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Lytic Transglycosylases: Concinnity in concision of the bacterial cell wall

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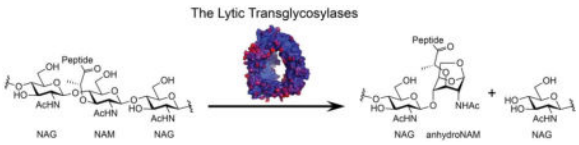
Abstract

The lytic transglycosylases (LTs) are bacterial enzymes that catalyze the non-hydrolytic cleavage of the peptidoglycan structures of the bacterial cell wall. They are not catalysts of glycan synthesis as might be surmised from their name. Notwithstanding the seemingly mundane reaction catalyzed by the LTs, their lytic reactions serve bacteria for a series of astonishingly diverse purposes. These purposes include cell-wall synthesis, remodeling, and degradation; for the detection of cell-wall-acting antibiotics; for the expression of the mechanism of cell-wall-acting antibiotics; for the insertion of secretion systems and flagellar assemblies into the cell wall; as a virulence mechanism during infection by certain Gram-negative bacteria; and in the sporulation and germination of Gram-positive spores. Significant advances in the mechanistic understanding of each of these processes have coincided with the successive discovery of new LTs structures. In this review, we provide a systematic perspective on what is known on the structure-function correlations for the LTs, while simultaneously identifying numerous opportunities for the future study of these enigmatic enzymes.

Graphical abstract



Keywords

Lytic Transglycosylase; Peptidoglycan; Muropeptide; Bacteria; Cell-Wall Recycling; Secretion System; AmpC; AmpR

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Introduction

Bacteria preserve the integrity of their cell envelope during growth and cell division by the integration of an array of interwoven pathways (Pazos *et al.*, 2017). A key component of this envelope and focus of these pathways is a peptidoglycan polymer—the cell wall—that fully surrounds the bacterium. The gross structure of the peptidoglycan is of strands of a $(NAG\text{-}NAM)_n$ (NAG: *N*-acetylglucosamine and NAM: *N*-acetylmuramic acid) polysaccharide, covalently interconnected to neighboring strands through peptide stems attached to the NAM saccharide. The intact polymer is called the murein sacculus, and the individual strands that comprise the sacculus are collectively referred to as muropeptides (Wientjes *et al.*, 1991; Silhavy *et al.*, 2010; Vollmer & Seligman, 2010). The peptidoglycan contributes directly to the shape of the bacterium (Young, 2010). The peptidoglycan of the monoderm Gram-positive bacterium is typically multi-layered (15–30 nm thick), while the peptidoglycan of the diderm Gram-negative bacterium is primarily mono- or bilayered (3–6 nm thick). The peptidoglycan is an exterior component of the Gram-positive cell envelope, while in the Gram-negative bacterium the peptidoglycan is located in the periplasm between the two membranes. The peptidoglycan is a structurally dynamic polymer that grows, remodels, and separates. For example, in the course of doubling the peptidoglycan in a single generation of exponential growth, the Gram-negative bacterium *Escherichia coli* remodels and recycles nearly half of its existing peptidoglycan (Goodell & Schwarz, 1985; Goodell, 1985; Park, 1993). As the Gram-negative bacterium ages and enters stationary growth, the extent of the cross-linking of its peptide stem increases, and the barrier function of the peptidoglycan is reinforced (Pisabarro *et al.*, 1985). The peptidoglycan prevents the undesired passage of macromolecules into and out of the cell, while also serving as a scaffold through which critical proteins of the bacterium interact (Typas *et al.*, 2012). A final and no less important function of peptidoglycan growth, and of the recycling of the muropeptides liberated during growth, is the provision of sensory mechanisms to detect the presence of cell-wall-targeted antibiotics (Park, 1995).

Superfamilies of the amide and glycoside hydrolases orchestrate the dynamic peptidoglycan structure to enable wall expansion during cell growth, for the splitting of the septum to permit separation of the divided cells, for the excavation of the cell wall for the insertion of protein complexes (for example, secretion systems, flagella, and pili), for the recycling of the muropeptides liberated during these processes, for endospore sporulation and germination, and for other functions that await discovery (Höltje, 1998; Koraimann, 2003; Keep *et al.*, 2006; Baron & Coombes, 2007; Scheurwater *et al.*, 2008; Uehara & Park, 2008; Scheurwater & Burrows, 2011; van Heijenoort, 2011; Alcorlo *et al.*, 2017). A key superfamily of enzymes at the nexus of both cell-wall recycling and cell-wall-antibiotic detection is the lytic transglycosylases (LTs). Although their structures define them as members of the glycoside hydrolase superfamily, the reaction catalyzed by the LTs is non-hydrolytic (Höltje *et al.*, 1975). The reactions of LTs fragment the polysaccharide of the peptidoglycan at the NAM-NAG glycosidic bond, by an intramolecular cyclization of the *N*-acetylmuramyl moiety to yield a 1,6-anhydro-*N*-acetyl-β-D-muramyl (1,6-anhydroMurNAc) product (Figure 1). This transformation is the hallmark of LT catalysis. In Gram-negative bacteria, the 1,6-anhydroMurNAc-containing muropeptides are transported from the

periplasm to the cytoplasm through the transmembrane protein AmpG (Jacobs *et al.*, 1994). These muropeptides are degraded in the cytoplasm and their constituent components are used for Lipid II biosynthesis. The Lipid II assembled in the cytoplasm is delivered to the periplasm for *de novo* synthesis of the peptidoglycan (Figure 2) (Barreteau *et al.*, 2008; Bouhss *et al.*, 2008; Vollmer & Bertsche, 2008; Butler *et al.*, 2013; Sieger *et al.*, 2013; Mohammadi *et al.*, 2014; Sham *et al.*, 2014; Meeske *et al.*, 2015; Scheffers & Tol, 2015; Ruiz, 2016; Kuk *et al.*, 2017; Leclercq *et al.*, 2017). The elegantly preserved balance among peptidoglycan synthesis, remodeling, and degradation sustains the integrity of the cell wall (Höltje & Heidrich, 2001).

Herein, we review the LT enzymes, with particular emphasis on their structure and their roles in the Gram-negative bacteria *E. coli*, *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, and *Stenotrophomonas maltophilia* and in the Gram-positive bacteria *Clostridium difficile, Bacillus anthracis*, and *Bacillus cereus.* This review discusses first the reaction products of LT catalysis, and progresses to the protein structural studies that organize the LT superfamily of the Gram-negative bacteria. We expand the previous six-family classification for the Gram-negative LTs (Herlihey & Clarke, 2016) and introduce two new families to accommodate the Gram-positive LTs. This latter section comprises the major topic of this review. We provide a concise mechanistic perspective, and discuss the different assay methods used to evaluate LT catalysis. Lastly, we promote the LTs as potentially important drug targets, and summarize efforts toward this objective.

The reaction products of LTs initiate both offensive and defensive mechanisms

Escherichia coli, the first organism for which LT activity was documented, recycles more than 90% of its LT reaction products (Uehara *et al.*, 2005). Other Gram-negative organisms are less efficient and release un-recycled muropeptides to the surrounding extracellular environment (Greenway & Perkins, 1985). The iconic products of LT catalysis, the 1,6-anhydroMurNAc muropeptides, contribute to bacterial virulence in many organisms (Adin *et al.*, 2009; Sorbara & Philpott, 2011; Boudreau *et al.*, 2012; Bertsche *et al.*, 2015). An example is the Gram-negative pathogen *Bordatella pertussis*, the notorious cause of whooping cough, that exports the “tetrapeptide” GlcNAc-1,6-anhydroMurNAc-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala muropeptide—appropriately named tracheal cytotoxin (TCT)—from the bacterium (Rosenthal *et al.*, 1987; Cookson *et al.*, 1989; Luker *et al.*, 1993; Chang *et al.*, 2006; Kawasaki *et al.*, 2008). This muropeptide causes both ciliostasis and extrusion of ciliated cells in infected patients. The mechanism for this cytotoxic effect is induction of interleukin-1 (IL-1), which subsequently upregulates nitric oxide (NO) production, a toxin to sensitive ciliated cells (Heiss *et al.*, 1994). Similarly, export of structurally related muropeptides (such as 1,6-anhydroMurNAc tripeptide, lacking the terminal D-Ala of the “tetrapeptide” muropeptide) by the Gram-negative *N. gonorrhoeae* during pelvic infection causes an inflammation-dependent loss of the ciliated cells of the fallopian tubes (Chan *et al.*, 2012; Chan & Dillard, 2016). These released muropeptides induce inflammatory cytokine (including IL-1β, IL-8, and TNF-α) production via NOD1 activation of the inflammasome (Philpott *et al.*, 2014; Wheeler *et al.*, 2014; Caruso & Núñez, 2016; Keestra-

Gounder *et al.*, 2016). Muropeptide-induced inflammatory dysfunction is associated also with infections caused by methicillin-resistant *Staphylococcus aureus* (Müller *et al.*, 2015), *Helicobacter pylori* (Suarez *et al.*, 2015), and the *Chlamydia* (Zou *et al.*, 2016).

LT reaction products enable bacterial defense mechanisms. An efficient resistance mechanism to β-lactam antibiotics—mainstays of antibacterial chemotherapy—in many Gram-negative bacteria (including *P. aeruginosa* and most *Enterobacteriaceae*) is the induction of an enzyme, a β-lactamase, to hydrolytically deactivate the β-lactam antibiotics. β-Lactams enter the periplasm of these bacteria through outer-membrane porins to encounter their biological target, the penicillin-binding proteins (PBPs) of peptidoglycan biosynthesis. β-Lactam antibiotics obstruct cell-wall synthesis by covalent inactivation of these enzymes. The β-lactam ring (of the penicillin, cephalosporin, and carbapenem antibiotics) mimics the D-Ala-D-Ala segment of the muropeptide stem of the peptidoglycan (Lee *et al.*, 2001; Lee *et al.*, 2003; Pratt, 2016). PBPs act on this same muropeptide stem as their substrate. The consequence of this mimicry—the inactivation of these PBPs—results in failed crosslinking of the peptidoglycan. The resulting aberrant peptidoglycan is degraded by the LTs. Notably, the soluble LT of *E. coli* (Slt70) enriches the periplasmic muropeptide pool of tripeptide and tetrapeptide containing muropeptides, preventing the misincorporation of nascent peptidoglycan into the cell wall by nonspecific transpeptidases in *E. coli* (Cho *et al.*, 2014). Other LTs enrich the pool of pentapeptide-containing muropeptides. These muropeptides are transported to the cytoplasm by the inner-membrane protein AmpG (Johnson *et al.*, 2013; Fisher & Mobashery, 2014). In the cytoplasm, pentapeptide-containing muropeptides derepress (in *P. aeruginosa* and many *Enterobacteriaceae*) the AmpR transcription regulator so as to initiate β-lactamase expression (among other effects) as a key resistance mechanism (Wiedemann *et al.*, 1998; Balcewich *et al.*, 2010; Balasubramanian *et al.*, 2015; Vadlamani *et al.*, 2015; Lee *et al.*, 2016a; Dik *et al.*, 2017). The enabling function of LTs intimately links muropeptide recycling to antibiotic resistance (Figure 2). Antibiotic resistance is a problem of profound societal consequence and contributes to an estimated 700,000 deaths per year globally from multidrug-resistant bacterial infections (O'Neill, 2016).

Other physiological responses in eukaryotes caused by muropeptide release include somnogenic, arthritogenic, and pyrogenic activities (Krueger *et al.*, 1984; Fleming *et al.*, 1986; Johannsen, 1993). Furthermore, muropeptides cause appetite suppression and weight loss in rats, and are suggested to be the cause of appetite loss during bacterial illness (Biberstine *et al.*, 1996).

A large diverse family of LTs has evolved

Bacteria depend on an array of cellular tasks performed by LTs, and encode a seemingly matching array of LTs for these purposes. Although each member is presumed to have specialized tasks, extensive study implicates functional redundancy so as to compensate for the loss of any single member. The inability to create a viable pan-LT knockout suggests that at least some of the LT functions are essential (Scheurwater & Clarke, 2008). Nonetheless (and accordingly) in many cases the primary cellular task performed by each LT is unknown. Six unique LT catalytic folds are known in Gram-negative bacteria, with each annotated as a conserved domain in the Pfam database (Figure 3). These six domains were organized

previously into six distinct families (Herlihey & Clarke, 2016). LTs may be further organized by their substrate preference for either exolytic or endolytic strand cleavage. Exolytic LT activity cleaves a terminal NAG-NAM disaccharide from the end of the glycan strand. Endolytic LT activity cleaves the chains internally and hence gives products that are at least four saccharides in length (Lee *et al.*, 2013; Lee *et al.*, 2017). These dual characteristics provide a basis for LT organization, and in turn provide insight into their tasks within the cell. In this review, we classify the known LTs (and LT homologues) of four Gram-negative and three Gram-positive organisms. Additionally, we classify recently discovered protein homologues that are not yet proven to have *bona fide* LT catalytic activity.

Gram-negative Family 1 Lytic Transglycosylases

Overview

The largest family of LTs is Family 1. Family 1 previously encompassed five subfamilies in *E. coli* (1A, 1B, 1C, 1D, 1E) and three subfamilies in *P. aeruginosa* (1A, 1D, 1E) (Blackburn & Clarke, 2002). It is herein expanded to six subfamilies in *E. coli* (1A, 1B, 1C, 1D, 1E, 1G), four subfamilies in *P. aeruginosa* (1A, 1D, 1E; and as a phage 1H), two subfamilies in *S. maltophilia* (1A, 1D), and four subfamilies in *N. gonorrhoeae* (1A, 1B, 1D, 1G). The specific LTs of the subfamilies include: (1A) *E. coli* Slt70, *P. aeruginosa* Slt, *S. maltophilia* Slt, *N. gonorrhoeae* LtgA; (1B) *E. coli* MltC, *N. gonorrhoeae* LtgB; (1C) *E. coli* MltE; (1D) *E. coli* MltD, *P. aeruginosa* MltD, *S. maltophilia* MltD1 and MltD2, *N. gonorrhoeae* LtgE; (1E) *E. coli* MltF, *P. aeruginosa* MltF and MltF2; (1F) *R. sphaeroides* SltF and their respective homologues in other Gram-negative organisms (see Table 1 for complete description). Additionally the Family 1 LTs include the herein newly assigned non-chromosomally encoded EtgA (1G) of EPEC *E. coli* (Garcia-Gomez *et al.*, 2011) and the LtgX (1G) residing in the gonococcal genetic island (GGI) of *N. gonorrhoeae* (Kohler *et al.*, 2007). Additionally, the endolysin of bacteriophage ΦKZ GP144 (1H) is a member of Family 1. Two proteins from *Mycobacterium tuberculosis*, resuscitation-promoting factor B and E, give structural evidence in support of their identification as LTs, but formation of the LT reaction product has not yet been shown (Squeglia *et al.*, 2013; Mavrici *et al.*, 2014; Sexton *et al.*, 2015; Ruggiero *et al.*, 2016). Family 1 is a diverse family in both sequence (Figure 4) and function (Lee *et al.*, 2013). Family 1 includes both primarily soluble subfamilies (1A, 1F, 1G, 1H) and lipoprotein (membrane-bound) subfamilies (1B, 1C, 1D, 1E). Each protein has a highly conserved catalytic domain, comprised primarily of α-helices (Figure 5).

Blackburn and Clarke previously defined four consensus motifs for LT assignment to Family 1. Motif I is the two residue ES, numbered as E64 and S65 in the MltE (ECK1181) LT of *E. coli*. This LT is chosen for representative numbering protein due to its structural simplicity, in that it lacks the additional domains of the other *E. coli* LTs. Motif I is invariant among Family 1 LTs (with the exception of ET in a few species encoding Family 1F). The ES dipeptide is positioned between substrate binding subsites –1 and +1 (Davies *et al.*, 1997; Alcorlo *et al.*, 2017). E64 is the primary catalytic residue of Family 1 LTs (Dijkstra & Thunnissen, 1994; Thunnissen *et al.*, 1994; van Asselt *et al.*, 1999b). The role of S65 in

catalytic function has not been elucidated. Motif II (G–L–M–Q, 1A, 1B, 1C), (G–I/L–W/M–Q, 1D), (G–L/M–M–M/Q, 1E), (G–C–M/F–Q, 1F), (G–L/I–M–G/Q, 1G), and (G–W–F–Q, 1H), appearing as G_{79}-L-M-Q_{82} in *E. coli* MltE is positioned on a loop at the +1 subsite. Motif III (A/G–Y–N, 1A), (A/R–Y–N, 1B), (Y–A–N, 1C), (A–Y–N, 1D, 1E, 1G), (A–Y–H, 1F), (A–H–F, 1H), appearing as Y_{146}-A-N_{148} in *E. coli* MltE, is positioned on an α-helix at the –2 subsite. In all but two LT subfamilies, Motif IV is a conserved Y192 flanked by a hydrophobic residue. Residue Y192 is positioned on an α-helix at the –1 subsite adjacent to the catalytic E64, and is believed to have a catalytic role. The sequence identity at the consensus motif sites of *N. gonorrhoeae* LtgB is less conserved, but the sequence similarity to *E. coli* MltC is indisputable. The consensus motifs of Family 1 LTs are shared with other members of glycoside hydrolase Family 23 (GH23). However, the diversity of structures (Figure 5) and functions of these enzymes has at this time prevented the identification of a consensus mechanism.

