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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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# Reactions of All *Escherichia coli* Lytic Transglycosylases with Bacterial Cell Wall

Mijoon Lee, Dusan Hesek, Leticia I. Llarrull, Elena Lastochkin, Hualiang Pi, Bill Boggess, and Shahriar Mobashery*

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

Supporting Information

**ABSTRACT:** The reactions of all seven *Escherichia coli* lytic transglycosylases with purified bacterial sacculus are characterized in a quantitative manner. These reactions, which initiate recycling of the bacterial cell wall, exhibit significant redundancy in the activities of these enzymes along with some complementarity. These discoveries underscore the importance of the functions of these enzymes for recycling of the cell wall.

Bacterial cell wall, also called the sacculus, is a cross-linked polymer that encases the organism and is critical for its survival. Due to the complexity and importance of the cell wall, the study of how the cell wall is assembled and maintained is an area of intense investigation.[1]

The peptidoglycan is the major constituent of the cell wall. This polymer is formed by the reaction of transglycosylases, which assemble repeats of the disaccharide *N*-acetylglucosamine (NAG)-*N*-acetylmuramic acid (NAM) having an appended stem peptide. Cross-linking of neighboring strands of peptidoglycan is performed by DD-transpeptidases. A number of other enzymes modify the assembled cell wall, and these processes are dynamic. These biosynthetic events go hand-in-hand with cell-wall recycling, which processes >50% of cell wall during the normal growth of bacteria, for reasons that are not entirely understood.[2] Recycling also takes place in response to cell-wall damage by antibiotics.[2]

Cell-wall recycling, first discovered in Gram-negative bacteria, also takes place in Gram-positives.[2,3] In Gram-negatives, the recycling commences by the action of lytic transglycosylases (LTs), which degrade the peptidoglycan in an unusual reaction that entails entrapment of the $C_6$-hydroxyl of the NAM moiety at the oxocarbenium species generated at the glycosidic carbon (Figure 1). The end product of the reactions of these LTs is the metabolite *N*-acetyl-$\beta$-D-glucosamine-(1→4)-1,6-anhydro-*N*-acetyl-$\beta$-D-muramyl-L-Ala-D-$\gamma$-Glu-*meso*-DAP-(D-Ala)$_n$ (**1**), with $n$ = 0, 1, 2 as typical, and $n$ = 1 as the most abundant. Metabolite **1** is internalized by the permease AmpG (Figure 1). Once in the cytoplasm, a series of reactions convert **1** into Lipid II, which is transferred to the surface of the plasma membrane for *de novo* synthesis of cell wall. The process is not as well understood in Gram-positives; however, they would appear to depend less on LTs and more on muramidases in degradation of cell wall.[3]

Why various bacteria possess multiple distinct LTs—seven in *Escherichia coli*—is not understood. It could be that their functions are either distinct and complementary or redundant and overlapping. The latter scenario arises if the functions of the enzymes are critical, so that redundancy is a safeguarding mechanism. It is interesting to note that inactivation of all seven *E. coli* LTs is not tolerated, but loss of individual enzymes is not lethal, implying existence of redundancy for the critical functions.[4] This observation indicates that broad-spectrum inhibition of all LTs might provide opportunities for antibiotic design. However, redundancy might not always be seen in LTs, as some organisms have fewer of these enzymes.[5] The LTs from *Neisseria gonorrheae*,[5a] *Helicobacter pylori*,[5b] and *Bacillus anthracis*[5c] would appear to play distinct functions (see Supporting Information (SI)).

