Lytic Transglycosylases

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From Genome to Proteome to Elucidation of Reactions for All Eleven Known Lytic Transglycosylases from *Pseudomonas aeruginosa*

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Abstract: *An enzyme superfamily, the lytic transglycosylases (LTs), occupies the space between the two membranes of Gram-negative bacteria. LTs catalyze the non-hydrolytic cleavage of the bacterial peptidoglycan cell-wall polymer. This reaction is central to the growth of the cell wall, for excavating the cell wall for protein insertion, and for monitoring the cell wall so as to initiate resistance responses to cell-wall-acting antibiotics. The nefarious Gram-negative pathogen Pseudomonas aeruginosa encodes eleven LTs. With few exceptions, their substrates and functions are unknown. Each P. aeruginosa LT was expressed as a soluble protein and evaluated with a panel of substrates (both simple and complex mimetics of their natural substrates). Thirty-one distinct products distinguish these LTs with respect to substrate recognition, catalytic activity, and relative exolytic or endolytic ability. These properties are foundational to an understanding of the LTs as catalysts and as antibiotic targets.*

Pseudomonas aeruginosa is an opportunistic bacterial pathogen. Its nearly 5700 open reading frames—a genetic complexity approaching the simple eukaryote *Saccharomyces cerevisiae*—encode a diversity of antibiotic-resistance mechanisms, few of which are fully understood.[1] Many of these resistance mechanisms focus on antibiotics that compromise cell-wall integrity. Although a polymer, the peptidoglycan of the bacterial cell wall is a dynamic structure that is in perpetual states of assembly and disassembly.[2] This dynamism requires numerous enzymes. A key process is the recycling of the cell-wall components that are liberated concurrent with cell-wall growth. This recycling not only conserves biosynthetic intermediates, but signals (by its perturbation) the presence of cell-wall active antibiotics.[3] Cell-wall recycling in *P. aeruginosa* PAO1 generates over 20 different cell-wall components (called muropeptides).[4]

The lytic transglycosylases (LTs) are essential catalysts of bacterial cell-wall function.[5] Genome analysis of *P. aeruginosa* PAO1 reveals at least 11 LT enzymes.[6,7] The function of the LTs is to truncate the glycan component of the cell wall. This polymer consists of glycan strands showing a repeating disaccharide motif, *N*-acetylglucosamine (NAG)–*N*-acetylmuramic acid (NAM), crosslinked to a neighboring glycan strand through the peptide stems of the NAM saccharide. The full-length uncrosslinked stem structure for *P. aeruginosa* is L-Ala–γ-D-Glu–*m*-DAP–D-Ala–D-Ala, where DAP is diaminopimelate (Figure 1). Neighboring strands of the peptidoglycan

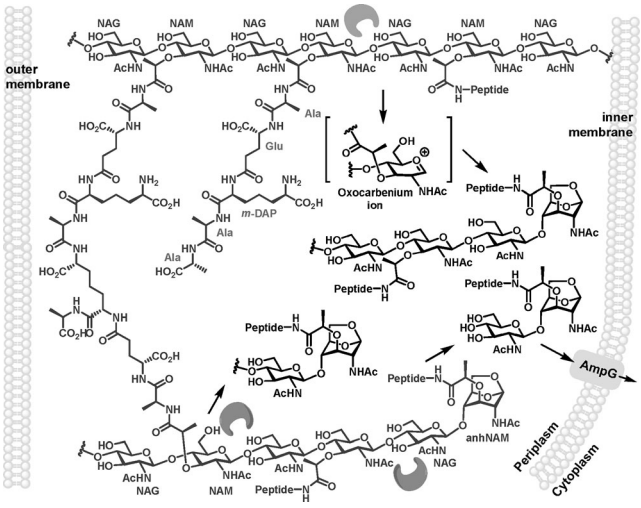

Figure 1. Cell-wall turnover by LTs within the periplasm of Gram-negative bacteria.

crosslink by acyl transfer of the penultimate D-Ala of one strand to the *m*-DAP of an adjacent strand. LTs cleave the glycosidic bond between NAM and NAG, through a NAM oxocarbenium-like intermediate that is trapped by the C_6 hydroxy of NAM. Muropeptides having the 1,6-anhydro-*N*-acetylmuramic acid (anhNAM) terminus, which results from LT activity, are transported from the periplasm through the AmpG transporter of the inner membrane, into the cytoplasm for subsequent transformations.