Family 1A

The founding member of the LT family is the soluble lytic transglycosylase Slt70 (73 kDa; ECK4384) of *E. coli* that was discovered in 1975 by Höltje *et al.* (Höltje *et al.*, 1975). The protein of the *slt* gene subsequently was cloned and purified in 1989 (Betzner & Keck, 1989; Rozeboom *et al.*, 1990). Slt70 was initially proposed to be a cytoplasmic enzyme (Mett *et al.*, 1980; Keck *et al.*, 1985). Keck *et al.* later discovered that the N-terminal amino-acid residues comprise a signal peptide that directs the protein to the periplasm, with loss of the signal peptide (Engel *et al.*, 1991). Slt70 is acknowledged as a representative exolytic enzyme of the LT superfamily (Kusser & Schwarz, 1980; Beachey *et al.*, 1981; Keck *et al.*, 1985; Betzner *et al.*, 1990). Although Slt70 is primarily exolytic, Lee *et al.* demonstrated that a lower level of endolytic activity was present. Slt70 also shows strong preference for non-crosslinked muropeptides (Lee *et al.*, 2013). A deletion mutant of the *slt* gene yielded *E. coli* without a growth defect phenotype (Roeder & Somerville, 1979; Templin *et al.*, 1992). The structure of Slt70 (Figure 5) was solved by X-ray crystallography in 1994 by Thunnissen *et al.* It has a “doughnut-shaped” three-domain structure, including a dual-domained “superhelical” ring of α-helices on its N-terminus (Thunnissen *et al.*, 1994; Thunnissen *et al.*, 1995a). The ring is not closed. This characteristic may allow the protein to navigate, by simple rotation, around peptide stems. The third and catalytic domain is at the C-terminus. This SLT domain (Pfam: PF01464) has a characteristic lysozyme-like fold with the catalytic glutamate found as E478. Thunnissen *et al.* later reported Slt70 structures having bound both 1,6-anhydromurotripeptide and the disaccharide-mimetic LT inhibitor bulgecin A, shedding light on a mechanism (Thunnissen *et al.*, 1995b; van Asselt *et al.*, 1999b). Enzyme affinity assays revealed protein-protein interactions between *E. coli* Slt70 and PBPs 1b, 1c, 2, 3, 7/8 (Romeis & Höltje, 1994; von Rechenberg *et al.*, 1996). *P. aeruginosa* Slt (PA3020), *N. gonorrhoeae* LtgA (NGO2135), and *S. maltophilia* Slt (Smlt4007) are homologous proteins to Slt70 of *E. coli* (33% (96%), 25% (78%), 27% (95%) for sequence identity (query coverage[1]), respectively). Unique to its family, LtgA is believed to localize to the inner leaflet of the outer membrane near the septum, due to the presence of a lipobox-processing site adjacent to the N-terminal signal peptide (Schaub *et*

1Query Coverage: The percent of the query sequence that is aligned based on an E Value optimization.

al., 2016). LtgA is the first Family 1 LT proven to cleave the glycosidic bond of a synthetic peptidoglycan tetrasaccharide (Schaub *et al.*, 2016). A crystal structure of LtgA was recently reported by Williams and colleagues, but is not yet available in the PDB bank (Williams *et al.*, 2017). A notable difference between the structure of LtgA and *E. coli* Slt70 is an overlap between the U-domain and the L-domain of the *Neisseria* protein that locks the protein in the "doughnut-shape". Knockout mutants of *N. gonorrhoeae* LtgA displayed substantial loss of peptidoglycan monomer production. However, like the *E. coli* Slt70 knockout, growth of the *N. gonorrhoeae* LtgA-knockout is unperturbed (Cloud & Dillard, 2002). Interestingly, in the presence of primary human neutrophils, an *N. gonorrhoeae* strain with both *ltgA* and *ltgD* (an enzyme with a similar cellular role, as discussed later) knockouts exhibited reduced viability. This consequence was suggested to imply a protective role for the two LTs in the maintenance of the envelope, so as to limit exposure to antimicrobial proteins (Ragland *et al.*, 2017). Nonetheless, LtgA and LtgD have essential roles in the cell-wall recycling of *N. gonorrhoeae*, even though they are not necessary for cell growth or division (Chan *et al.*, 2012). Recent studies demonstrated that LtgA-produced peptidoglycan monomers are recycled at a higher rate than LtgD-produced peptidoglycan monomers (Schaub *et al.*, 2016). Yet, the LtgA mutant was fortified compared to its wild-type counterpart, proving to be more resistant to autolysis and cell death in its stationary phase of growth (Cloud & Dillard, 2002). Similarities between LtgA and other Slt70 homologues have been observed. In *Helicobacter pylori*, Slt70 homologue knockouts produced significantly less peptidoglycan monomer (40% of wild-type) resulting in reduced induction of IL-8 in human (HEK293) cells (Viala *et al.*, 2004), and likewise in *Shigella flexneri* an Slt70 homologue is required for full virulence (Bartoleschi *et al.*, 2002).

Family 1B

Membrane-bound lytic transglycosylase C (MltC; ECK2958) of *E. coli* was first reported by Dijkstra and Keck in 1996 on the basis of sequence alignment with *E. coli* Slt70 (Dijkstra & Keck, 1996). *E. coli* MltC gene encodes an N-terminal signal peptide and Cys17 lipoyl anchor (LISCSTT) lipobox sequence to enable transport of the enzyme to the inner leaflet of the outer membrane for subsequent incorporation as an *S*-lipidated lipoprotein. MltC has the catalytic SLT domain (Pfam: PF01464) as its C-terminus and a DUF3393 (Domain of Unknown Function) domain (Pfam: PF11873) as its N-terminus. The three-dimensional structure of apo MltC (Figure 5) revealed that the DUF3393 domain comprised a five-stranded anti-parallel β-sheet, flanked by a single α-helix on one side and two α-helices on the other (Artola-Recolons *et al.*, 2014). The catalytic acid is E218. Using the sacculus as a substrate, MltC processed both crosslinked and non-crosslinked peptidoglycan. Furthermore, MltC turned over oligomeric sugars (endolytic product) and also formed hydrolytic reaction products (lacking the 1,6-anhydro moiety). Both reactions were minor contributors to the overall reaction products (Lee *et al.*, 2013). *N. gonorrhoeae* LtgB (NGO1033) is homologous to *E. coli* MltC (29% sequence identity and 54% query coverage), yet lacks an N-terminal DUF3393 domain (Kohler *et al.*, 2005). The *ltgB*-knockout *N. gonorrhoeae* strain was viable and was unaffected with respect to release of peptidoglycan fragments, notwithstanding previous studies that implicated a role in peptidoglycan degradation. LtgB mutant analysis demonstrated that loss of the "catalytic residue" (E117A, inferred from homology) did not reduce activity, while loss of a (seemingly) non-conserved glutamate

(E115A) in LtgB homologues was inactive (Kohler *et al.*, 2005). Understanding the apparent deviation from conserved sequence of the protein, and the identification of the specific role of LtgB in *N. gonorrhoeae*, awaits further study.

Family 1C

The membrane-bound lytic transglycosylase E (MltE; ECK1181) of *E. coli* was first reported as a 22 kDa single-domain LT, named initially after its gene *emtA* (Kraft *et al.*, 1998). The MltE gene encodes an N-terminal signal peptide, a lipobox sequence (LAGCSSK), and an SLT transglycosylase domain (Pfam: PF01464). MltE is the primary endolytic LT of *E. coli* with oligomeric saccharides containing a 1,6-anhydroMurNAc terminus comprising more than 33% of its reaction products (Lee *et al.*, 2013). Its endolytic ability is attributed to a fully accessible peptidoglycan-binding groove that lacks the steric clash provided by a transverse loop at the +2 binding site or provided by a separate domain (van Asselt *et al.*, 1999b; van Asselt *et al.*, 2000; van Straaten *et al.*, 2007; Fibriansah *et al.*, 2012). The small size of MltE likely contributes to its endolytic activity, as the enzyme is small enough to navigate the complex peptidoglycan meshwork. *E. coli* MltE processes crosslinked, non-crosslinked, and peptide-free peptidoglycan. It appears to favor substrates that lack peptide crosslinking. The cellular role of MltE in a pathogenic *E. coli* strain has been elucidated. *E. coli* MltE is recruited by the periplasmic domain of TssM, a structural component of the Type VI secretion system, for excavation of the cell wall at the Type VI secretion system insertion site (Santin & Cascales, 2017). The structure of MltE (Figure 5) was first reported in apo form (Artola-Recolons *et al.*, 2011a; Artola-Recolons *et al.*, 2011b) and subsequently (as the E64Q catalytic mutant) in complex with bulgecin A and a murodipeptide, and in complex with chitopentaose (Fibriansah *et al.*, 2012). These structures reveal the ability of MltE to accommodate long oligomeric saccharides. Its structural simplicity (compared to its Family 1 counterparts) and the availability of these structures distinguish MltE for mechanistic study. The other Gram-negative organisms included within the focus of this review (*P. aeruginosa*, *S. maltophilia*, and *N. gonorrhoeae*) do not encode an MltE homologue.

Family 1D

The structure of a Family 1D LT, exemplified by membrane-bound lytic transglycosylase D (MltD; ECK0211) of *E. coli*, has yet to be solved. However, there is sequence homology between Slt70 and MltD (gene product of *yafG*) (Koonin & Rudd, 1994). *E. coli* MltD is a 49 kDa protein that has an N-terminal SLT domain (Pfam: PF01464) and two C-terminal LysM domains (Pfam: PF01476). Note, however, that some Family 1D LTs encode one or three lysM domains (Figure 3). MltD of *E. coli* is a lipoprotein (anchor residue Cys16), in contrast to *P. aeruginosa* MltD (PA1812) that encodes a presumptive Lol avoidance lipobox (LAGCQGSG) sequence that would retain the protein in the outer leaflet of the inner membrane (Lewenza *et al.*, 2008). A structure of the LysM domain of MltD (~40 residues, Figure 5) was reported in 2000 by Bateman and Bycroft as a representative LysM domain structure. LysM domains are peptidoglycan-binding modules that are common in proteins that facilitate bacterial pathogenesis and degrade the murein sacculus (Bateman & Bycroft, 2000; Mulder *et al.*, 2006). Although the cellular role of *E. coli* MltD is not known, its interaction with peptidoglycan has been investigated. *E. coli* MltD has the uncommon ability

to act on non-crosslinked peptidoglycan preferentially, but not exclusively, by exolytic cleavage. MltD reactions also form a significant proportion of hydrolytic products (Lee *et al.*, 2013). *E. coli* MltD is homologous to the aforementioned MltD of *P.aeruginosa* (38% sequence identity, 96% query coverage), two LTs of *S. maltophilia*, MltD1 (Smlt0994: 29% sequence identity, 72% query coverage) and MltD2 (Smlt3434: 33% sequence identity, 44% query coverage), and *N. gonorrhoeae* LtgE (NGO0608: 35% sequence identity, 86% query coverage). The LtgE-knockout of *N. gonorrhoeae* showed no change in peptidoglycan monomer release, similar to the LtgB-knockout (Cloud-Hansen *et al.*, 2008). An MltD-knockout of *H. pylori* showed reduced autolysis in stationary-phase cells. MltD is believed to act primarily toward rearrangement of the peptidoglycan layer (Chaput *et al.*, 2007). However, the precise cellular function of the Family 1D LTs is unknown.

Family 1E

A distinguishing characteristic of the Family 1E LTs is their regulation by an allosteric mechanism. This property is likely true (but has yet to be demonstrated) for the other LTs that have secondary peptidoglycan-binding domains, such as the SPOR domain of RlpA and the LysM domain of MltD. Family 1E includes the membrane-bound lytic transglycosylase F (MltF; ECK2556) of *E. coli*. The *E. coli* MltF protein was first reported as a putative LT under the gene name *yfhD* (Koonin & Rudd, 1994) and was proven so in 2008 (Scheurwater & Clarke, 2008). *E. coli* MltF is a 58 kDa lipoprotein of the outer membrane (Scheurwater & Clarke, 2008). While the MltF of *P. aeruginosa* (38% sequence identity, 86% query coverage) is homologous, it appears to have a Lol avoidance lipobox sequence (Tokuda & Matsuyama, 2004), possibly resulting in its retention in the outer leaflet of the inner membrane. *P. aeruginosa* has a second Family 1E LT, MltF2 (PA2865), whose sequence closely resembles that of *E. coli* MltF, and thus is likely a lipoprotein of the inner leaflet of the outer membrane. MltF2 (26%, sequence identity, 72% query coverage compared to *E. coli* MltF) was studied recently (Lee *et al.*, 2017). The domain architecture of *E. coli* MltF consists of an N-terminal SBP_bac_3 (Bacterial extracellular solute-binding proteins, Family 3) domain (Pfam: PF00497) and a C-terminal classic Family 1 SLT domain (Pfam: PF01464). MltF acts entirely on non-crosslinked peptidoglycan, both exo- and endolytically, consistent with an assignment as a specialized LT activated through its allosteric mechanism (Lee *et al.*, 2013). MltF lacks function either in side-wall growth or in septum formation in both *E. coli* and *P. aeruginosa* (Scheurwater & Clarke, 2008; Lamers *et al.*, 2015). Accordingly, its assignment may be for cell-wall excavation so as to enable the passage of the pilli, fimbriae, and other macromolecular complexes through the peptidoglycan (Dominguez-Gil *et al.*, 2016). Structures of the apo form of *P. aeruginosa* MltF (PA3764), and its complexes with bulgecin, muropeptide, and various amino acids (cysteine, valine, leucine, and isoleucine) in its ABC-transporter domain were deposited by Thunnissen in 2015, but without accompanying publication (Madoori & Thunnissen, 2010). Complementary structures of active and inactive *P. aeruginosa* MltF (Figure 5) in complex with NAG-anhNAM-pentapeptide, NAM-pentapeptide, tetrasaccharide and tetrapeptide, and tetrapeptide gave insight into its allosteric regulation (Dominguez-Gil *et al.*, 2016). The structure of the N-terminal SBP_bac_3 regulatory module of MltF, comprising of two subdomains connected by a flexible linker, resembles that of an ABC-transporter domain. Structural studies accompanied by mass-spectrometry analysis demonstrated that binding of

a muropeptide (L-Ala-γ-D-Glu-L-Lys-D-Ala) to this regulatory module prompted a dramatic and long-distance (40 Å) conformational change (55 Å movement) across its RM-linker-CM domains, exposing the active site of the Family 1 SLT domain of MltF for catalysis. Analysis of the domain composition of other LTs suggests that this regulation may not be unique to Family 1E.

Family 1F

The one current member of Family 1F is the soluble lytic transglycosylase F (SltF; not a soluble form of MltF) of *Rhodobacter sphaeroides* (de la Mora *et al.*, 2007). No homologous protein to SltF is found in *E. coli*, *P. aeruginosa*, *N. gonorrhoeae*, or *S. maltophilia.* The protein was discovered from a phenotypic screen in which an N-terminal deletion mutant of SltF lacked motility. Further studies revealed that SltF interacts with FlgJ to excavate the cell wall for the insertion of the flagellum protein edifice through the sacculus (de la Mora *et al.*, 2007). Herlihey and Clarke assigned SltF to the new subfamily 1F of Family 1 based on sequence analysis (Herlihey & Clarke, 2016). The Pfam database recognizes the catalytic domain of SltF as a Family 1 SLT domain (Pfam: PF01464). However, the catalytic domain of SltF is seemingly larger than that of the other Family 1 LTs, possibly due to additional amino acids within disordered loop regions. As noted earlier, the glutamate in Motif I of *R. sphaeroides* SltF is flanked by a threonine rather than a serine. Homologous SltF proteins in other Gram-negative organisms retain the more common ES Motif I (Herlihey & Clarke, 2016). The E57A and E83A mutants of SltF lacked activity (de la Mora *et al.*, 2007). Three other consensus motifs are moderately conserved and give further credence to the Family 1F subfamily assignment. Moreover, a homology model of SltF displayed the classic Family 1 LT catalytic fold. More recently, its catalytic activity was elucidated (confirming it as an endolytic LT) through MS analysis of the products released from the sacculus by a C-terminal SLT domain construct (Herlihey *et al.*, 2016). Its LT activity is regulated by two proteins (FlgB and FlgF), which stabilize SltF and increase its catalytic activity.