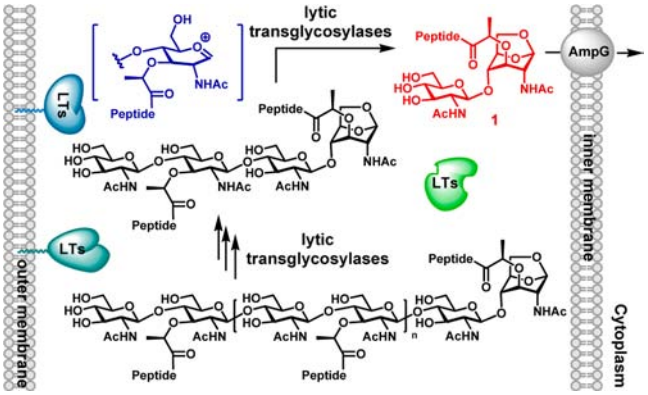


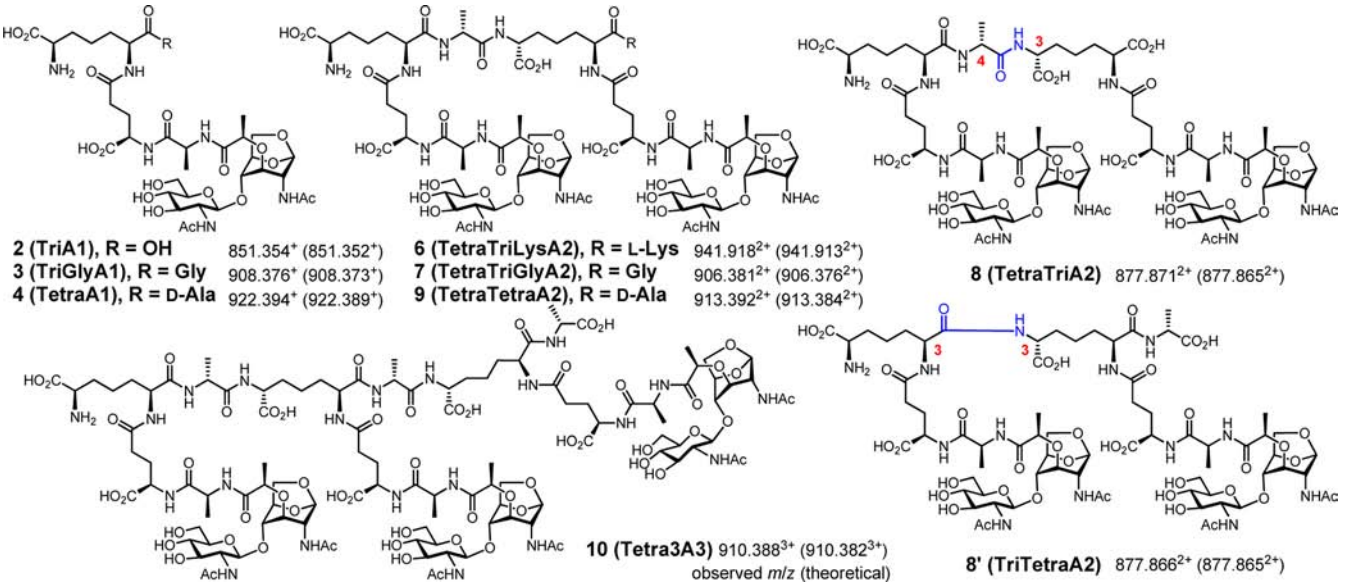

**Figure 1.** Degradation of cell wall by lytic transglycosylases initiates the early events in cell-wall recycling in Gram-negative bacteria.

Since the discovery of the first LT in 1975,[6] the enzymes of *E. coli* have been most studied.[4,7,8] However, the earlier studies focused on individual enzymes, which identified a few reaction products. The full scope of reactions of LTs and their side-by-side comparison have not been investigated. The difficulty is twofold. First, the substrate for these enzymes is a complex polymer, which in *E. coli* has been estimated to be larger than the chromosome.[9] Second, sensitive methods are needed to identify and characterize the reaction products. We have addressed both of these challenges in our present study by using preparations of cell wall from *E. coli* as substrate for all seven recombinant LTs and by employing LC/MS and LC/MS/MS for elucidating products of each of the LTs of *E. coli* at the low picomole level of sensitivity. The seven *E. coli* LTs are

**Received:** September 11, 2012
**Published:** February 19, 2013

   dx.doi.org/10.1021/ja309036q | *J. Am. Chem. Soc.* 2013, 135, 3311−3314

**Chart 1. Chemical Structures of the Major Products from the Reaction of the Stationary-Phase Bacterial Sacculus with MltA**




designated MltA, MltB, MltC, MltD, MltE, MltF, and Slt70. The first six are membrane bound, and Slt70 is soluble.[4b,6−8] We also prepared the *E. coli* sacculus. As the *E. coli* cell wall is cross-linked, the sacculus is a single entity of dimensions of 2 $\mu$m × 1 $\mu$m × 1 $\mu$m, which by microscopy appears as a ghost of the bacterium.[10] For this study, sacculus was prepared from *E. coli* at both the log and stationary phases of growth. Sacculus was exposed to each of the *E. coli* LTs one by one. We then characterized the resultant products by LC/MS and/or LC/MS/MS. The use of a mass analyzer with high resolving power (>10 000) permitted the determination of elemental compositions for ions from high-molecular-mass reaction products (>2000 Da). This provided the opportunity for direct comparisons of all reaction products. In all reactions, the amounts of the enzyme and of the sacculus and the reaction times were kept constant.

