via the cleavage of the terminal D-Ala, transpeptidases can either accept the stem peptide from the adjacent strand or a D-amino acid. Most low molecular weight PBPs are carboxypeptidases that remove the terminal D-alanine via hydrolysis, but a subset of low molecular weight PBPs are able to catalyze the D-amino acid exchange.^{34,36} **(b) D-amino acid probes for peptidoglycan labeling.** Biotin-D-lysine (BDL)³⁴, FDAAs (HADA, NADA & FDL)⁴⁶, carboxamide FDAAs (FDL-NH₂)⁵⁰, and rotor-fluorogenic D-amino acid (Rf470DL)⁵² are shown. **(c) MurNAc derivatives for peptidoglycan labeling.** MurNAc variants containing an azide or alkyne handle that were incorporated into the cell wall are shown.⁵³