Family 1G

The 1G subfamily of the LTs exemplifies the smallest (typically a mere 17 kDa) of the LT enzymes. Their single domain is identified as an SLT domain (Pfam: PF01464). Family 1G LTs include the EtgA enzyme of enteropathogenic *E. coli* (EPEC) and the LtgX enzyme of *N. gonorrhoeae*. LtgX is one of two LTs (with AtlA in Family 4) that is encoded on the gonococcal genetic island (GGI) acquired by horizontal gene transfer (Kohler *et al.*, 2007). This 57 kb gonococcal chromosome is found in 80% of *N. gonorrhoeae* strains for the collective purpose of facilitating chromosomal DNA secretion through a type IV secretion system (Chan *et al.*, 2012). Knockout of the LtgX activity severely decreased DNA secretion (Kohler *et al.*, 2007). The locus for enterocyte effacement (LEE) plasmid of EPEC encodes EtgA, a LT that functions in partnership with 20 other proteins encoded by the LEE plasmid to assemble a Type 3 secretion system assembly (Kubori *et al.*, 1998; Blocker *et al.*, 2001; Mueller *et al.*, 2005; Mueller *et al.*, 2008; Garcia-Gomez *et al.*, 2011). EPEC bacteria adhere to the epithelium of the small intestine, with effacement of the microvilli and formation of actin pedestals at the attachment site. Since the *E. coli* peptidoglycan creates a barrier for proteins exceeding a molecular weight of 50 kDa, EtgA seemingly excavates the cell wall for the insertion of the entire secretion system, to enable delivery of virulence factors from

the bacterium to the host. The structure of the EtgA LT domain (D60A mutant, Figure 5) resembles the SLT domain of Family 1 LTs (Burkinshaw *et al.*, 2015). EtgA localizes to the periplasm and forms a 1:1 complex with EscI, a protein involved in the assembly of the inner rod component of the type 3 secretion system (Garcia-Gomez *et al.*, 2011; Burkinshaw *et al.*, 2015). The catalytic activity of EtgA increases significantly upon complexation with EscI. This purpose of EscI may be to recruit EtgA to secretion system assembly, thus protecting the cell from nonspecific peptidoglycan degradation. The reaction products of the Family 1G LTs with respect to the sacculus have not been determined. LtgX and EtgA are homologous to geneX (encoded in the *E. coli* F-plasmid on orf169) and VirB (also known as VirB1). Both are presumptive Family 1G LTs (Llosa *et al.*, 2000; Ward *et al.*, 2002; Höppner *et al.*, 2005; Zahrl *et al.*, 2005; Zupan *et al.*, 2007; Kohler *et al.*, 2017).

Family 1H

GP144 of the pseudomonal bacteriophage ΦKZ is a Family 1 LT encoding an SLT domain (Pfam: PF01464), as supported by structural comparisons of GP144 and the Family 1 LTs (Fokine *et al.*, 2008). Previously, GP144 was assigned as a Family 4 LT only on the basis of its bacteriophage origin (Domínguez-Gil *et al.*, 2016). The distinction between Family 1 LTs and Family 4 LTs (such as *E. coli* bacteriophage lambda lysozyme) is evident from structure analysis of the catalytic domains. In addition to its C-terminal SLT domain, GP144 has an N-terminal PG_binding_1 domain (Pfam: PF01471) that is homologous to the peptidoglycan-binding domain of Family 3B LTs, which is discussed later. GP144 was discovered in 2007 and demonstrated as a LT (Paradis-Bleau *et al.*, 2007). Its structure was solved shortly thereafter (Figure 5). The catalytic acid residue is E115 (Fokine *et al.*, 2008). The catalytic groove of GP144 accommodates five saccharides, as reflected by successful complexation of chitotetraose as a substrate mimic. Interestingly, other evidence suggests that GP144 has two active sites and identifies both E115 and E178 as catalytic residues (Chertkov *et al.*, 2017). GP144 interacts with anionic membranes and causes membrane disruption and eventual lysis, effects not seen in a zwitterionic membrane model (Cloutier *et al.*, 2010). Furthermore, in solution the protein exists in a monomer, dimer, and trimer equilibrium (Miroshnikov *et al.*, 2006). Interestingly, bacteriophage lysozymes that attack *E. coli*, *N. gonorrhoeae*, and *S. maltophilia* encode instead a Phage_lysozyme LT domain (Pfam: PF00959). How these catalytic domains function differentially, both mechanistically and physiologically, is unknown. At this time, GP144 is the sole member of Family 1H LTs.

Gram-negative Family 2 Lytic Transglycosylases

Overview

The LT Family 2 is less expansive than the LT Family 1. In the four Gram-negative species that are the focus of this analysis, a single Family 2 LT is present. This LT is named MltA in *E. coli*, *P. aeruginosa*, and *S. maltophilia*; and LtgC in *N. gonorrhoeae*. The sequence conservation of Family 2 LTs is less evident than that of the Family 1 LTs (Figure 6). Although the specific residues corresponding to the identification to each motif are less clearly defined compared to their Family 1 counterparts, Blackburn and Clarke identified six consensus motifs in the Family 2 LTs (Blackburn & Clarke, 2001). These motifs are Motif I (Residues Q–G–X_8–G); Motif II (G); Motif III (no conserved residues); Motif IV (N–X_5–F);

Motif V (P–X_5–A–X_1–D); Motif VI (D–X_1–G–X_1–A–X_6–D–X_3–D–X_3–G–X–G–X_3–G–X_2–A–G). MltA is a member of the glycoside hydrolase GH102 Family. LtgC of *N. gonorrhoeae* is differentiated from the other MltA enzymes by an amino-acid insertion (residues 172–205) of unknown function (Figure 7). This insertion may warrant the creation of a new Family 2 subfamily (family 2B). However, as the solitary example of this insertion among the LTs covered (and therefore not identifying with a domain in the Pfam database) and lacking a functional designation for this insertion, LtgC here is kept as a Family 2 LT.

Family 2A

E. coli MltA (ECK2809) was identified and named Mlt38 (38 kDa in size, after signal peptide removal) in 1994 (Ursinus & Höltje, 1994). It was later renamed as MltA (Ehlert *et al.*, 1995). MltA is a two-domain lipoprotein of the periplasm. MltA shares with the RlpA LT of Family 6 (discussed later) the absence of the quintessential catalytic glutamate (homologous to E64 of *E. coli* MltE). Its first domain is an N-terminal MltA domain (Pfam: PF03562, and conserved in all four MltAs) that assists in peptidoglycan binding (Figure 3). The catalytic 3D domain (Pfam: PF06725) at the C-terminus has two aspartates (*E. coli* numbering: D317 and D328) assigned as a catalytic pair (Powell *et al.*, 2006). *P. aeruginosa* MltA (PA1222), *N. gonorrhoeae* LtgC (NGO2048, and previously named GNA33 as the genome-derived *Neisseria* antigen-33), and *S.maltophilia* MltA (Smlt0155) have respective sequence similarities of 31%, 28%, and 30% (and query coverage of 97%, 97%, and 93%), compared to MltA of *E. coli.*

The apo-crystal structure of *E. coli* MltA was solved in 2005 (Van Straaten *et al.*, 2004; van Straaten *et al.*, 2005) (Figure 7), and re-solved subsequently as a catalytically-inactivate mutant (labeled D308A; residue D328 of the full-length protein) in complex with chitohexaose as a substrate mimetic (van Straaten *et al.*, 2007). The catalytic domain has a double-psi β-barrel fold. Unlike the lysozyme-like fold of the Family 1 LTs, the double-psi β-barrel fold of Family 2 LTs is similar to the catalytic domain of the barwin-like endoglucanase superfamily (van Straaten *et al.*, 2005). Solution of the structure of *N. gonorrhoeae* LtgC allowed comparison to *E. coli* MltA (Figure 7) (Powell *et al.*, 2006). While both catalytic and substrate-binding residues are conserved in the active site, the morphology of the domains differs. The cleft of the catalytic domain of MltA is much wider than the cleft seen in the *N. gonorrhoeae* LtgC. This difference defines the breadth of movement available to MltA during substrate binding (Powell *et al.*, 2006). A shift in subdomain 2 relative to subdomain 1 opens the active site to allow the peptidoglycan to bind. However, the shift is so large (37° rotation) that domain closure once the peptidoglycan has bound may occur. This rotation results in a narrowing of the active-site cleft and a synchronous increase in cleft depth (as seen in MltA bound to chitohexaose) (van Straaten *et al.*, 2007). Both *E. coli* MltA and *N. gonorrhoeae* LtgC engage in a protein-protein interaction with penicillin-binding proteins. *E. coli* MltA immobilized on a Sepharose column retained four PBPs: 1b, 1c, 2 and 3 as well as five different non-PBP proteins (von Rechenberg *et al.*, 1996). Surface-plasmon resonance analysis of the MltA interaction with PBP1b and MipA (MltA-interacting protein) suggested a possible 1:2:1 stoichiometry, respectively, for the multiprotein complex (Vollmer *et al.*, 1999). LtgC interacts with PBP2 of *N. gonorrhoeae* as well (Jennings *et al.*, 2002). However, no

interaction of LtgC with a MipA homologue was detected. Powell proposed that the difference in affinity for MipA between the two LTs may be due to an insertion (referred to as Domain 3 by Powell and colleagues) in LtgC. Based on the sequence conservation between PBP2 of *N. gonorrhoeae* and PBP3 of *E. coli*, the LtgC-PBP2 complex may localize to the septum of *N. gonorrhoeae* (the location for *E. coli* PBP3) (Park & Burman, 1973; Schmidt *et al.*, 1981). This conclusion is supported by the inability of LtgC-knockout strains to complete separation into daughter cells during bacterial division (Adu-Bobie *et al.*, 2004; Cloud & Dillard, 2004). However, this phenotype was not seen for the *E. coli* MltA-knockout strain (Lommatzsch *et al.*, 1997). The interaction network for the MltA LTs of *P. aeruginosa* and *S. maltophilia* has not been studied.

Studies of MltA as a catalyst in *E. coli* are limited to a single analysis comparing the ability of seven LTs to digest the *E. coli* sacculus. *E. coli* MltA catalyzes, with near equal ability, the release of both cross-linked and non-crosslinked muropeptides from the *E. coli* sacculus as a substrate. This enzyme showed exolytic activity, and (in contrast to the other LTs) there was no evidence for the formation of hydrolytic products (Lee *et al.*, 2013). This observation opens the possibility that the mechanism of MltA is different from the Family 1 LTs.

Gram-negative Family 3 Lytic Transglycosylases

Overview

Family 3 LTs encompass both soluble and membrane-bound enzymes possessing a range of cellular functions. Although the Family 3 LTs share the same consensus motifs of the Family 1 LTs, numerous amino-acid insertions within the catalytic module (Figure 8) distinguish Family 3 structurally, and also likely functionally. The representative Family 3 LT is the MltB lipoprotein of *E. coli*, assigned by the CAZy database as a member of the glycoside hydrolase 103 (GH103) Family. This MltB undergoes proteolytic truncation in the periplasm to yield a soluble enzyme named Slt35. Individual point mutations of either R187 or R188 to an Ala significantly impaired the ability of peptidoglycan to bind to the catalytic site. R188 (10-fold decrease in catalytic activity of R188A) is suggested to hydrogen bond with the peptidoglycan lactyl carbonyl, and so complement the salt-bridge interaction between R187 (100-fold decrease in catalytic activity of R187A) and the substrate at the –1 subsite (Reid *et al.*, 2006). Sequence alignments of MltB homologues in more than 40 organisms revealed that R188 is conserved in all examined enzymes, while R187 is less conserved (~70%). Reid *et al.* propose that the presence of R187 should distinguish two Family 3 subfamilies. Family 3A has arginine at position 187, whereas Family 3B LTs would not. All MltB homologues with the absent of R187 (~30%) are Gram-negative. It is suggested that these enzymes are predatory enzymes, functioning akin to a bacteriophage endolysin (Kadurugamuwa & Beveridge, 1996; Kadurugamuwa & Beveridge, 1997; Kadurugamuwa *et al.*, 1998; Li *et al.*, 1998; Kadurugamuwa & Beveridge, 1999). However, division of Family 3 LTs into two subfamilies based on a few residues is inconsistent with the nomenclature used to distinguish the subfamilies of the LT Family 1. Family 1 LTs have a conserved core catalytic domain and are divided into subfamilies on the basis of their peripheral domains (Figure 3). Applying this same criterion to Family 3 keeps all of the MltBs (and SltB1 of *P. aeruginosa*) in one sub-family, and would separate the two *P. aeruginosa* LTs, SltB2 and SltB3 (and the

S. maltophilia MltB2), into the sub-family 3B. These latter three LTs have a C-terminal putative peptidoglycan-binding domain (annotated as PG_binding_1, Pfam: PF01471), whereas *E. coli* MltB does not (Figure 9). Our preference is this sub-family division. In this resulting sub-family 3B, only *P. aeruginosa* SltB1 lacks R187 (R187P), therefore the mechanistic implications of this absence will not be representative of its own subfamily. The recent discovery of Family 3 LTs in other bacteria, including plant bacteria, may at a later time allow for expansion of Family 3 (Guglielmetti *et al.*, 2014; Neudorf & Yost, 2017). The consensus motifs of Family 3 LTs were defined by Blackburn and Clarke as Motif I (Residues V–X_{11}–E–S, 3A as defined herein and V–X_{11}–E–T, 3B as defined herein); Motif II (L); Motif III (G–S– X_1–A–X_1–A–X_1–G–X_3–F); Motif IV (EF-hand) (D); Motif V (S–X_2–N–X_5–G–W). Structural differences, resulting from amino-acid insertions in the Family 3 primary structure as compared to Family 1, are believed to have mechanistic consequences (possibly invoking an oxazolinium intermediate; described later), notwithstanding the shared substrate-binding and catalytic residues between the two families.

Family 3A

MltB of *E. coli* (ECK2696) is an *S*-lipidated (LAACSS lipobox sequence, anchored at Cys19 to the inner leaflet of the outer membrane) lipoprotein of the periplasm (Ehlert *et al.*, 1995). Its proteolytically-processed form (releasing a single-domain soluble enzyme of 35 kDa, named Slt35) was identified prior to the lipoprotein form (Engel *et al.*, 1992). The site of proteolytic cleavage is believed to be between residues F39 and L40 of MltB (Dijkstra *et al.*, 1995). The single domain of MltB is a SLT_2 (Pfam: PF13406) domain containing a catalytic glutamate at position 162. E162 is homologous to the catalytic glutamate of Family 1 LTs (E64 of MltE) and likewise demarcates the –1 and +1 saccharide-binding subsites (Reid *et al.*, 2006). The structure of Slt35 was first reported in 1998 (shown in Figure 9 as MltB) (van Asselt *et al.*, 1998). Although prediction tools recognize Slt35 as single-domain LT, van Asselt *et al.* subdivide the structure into three; an α-domain, a core (LT) domain, and a β-domain (van Asselt *et al.*, 1999a). The core domain sits between the α and β domains, includes the substrate-binding site, and resembles the lysozyme-like fold of the goose egg-white lysozyme. At the C-terminal end of the core domain is an EF-hand motif that binds a calcium ion. The calcium ion is presumed to have a structural, rather than regulatory or catalytic role. *E. coli* MltB has broad specificity with respect to digestion of the *E. coli* sacculus (Lee *et al.*, 2013), accommodating both crosslinked and non-crosslinked peptidoglycan substrates. Although MltB is primarily exolytic, it processes oligomeric substrates at a similar rate to MltD of *E. coli.* Catalysis of turnover of peptidoglycan by MltB is disciplined, as the hydrolytic reaction product is only rarely formed. An early study of the binding partners of *E. coli* MltB revealed protein-protein interactions with PBP 1b, 1c, and 3 (von Rechenberg *et al.*, 1996). The other Family 3A LTs include *P. aeruginosa* MltB (PA4444) and SltB1 (PA4001), *N. gonorrhoeae* LtgD (NGO0626), and *S. maltophilia* MltB1 (Smlt4052). These four have, respectively, 68%, 47%, 35%, and 41% sequence identity (and, respectively, 89%, 77%, 90%, and 74% query coverage) to MltB of *E. coli.* The structure of SltB1 was solved by Nikolaidis *et al.* in 2012 and showed high similarity to that of *E. coli* MltB (Figure 9).

Blackburn and Clarke reported that *P. aeruginosa* SltB1 is more catalytically efficient than its membrane-anchored partner MltB, notwithstanding the findings that both proteins produce identical reaction products (Blackburn & Clarke, 2002). SPR analysis of *P. aeruginosa* SltB1 demonstrated that it binds to PBP2 (also of *P. aeruginosa*) with 1:1 stoichiometry (Nikolaidis *et al.*, 2012). Complex formation required calcium. Cloud-Hansen and colleagues studied the *N. gonorrhoeae* lipoprotein LtgD, the homologue of MltB. The enzyme functions as an exo-muramidase, similar to MltB (Cloud-Hansen *et al.*, 2008). LtgD produces identical reaction products to *N. gonorrhoeae* LtgA (the Slt70 homologue, previously described), though it is less active. Interestingly, the products from each of the two LT have distinct fates: the reaction products of LtgA are recycled into the cytoplasm, while the reaction products of LtgD are released from the bacterium for the purpose of virulence via the inflammatory response initiated by their binding to NOD1 (Mavrogiorgos *et al.*, 2014; Schaub *et al.*, 2016). LtgA and LtgD mediate bacterial survival in neutrophils by reinforcing the *N. gonorrhoeae* cell-wall envelope so as to improve its resistance to lysozyme degradation, independent of their roles in peptidoglycan monomer release (Ragland *et al.*, 2017).