The 11 LTs of *P. aeruginosa* are MltA, MltB, MltD, MltF, MltF2, MltG, RlpA, Slt, SltB1, SltB2, and SltB3 (Table 1); Mlt stands for "membrane-bound LT", Rlp for "rare lipoprotein" and Slt for "soluble LT". The genes were cloned and each LT was expressed as a soluble protein (by exclusion of the signal peptide, and where applicable also the membrane-anchoring lipobox site) and purified to >95% purity (Table S1 and Figure S1 in the Supporting Information).

Functional studies of five LTs were reported. Three *P. aeruginosa* LTs (MltB and SltB1,[8] RlpA[9]) were studied by others, and two LTs (SltB3[10] and MltF[11]) by ourselves. As these studies used different substrates and different analytical methodologies, we report here the first systematic and

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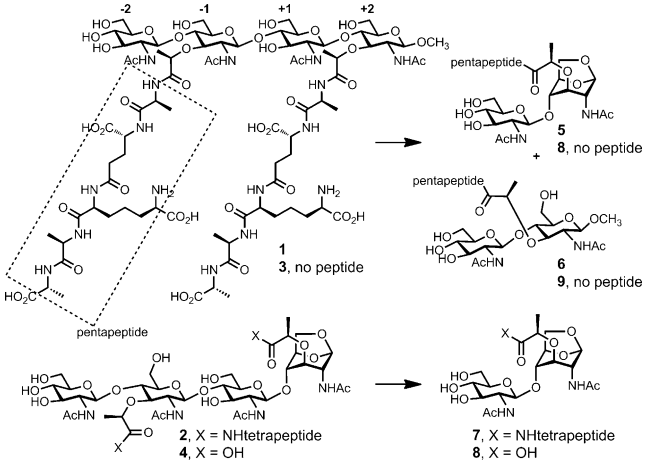
quantitative comparison of the 11 known LT ensemble of *P. aeruginosa*. Each LT was evaluated using four synthetic substrates as well as the intact cell-wall polymer—the sacculus—as substrates.

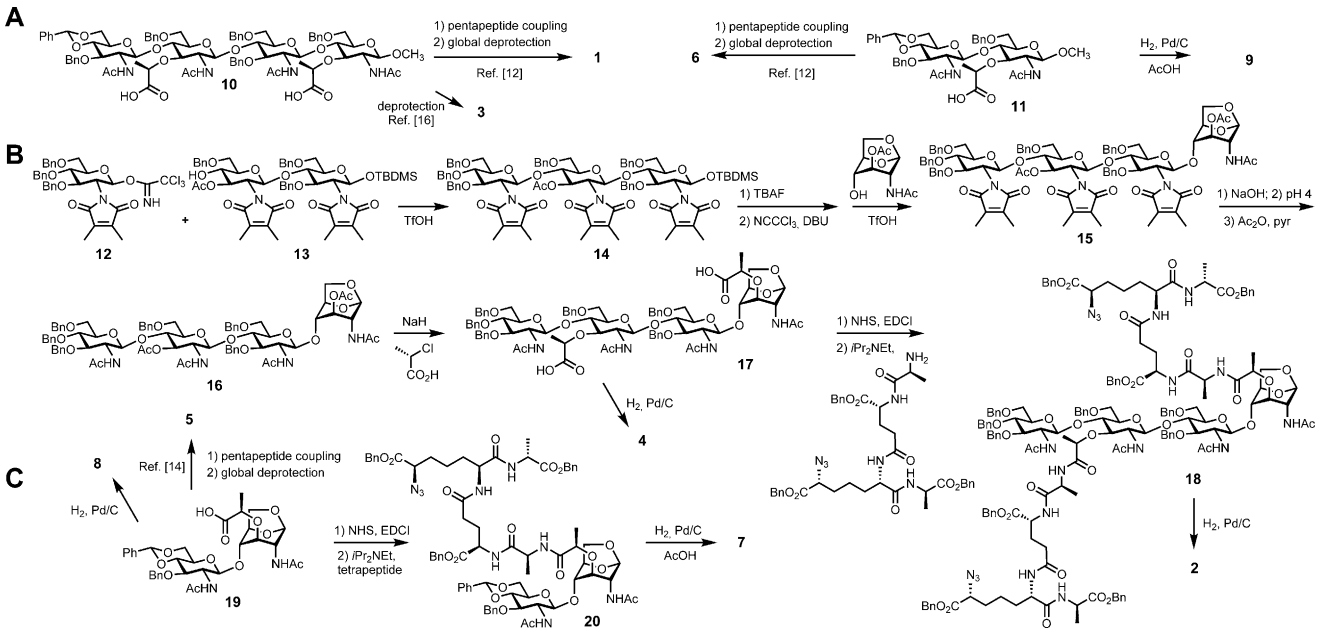
Our first synthetic substrate, NAG–NAM(pentapeptide)–NAG–NAM(pentapeptide) **1**, presents the minimal motif for an LT substrate (Scheme 1). We reported previously its 63 step convergent synthesis (Scheme 2 A).[12] Surprisingly, only MltB and its subfamily (SltB1, SltB2, and SltB3) recognized



