Published in final edited form as:

Chembiochem. 2017 September 05; 18(17): 1696–1702. doi:10.1002/cbic.201700293.

Deciphering the Nature of Enzymatic Modifications of Bacterial Cell Wall

Dr. Mijoon Lee, Dr. Dusan Heseck, Ms. Elena Lastochkin, Mr. David A. Dik, Dr. Bill Boggess, and Prof. Shahriar Mobashery

Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556, United States

Abstract

The major constituent of bacterial cell wall is peptidoglycan, which in its crosslinked form is a polymer of considerable complexity that encases the entire bacterium. A functional cell wall is indispensable for the survival of the organism. There are several dozens of enzymes that assemble and disassemble the peptidoglycan dynamically within each bacterial generation. Understanding of the nature of these transformations is critical knowledge on these events. Octasaccharide peptidoglycans were prepared and studied with seven recombinant cell-wall-active enzymes (SltB1, MltB, RlpA, mutanolysin, AmpDh2, AmpDh3 and PBP5). With the use of highly sensitive mass spectrometry methods, we describe the breadth of reactions that these enzymes catalyze with the peptidoglycan and shed light on the nature of the cell-wall alteration performed by these enzymes. The enzymes exhibit broadly distinct preferences for their substrate peptidoglycans in the reactions that they catalyze.

TOC Image

Homogeneous and heterogeneous octasaccharide peptidoglycans were synthesized for characterization of reactions of seven enzymes that modify the bacterial cell wall – SltB1, MltB, RlpA, mutanolysin, AmpDh2, AmpDh3, and PBP5. Three lytic transglycosylases and mutanolysin exhibit broadly distinct preferences for their substrate peptidoglycans in the reactions that they catalyze.

Correspondence to: Shahriar Mobashery.
mobashery@nd.edu.

Supporting information for this article is given via a link at the end of the document.

Keywords

cell wall; peptidoglycan; oligosaccharide; antibiotic resistance; substrate specificity

Bacterial peptidoglycan is the major constituent of the cell wall, which is comprised of a repeating backbone of *N*-acetylglucosamine (NAG)-*N*-acetylmuramic acid (NAM), connected by β -1 $\rightarrow$ 4 glycosidic linkages. A pentapeptide, L-Ala- γ -D-Glu-*m*-DAP-D-Ala, is appended to the NAM unit, which serves as the site of crosslinking of two neighboring peptidoglycan strands. Notwithstanding the structural complexity, cell wall is a dynamic polymer with dozens of enzymes involved in both its biosynthesis and recycling. In excess of 50% of the cell wall undergoes recycling during homeostasis in each bacterial generation for reasons that we do not entirely understand.^[1] Furthermore, once damage is inflicted to the cell wall, for example due to exposure to cell-wall-active antibiotics, the repair entails degradation and biosynthesis.^[2–5]

The investigations of bacterial cell wall and its modifications have been difficult not merely because the substrate is a polymer, but also because of its heterogeneity. Furthermore, the overall outcome of the transformations is complex, so interpretation of the data often is not straightforward. A serious impediment has been the lack of larger synthetic peptidoglycan samples that possess the complexity inherent to the natural substrate, yet would produce simpler reaction outcomes. We describe herein the synthetic preparation of two octasaccharide peptidoglycans (**1** and **1p**) for characterization of the reactions of LTs (Fig. 1B). These compounds were prepared for this study in as many as 58 convergent synthetic steps. We also prepared **1a** from fragmentation of the cell wall. The qualifiers “a” and “p” stand for compounds with the anhNAM or the peptide stem(s), respectively.