Family 3B

Blackburn and Clarke discovered the enzymes SltB2 (PA1171) and SltB3 (PA3992) in *P. aeruginosa* in 2002, marking a second subfamily of the Family 3 LTs (Blackburn & Clarke, 2002). Although Family 3B lacks a counterpart protein in either *E. coli* or *N. gonorrhoeae*, sequence analysis reveals a Family 3B representative in *S. maltophilia* (MltB2, Smlt4650) (Wu *et al.*, 2016). Family 3B LTs have the following sequence identity (query coverage) to *E. coli* MltB: *P. aeruginosa* SltB2 33% (75%), *P. aeruginosa* SltB3 34% (58%), and *S. maltophilia* MltB2 33% (66%). The catalytic glutamate was assigned to residue 172 of SltB3 based on structural alignments. The catalytic activity of *P. aeruginosa* SltB3 was similar to that of *E. coli* MltA, showing exolytic digestion of the sacculus without formation of endolytic products (Lee *et al.*, 2016b). The crystal structures of apo SltB3 (Figure 9), and SltB3 in complex with NAG-anhNAM-pentapeptide and NAG-NAM-pentapeptide were reported as well (Lee *et al.*, 2016b). The catalytic domain of SltB3 displays folds that are nearly identical to *E. coli* MltB. The C-terminal domain of SltB3 is a PG_binding_1 domain (previously described). The peptidoglycan-binding domain of Family 3B LTs are homologous to the peptidoglycan-binding domain of the peptidoglycan amidases, including *P. aeruginosa* AmpDh2 and AmpDh3, among others (Lee *et al.*, 2013; Martínez-Caballero *et al.*, 2013). However, no functional regulation has been attributed to this domain in Family 3B LTs and the cellular role(s) of these LTs remains unknown.

Gram-negative Family 4 Lytic Transglycosylases

Overview

Family 4 LTs are commonly the LT enzymes of the bacteriophages. There are, as yet, no chromosomally encoded Family 4 LT enzymes. These LTs are presumed to function in the assembly of a secretion system to facilitate host-cell pathogenicity (Walmagh *et al.*, 2013). Bacteriophages encoding LTs with this domain that infect *E. coli*, *S. maltophilia*, and *N. gonorrhoeae* are known. The Family 4 LTs are single-domain enzymes annotated as a Phage_Lysozyme (Pfam: PF00959) catalytic domain. The four consensus motifs of this

domain, as identified by Blackburn and Clarke, are Motif I (Residues A–X_7–S–E); Motif II (Y–X_4–G–X_5–D–X–S–X–HP); Motif III (S–T– X_4–G–R–Y–Q–X_5–W); and Motif IV (W–X–S) (Blackburn & Clarke, 2001). The catalytic glutamate in Motif I has a preceding serine, in contrast to Family 1 LTs, where the serine follows the catalytic glutamate (Figure 10). All known Family 4 LTs are in a single subfamily, termed Family 4A.

Family 4A

The LT of bacteriophage endolysin λ (“λ-lysozyme” or “LAL”) was one of the earliest LTs discovered (Taylor *et al.*, 1975). The structure of apo λ-lysozyme (Evrard *et al.*, 1998) and its complex with chitohexasaccharide as a substrate mimic (Leung *et al.*, 2001) are shown in Figure 11. Family 4 LTs have different structural folds from the other LT families. A search for homologous proteins to the bacteriophage endolysin λ in *N. gonorrhoeae* revealed a second Family 4 LT (38% sequence identity, 84% query coverage) encoded by the GGI of *N. gonorrhoeae.* This LT (AtlA, a λ-lysozyme homologue) was first identified as an autolysin and named autolysin A (Dillard & Seifert, 1997). AtlA is involved in type IV secretion system assembly and hence has a similar function to the GGI LT LtgX. Mutation of the catalytic E48 of AtlA significantly reduced type IV secretion system dependent DNA secretion (Kohler *et al.*, 2007). In this respect, it is similar to endolysin λ of *E. coli*. The gonococci expressing AtlA, however, do not experience cell lysis. A bacteriophage (maltocin P28) isolated from *S. maltophilia* encodes among its 23 ORFs a Family 4 LT (Endolysin P28) in its ORF8 having 57% sequence identity, 84% query coverage, compared to endolysin λ of *E. coli.* Endolysin P28 degrades peptidoglycan with sufficient catalytic competency as to show bactericidal activity against many Gram-negative bacteria (Dong *et al.*, 2015).

Gram-negative Family 5 Lytic Transglycosylases

Overview

The recently discovered Family 5 LTs are the first examples of an LT family found in both Gram-negative and Gram-positive bacteria. They are commonly called MltG. The presumptive function of MltG is glycan-chain termination during the biosynthesis of the cell-wall peptidoglycan (Yunck *et al.*, 2016). It has long been believed that LTs function in the chain termination of nascent peptidoglycan, given the relative scarcity of reducing saccharide termini in muropeptides obtained from amidase-catalyzed degradation of sacculi. The seven consensus motifs of the Family 5 LTs are Motif I (residues K–X_7–G–T–Y); Motif II (L–X_4–G–K–E–X–Q–X_6–E–G); Motif III (E–G–X_3–P–X–T–X_2–Y–X_5–D–X_3–L–X); Motif IV (A–S–I–X–E–K–E); Motif V (E–R–X_2–V–X–S–V–F–X_2–N–R–L–X_3–M–X–L–Q–T–D–V–I–Y–G–X–g); Motif VI (G–X_5–D–L–X_5–Y–N–T–Y–X–I–X–G–L–P–P); and Motif VII (L–X–A–X–A–X–P–X_2–T–X_3–Y–F–V–A–D–G–X_3–G–G–H–X–F–X–L–X_2–H–N) (Herlihey & Clarke, 2016). The catalytic glutamate in Motif IV is preceded by a conserved EK pair and is followed commonly by a threonine residue (Figure 12), similar to Family 3 LTs. All known Family 5 LTs form the single Family 5A subfamily.

Family 5A

The crystal structure of MltG was determined prior to its recognition as a LT (Figure 13). A PDB-deposited structure of MltG was solved in 2007 by Patskovsky *et al.* and annotated as an aminodeoxychorismate lyase based on the protein sequence. MltG is 38 kDa in size, has E218 as its catalytic acid, and is located in the outer leaflet of the inner membrane (Yunck *et al.*, 2016). The catalytic domain of *E. coli* MltG is structurally alike to the catalytic domain of the Gram-positive LT SleB (Figure 13). However, Pfam database annotates its catalytic domain as a YceG (Pfam: PF02618) domain, while the domain of SleB is annotated as a PG_Hydrolase_2 (Pfam: PF07486) domain. Further elucidation of the reaction products is required to understand whether or not the two domains are mechanistically identical. Yunck *et al.* first reported *E. coli* MltG as an LT when a multi-copy MltG-encoding (gene *yceG*) plasmid exhibited a lethal phenotype in a PBP1b-knockout strain (Yunck *et al.*, 2016). MltG is the first LT from *E. coli* localized in the inner membrane. In this location, MltG is suitably located to act in peptidoglycan chain termination. Strong circumstantial evidence that MltG regulates glycan strand length was obtained from an MltG-knockout strain where the average length of the glycan strands (obtained upon sacculus degradation through amidase cleavage of the peptide crosslinks of this polymer) were longer than for the wild-type strain. Although the specific reaction products of *E. coli* MltG have not been elucidated, the enzyme has been shown to be endolytic. *E. coli* MltG interacts with PBP1b, lending credence to the initial finding that PBP1b was required for the non-lethal phenotype. MltG is a widely conserved protein. Homologues to *E. coli* MltG are identified by sequence analysis in *P. aeruginosa* (38% sequence identity, 97% query coverage), *S. maltophilia* (46% sequence identity, 77% query coverage), and *N. gonorrhoeae* (42% sequence identity, 83% query coverage). The *N. gonnorhoeae* homologue may be named as LtgG (rather than as an MltG) for consistency with the previous LT nomenclature developed for the *N. gonorrhoeae* LTs by the Dillard group. An MltG homologue was recently identified in the Gram-positive bacterium *Streptococcus pneumoniae* from sequence analysis (Tsui *et al.*, 2016). The assignment of this protein as an LT awaits experimental verification.

Family 6 Lytic Transglycosylases

Overview

Family 6 LTs have three conserved aspartates, including two believed to be involved in catalysis, akin to MltA of the Family 2 LTs (previously described). The four consensus motifs of the Family 6 LTs are Motif I (residues T–X_2–G–E–X_2–D); Motif II (A–A–H–X–T–L–P–X–P–S–X_4–T–N–X_2–N–G); Motif III (R–X–N–D–R–G–P); and Motif IV (R–X–I–X–L–S–X–A–X–A–X_2–L) (Herlihey & Clarke, 2016). Although a mutant of the second aspartate (D157A) abolished catalytic activity, sequence alignment to the Family 2 LTs suggests that the third aspartate (D168) may be the primary catalytic residue in *P. aeruginosa* (Jorgenson *et al.*, 2014). Notably, in *E. coli* the third aspartate is a serine (S147) (Figure 14). Family 6 LTs have a catalytic DPBB_1 domain (Pfam: PF03330) and a structure of a domain with this designation is solved (PDB code: 4AVR) for a *P. aeruginosa* protein (locus tag PA4485 in strain PAO1) (Moynie *et al.*, 2013). However, a Family 6 LT structure has not yet been solved, and the catalytic activity of PA4485 is yet unproven. The DPBB_1 domain of PA4485 shows distant similarity to cellulose-binding domains, and to the 3D domain of

MltA described earlier. The PA4485 protein is only 13 kDa in size. Although it lacks the SPOR domain of Family 6A proteins, should future studies confirm it as a Family 6 LT, it would be assigned to a new subfamily (Family 6B).

Family 6A

A highly-specialized LT, the so-called rare-lipoprotein A (RlpA; PA4000) from *P. aeruginosa*, is a 37 kDa protein involved in cell division. *P. aeruginosa* RlpA is an outer-membrane lipoprotein (lipobox motif: LSSCSS) comprised of two domains, a catalytic N-terminal DPBB_1 (Pfam: PF03330) domain and a C-terminal Sporulation-related (SPOR) domain (Pfam: PF05036). RlpA localization experiments showed that the protein localizes to the septal ring during cell division (Gerding *et al.*, 2009; Arends *et al.*, 2010). This finding is expected as all known proteins possessing SPOR domains are either involved in cell division or morphogenesis. SPOR domains are widely recognized as peptidoglycan-binding domains (Mishima *et al.*, 2005; Gode-Potratz *et al.*, 2011; Yahashiri *et al.*, 2015, Yahashiri *et al.*, 2017). In contrast to the three other cell-wall proteins encoding a SPOR domain in *E. coli*, RlpA is the only one that is non-critical (Gerding *et al.*, 2009; Arends *et al.*, 2010). Interestingly, *E. coli rlpA* shares an operon with the peptidoglycan-synthesizing proteins PBPa and RodA (Matsuzawa *et al.*, 1989; Mohammadi *et al.*, 2011; Banzhaf *et al.*, 2012). Neighboring this operon is the gene *dacA*, which encodes a peptidoglycan hydrolase (PBP5) also implicated in cell division (Potluri *et al.*, 2012). *P. aeruginosa* RlpA is a *bona fide* LT with preference for glycan strands lacking peptide stems, sometimes referred to as “naked glycan” (Jorgenson *et al.*, 2014). Comparison of *E. coli* RlpA to *P. aeruginosa* RlpA presents a dilemma. Although the two proteins have sequence similarity (45% sequence identity, 36% query coverage), LT activity has not been shown for the *E. coli* RlpA (Jorgenson *et al.*, 2014). A possible explanation is the presence of a serine (S147) in *E. coli* RlpA in contrast to an aspartate (D168)—the presumptive catalytic aspartate—at the equivalent position in *P. aeruginosa* RlpA. Careful assay will be required to show whether this difference abolishes catalytic activity.

P. aeruginosa RlpA-knockout strains contain longer naked glycan strands than the wild-type strain (Jorgenson *et al.*, 2014). This finding suggests that RlpA activity is linked to periplasmic activity of the *P. aeruginosa* AmpDh2 and AmpDh3 amidases. RlpA-knockout strains displayed no phenotype when grown in lysogeny broth (LB). However, in LB without NaCl the knockout failed to produce single colonies when plated. The knockout grew slowly and formed long chains of cells, each with a visible septum, but with incomplete cell division. In addition, these cells were 50% shorter in length and were 20% wider. Replacing NaCl with proline (or sucrose) in the media recovered the wild-type phenotype, suggesting that the long-chain phenotype of the knockout was osmotic-stress dependent. However, induction of RlpA is not linked to osmotic-stress response. Sequence analysis reveals RlpA homologues in both *N. gonorrhoeae* (39% sequence identity, 46% query coverage) and *S. maltophilia* (45% sequence identity, 50% query coverage to *E. coli* RlpA). The low query coverage may be the result of comparably low sequence identity of the SPOR domains among the species. More experiments will be required to understand the role of Family 6A LTs in their respective organisms.

The Gram-positive LTs

The cell wall of Gram-positive bacteria is very different than its Gram-negative counterpart. Although the Gram-positive and Gram-negative peptidoglycan have very similar gross structural chemistries, the Gram-positive cell wall is a thicker, multi-layered exoskeleton. In addition, other polymeric structures (notably those of the lipoteichoic acids, the wall teichoic acids, and the capsular polysaccharide) are covalently linked to the Gram-positive cell wall. Although remodeling of the cell wall of Gram-positive organisms is documented, only recently has the contribution of muropeptide recycling been assessed (Reith & Mayer, 2011). The Gram-positive *B. subtilis* has a chromosomally encoded six-gene cluster implicated in cell-wall recycling (Litzinger *et al.*, 2010). However, none of the proteins encoded by these genes is an LT. The LT activities of the Gram-positive bacteria appear to be limited to glycan-strand sizing during peptidoglycan biosynthesis (MltG activity) (Tsui *et al.*, 2016) and for the Gram-positive spore-forming bacteria, the separate processes of spore formation and of germination. Spore-forming bacteria (including the *Bacillus* and *Clostridium* species) undergo sporulation in the face of nutrient challenge (Moir & Cooper, 2015). The bacterial spore preserves the genetic identity of the bacterium in the face of extreme temperatures, dehydration, environmental chemicals, and radiation (Setlow, 2006). Bacterial spores are the ultimate survival mechanism. They retain a highly transmissible capacity even after a dormancy period of years. When a favorable environment for growth is encountered, the spores transform by germination (or desporulation) for the restoration of their bacterial state (Setlow, 2014). Spores are a challenge in the food industry as they are primary agents for spoilage and the spread of disease. Equally important is the threat of bioterrorism through release of the spores of a pathogenic Gram-positive bacterium, such as *Bacillus anthracis.* The transition from the spore state of dormancy to the bacterium (and the reverse process) involves extensive remodeling of the cell-wall peptidoglycan. The unique structure of the peptidoglycan in the spore is named the peptidoglycan cortex, wherein a portion of the NAM saccharides of the $(NAG\text{-}NAM)_n$ glycan chains are converted to the muramic-δ-lactam structure and are primarily linked by L-Ala (in contrast to the oligopeptide crosslinking of the Gram-positive bacterium) (Atrih & Foster, 1999; Setlow, 2003; Bernhards *et al.*, 2015). As such, the peptidoglycan cortex contains significantly fewer cross-linked peptides relative to the bacterial cell-wall peptidoglycan (Popham, 2002). During germination the peptidoglycan cortex of the spore degrades (Moir, 2006).

Gram-positive Family 1 Lytic Transglycosylases

Overview

The initiation of cell-wall degradation during peptidoglycan outgrowth of *Bacillus* species requires the two enzymes, SleB and CwlJ (Paidhungat *et al.*, 2001; Chirakkal *et al.*, 2002; Hu *et al.*, 2007; Giebel *et al.*, 2009; Heffron *et al.*, 2009; Setlow *et al.*, 2009; Heffron *et al.*, 2010). SleB is a LT. The specific activity of CwlJ is unknown. Both enzymes cleave muramic-δ-lactam-containing peptidoglycan, and are therefore peptidoglycan cortex-specific. A spore containing a single knockout of either protein will germinate, although at a much slower rate (Ishikawa *et al.*, 1998; Moriyama *et al.*, 1999; Boland *et al.*, 2000; Giebel *et al.*, 2009; Heffron *et al.*, 2009). Spores with knockouts of both SleB and CwlJ exhibit poor

germination and were less viable. The SleB enzyme was the first LT identified in Gram-positive bacteria, and its structure defines a new Family of bacterial LTs (Figure 15). We assign SleB as a Gram-positive Family 1 LT. The LT domains of Gram-positive Family 1 LTs are structurally similar to the LT domain of Gram-negative Family 5 LTs (as previously described; Figure 13). Sequence analysis identifies a homologue to the *Bacilli* SleB in *C. difficile* (Sebaihia *et al.*, 2006; Burns *et al.*, 2010). This homologue shares a C-terminal Hydrolase_2 domain, but lacks the N-terminal PG_binding_1 domain of SleB. Importantly, recent studies suggest that the molecular events of *Bacillus* germination may be very different from those of *C. difficile*, and therefore these homologous proteins may not share identical functions (Paredes-Sabja *et al.*, 2008; Paredes-Sabja *et al.*, 2009). This conclusion is consistent with the discovery that *C. difficile sleB* gene knockout mutants exhibited no ill effects, where instead SleC (discussed below) has assumed the functional attribution of both SleB and CwlJ (Kumazawa *et al.*, 2007). Regardless, if the *sleB* homologue in *C. difficile* is shown to have LT activity, it should be assigned to the Gram-positive Family 1 LTs. The sequence of the Hydrolase_2 domain from this protein is included in Figure 16 as SleB (*C. difficile*) for comparative purpose. Catalysis by Gram-positive Family 1 LTs uses a conserved catalytic Glu, flanked by a Ser in the *Bacillus* species and by an Ala in *C. difficile*.