Scheme 1. Turnover of four synthetic substrates (**1**–**4**) by LTs.

1 as a substrate, to yield **5** and **6** as products (Table 1 and Figure S2). The possibility that an anhNAM-terminus at the +2 position (two sugars to the right of the scissile bond) was required for substrate recognition by the other seven LTs was evaluated with our second synthetic substrate, NAG—NAM-(tetrapeptide)–NAG–anhNAM(tetrapeptide) **2** (Scheme 1), herein reported for the first time. While syntheses of large (8 saccharide) muropeptides having a NAM-terminus are known,[13] those of muropeptides with an anhNAM-terminus are rarer and heretofore were limited to the NAG–anhNAM disaccharide.[14,15] As outlined in Scheme 2 B, our synthesis of **2** used dimethylmaleoyl (DMM) amine protection to both direct β-glycosidic bond formation and to provide improved solubility, *O*-TBDMS protection at C-1 of the NAG, trichloroacetimidate glycoside activation, and catalytic hydrogenation for the final global deprotection. Key tetrasaccharide **15** was prepared by consecutive glycosylations of disaccharide **13**. The DMM groups in **15** were removed by sequential base/acid treatment. Acetylation of the amine gave **16**. Deprotection at C-3, lactyl attachment, and catalytic hydrogenation yielded substrate **2**. Two additional substrates (**3** and **4**), each devoid of peptide stems, were prepared by catalytic hydrogenation of **10** and **17**, respectively. Synthetic standards for the LT products (**5**–**9**) also were synthesized (Scheme 2). The syntheses of **5** and **6** were reported previously by our laboratory.[12,14] We note that **7** ("tracheal cytotoxin") is not only the turnover product of **2**, but is also a major turnover product of the sacculus by LTs. Although its synthesis was reported earlier,[15] we now provide its full spectral characterization.

All four LTs that turned over **1**—MltB, SltB1, SltB2, and SltB3—also turned over **2** (100 % conversion to **7**). Whereas MltA fully converted **2** to **7**. The rest of LTs did so to a lesser extent (4–41 %: Table 1 and Figure S3). Nonetheless, **2** is a more general LT substrate than **1**. Fewer LTs turned over the two tetrasaccharide substrates lacking peptide stems, **3** (NAG–NAM–NAG–NAM) and **4** (NAG–NAM–NAG–anhNAM), as compared to **1** and **2** (Figures S4 and S5, Table 1). Comparison of the four structurally distinct substrates gave these conclusions. MltA requires a +2 anhNAM (prior product of an LT reaction). MltB requires a peptidyl stem. SltB1, SltB2, and SltB3 accept all tetrasaccharide



Scheme 2. Syntheses of substrates (**1**–**4**) and of products of LT catalysis (**5**–**9**).