We characterized turnover of these peptidoglycans by two groups of enzymes, one processing the glycan backbone and the other the peptide stem. Four glycan-processing enzymes were studied, three lytic transglycosylases (MltB, SltB1 and RlpA) from

Pseudomonas aeruginosa, and an *N*-acetylmuramidase (mutanolysin from *Streptomyces globisporus*). These enzymes fragment the glycan backbone by cleaving the glycosidic bond between NAM-NAG, but they are mechanistically distinct. Mutanolysin is a hydrolytic enzyme that produces a terminal NAM as a product, whereas lytic transglycosylases (LTs) produce a terminal *N*-acetyl-1,6-anhydromuramyl moiety (anhNAM). Both are assumed to go through an oxocarbenium species at the NAM. These catalyze exolytic and endolytic reactions. The former is cleavage of a disaccharide from the peptidoglycan termini (Fig. 1A, the green arrow) and the latter is the reaction in the middle of it (Fig. 1A, the blue arrow).^[6] These enzymes have evolved also preferences for the presence or absence of stem peptides on the NAM, crosslinking, the length of the peptidoglycan and the existence of anhNAM at one end of the peptidoglycan. Three peptide-hydrolyzing enzymes were studied, two amidases (AmpDh2 and AmpDh3) from *P. aeruginosa* and a DD-carboxypeptidase (penicillin-binding protein 5) from *Escherichia coli*. AmpDh2 and AmpDh3 removed the peptide stem attached at the NAM by hydrolyzing the amide bond between D-Lac and L-Ala. On the other hand, PBP5 recognizes the D-Ala-D-Ala in the pentapeptide stem and removes the terminal D-Ala.

The synthetic route to octasaccharides **1** and **1p** is outlined in Scheme 1. We took advantage of our synthetic strategy that was used for construction of a smaller tetrasaccharide previously.^[7] Trichloroacetimidate activation was used for the formation of the β -1,4-glycosidic bond, along with the use of the dimethylmaleoyl (DMM) amine protective group at C-2. Four D-lactates for the muramyl moieties were introduced when the glycan backbone was in hand. Despite use of known standard methodology, the difficulties in preparation of these peptidoglycans are numerous. Regardless of the fact that the disaccharide unit repeats itself four times, the challenges include construction of bonds with the correct stereochemical fidelity (β for the glycosidic bonds and D-lactate for the muramyl moiety), taking into account that the final molecules are not symmetrical, as the terminal sugars are distinct (an unsubstituted NAG to the left and a methyl glycoside to the right; structural handles for the characterization effort, as described below). These structural considerations would mandate the use of distinct building units for each of the sugars from left to right, whose syntheses were reported previously.^[6, 7] In our case, it was to our advantage to construct a trisaccharide (**9**) and a pentasaccharide (**8**) separately, which then were coupled to give the final octasaccharide (**11**). Deprotection of the dimethylmaleoyl (DMM) group from the amines by adjustment of pH is typically facile for small molecules. However, to perform this reaction at each of the eight sites in intermediate **11** proved to be challenging, which ultimately was resolved.

Following acetylation of the amines (**12**), we removed the acetyl esters at C3 to introduce the lactyl moiety there with an attendant inversion of stereochemistry to install the four NAM saccharides (**13**). The introduction of lactate at the four sites was also a challenging reaction. This reaction was repeated with monitoring by mass spectrometry until completed. We noted some formation of the lactyl over-reaction product (**14**), which was eliminated by saponification of the ester bond to revert it to the pool of **13**. In the case of **1p**, the pentapeptide was made in a suitable protected form,^[8] before it was coupled to the four

lactyl moieties. We note that peptidoglycans have also been made by others by different synthetic strategies.^[9, 10]