Family 1A

The founding member of the Gram-positive LTs and herein assigned to Family 1A is SleB of *B. cereus.* The gene for this enzyme encodes a 28 kDa protein having an N-terminal signal peptide for initial delivery to the peptidoglycan-membrane interstitial space (Moriyama *et al.*, 1996). SleB remains inactive until the spore begins germination (Boland *et al.*, 2000; Heffron *et al.*, 2009). The specific localization of SleB within the spore has been debated (Moriyama *et al.*, 1999; Atrih & Foster, 2001; Chirakkal *et al.*, 2002). As previously described, SleB has an N-terminal PG_binding_1 domain (Pfam: PF01471) and a C-terminal Hydrolase_2 domain (Pfam: PF07486). The peptidoglycan-binding domain increases the protein's affinity for peptidoglycan and the rate of peptidoglycan cleavage. However, it is not necessary for catalysis (Heffron *et al.*, 2009). A mutation study performed with the *Bacillus megaterium* SleB demonstrated that germination and outgrowth proceeded correctly in the absence of the C-terminal hydrolase domain. Interestingly, mutants encoding only the N-terminal peptidoglycan-binding domain were more efficient at facilitating outgrowth and germination than mutants encoding only the C-terminal hydrolase domain. The authors infer that both domains have LT activity, an intriguing observation considering the prevalence of the PG_binding_1 domain in the LT superfamily (Christie *et al.*, 2010). This phenomenon has also been reported in the protein SleC from *Clostridium perfringens*. SleC only encodes a single C-terminal PG_binding_1 domain, yet recent reports demonstrate by MALDI mass spectrometry analysis of sacculus digestion products that SleC functions as an exolytic LT (Gutelius *et al.*, 2014). Notably, this activity has not been assigned to this same PG_binding_1 domain of the Gram-negative Family 1H or 3B LTs. The SleB gene-knockout strain failed to produce in the germination exudate the anhydromuropeptide products that are characteristic of the LT reaction (Boland *et al.*, 2000; Atrih & Foster, 2001; Heffron *et al.*, 2009; Christie *et al.*, 2010). Furthermore, LT activity of purified recombinant SleB was demonstrated *in vitro* (Heffron *et al.*, 2011; Li *et al.*, 2013). The structure of the C-terminal

domain of SleB reported in 2012, revealed the position of the catalytic glutamate (E157 of *B. cereus*) in the active site (Li *et al.*, 2012). The topological arrangement of this LT is different from that of any previous LT discovered at that time (Li *et al.*, 2013). However, recent identification of the Gram-negative Family 5 LTs reveals a near identical catalytic domain to that of the MltG protein from *E. coli* (Figure 13). Conservation of this secondary structure is also seen in the structure of the *B. anthracis* SleB (Jing *et al.*, 2012). In this structure, a metal-binding site at the entrance of the substrate-binding pocket was discerned. The identity of the metal was not identified, but is believed to be either a Ca^{2+} or Na^{+} ion. SleB from *B. anthracis* and *B. cereus* are nearly identical (97% sequence identity, 100% query coverage). SleB is believed to localize with the protein YbeP. Although protein-protein interactions were not seen *in vitro*, YbeP-mediated inhibition of SleB has been shown (Li *et al.*, 2013). The intricacies of YbeP regulation of SleB are not well understood.

Gram-positive Family 2 Lytic Transglycosylases

Overview

Gram-positive Family 2 LTs have also found a niche among the spore-forming Gram-positive organisms. Particularly, Gram-positive Family 2 LTs are involved in sporulation (spore formation), in contrast to the Gram-positive Family 1 LTs, which are involved in germination. Sporulation occurs following the formation of a septum near a pole of the cell, dividing the cell into one large and one small cell structures. The smaller structure—the forespore—ultimately becomes the spore. The larger compartment (named the mother cell) is doomed to lysis. The forespore engulfs the desired components of the cell and pinches closed, thereby releasing itself from the mother cell. Processing by the mother cell coats the forespore with protective proteins, whereby it can be exported as a mature spore. Formation of the spore requires large-scale remodeling of the cellular membrane and sacculus. Family 2 LTs (in collaboration with other cell-wall remodeling enzymes) participate in this process (Gutierrez *et al.*, 2010). Gram-positive Family 2 LTs each encode a single SpoIID catalytic domain (Figure 15) that has a unique structural topography (Figure 18) in comparison to the previous LT structures that we have discussed (Nocadello *et al.*, 2016). They possess a catalytic glutamate (E87 of *B. anthracis*) flanked by a conserved methionine (Figure 17).

Family 2A

It was widely assumed that Gram-positive bacteria did not encode a LT until the discovery of SleB (see Gram-positive Family 1 LTs) in 2012. A second LT, Stage II sporulation protein D (SpoIID), was discovered subsequently in Gram-positive spore-forming bacteria. SpoIID has a single catalytic SpoIID domain (Pfam: PF08486). SpoIID forms macromolecular complexes with a series of related proteins, similar to Gram-negative Family 1C and 1G, among others. SpoIID interacts with both SpoIIM and SpoIIP to breakdown peptidoglycan and to allow for spore engulfment (Abanes-De Mello *et al.*, 2002; Gutierrez *et al.*, 2010). In this process, SpoIIM serves as a scaffold protein that congregates both SpoIID and SpoIIP (Chastanet & Losick, 2007; Gutierrez *et al.*, 2010; Morlot *et al.*, 2010). SpoIIP is a bifunctional enzyme that performs both amidase and endopeptidase activities. Subsequently, SpoIID cleaves the glycosidic bond of the resulting peptide-free glycan strands, forming the 1,6-anhydroMurNAc LT reaction products (Gutierrez *et al.*, 2010; Morlot *et al.*, 2010;

Nocadello *et al.*, 2016). SpoIIP and SpoIID are single-pass transmembrane proteins. In *C. difficile*, the catalytic residue E101 has been verified in a single-mutant study resulting in a complete loss of enzyme activity. This residue is located in the signature position for the catalytic glutamate of Gram-negative Family 1 LTs between the –1 and +1 subsites. The apo structure of *C. difficile* and *B. anthracis* SpoIID were reported in 2016 by Nocadello *et al.* Crystals of the *C. difficile* SpoIID only formed in complex with triacetylchitotriose (Nocadello *et al.*, 2016). The two homologous proteins (43% sequence identity, 79% query coverage between the *B. anthracis* and *C. difficile* enzymes) share features in their catalytic domain, but also differences. Notably, (and similar to Family 3 LTs) *C. difficile* SpoIID coordinates with zinc, while a zinc coordination site is not seen in *B. subtilis* SpoIID. Sequence analysis found that the four amino acids (H134, C140, H145, and C146) involved in zinc coordination by *C. difficile* SpoIID are not conserved either in the *B. anthrancis* or *B. subtilis* SpoIID LTs. Interestingly, single mutations of C140, H145, and C146 debilitated the *C. difficile* SpoIID. Why *C. difficile* SpoIID requires metal coordination but the *Bacillus* SpoIID does not remains unclear (Nocadello *et al.*, 2016). Nonetheless, we can infer from the Gram-negative Family 3 LTs that the zinc-ion coordination by the protein in this case is likely involved in stabilization of the folded enzyme.

LTs Catalyze a Unique Transacetalization Reaction

Families 1, 3, and 4 of the Gram-negative LTs have been classified by the CAZy database as belonging to the enigmatic goose-type (G-type) lysozyme sub-families. Family 1 and 4 belong to the GH23 sub-family, and Family 3 belongs to the GH103 subfamily. Family 2 belongs to the non-G-type GH102 sub-family. The well-studied chicken-type (C-type) lysozyme belongs to the GH22 sub-family (Gloster & Davies, 2010; Wohlkonig *et al.*, 2010; Domínguez-Gil *et al.*, 2016). The C-type lysozymes use a dual catalytic acid-catalytic base mechanism. The acid facilitates departure of the GlcNAc leaving group, resulting in an oxocarbenium transition state, that is trapped by the base (Asp) as a nucleophile forming a covalent acyl-enzyme intermediate. Catalysis is completed by water hydrolysis, in a net stereochemistry-retaining mechanism (Vocadlo *et al.*, 2001). In contrast, the mechanism of the LTs is uncertain (Gloster & Davies, 2010). The Gram-negative GH23/GH103 LTs have a general acid catalytic residue (Glu in Gram-negative families 1, 3, 4, 5 and Asp in families 2, 6), and also show retention of stereochemistry. As such their mechanism likely uses a similar activation for breaking of the glycosidic bond and GlcNAc departure, with formation of an oxocarbenium species. However, in the GH23 and GH103 Families, no residue equivalent to the GH22 carboxylate nucleophile appears (Weaver *et al.*, 1995). Possible LT mechanisms have been proposed without covalent intermediate formation, invoking substrate-assisted catalysis from the MurNAc *N*-acetyl group whereby the oxygen of the *N*-acetyl group on C2 stabilizes the oxocarbenium species through bonding to C1 with formation of an oxazolinium intermediate (Figure 19) (Reid *et al.*, 2007; Fibriansah *et al.*, 2012). Efforts to mimic this oxazolinium intermediate using a NAG-thiazoline analog are discussed in the following section (Reid *et al.*, 2004a; Reid *et al.*, 2004b). Whether this oxazolinium intermediate is formed, or if stabilization of the oxcarbenium occurs following near-synchronous formation of the 1,6-anhydro species and forgoing the oxazolinium intermediate, awaits further study. Regardless, the reaction concludes with deprotonation of

the C6-hydroxyl, assisted by the catalytic glutamate now serving as a general base, enabling bond formation between the C6 oxygen and C1 to form the 1,6-anhydroMurNAc product. It is by no means clear whether a single universal mechanism pertains to all LTs. The reaction mechanism of the Gram-positive LTs on the muramic-δ-lactam of the peptidoglycan cortex has not been studied.

Methods for LT analysis

The idealized *sine qua non* for the study of an enzyme is an assay method that is reliable, quantifiable, sensitive, and continuous. Although several LT assays exist, none encompasses all of these criteria. Two reasons explain this limitation. The first reason is the substantial synthetic effort required to prepare the $(NAG\text{-}NAM)_n$ oligosaccharide as a discrete substrate. This difficulty is further compounded by the certainty (as established by the existing assay methods) that the different LTs respond to changes to this structure differently (for example, the value for n in terms of preferential endolytic or exolytic cleavage; the presence or absence of the peptide stem; whether the stem is or is not crosslinked). Moreover, the simple $(NAG\text{-}NAM)_n$ oligosaccharide lacks a reporting chromophore. While such a chromophore could surely be introduced (for example, in the departing NAG saccharide, or alternatively as a pair of chormophores for FRET assay), these additional synthetic objectives would add to an already herculean synthetic commitment. Given the incomplete understanding with respect to substrate recognition by these enzymes, multiple chromophore-labeled substrates might well be required to unmask the particular $(NAG\text{-}NAM)_n$ structure matching to recognition by the particular LT under study. The second reason is the proven multi-protein regulation of the membrane-bound LTs. It is highly probable that all membrane-bound LTs use protein-protein interaction both for localization and catalytic activation. We are only just now beginning to grasp (as opposed to understand) the role of LT interaction networks in the control of their function (Garcia-Gomez *et al.*, 2011; Santin & Cascales, 2017). Although the soluble LTs may be exceptional (compared to the membrane-bound LTs) in having a simpler regulation by protein-protein interaction, their ability to complex with PBPs (Romeis & Höltje, 1994; Höltje, 1996; von Rechenberg *et al.*, 1996; Legaree *et al.*, 2007; Legaree & Clarke, 2008; Gutierrez *et al.*, 2010; Nikolaidis *et al.*, 2012) strongly suggests that their activity too is regulated by protein-protein interaction. The reliable (but again, exceedingly laborious) synthesis of $(NAG\text{-}NAM)_n$ structures is now possible (Wang *et al.*, 2017). In this concise section, we summarize the strengths and limitations of the five LT assay methods in current practice. These five assays are the bacterial phenotypic assay, the qualitative gel-based assay, the semi-qualitative continuous sacculus-based turbidity assay, the sacculus-based fluorescence-perturbation continuous (or release, discontinuous) assay, and the synthetic substrate (and sacculus) quantitative and discontinuous chromatographic-mass spectrometry (LC-MS) assay.

The use of the bacterial sacculus, either as isolated from bacteria or reporter group-labeled, as the substrate is common to many of these assays. The procedure for the isolation of sacculi following bacterial culture is straightforward (but painstaking). Sacculus preparation involves dissolution of the non-polymeric compounds of the bacterium using hot aqueous SDS, repetitive wash and high-speed centrifugal sedimentations to remove the SDS (essential!), and possibly with intervening HF or protease digestion to remove the proteins

and lipids covalently-attached to the peptidoglycan. Once these steps are practiced to perfection (for example, the thinner and therefore lighter *P. aeruginosa*-derived sacculus requires an entirely different centrifugal wash procedure than is required for the *E. coli* sacculus), isolation of an SDS-free sacculus is a reliable preparation. An alternative SDS-free procedure for the preparation of Gram-negative sacculi is described (Kühner *et al.*, 2014), but comparative data on these sacculi for LT analysis is not available. Three caveats require emphasis. For *E. coli* the peptidoglycan structure is extensively remodeled in the transition from log growth to stationary phase. Accordingly, sacculi isolated from early log-phase, late-log phase, and stationary phase must be regarded as chemically distinct entities. Second, even the well-prepared sacculus is chemically heterogeneous. Muropeptide release from sacculi that have been radiolabeled (typically by glucosmine or diaminopimelate incorporation) was a historical method of LC assay. This assay method is now largely supplanted by the simpler fluorescent assays, or the much more informative LC-MS assay of released muropeptides, as discussed below. While the value of all of these sacculus-based assays must not be underestimated, the use of “bulk” sacculus as the substrate may obscure mechanistically important distinction with respect to muropeptide recognition by the membrane-bound LTs. The presentation of the entire (that is, intact single-molecule) sacculus to a solubilized LT that in the bacterium localizes to the septum, likely will competitively release simultaneously septal-, cap-, and sidewall-derived muropeptides. Moreover, the assay leaves invisible possibly significant glycan cleavage events that do not release muropeptides. With respect to the MltG (Gram-negative Family 5) LTs and the soluble LTs in particular, the sacculus polymer may be a misleading substrate. Without further acknowledging the potential limitations of the sacculus as the assay substrate, the principle LT assay methods are summarized.

Phenotypic Evaluation

The application of microscopy for the phenotypic characterization of LT function, typically as a consequence of gene knockout, is a powerful means of analyzing LT function. Numerous studies affirm the interpretive value of microscopic analysis, as exemplified by a series of reports (Heidrich *et al.*, 2002; Meisel *et al.*, 2003; Viollier & Shapiro, 2003; Reid *et al.*, 2004b; Hett *et al.*, 2010; Roure *et al.*, 2012; Jorgenson *et al.*, 2014; Yunck *et al.*, 2016). Important complementary information is also provided from the measurement of the MIC values for β-lactam antibiotics, as exemplified by the additional publications (Templin *et al.*, 1999; Costa & Anton, 2006; Cavallari *et al.*, 2013; Lamers *et al.*, 2015; Zeng *et al.*, 2015). Fluorescent microscopy of LT fusion proteins can provide information (in rod-shaped bacteria) as to septal or sidewall localization (Schaub *et al.*, 2016; Yunck *et al.*, 2016). While these studies have powerful interpretive value as to LT function, they are not LT assay methods.

Zymographic Assays

Impregnation of a gel with sacculi enables the zymographic detection of LT activity as measured by the clearing that follows digestion of the polymer (Strating & Clarke, 2001). Although the generally very slow turnover of LTs (as compared to muraminidases) demands typically long incubation times (and thus careful control assays), the assay is reliable (Watt & Clarke, 1994; Blackburn & Clarke, 2002; de la Mora *et al.*, 2012). For example, this assay

differentiates easily between catalytically active and inactive mutant LTs (Weadge & Clarke, 2006). The limitations of this assay are its extensive time requirement and its essentially binary (active/inactive) output.

Suspended-Sacculus Turbidity Assay

This assay is a widely used spectrophotometric variation of the zymographic assay. Sacculi are suspended in solution and the progressive change in light scattering upon addition of the LT (generally seen as a clearance of turbidity) is measured over time (≤ 15 min, as the effect of the settling of the sacculi on the light scattering contributes increasingly at longer reaction times) (Hash, 1967; Scheurwater & Clarke, 2008). This assay validated successfully the Ivy protein as an LT inhibitor (Clarke *et al.*, 2010), gave confirmation that *O*-acetylation of the peptidoglycan was protective against LT degradation (Weadge & Clarke, 2006; Clarke *et al.*, 2010; Moynihan & Clarke, 2011), and enabled generation of a pH-rate profile for LT catalysis (Herlihey *et al.*, 2014). The strength of the assay is its relative ease of performance and its rapidity. Nonetheless, in our hands this assay has significant shortcomings with respect to reproducibility (reflecting perhaps a high degree of sensitivity to the method used for sacculus preparation) and interpretation. Importantly, the assay is often performed with commercially available Gram-positive *Micrococcus luteus* sacculus. Although the use of this commercial sacculus improves experimental reproducibility, it otherwise does not address the challenge of interpreting the activity of a Gram-negative enzyme on a Gram-positive substrate (Scheurwater & Clarke, 2008; Fibriansah *et al.*, 2012). The transmittance/absorbance change in the spectrophotometric signal does not translate as a quantitative measure of LT catalytic activity. Like the zymographic assay, the interpretation of the spectrophotometric turbidity assay does not extend beyond interpretation against a carefully matched control.