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Table 1: Reactions of 11 LTs with synthetic substrates **1**–**4**.[a,b,c]

LT Name	MltA	MltB	MltD	MltF	MltF2	MltG	RlpA	Slt	SltB1	SltB2 (SltG)	SltB3 (SltH)
Locus tag	PA1222	PA4444	PA1812	PA3764	PA2865	PA2963	PA4000	PA3020	PA4001	PA1171	PA3992
1	NR	100	NR	NR	NR	NR	NR	NR	100	100	100
2	100	100	10	6	5	17	4	41	100	100	100
3	NR	NR	NR	NR	NR	NR	NR	NR	100	NR	NR
4	100	NR	3	12	NR	68	NR	NR	100	14	86

[a] Percentage of product is given. This was converted to specific activity in nmol product min^{-1} mg^{-1} protein in Table S2 in Supporting Information. Reactions were carried out in 20 mM HEPES, 0.1 M NaCl, 0.1 % Triton X, pH 7.0 for 2 h at 37 °C. [b] Gray highlights mean avid turnover of a given substrate. [c] "NR" means no reaction.

substrates. The rest of the LTs appear to have additional requirements for substrate recognition, such as a glycan strand length of greater than four saccharides. A limitation of the tetrasaccharide substrates is their inability to differentiate between intrinsic exolytic (degradation from a terminus) or endolytic activity. This preference was examined by the reaction of the LTs with the purified *P. aeruginosa* sacculus. LT degradation of the sacculus releases a set of soluble muropeptides (Figure 2, Tables 2 and S3).

A total of 31 muropeptides were identified, at picomole sensitivity by LC/MS analysis, per earlier methodology.[16] The complete list of LT products is given in Table S3, and the 12 most abundant products are shown in Figure 2 A. In a second assay, the sacculus was first allowed to react with the LT, and the products of this reaction then reacted with the AmpDh3 amidase (hydrolysis at the lactyl moiety of NAM)[17] to remove both crosslinked and uncrosslinked stem peptides.

We further examined oligomeric substrates, (NAG–NAM)$_n$–NAG–anhNAM (**22**, where $n = 2$–9, and lacking peptide stems) which interrogates the requirement for the presence of the stem peptide for recognition, as well as the exolytic or endolytic preference. The results are summarized in Table 2. Figure 2 shows chromatograms for the reactions of MltA and RlpA (representing two LT families) with the sacculus. A detailed summary of each reaction is given in the Supporting Information

Unexpectedly, all 11 enzymes have endolytic and exolytic capabilities. The LTs distinguished for endolytic capability (MltD, MltF2, RlpA, and Slt) are highlighted in dark gray. The LTs distinguished for exolytic activity (MltA, SltB1, SltB2, and SltB3) are highlighted in light gray (Table 2). MltB,