We devised a nomenclature based on a variation of a known method.[10,11] As the smallest unit for the products of the LT reactions with sacculus is a NAG-anhydroMur disaccharide (such as compounds **1**), this minimal motif is designated as A1. The full peptide stem in *E. coli* is a pentapeptide: L-Ala$^1$-D-$\gamma$-Glu$^2$-*meso*-DAP$^3$-D-Ala$^4$-D-Ala$^5$. As this sequence is shortened from the C-terminus in the events leading to cell-wall maturation, the remaining peptide is defined as Penta, Tetra, Tri and Di. For example, "TetraA1" is NAG-1,6-anhydroMur with a tetrapeptide for the stem (**4** in Chart 1). As will be described later, glycine and lysine are introduced into the *E. coli* sacculus in place of D-Ala, as minor components.[10,12] Thus, "TriGlyA1" indicates NAG-1,6-anhydroMur with the usual sequence for the first three amino acids and terminating in Gly (a tetrapeptide stem, **3** in Chart 1). In cases when peptide stems are cross-linked, the donor strand is given before the acceptor strand ("TetraTriA2" indicating a tetrapeptide donor and tripeptide acceptor and two NAG-1,6-anhydroMur units; **8** in Chart 1).

We give here a representative reaction and its analysis. The preparation of *E. coli* sacculus from the stationary-phase culture was incubated with MltA for 24 h, at which time the reaction was terminated and the mixture was analyzed by LC/MS. Figure 2 shows the total-ion chromatogram for mass

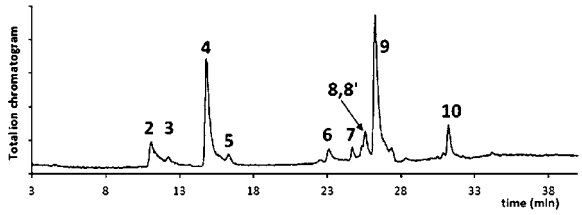



**Figure 2.** MS total-ion chromatogram of reaction of MltA.

spectrometric detection, which paralleled that of UV detection at 205 nm (see SI). The products ionized well with electrospray ionization (ESI), which suggested that structurally related, but less abundant, products should be detected. The ten most abundant products were readily observed by UV, but not so for the less abundant ones. However, the less abundant products were detected in the mass spectra. The structures of 10 most abundant reaction products were assigned and are given in Chart 1. An important observation was that the two most abundant products are TetraA1 (**4**) and TetraTetraA2 (**9**). Furthermore, only four of the 10 products were not cross-linked.

The cross-linking of the peptides in cell wall takes place via the side chain of diaminopimelate (DAP; position 3), with the main chain containing D-Ala at the 4-position (a conventional 3,4-cross-linking), or via the side chain of DAP (position 3) and the main-chain backbone of another DAP (also at position 3; the less common 3,3-cross-linking). We observed both arrangements, for which the order of attachment (acceptor given after donor) was assigned by LC/MS/MS experiments (Chart 1 and SI). The amounts of the individual products were quantified by integration of the peak areas of extracted-ion chromatograms (EICs), and these amounts are given as percentages of the total in Table 1. As indicated earlier, we also identified minor products by MS that were not detectable by UV. The EICs of corresponding $m/z$ values revealed 18 additional minor products (Table S1). These are mostly the less common variants containing lysine and glycine, as outlined in SI. This analysis was repeated for the other six *E. coli* LTs, and their products were assigned (Table 1 and Table S1). The


dx.doi.org/10.1021/ja309036q | *J. Am. Chem. Soc.* 2013, 135, 3311−3314

**Table 1. Major Products from Reaction of the Stationary-Phase Sacculus with LTs and Their Percent Relative Abundance[a]**