Suspended Sacculus Fluorescent Response Assay

This further variation of the turbidity assay, now available as a commercial assay kit, is the most reliable turbidity assay. In the commercial kit sacculi obtained from the Gram-positive bacterium *Micrococcus luteus* are purified and impregnated with a fluorescent reporter. The catalytic action of an LT on the suspended sacculi gives a time-dependent perturbation of the emissive intensity of the fluorophore by continuous assay. Effects of sacculus sedimentation are reduced compared to the non-fluorescent methodology due to its use of a lower concentration of suspended sacculus. An alternative assay uses discontinuous measurement of fluorophore release (Zhou *et al.*, 1988; Nocadello *et al.*, 2016; Yunck *et al.*, 2016). This latter assay is generally applicable to all sacculi (from different bacteria, or from the same bacterium as obtained under different growth stages or from different enzyme expression levels). As is the case with the other turbidity assays, these fluorescence assays are proportional rather than quantitative. For example, in the fluorescence perturbation assay the slope is not proportional to amount of enzyme added; the slope is not quantifiable with respect to the substrate, and neither v nor K_M values may be extracted from the line shape. A further limitation of this assay is its poor translation from fluorescence measurement within a cuvette (good reproducibility) to fluorescent measurement using a microplate (poor reproducibility).

LC-MS Evaluation of Sacculus and Synthetic Peptidoglycan Substrate Degradation

The analysis of the muropeptide composition of the sacculus by LC-MS analysis is unquestionably the dominant assay method for the quantitative evaluation of LT activity. Although the sacculus lacks an intrinsic chromophore, the sensitivities and resolving powers of both the LC and MS methods give great value to this assay. The assay is appropriate to the use of both the sacculus and synthetic muropeptides as substrates. The assay is reliable, robust, quantifiable, sensitive, and interpretable. Its limitations are its discontinuity, cost, and low volume. Nonetheless, this assay method has evolved from its origins (Glauner *et al.*, 1988; Glauner, 1988; Blackburn & Clarke, 2000) to high levels of sophistication and automation (Kühner *et al.*, 2014; Desmarais *et al.*, 2015; Alvarez *et al.*, 2016; Espaillat *et al.*, 2016; Bern *et al.*, 2017). Its value to LT analysis is showcased by its application to the comparative study of the LTs of *E. coli* (Lee *et al.*, 2013), *N. gonorrhoeae* (Schaub *et al.*, 2016) and *P. aeruginosa* (Lee *et al.*, 2017), wherein the assay distinguishes between exolytic and endolytic preference, stem peptide or stem-peptide-free preference, and tolerance for the presence of cross-link. The assay can be adopted to provide v and K_M values.

Opportunities for LT Assay Development

The absent assay method is the one that encompasses the strengths of the LC-MS assay, but is rapid, sensitive, and continuous. The equivalent assay for the muraminidases has a visible light or fluorescent reporter as the leaving group. Although the LTs very likely share with the muraminidases a requirement for oligosaccharide presentation for optimal catalytic efficiency, in principle the presentation on a MurNAc monosaccharide of a reactive leaving group (as is customary to many reporter groups in such assays) that exploits the sensitivity of fluorescence detection might address this need. A possible limitation (and key difference between the muraminidases and the LTs) is the likelihood that the membrane-bound LTs are not, as the soluble enzyme and in the absence of their regulatory protein partner, robust catalysts. Accordingly, even the provision of a good leaving group for sensitive detection may prove inadequate. As attention turns to assessing the value of LT inhibitors as adjuvants of the β-lactam antibiotics, efforts toward the development of new LT assays will continue.

LTs as Targets for Antibiotic Development

The combination of β-lactam antibiotics and β-lactamase inhibitors has extended by decades the clinical efficacy of the β-lactams. In the current time of rampant bacterial resistance, when the efficacy of this combination is threatened by the exchange of increasingly powerful β-lactamases between and among species, the identification of new adjuvants for the β-lactams is highly desirable. One very attractive possibility is a combination of an LT inhibitor with the β-lactam. The value of this strategy for further extending the clinical life of β-lactams arises from the appreciation that LTs and PBPs cooperate during cell-wall biosynthesis, in a homeostatic balancing act between construction and demolition of the cell wall (Johnson *et al.*, 2013). The inhibition of PBPs by β-lactams disturbs this equilibrium, and in many pathogenic Gram-negative bacteria the resulting change in the pool of the recycling cell-wall fragments triggers β-lactamase expression (Jacobs *et al.*, 1994; Fisher & Mobashery, 2014; Vadlamani *et al.*, 2015; Lee *et al.*, 2016a; Dik *et al.*, 2017) These observations raise two central questions with respect to LT inhibition. The first is whether an

LT inhibitor might suppress the ability of these Gram-negative bacteria to induce β-lactamases. The second is whether LT inhibition might further synergize with PBP inhibition to secure the ultimately bactericidal compromise of peptidoglycan integrity. Compelling evidence already in the literature points to a positive answer to both questions.

LT-knockout studies yield insight into the enzymatic roles of the LTs and suggest the possible clinical value of LT inhibition. With respect to the answer for the first question, the triple LT (MltA, MltB and Slt70) knockout in an *ampR*-reconstituted *E. coli* strain showed unimpaired *in vitro* growth, but substantially reduced cell-wall turnover (by approximately 70%) and removing these proteins reduced the bacterium's ability to induce β-lactamase expression (Kraft *et al.*, 1998; Korsak *et al.*, 2005). The Slt70-deletion strain of *E.* coli likewise lacked a growth defect, yet was more sensitive to the β-lactam mecillinam (Templin *et al.*, 1992). An explanation for this result was offered by Cho *et al.* (Cho *et al.*, 2014) in their assignment of a "quality control" role to Slt70. Slt70 is suggested to remove the non-crosslinked peptidoglycan that is the result of β-lactam inhibition of PBP transpeptidation, thus preventing misincorporation of peptidoglycan into the cell wall. Therefore, Slt70 may be responsible in part for repairing cell-wall damage caused by β-lactam antibiotics. In such a role, this LT could be an advantageous drug target. Simultaneous genetic deletion of six LT genes (*slt70*, *mltA-E*) still did not inhibit cell growth (Heidrich *et al.*, 2002), yet a viable strain could not be isolated with a seventh (*mltF*) LT-knockout (Scheurwater & Clarke, 2008). As a clear functional redundancy exists among the LTs (Lee *et al.*, 2013), a broad-spectrum LT inhibitor might be necessary.

Additional studies in other bacteria, however, suggest that even when such an inhibitor is in hand, the value of its activity may not be general. Both insertional and deletion knockouts of the LTs of *Pseudomonas aeruginosa* identified MltB and SltB1 (both family 3) knockout strains as showing *increased* resistance to β-lactam drugs and further suggested that SltB3 (SltH), MltD, or MltF2 knockout strains displayed a similar effect (Cavallari *et al.*, 2013; Lamers *et al.*, 2015). The loss of both MltB and SltB1 together resulted in an even higher MIC of β-lactams (although this effect was unrelated to β-lactamase induction). These studies suggest that impairing the activity of (at least certain) LTs could be "protective" by reducing the ability of the bacterium to undergo lysis following β-lactam exposure. In contrast, both analyses showed that Slt-knockout strains had increased susceptibility to β-lactams (identical to the earlier studies with Slt70 in *E. coli*). The strain with insertional inactivation of MltF also displayed lower MICs for β-lactams. Moreover, knocking out the Family 1 LTs (MltD, MltF, MltF2, Slt) or several Mlts (MltA, MltB, MltD, MltF, MltF2) increased susceptibility to β-lactams. Finally, two single LT-knockout strains, (separately MltA and SltB2 (SltG)) did not affect the MICs of β-lactam antibiotics. While the deletion of specific LTs in *E. coli* reduced β-lactamase expression, single-knockout strains of *Pseudomonas* gave a different conclusion. Individual deletion of MltB, MltF, Slt, and SltB1 mutant strains had no significant effect on either basal or cefoxitin-induced AmpC expression compared to the wild-type strain (Cavallari *et al.*, 2013). Similarly, LT-gene knockouts in *Stenotrophomonas maltophilia* (including Slt, MltA, MltB1, MltB2, MltD1, and MltD2) explored the effects of loss of LT function in this species (Huang *et al.*, 2015; Wu *et al.*, 2016). Removal of one LT (MltD1) yielded high levels of uninduced β-lactamase activity, an undesirable phenotype. Yet, inactivation of this single LT as well as MltB1,

MltD2, or Slt resulted in strains that were more susceptible to macrolide antibiotics compared to the wild-type strain. Deletions of *slt* or *mltB1* had a similar effect with aminoglycoside antibiotics, suggesting an increase in outer-membrane permeability (and thus antibiotic susceptibility) as a result of loss of LT function (Wu *et al.*, 2016; Wu *et al.*, 2016). An "appropriate" LT inhibitor could thus be beneficial for combination with antimicrobials outside of the β-lactam family. LT mutational analysis has also studied LT function in *N. gonorrhoeae*, where extracellular anhydroMurNac muropeptides directly contribute to pathogenicity (Cloud & Dillard, 2002; Chan *et al.*, 2012). Two LTs (LtgA, a homolog of *E. coli* Slt70; and LtgD, a homolog of *E. coli* MltB) are involved in the release of the toxic muropeptides (Kohler *et al.*, 2005; Cloud-Hansen *et al.*, 2008). The remaining (known) LTs [LtgC (MltA), LtgB (MltC), LtgE (MltD), LtgX, and AltA] do not contribute. Although LtgA and LtgD are not essential for *in vitro* planktonic growth (Cloud-Hansen *et al.*, 2008), their inhibition could reduce the virulence of *N. gonorrhoeae* infections.

Despite their potential value, the development of inhibitors targeting the LTs has been slow, reflecting in part the perception of extensive LT functional redundancy and the lack of a simple assay for LT activity. However, one natural product is a distinguished LT inhibitor (Williams *et al.*, 2017). In the early 1980s, the exploration of the monobactam antibiotics produced by soil-dwelling *Pseudomonas* strains at Takeda Chemical Industries coincided with the observation that these bacteria also produced compounds that gave a "bulge" phenotype in *E. coli* cells when co-administered with a β-lactam (Imada *et al.*, 1982). These aptly named compounds, the "bulgecins" (Figure 20A) (Shinagawa *et al.*, 1985), were strongly synergistic with β-lactam antibiotics (Nakao *et al.*, 1986). The nearly identical observation—bulge formation—was made subsequently with an *E. coli* Slt70-deletion strain, which also showed increased sensitivity to β-lactams (Templin *et al.*, 1992). The bulgecins were postulated (and then confirmed) as Slt70 inhibitors (IC_{50} of 0.4 μM). The additional observation that "a crude preparation of solubilized membrane-bound LT was not inhibited by bulgecin," suggested specificity of the bulgecins for Slt70 (Templin *et al.*, 1992). The particular targeting of Slt70 by the bulgecins is further supported by the observation that the bulgecins no longer displayed synergy with β-lactams in the Slt70 deletion strain (Kraft *et al.*, 1999). However, bulgecin-A-complexed crystal structures were solved subsequently for several LTs including Slt70 (Thunnissen *et al.*, 1995b), Slt35 (van Asselt *et al.*, 2000), and MltE (Fibriansah *et al.*, 2012). The seeming discrepancy between the *in vivo* (apparent) selectivity and the *in vitro* ability to bind to multiple LTs may reflect both different individual affinities and how the LT activity is regulated *in vivo*. In these LT structures, bulgecin A acts as an apparent standard iminosaccharide GH inhibitor, wherein both the hydroxymethyl moiety and the nitrogen of the pyrrolidine are in near proximity to the catalytic glutamic acid, substantiating a competitive mode of inhibition. The mode of inhibition might be characterized as mimicry of the transient oxocarbenium species in the mechanism of substrate turnover. In addition to the previously cited studies in *E. coli*, crude bulgecin extracts have demonstrated potential when combined with β-lactams *in vitro* against clinical strains of both *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Skalweit & Li, 2016). Bulgecin A likewise synergizes with amoxicillin against *Helicobacter pylori* (Bonis *et al.*, 2012) and is effective at reestablishing β-lactam vulnerability in resistant *Neisseria* species (Williams *et al.*, 2017).

The Family 3 LTs are proposed to use a substrate-assisted mechanism involving an oxazolinium intermediate (Reid *et al.*, 2004a; Reid *et al.*, 2004b). A GlcNAc-based thiazoline inhibitor, commonly understood to mimic the oxazolinium intermediate (Knapp *et al.*, 2009) (Figure 20B), inhibited MltB from *P. aeruginosa* with a millimolar IC_{50} (Reid *et al.*, 2004b). At a similar concentration (1 mg/mL, equal to 4.6 mM), this same NAG-thiazoline induced morphological changes in *E. coli*, but did not prevent growth (Reid *et al.*, 2004a). In fact, NAG-thiazoline prevented β-lactam-induced cell lysis, consistent with the LT-housekeeping hypothesis (Cho *et al.*, 2014). A similar phenotype was noted also in certain *Pseudomonas* LT knockout strains, including the MltB and SltB1 LT deletions mentioned above (Lamers *et al.*, 2015). Regardless, NAG-thiazoline appears insufficiently potent for consideration as a β-lactam adjuvant. Several piperidine-based iminosaccharides based on MurNAc (Figure 20C) were evaluated as inhibitors of *E. coli* MltB (Yamaguchi *et al.*, 2012). The nitrogen of the piperidine in these inhibitors serves the same function as the nitrogen of the pyrrolidine of bulgecin: as a (possibly) positively charged mimic of an oxocarbenium transition-state entity. The best compound had saturable binding with a 0.2 mM K_d value. As successful iminosaccharide inhibition of GH enzymes coincides with precise complementation of the inhibitor (ring size, stereochemistry, and substitution) and enzyme target (Stütz & Wrodnigg, 2011), the possibility of further exploration of the iminosaccharide class for LT inhibition remains open. Iminocyclitols **1** and **2** (Figure 20D) have nanomolar K_i values against hexosaminidases (Liu *et al.*, 2001) and *O*-GlcNAcase (Bergeron-Brlek *et al.*, 2015), offering exemplification of the level of potency that can be achieved upon optimization of the iminosaccharide structure. As the idealized LT inhibitor might interfere with the induction of the AmpC β-lactamases; synergize both with β-lactams and antimicrobials outside of the β-lactam family; and suppress the virulence of muropeptide-producing bacteria, future chemistry efforts toward the identification of LT inhibitors offers considerable opportunity.

Conclusion

The LTs defy generalization. The eleven LT enzyme ensemble encoded by *P. aeruginosa* (as an exemplary bacterium) includes enzymes that are small and single domain, and large and multidomain; lipoproteins and soluble enzymes; enzymes of peptidoglycan synthesis and enzymes of peptidoglycan processing; enzymes presumptively of the sidewall and of the septum; enzymes without direct evidence of allosteric regulation and enzymes with direct evidence of allosteric regulation; and enzymes with (apparently) either one or two critical catalytic residues. The LTs accomplish a diversity of cellular functions that credibly may be surmised to correspond to selective assignment within the ensemble, yet have proven functional redundancy. Strong circumstantial evidence argues that the inability to accomplish at least some of these functions is lethal. We have a credible perspective on the breadth of the LT landscape.

Nonetheless, within this landscape key answers are missing with respect to their structure, mechanism, and function. A partial enumeration of unanswered questions follows. Which LTs match to which function? Which function(s) are essential LT function(s)? Which LT functions synergize with β-lactam inactivation of the PBPs, and which antagonize? Here, of course, the diversity of β-lactam structure within a breath of PBP functions may exclude a

simple answer. What is the true substrate(s) recognized by each LT? Here, there is a structural chasm among the peptidoglycan structures embedded in a polymeric bacterial exoskeleton (the sacculus) and the peptidoglycan structures of bacterial catabolism, and the uncertainty whether the select structures that may be crafted by *de novo* synthesis, align with either. If MltG is an enzyme of peptidoglycan synthesis, how do we reconcile the presumptive neighboring presence of other LTs, as suggested by the ability of these LTs to form protein-protein complexes with PBPs? Which LTs have regulatory partners, and what is the identity of these partners? For which LTs is their catalysis regulated by simultaneous contact with a regulatory partner and allosteric occupancy of a peripheral domain? Are the extents of LT structures (whether with or without ligand) only approximations of the true catalytic conformations, given the ability of their partners to stimulate their turnover chemistry? What value might be offered by an LT inhibitor, and does this value rest with an inhibitor that targets preferentially LT participation in peptidoglycan synthesis, peptidoglycan recycling, or peptidoglycan-dependent virulence?