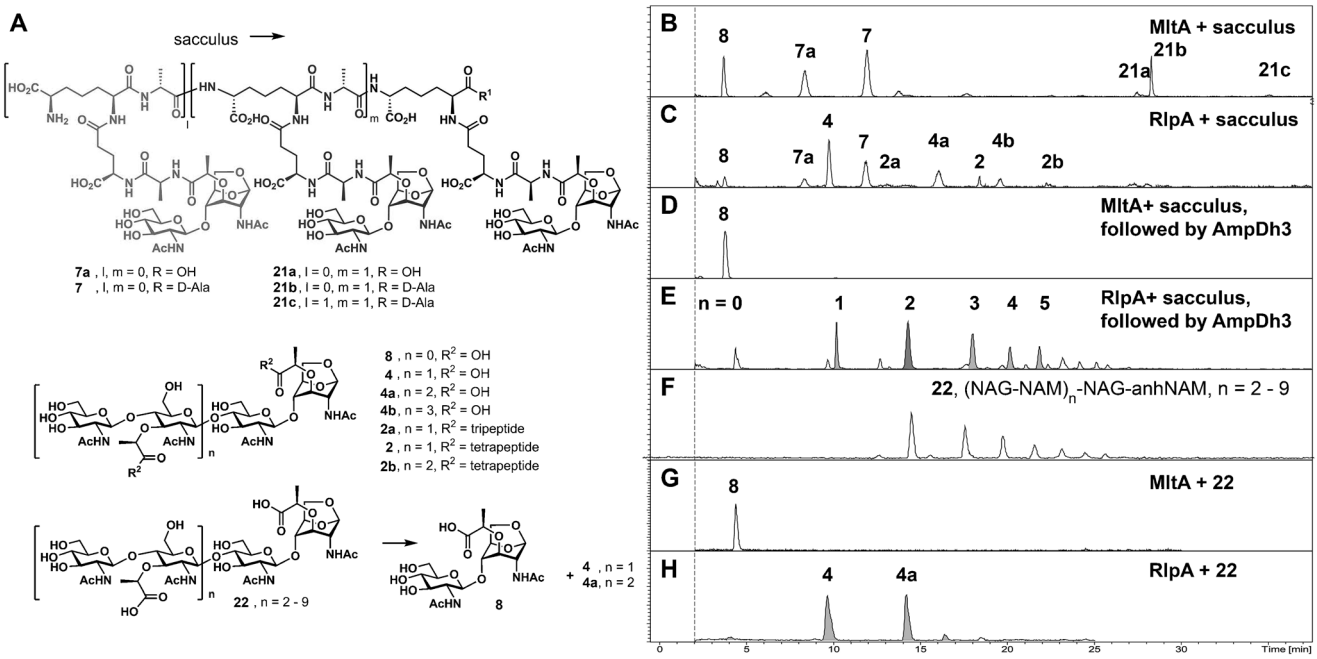

Figure 2. A) Reactions of LTs with various substrates used in this study. B–H) LC/MS extracted-ion chromatograms of reactions of MltA and RlpA. Reactions with the sacculus (B, C), with the sacculus, followed by AmpDh3 (D, E), with **22** (F, G, H).

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Table 2: Summary of reactions of 11 LTs of *P. aeruginosa* with sacculus.[a,b]

Name	MltA	MltB	MltD	MltF	MltF2	MltG	RlpA[c]	Slt	SltB1	SltB2	SltB3
Reactions with sacculus											
% of endolytic products	0.5 ± 0.1	4 ± 0.6	15 ± 1	5 ± 3	26 ± 5	12 ± 0.7	16 ± 8 (58 ± 8)	12 ± 0.4	0.1 ± 0.02	0.3 ± 0.1	0.1 ± 0.03
Relative activity[d]	0.2	0.13	0.02	0.08	0.02	0.07	0.04 (0.07)	0.04	1	0.2	0.9
Non/crosslinked	3 ± 0.1	5 ± 1	14 ± 1	7 ± 1.4	26 ± 2	5 ± 0.1	22 ± 1 (22 ± 3)	15 ± 3	6 ± 0.2	9 ± 0.3	6 ± 0.6
% of products with a reducing NAM-terminus	2 ± 1	3 ± 0.8	27 ± 10	6 ± 3	40 ± 5	12 ± 0.2	8 ± 8 (4 ± 4)	17 ± 2	3 ± 0.2	14 ± 0.1	3 ± 0.5
Reaction with the sacculus, followed by AmpDh3											
% of NAG-anhNAM (**8**)	98 ± 0.1	12 ± 1	6 ± 1	8 ± 0.2	5 ± 1	7 ± 1	5 ± 0.1	5 ± 1	37 ± 0.2	16 ± 1	38 ± 4
Reaction with of (NAG-NAM)n-NAG-anhNAM (**22**, n = 2 - 9)											
% of $(NAG\text{-}NAM)_{0,1}$-NAG-anhNAM (**8**+**4**)	100	0	21	43	8	90	52 (**4**, **4a**)	19	100	54	100

[a] Average of two runs with standard deviations for reactions with the sacculus. [b] Data indicating endolytic activity are highlighted in dark gray and those for exolytic activity are in light gray. [c] These numbers are for saccharides with stem peptides and those in parentheses are for total saccharides. [d] Activities are normalized to the value for SltB1.