| | | MltA | MltB | MltC | MltD | MltE | MltF | Slt70 |
|---|---|---|---|---|---|---|---|---|
| TriA1 | (**2**) | 10.6 | 8.5 | 14.1 | 23.0 | 18.9 | 15.7 | 13.6 |
| TriGlyA1 | (**3**) | 1.6 | 1.2 | 1.9 | 2.5 | 1.3 | 3.0 | 1.0 |
| TetraA1 | (**4**) | 41.8 | 47.2 | 51.5 | 32.6 | 28.5 | 32.0 | 39.2 |
| Tri2A1 | | | 0.9 | | 1.1 | 5.9 | | 4.0 |
| DiA1 | (**5**) | 1.6 | 2.7 | 4.4 | 6.4 | 4.1 | 11.6 | 3.3 |
| TetraTriA1 | | | 0.3 | | | 0.9 | | 1.3 |
| TriTetraA1 | | | 0.4 | | | 1.5 | | 0.9 |
| Tetra2A1 | | | 4.3 | 0.4 | 4.4 | 15.3 | | 13.9 |
| TetraTriLysA2 | (**6**) | 2.0 | 0.5 | 0.9 | | | | |
| Tetra3A1 | | | 1.0 | 0.1 | 1.8 | 7.0 | | |
| TetraTriGlyA2 | (**7**) | 1.4 | 0.7 | 0.7 | | | | |
| TetraTriA2 | (**8**) | 2.3 | 1.4 | 1.8 | | | | |
| TriTetraA2 | (**8′**) | 4.6 | 2.1 | 2.1 | | | | |
| Tetra2A2 | (**9**) | 25.9 | 20.7 | 16.6 | | 2.1 | | 3.0 |
| Tetra3A2 | | | 2.0 | 0.3 | | 0.8 | | 1.8 |
| Tetra3A3 | (**10**) | 3.8 | 1.3 | 1.3 | | | | |
| TetraA0 | | | 0.5 | 0.4 | 8.6 | 2.8 | 21.9 | 6.1 |
| Tetra2A0 | | | 0.5 | 0.6 | 15.6 | 6.1 | 15.8 | 8.7 |
| Tetra3A0 | | | 0.1 | 0.1 | 3.9 | 1.2 | | 1.8 |
| relative activity[b] | | 1.0 | 0.3 | 0.1 | 0.006 | 0.009 | 0.001 | 0.01 |
| non-cross-linked | | 56 | 59 | 72 | 65 | 53 | 62 | 57 |
| cross-linked | | 40 | 29 | 23 | 0 | 3 | 0 | 5 |
| Other 1[c] | | 0 | 1 | 1 | 28 | 10 | 38 | 17 |
| Other 2[d] | | 0 | 7 | 0.5 | 7 | 33 | 0 | 20 |
| minor products | | 4 | 4 | 4 | 0 | 4 | 0 | 1 |

[a]Amounts are expressed as a percentage of the total EIC peak area. [b]Total peak area of muropeptides found for each enzyme normalized to that of MltA. [c]Products without anhydroMur (A0). [d]Products with oligomeric sugars containing one anhydroMur.

major products account for >96% of total. Control experiments (sacculus in the absence of LT) did not produce any detectable muropeptide.

These analyses led us to several observations on shared attributes for the reactions of LTs, and we also identified points of distinction. First, *all* LTs produce TetraA1 (i.e., product **4**). This statement is inclusive of MltE, which previously had been proposed as the only endolytic LT,[7d] while the rest were thought to be exolytic.[4b,6−8] Another important observation is that some of the LTs actually discriminate based on the presence or absence of cross-links (Table 1). We were not able to detect any cross-linked products for the MltD and MltF reactions, while those of MltE and Slt70 generated <5% of cross-linked products.

In addition, the MltA reaction appeared to produce the largest quantities of products (i.e., high specific activity) compared to those of other enzymes. We give the order of activity of these enzymes as MltA > MltB > MltC ≫ Slt70 > MltE > MltD > MltF (Table 1), based on the total muropeptides found for each enzyme. We also identified reaction products containing a reducing end (compounds with "A0" and "Other 1" in Table 1). The quantities of these were relatively small, but some LTs with lower activities (such as MltD, MltF, and Slt70) produced more of this type of product. Why these products are produced is not presently known, but one possibility is the potential for partitioning between entrapment of the NAM $C_6$-hydroxyl and a water molecule at the transient oxocarbenium species. This reaction for MltD, MltF, and Slt70 might be construed as a muramidase-like activity, which has been noted recently in an LT from *Bacillus subtilis*.[13]