Recent clever interdisciplinary study has clarified the identities of the proteins and enzymes that assemble to enable cytoskeleton-driven peptidoglycan synthesis (Bisson-Filho *et al.*, 2017; Egan *et al.*, 2017; Rued *et al.*, 2017; Yang *et al.*, 2017). Their identities are captured in imaginative cartoons suggesting the complexity of the protein-protein interaction network (Egan & Vollmer, 2013; Massidda *et al.*, 2013; Szwedziak & Lowe, 2013; Fleurie *et al.*, 2014; Egan & Vollmer, 2015; Leclercq *et al.*, 2017; Lewis, 2017). A credible measure of the inchoate understanding of LT function is the absence of an LT in all of these cartoons. Our observation of this absence is not criticism. We simply do not know where, in this specific function, the LTs properly fit. Efforts toward finding this fit (while yet to supply the answer) have been no less clever, and the foundational efforts of Bernhardt, Boneca, Clarke, Dillard, Dijkstra, Hermoso, Höltje, Keck, Thunnissen, Vollmer and others have magnified into an explosion of recent studies on LT identity, structure, and function. This review is, therefore, a timely assimilation of these studies into a coherent framework, to support future efforts to place properly each member into the intriguing LT enzyme superfamily landscape.

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Biographies

David Dik is an alumnus of Hillsdale College (B.Sc., 2013). He is now working toward his doctoral degree in Chemical Biology at the University of Notre Dame under the supervision of Shahriar Mobashery with particular interest in the biochemical signaling pathways that manifest bacterial resistance to β-lactam antibiotics.

Daniel Marous received his undergraduate degree from Wittenberg University before completing his doctoral studies with Craig Townsend at the Johns Hopkins University. During his postdoctoral fellowship with Shahriar Mobashery at the University of Notre Dame, he seeks to develop innovative treatments of Gram-negative pathogens.

Four decades after defining (with Jeremy Knowles) the acyl-enzyme mechanism for catalysis by, and inactivation of, the Class A β-lactamases, Fisher has returned to the compelling enzymology of the micrometer-sized carnivores.

Shahriar Mobashery did his undergraduate and doctoral studies, followed by postdoctoral work, at the University of Southern California, the University of Chicago, and the Rockefeller University, respectively. He holds the Navari Chair in the Department of Chemistry and Biochemistry at the University of Notre Dame.

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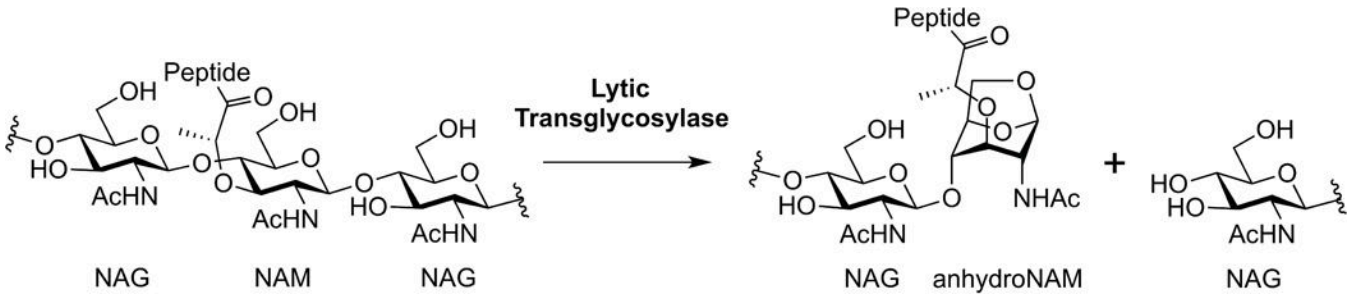

Figure 1.
The hallmark reaction of the lytic transglycosylases.

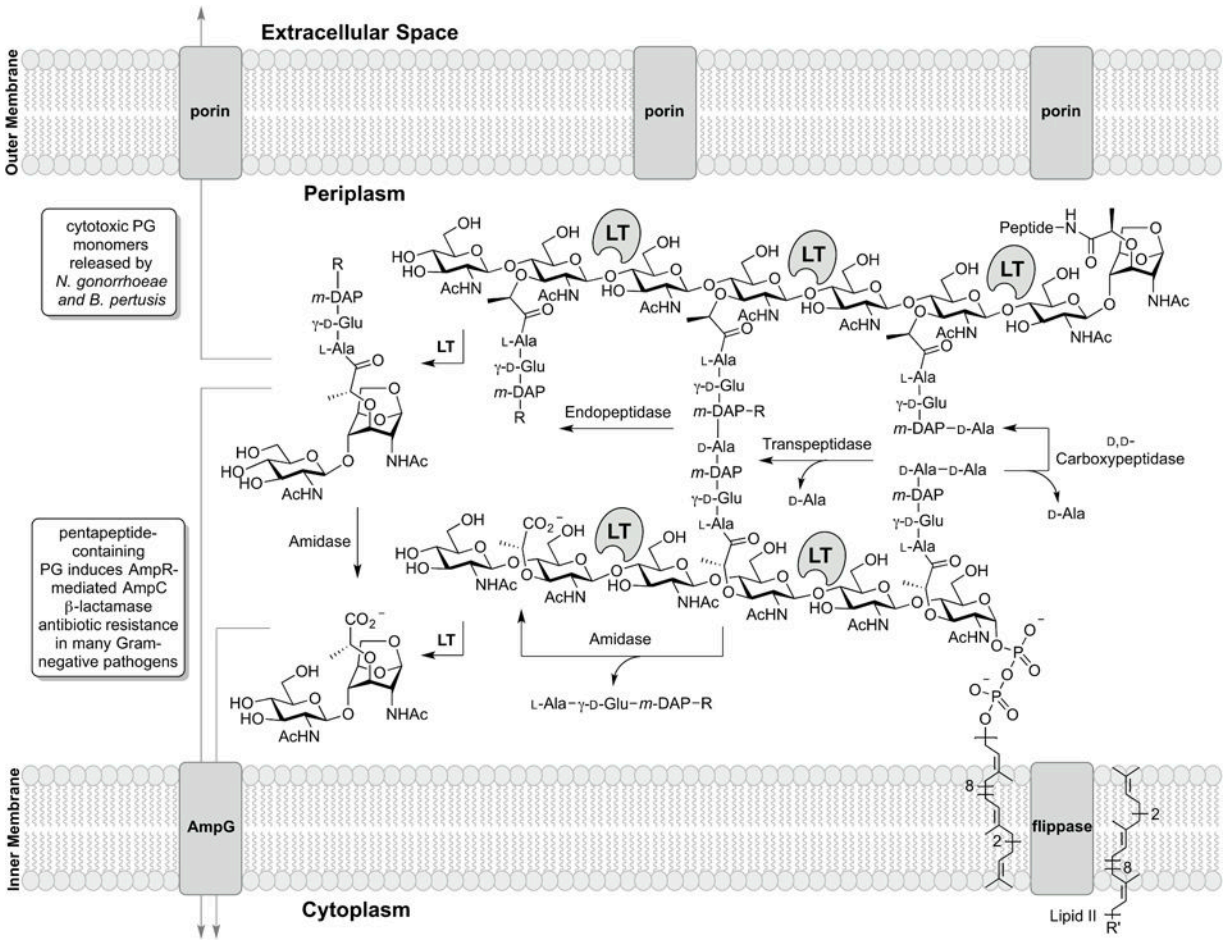

Figure 2.

The periplasmic events (where the LTs exist) of Gram-negative cell-wall recycling are depicted. The membrane anchoring of the LTs is not represented in the figure. The LT reaction sites are shown. In the scheme R = D-Ala-D-Ala (penta), or D-Ala (tetra), or H (tri) and R' = diphosphoryl-*N*-acetyl muramoyl (glucosamine)-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala-D-Ala.

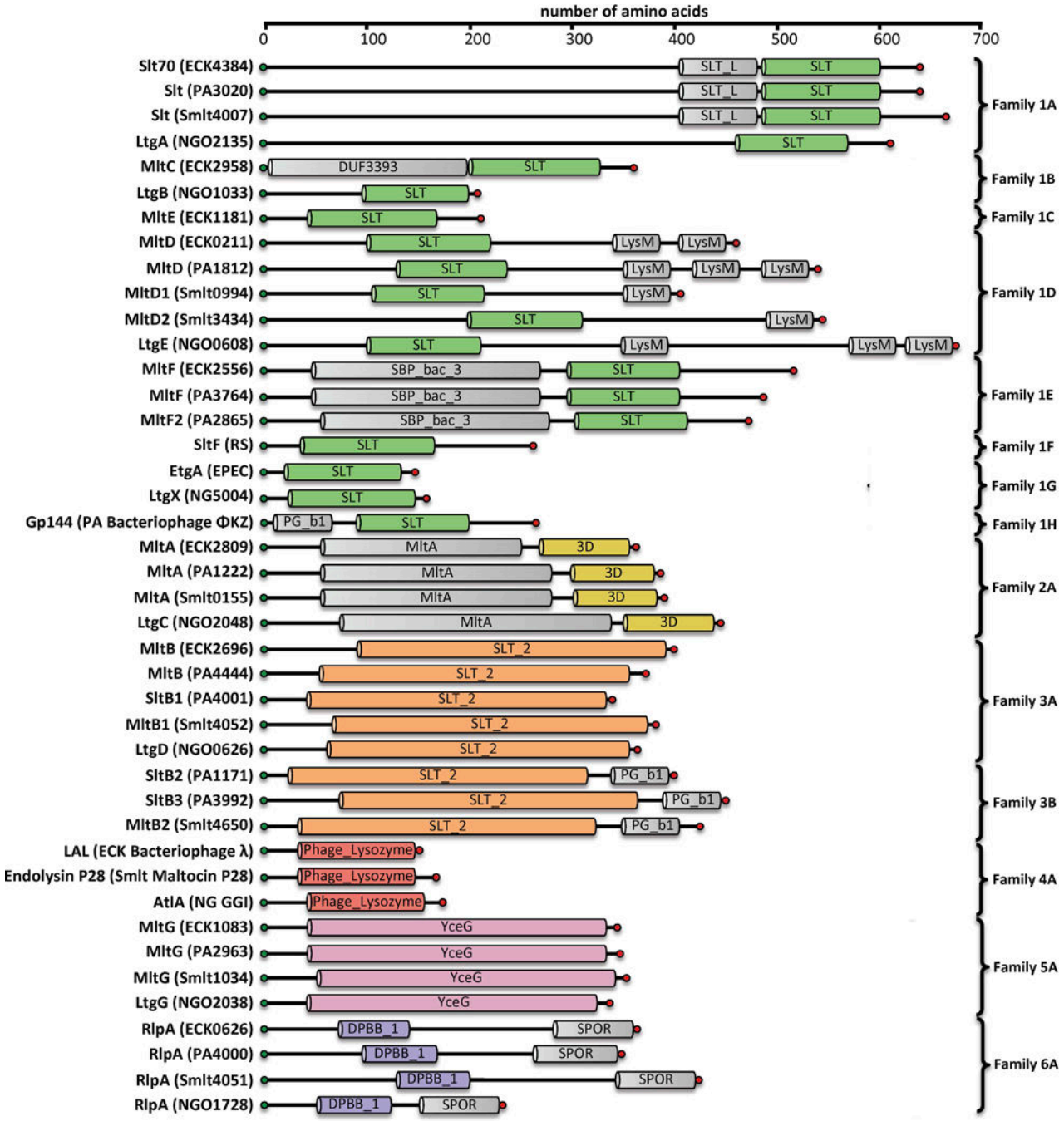

Figure 3.

Domain architecture of Gram-negative LTs. Domains were assigned based on consensus analysis of Pfam Database and NCBI conserved domain database. Protein name and locus tag are given for *E. coli* K12 (ECK…), *P. aeruginosa* PAO1 (PA…), *S. maltophilia* KJ (Smlt…), and *N. gonorrhoeae* FA1090 (NGO…). Locus tags are not given for Enteropathogenic *E. coli* (EPEC) or *Rhodobacter sphaeroides* (RS). For the bacteriophage LTs (family 1H and 4), the name of the infected host organism (ECK, PA, Smlt, and NG) and the identities of the bacteriophages encoding these LTs are provided. The PG_binding_1

domain of Family 1H and 3B is abbreviated as PG_b1. A color version of this figure is available at www.tandfonline.com/ibmg.

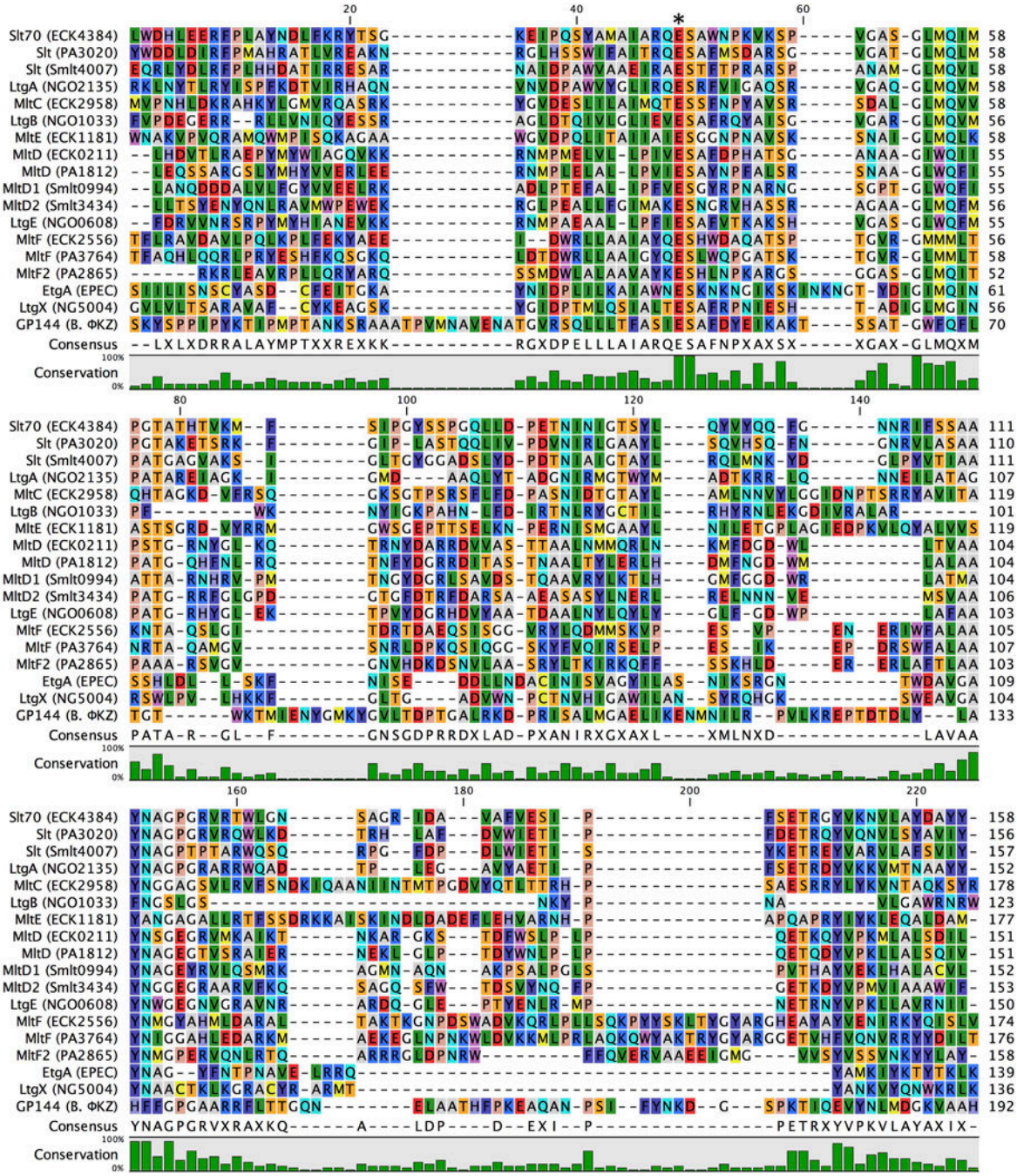

Figure 4.

Multiple-sequence alignment displaying the LT domains of Gram-negative Family 1 LTs. In consideration of the size of the Family 1 alignment, *R. sphaeroides* SltF is not included as the SLT domain of the protein is considerably larger than the other Family 1 members. The asterisk denotes the position of the catalytic residue. A color version of this figure is available at www.tandfonline.com/ibmg.