MltF, and MltG have dual-activity. The total muropeptides released from the sacculus gave this order of overall activity: (most active) SltB1, SltB3 > MltA, SltB2 > MltB > MltF, RlpA, MltG > Slt > MltD, MltF2 (least active).

While more uncrosslinked (**7a** and **7**) than crosslinked (**21a**, **21b**, **21c**) products were seen for each LT, all LTs turnover both. We also observed minor products having a reducing muramyl terminus, arising from partitioning of the transient oxocarbenium species of LT catalysis between interception by the C_6 hydroxy (major product) and water. The amount of water-derived product is greater for the two enzymes with the lowest specific activities (MltD and MltF2). MltB is unique in its requirement for the presence of the peptide stem for substrate recognition (**22** is unreactive). In contrast MltA, MltF, MltG, RlpA, SltB1, SltB2, and SltB3 show up to 100 % conversion of **22**. RlpA prefers muropeptide substrates lacking the peptide stem, as first observed by Jorgenson et al.,[9] yielding the di- (**8**), tetra- (**4**), hexa- (**4a**), and octasaccharide (**4b**) product ensemble. With the sole exception of MltB, all other LTs produced NAG–anhNAM (**8**) to the extent of 1–13 % (Table S3), but not the larger tetra-, hexa- and octasaccharides (**4**, **4a** and **4b**).

These data confirm the presence of a superfamily of at least 11 LTs in the periplasm of *P. aeruginosa* PAO1. The LTs of *E. coli* were classified on the basis of sequence,[5,18] but continuing study of the *P. aeruginosa* and *Escherichia coli* genomes and proteomes has increased the number and the diversity of the LT superfamily.[5,7,19] This study—the first comprehensive comparison of substrate recognition and processing by the individual members of the *P. aeruginosa* LT superfamily—provides important insight toward functional LT classification. A key discovery is the requirement of some LTs for a +2 anhNAM residue for substrate recognition. All LTs show a subtle interplay among glycan length, stem presence, and exo/endolytic preference. This subtlety is substantiated by the unperturbed planktonic growth of multiple LT gene knockouts,[5,7] implicating not only a redundancy of function, but a redundancy arising in response to critical biological function.

Although breadth of LT function is now proven, the complete functional assignment within the LT superfamily is the outstanding challenge. *P. aeruginosa* SltB1 and MltF have primary roles in the muropeptide-recycling pathway that connects, through PBP4 inhibition by β-lactam antibiotics, to AmpC β-lactamase induction (whereas, seemingly paradoxically, loss of either Slt or MltF decreases β-lactam resistance).[7] The SPOR domain of RlpA directs this LT to the septum for roles in both the rod shaping and daughter-cell separation of *P. aeruginosa*.[9] MltG is assigned as the glycan-sizing LT of peptidoglycan glycan strand elongation.[20] The robust syntheses that yielded the four key tetrasaccharide substrates used in this study have the power for further probing LT substrate identity. This foundational study of the intricacies of substrate recognition and catalysis within the LT family of *P. aeruginosa* is a prelude to further design of LT substrates for ultimate correlation with LT critical function.

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Conflict of interest

The authors declare no conflict of interest.