The reaction of MltE would appear to be distinct, as it produces linear oligomeric sugars containing one anhydromuramyl moiety at the end (compounds **11**, Figure 3). For example, the product mixture exhibited multiple chromatographic peaks for EIC at $m/z$ 922.389 (Figure 3). By contrast, the MltA reaction produced only one EIC peak at $m/z$ 922.389 with retention time of 14 min. The MltE reaction produced five EIC peaks at $m/z$ 922.389 with retention times of 13.5, 20, 24, 26, and 27 min. Positive-ion mode ESI generated ions with charged states of +1, +2, +3, +4, and +5, which corresponded to neutral molecular masses of 921, 1843, 2764, 3686, and 4607 Da, respectively. This indicates that the products have the general formula, $(\text{NAG-NAM-tetrapeptide})_n$-NAG-1,6-anhydroMur-tetrapeptide, consistent with structure **11** (Figure 3). Mass alone cannot differentiate between a linear oligosaccharide and a cross-linked one (e.g., **11** vs **12**—see SI). However, the use of LC/MS/MS allowed us to differentiate between these two possibilities, ruling out the cross-linked structure (SI). This outcome was due to the aforementioned endolytic

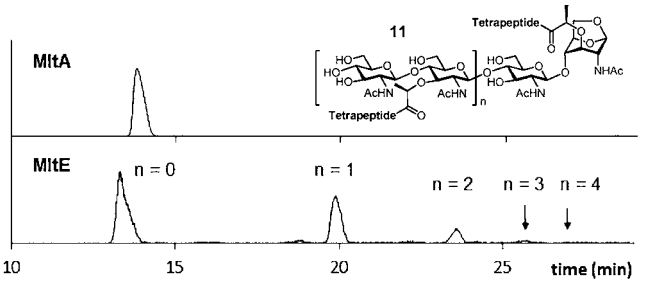



**Figure 3.** EIC at $m/z$ 922.389 of MltA and MltE.


dx.doi.org/10.1021/ja309036q | J. Am. Chem. Soc. 2013, 135, 3311−3314

activity for MltE. The origin of the endolytic activity for MltE was the observation of turnover of $(NAG\text{-}NAM)_7$ (devoid of peptide stems), which produced products consistent with this reaction.[7d] However, the authors could not document activity of the enzyme, when added to the sacculus. Importantly, we observe for the first time this endolytic activity with MltB, MltD, and Slt70 of *E. coli*, which previously were assumed to be exclusively exolytic enzymes. MltA and MltF did not produce this type of product, while MltC gave <0.5% of the endolytic product ("Other 2" in Table 1). MltD of *H. pylori* is reported to have endolytic activity, whereas that organism's Slt is exolytic.[5b] These analyses were done by gene ablation and not by monitoring the reaction of the purified enzymes with the sacculus.

We repeated the same experiments with sacculus isolated from log-phase cultures. Whereas we did not note any major differences in the product profiles between the log and stationary phases, the quantity of products formed was significantly higher when the log-phase sacculus was the substrate. The quantities of products were increased as much as 3-fold for MltA, MltD, MltE, and MltF, 10-fold for MltB and MltC, and 200-fold for Slt70. Product profiles and quantifications are given in Tables S2 and S3. The difference could be due to differing degrees of complexity, rigidity, and steric encumbrance of the cell wall. For example, it is known that there is a higher degree of cross-linking in the stationary-phase *E. coli* sacculus and that longer chain peptidoglycans are found in the log-phase sacculus.[10]

In summary, this is the first study that has undertaken a side-by-side analysis of the reactions of all LTs from the same organism, *E. coli*. Furthermore, the methodology was highly sensitive and was applied uniformly across all seven enzymes with samples of the sacculus from two distinct growth phases of *E. coli*. What is reported here is the elucidation of the propensity of the sacculus to undergo specific reactions catalyzed by these enzymes. Because six of these LTs are membrane bound, their access to the cell wall might not be as uniform as would be expected for the reactions in solution. Furthermore, the copy number of these proteins in *E. coli* is not known, and this presents a regulatory level of control on the outcome of the reactions. Regulation of the activities of LTs could also be manifested in the cases of multiprotein complexes, examples of which have been reported for LTs.[8,14]

Our study reveals that the seven LTs of *E. coli* exhibit redundancy—broad ability to perform exolytic reactions—but they also have unique distinctions, such as their preferences for non-cross-linked versus cross-linked cell wall and their ability to perform endolytic reactions.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Experimental procedure, tables of products formed by reaction of LTs, and MS/MS analyses of products. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

### Corresponding Author

mobashery@nd.edu

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

This work was supported by NIH grant GM61629. The Mass Spectrometry & Proteomics Facility of the University of Notre Dame is supported by grant CHE0741793.

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