We are aware that long peptidoglycans from bacteria usually end with the anhNAM moiety.^[11] We purified a small sample of the octasaccharide terminating with an anhNAM from this mixture by HPLC (**1a**).^[12] With compounds **1**, **1p** and **1a** in hand (Fig. S1), we were ready to study their properties with recombinant LT enzymes from *P. aeruginosa* to demonstrate their utility in deciphering how these enzymes modify their substrates.^[6, 13, 14] As shown in Fig. 1, there are a maximum of three possible reaction sites in **1**, **1a** and **1p**, which potentially could yield six different products as a consequence of the LT reaction, as shown in Fig. 2A (**II**, **IV**, **VI**, **IIa**, **IVa**, **VIa** from **1** and **IIp**, **IVp**, **VIp**, **IIpa**, **IVpa**, **VIpa** from **1p**). As an explanation for the nomenclature, **II** refers to a disaccharide product and **IV** a tetrasaccharide, and so on. On mixing of the substrate with a given LT, we monitored the reaction by LC/MS. Products were characterized by comparison to authentic synthetic standards, of which we prepared several by the methodology from our own lab: **II**,^[6] **IIa**,^[6] **IIp**,^[8] **IIpa**,^[15] **IV**,^[8] **IVa**,^[6] **IVp**,^[8] and **VIa**.^[12] In the two cases that we did not have authentic synthetic products (compounds **VI** and **VIp**), we resorted to analyses by HRMS, LC/MS, and LC/MS/MS (Figs. S2–S7).

The reactions of substrate **1** with the three LTs are given in Fig. 2. SltB1 turned over **1** almost completely to give two main end products, **II** and **IIa**, along with a very small amount of **VI**. In contrast, the reaction outcome for RlpA produced **IVa** and **IV** as a result of cleavage by route **b** (Fig. 2A). We also saw products **VIa** and **II** due to cleavage by route **c**. As products **IIa** and **VI** were not seen, cleavage by RlpA does not proceed by route **a**. To underscore this point, the observed products **IVa**, **IV** and **VIa** themselves do not serve as substrates for RlpA, as we do not see any formation of **IIa**. Hence, RlpA is a *bona fide* endolytic LT. MltB did not take **1** as a substrate.

The reactions of **1a**, a strand terminating with anhNAM, are similar to those of **1**, yet they give much simpler reaction outcomes (Fig. 2C). For example, SltB1 gave a sole product **IIa**. RlpA produced three products, **IIa**, **IVa**, and **VIa** along with the unreacted substrate **1a**. Because of the presence of the anhNAM in all products and the substrate, one cannot distinguish the chosen route by the enzyme in these transformations. The methyl glycoside handle at the NAM at far-right end of substrate **1** was instrumental in differentiation of the routes that the enzyme pursued. On the other hand, MltB did not accept **1a** as a substrate.

Compound **1p** is different from **1** by having the four pentapeptide stems. SltB1, again produced two end products (**IIp** and **IIpa**) as major components along with small amounts of **VIp**, similar to the pattern for the reaction with **1**. MltB, now turns over **1p** to several products, **IIp**, **IVp**, **VIp**, and **IIpa**. This is in sharp contrast to the case of **1**, which did not serve as a substrate for MltB. Therefore, MltB requires the presence of stem peptides in its substrate. RlpA did not form significant amount of any product with compound **1p**, which supports the statement that RlpA prefers the absence of stem peptide in substrates.

When we carried out the reaction to append the protected pentapeptide to the protected octasaccharide **13**, we had discovered that one half of the products had been incorporated

with four pentapeptides, which led to the synthesis of compound **1p**. The second half of the products was comprised of the octasaccharide incorporated with only three pentapeptides. After deprotection, this product resulted in **15**. Analysis by LC/MS/MS indicated that **15** is a mixture of three isomers (Fig. S1), which did not separate under our LC condition (Fig. 3A). The NAM at position 4 had the pentapeptide, but the three compounds miss a peptide at NAM positions 1, 2, or 3 (for analysis see Fig. S1). Availability of compounds **15** presented a unique opportunity in exploring a more complex heterogeneous peptidoglycan substrate for these enzymes. This is actually closer to reality, as uniformity in regards to the presence or the length of the stem peptide is not seen in the cell wall. Compounds **15**, as a mixture, were used in experiments with the LTs (Fig. 3). The reaction with SltB1 gave three exolytic products, **IIa**, **IIp** and **IIpa**, following the previous trends with compounds **1**, **1a** and **1p** (Fig. 3B). RlpA did not turnover **15** (Fig. 3C). However, compounds **15** served as substrates for MltB and the results are shown in the chromatogram of Fig. 3D, which indicates the formation of **IIp**, **IIpa** and two unique tetrasaccharides.