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[1] P. D. Lister, D. J. Wolter, N. D. Hanson, *Clin. Microbiol. Rev.* **2009**, *22*, 582; S. Wagner, R. Sommer, S. Hinsberger, C. B. Lu, R. W. Hartmann, M. Empting, A. Titz, *J. Med. Chem.* **2016**, *59*, 5929.
[2] R. D. Turner, W. Vollmer, S. J. Foster, *Mol. Microbiol.* **2014**, *91*, 862.
[3] J. F. Fisher, S. Mobashery, *Bioorg. Chem.* **2014**, *56*, 41; J. W. Johnson, J. F. Fisher, S. Mobashery, *Ann. N. Y. Acad. Sci.* **2013**, *1277*, 54–75; R. P. Lamers, L. L. Burrows, *Future Med. Chem.* **2016**, *8*, 975; B. L. Mark, D. J. Vocadlo, A. Oliver, *Future Microbiol.* **2011**, *6*, 1415.
[4] M. Lee, S. Dhar, S. De Benedetti, D. Hesek, B. Boggess, B. Blazquez, K. Mathee, S. Mobashery, *Angew. Chem. Int. Ed.* **2016**, *55*, 6882; *Angew. Chem.* **2016**, *128*, 6996.
[5] E. Scheurwater, C. W. Reid, A. J. Clarke, *Int. J. Biochem. Cell Biol.* **2008**, *40*, 586.
[6] J. F. Cavallari, R. P. Lamers, E. M. Scheurwater, A. L. Matos, L. L. Burrows, *Antimicrob. Agents Chemother.* **2013**, *57*, 3078.
[7] R. P. Lamers, U. T. Nguyen, Y. Nguyen, R. N. C. Buensuceso, L. L. Burrows, *MicrobiologyOpen* **2015**, *4*, 879.
[8] N. T. Blackburn, A. J. Clarke, *Biochemistry* **2002**, *41*, 1001.
[9] M. A. Jorgenson, Y. Chen, A. Yahashiri, D. L. Popham, D. S. Weiss, *Mol. Microbiol.* **2014**, *93*, 113.
[10] M. Lee, T. Domínguez-Gil, D. Hesek, K. V. Mahasenan, E. Lastochkin, J. A. Hermoso, S. Mobashery, *ACS Chem. Biol.* **2016**, *11*, 1525.
[11] T. Domínguez-Gil, et al., *Structure* **2016**, *24*, 1729.
[12] M. Lee, D. Hesek, I. M. Shah, A. G. Oliver, J. Dworkin, S. Mobashery, *ChemBioChem* **2010**, *11*, 2525.
[13] S. Inamura, K. Fukase, S. Kusumoto, *Tetrahedron Lett.* **2001**, *42*, 7613.
[14] D. Hesek, M. Lee, W. Zhang, B. C. Noll, S. Mobashery, *J. Am. Chem. Soc.* **2009**, *131*, 5187.
[15] A. Kawasaki, Y. Karasudani, Y. Otsuka, M. Hasegawa, N. Inohara, Y. Fujimoto, K. Fukase, *Chem. Eur. J.* **2008**, *14*, 10318.
[16] M. Lee, et al., *J. Am. Chem. Soc.* **2013**, *135*, 12604; M. Lee, D. Hesek, L. I. Llarrull, E. Lastochkin, H. Pi, B. Boggess, S. Mobashery, *J. Am. Chem. Soc.* **2013**, *135*, 3311.
[17] W. Zhang, M. Lee, D. Hesek, E. Lastochkin, B. Boggess, S. Mobashery, *J. Am. Chem. Soc.* **2013**, *135*, 4950.
[18] N. T. Blackburn, A. J. Clarke, *J. Mol. Evol.* **2001**, *52*, 78.
[19] F. A. Herlihey, M. Osorio-Valeriano, G. Dreyfus, A. J. Clarke, *J. Bacteriol.* **2016**, *198*, 1847.
[20] R. Yunck, H. Cho, T. G. Bernhardt, *Mol. Microbiol.* **2016**, *99*, 700.

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Communications

Lytic Transglycosylases

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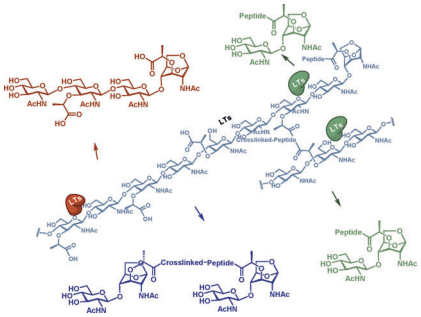
From Genome to Proteome to Elucidation of Reactions for All Eleven Known Lytic Transglycosylases from *Pseudomonas aeruginosa*



The lytic transglycosylases (LTs) are glycoside-cleaving enzymes found in Gram-negative bacteria. Their diversity of structure contrasts with a common substrate: the peptidoglycan of the bacterial cell wall. By the systematic evaluation of synthetic peptidoglycans and the polymeric peptidoglycan, 11 LTs of *Pseudomonas aeruginosa* are now characterized with respect to their substrate recognition, catalytic activity, and exolytic or endolytic preference.

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