In the interest of brevity, the analyses for **15a** are given in Fig. 3E and those for **15b** and **15c** are in Fig. S8. The crossed arrows are the routes that were not seen. The boxed compounds are those that we detected for the reactions. There are three potential sites of LT reaction in **15a**, shown by the curved arrows. Cleavage by route **a** results in **IIpa** and the given hexasaccharide, which then experiences further cleavage to give more of **IIpa** plus the tetrasaccharide **16**. The formation of this tetrasaccharide was detected (Figs. 3D and S7) and it is not processed further (absence of **IIa**). The alternative fragmentation of the hexasaccharide was not seen. Peptidoglycan **15a** was also fragmented by route **b**. This fragmentation gave **IVpa** plus the given tetrasaccharide. **IVpa** is processed to two molecules of **IIpa**, but the tetrasaccharide **16** is not processed further. The final possibility, route **c**, simply does not take place. This same analysis was performed for compounds **15b** and **15c** (Fig. S8). Overall, the combination of concurrent reaction outcomes of three isomers, **15a**–**15c** by MltB accounts for formation of all four products in Fig. 3D. MltB prefers to have peptide stems, but not necessarily at all NAM units in the substrate. However, this complex analysis elucidates that MltB requires a peptide stem at the NAM at subsite –1, but tolerates the absence of peptide at +2 subsite. Beyond these positions flanking the seat of the reaction, the enzyme does not have a strict requirement.

The summary of conclusions on these reactions is that the SltB1 is an exolytic enzyme with no preference for the presence or absence of the stem peptide in the peptidoglycan. MltB exhibits both the endolytic and exolytic reactions with a strong preference for the presence of the stem peptide, yet it tolerates absence of peptide stems. RlpA is a true endolytic LT and it does not like the presence of stem peptides. The three enzymes are clearly very distinct in the reactions that they catalyze with the peptidoglycan.

Mutanolysin, which is commercially available and is widely used for peptidoglycan analysis, turned over **1p** almost completely to give two main end products, **IIp-OH** and **IIp**, along with a very small amount of **VIp** (Figs. 4 and S9). Interestingly, mutanolysin did not take **1** as substrate, hence the presence of the stem peptide is a requirement in the substrate. We also investigated reactions of two peptidoglycan amidases, AmpDh2 and AmpDh3,^[16]

with **1p** (Figs. 4 and S10). Compound **1p** was cleanly transformed to **1** (removal of four stem peptides) by AmpDh3. On the other hand, AmpDh2 turned over **1p** to the mixture **15** as the prominent product(s), along with minor products that had more than one stem peptide removed.

Notwithstanding similarities of the two amidases, the two have distinct quaternary structures. One is a dimer and the other a tetrameric protein.^[17, 18] The distinct structures are commensurate with discrete reactivity in each case. Reaction of PBP5^[19] with **1p** gave a series of products with tetrapeptide (**17**, **18**, and **19** with four, three and two tetrapeptide stems, respectively) as a result of DD-carboxypeptidase activity (Fig. 4 and Fig. S11).

We have described in this report the preparations of long synthetic peptidoglycans, which were used in elucidating the nature of transformations by seven distinct bacterial recombinant enzymes that modify the cell wall, which underscores the diversity of structures that one sees in the complexity of the polymeric bacterial sacculus. The heterogeneity of the cell wall was recapitulated by unsymmetrical samples with variations in the muramyl sites (lack of peptide or different distributions of it). We described a mass spectrometry analysis paired with authentic synthetic samples for the delineation of the various reactions. This type of analysis presents opportunity in elucidating the preferences of these enzymes in a process of considerable complexity, both in enzyme catalysis and in selection of the sites of modification in a polymeric substrate of significant heterogeneity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the NIH (GM61629 and T32GM075762). DAD acknowledges support by the ECK Institute for Global Health.

References

1. Park JT, Uehara T. Microbiol Mol Biol Rev. 2008; 72:211. [PubMed: 18535144]
2. Lamers RP, Burrows LL. Future Med Chem. 2016; 8:975. [PubMed: 27228070]
3. Fisher JF, Mobashery S. Bioorg Chem. 2014; 56:41. [PubMed: 24955547]
4. Johnson JW, Fisher JF, Mobashery S. Ann NY Acad Sci. 2013; 1277:54–75. [PubMed: 23163477]
5. Mark BL, Vocadlo DJ, Oliver A. Future Microbiol. 2011; 6:1415. [PubMed: 22122439]
6. Lee M, Hesek D, Dik DA, Fishovitz J, Lastochkin E, Boggess B, Fisher JF, Mobashery S. Angew Chem, Int Ed. 2017; 56:2735.
7. Hesek D, Lee M, Morio KI, Mobashery S. J Org Chem. 2004; 69:2137. [PubMed: 15058963]
8. Lee M, Hesek D, Shah IM, Oliver AG, Dworkin J, Mobashery S. ChemBioChem. 2010; 11:2525. [PubMed: 21117117]
9. Wang N, Hasegawa H, Huang CY, Fukase K, Fujimoto Y. Chem Asian J. 2017; 12:27. [PubMed: 27868361]
10. Inamura S, et al. Org Biomol Chem. 2006; 4:232. [PubMed: 16391765]
11. Harz H, Burgdorf K, Hölte JV. Anal Biochem. 1990; 190:120. [PubMed: 2285138]
12. Artola-Recolons C, et al. ACS Chem Biol. 2014; 9:2058. [PubMed: 24988330]
13. Blackburn NT, Clarke AJ. Biochemistry. 2002; 41:1001. [PubMed: 11790124]

14. Jorgenson MA, Chen Y, Yahashiri A, Popham DL, Weiss DS. Mol Microbiol. 2014; 93:113. [PubMed: 24806796]
15. Heseck D, Lee M, Zhang W, Noll BC, Mobashery S. J Am Chem Soc. 2009; 131:5187. [PubMed: 19309146]
16. Zhang W, Lee M, Heseck D, Lastochkin E, Boggess B, Mobashery S. J Am Chem Soc. 2013; 135:4950. [PubMed: 23510438]
17. Martínez-Caballero S, et al. J Am Chem Soc. 2013; 135:10318. [PubMed: 23819763]
18. Lee M, et al. J Am Chem Soc. 2013; 135:12604. [PubMed: 23931161]
19. Heseck D, Suvorov M, Morio KI, Lee M, Brown S, Vakulenko SB, Mobashery S. J Org Chem. 2004; 69:778. [PubMed: 14750804]

Figure 1.
 (A) Reactions catalyzed by LTs. The subsites for saccharide binding are indicated as $-n$ to $+n$, flanking the seat of the reaction. (B) The structures of the three octasaccharide substrates for LTs.

Figure 2.

(A) All possible turnover routes of **1** by LTs. Representative LC/MS chromatograms of reactions with (B) **1**, (C) **1a**, and (D) **1p**.

Figure 3. (A–D) Representative chromatograms of reactions of **15** with SltB1, RlpA, and MltB. (E) All permutations for reaction routes with **15a** by MltB are depicted. The actual reaction products that were found are boxed in purple.

Figure 4. Turnover of **1** and **1p** by four peptidoglycan-processing enzymes. Reactions of peptide-hydrolyzing enzymes are colored in red and orange. That of mutanolysin is colored in blue.

Scheme 1.

Syntheses of octasaccharides **1** and **1p**. Glycosyl donors are in blue and glycosyl acceptors are in red.