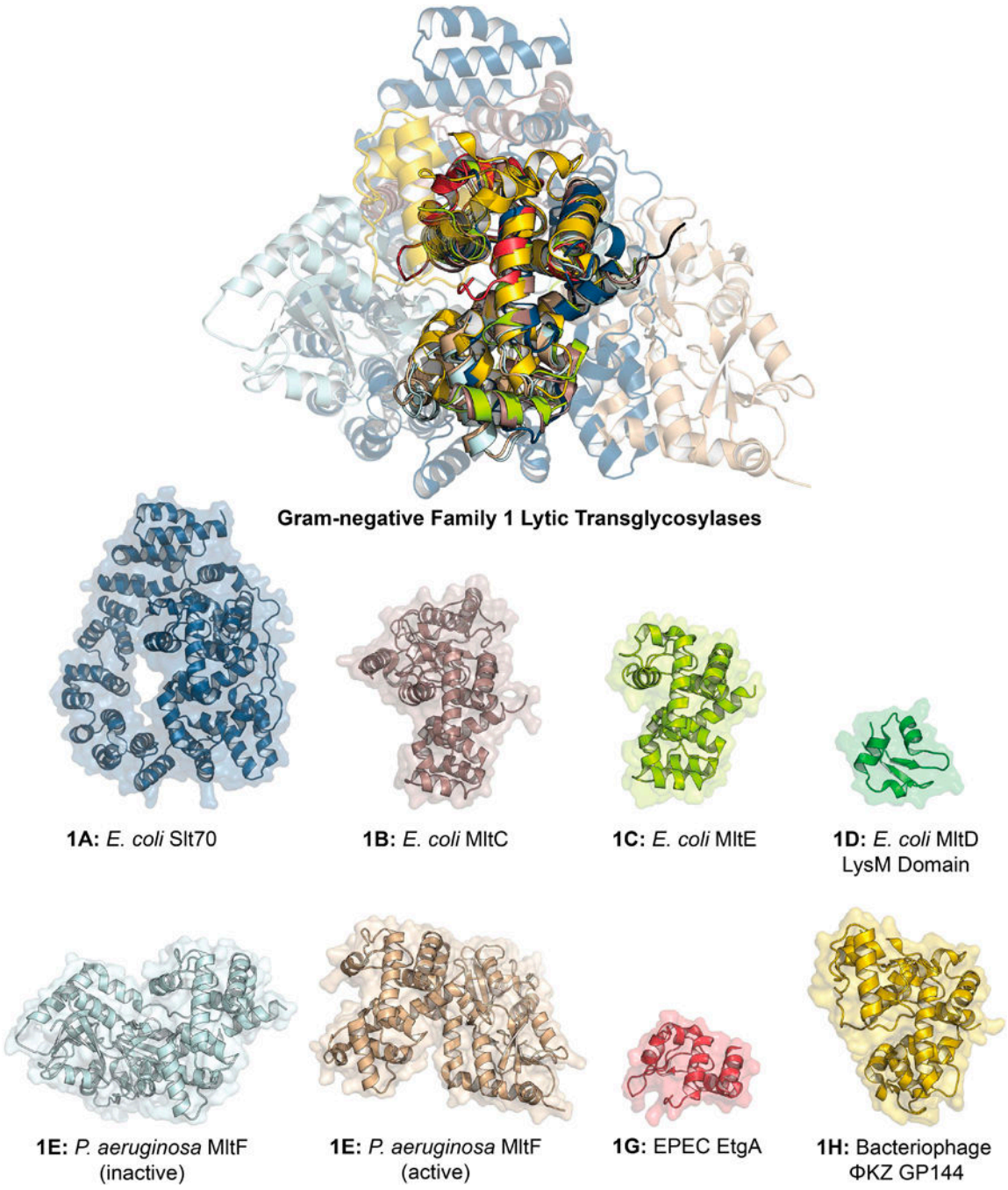

Figure 5.

X-ray structure alignment of Family 1 LTs, displaying the conservation of the core SLT domain (Pfam: PF01464) and the diversity of the peripheral domains. The ribbon representation of each apo LT crystal structure is displayed below with a transparent surface representation. The structure of an LT domain of Family 1D has not been solved, therefore only the peripheral domain is displayed. Family 1E is displayed in both the active and inactive conformations. Refer to table 1 for PDB codes. A color version of this figure is available at www.tandfonline.com/ibmg.

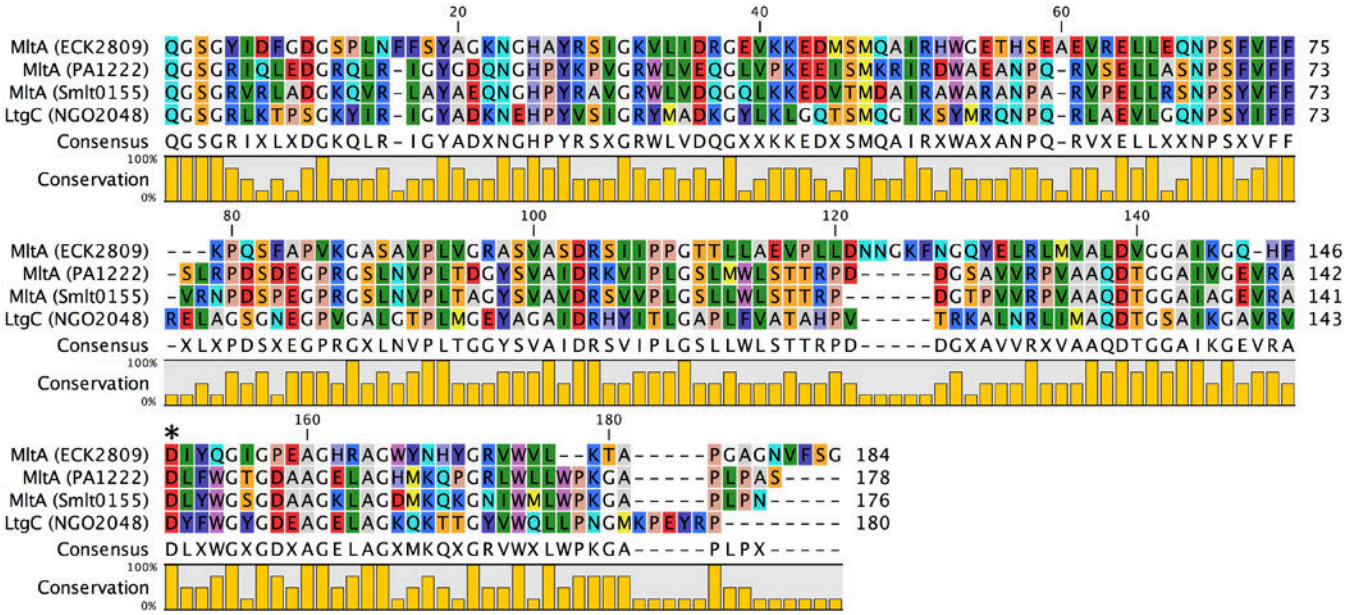

Figure 6.

Multiple-sequence alignment displaying the LT domain of Gram-negative Family 2 LTs. The asterisk denotes the position of the catalytic residue. A color version of this figure is available at www.tandfonline.com/ibmg.

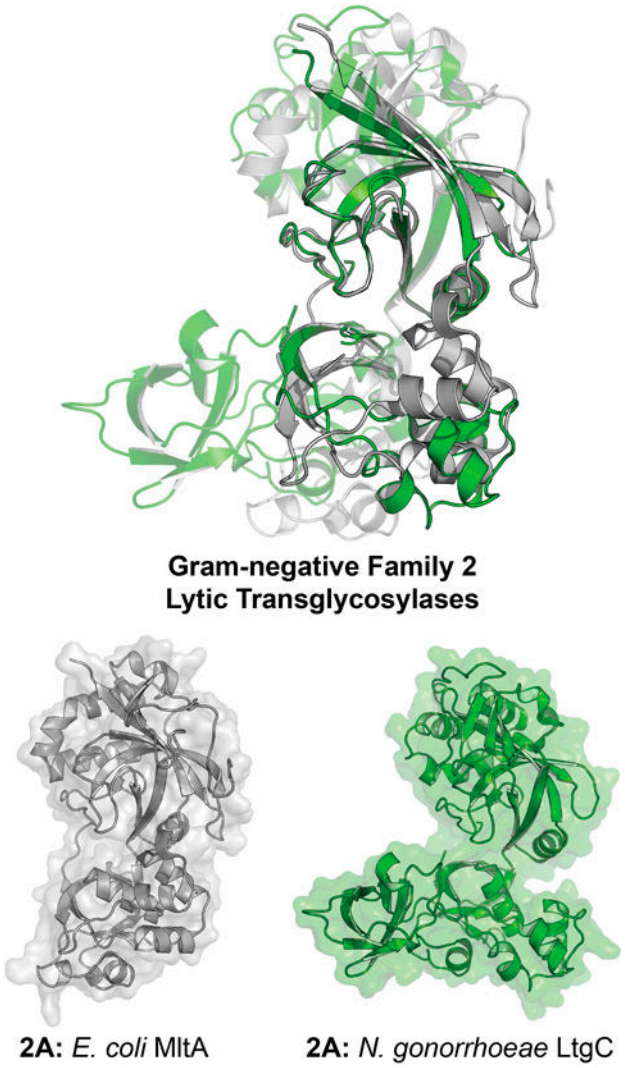

Figure 7.

X-ray structure alignment of Gram-negative Family 2 LTs, displaying conservation of the 3D domain (Pfam: PF06725). The 3D domain is comprised of two subdomains linked by a hinge. The two structures show an open (*E. coli* MltA) and a closed (*N. gonorrhoeae* LtgC) protein conformation. The structure overlay aligns the top sub-domain. The bottom sub-domains are also conserved, but align differently in the open and closed states. The ribbon representation of each apo LT crystal structure is displayed to the right with a transparent surface representation. A color version of this figure is available at www.tandfonline.com/ibmg.

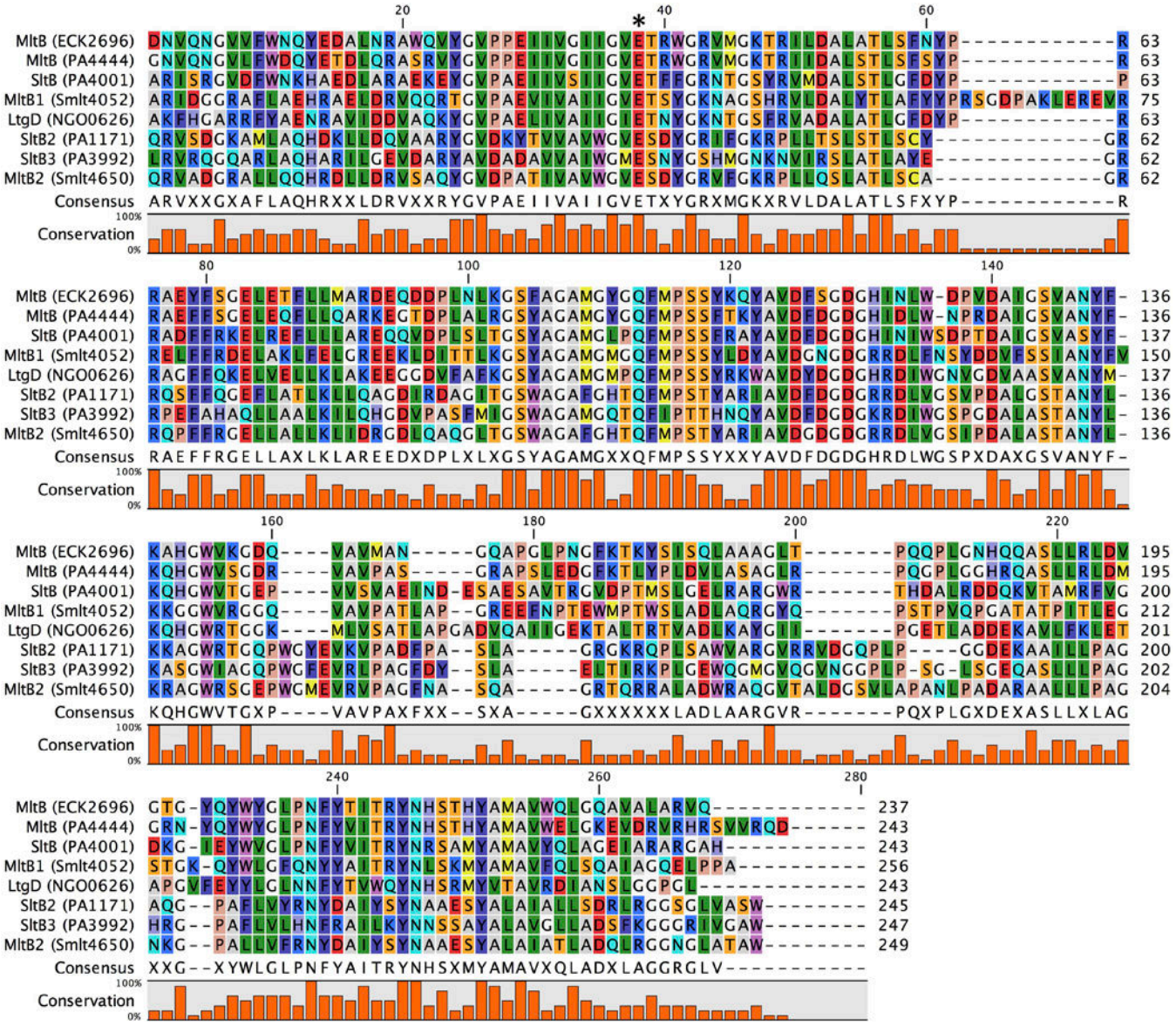

Figure 8.

Multiple-sequence alignment displaying the LT domain of Gram-negative Family 3 LTs. The asterisk denotes the position of the catalytic residue. A color version of this figure is available at www.tandfonline.com/ibmg.

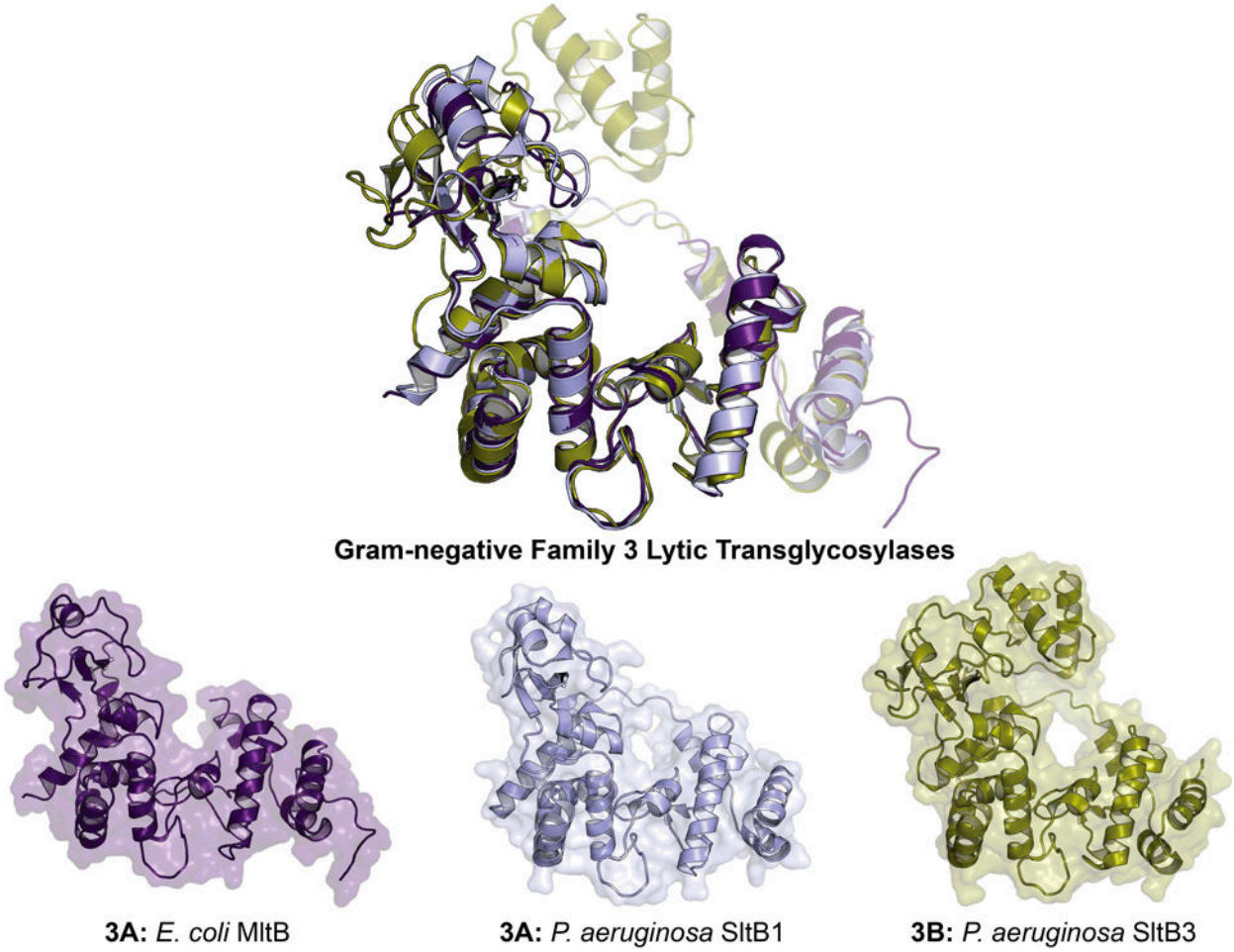

Figure 9.

X-ray structure alignment of Gram-negative Family 3 LTs, displaying the conservation of the Slt_2 domain (Pfam: PF13406). The ribbon representation of each apo LT crystal structure is displayed below with a transparent surface representation. A color version of this figure is available at www.tandfonline.com/ibmg.

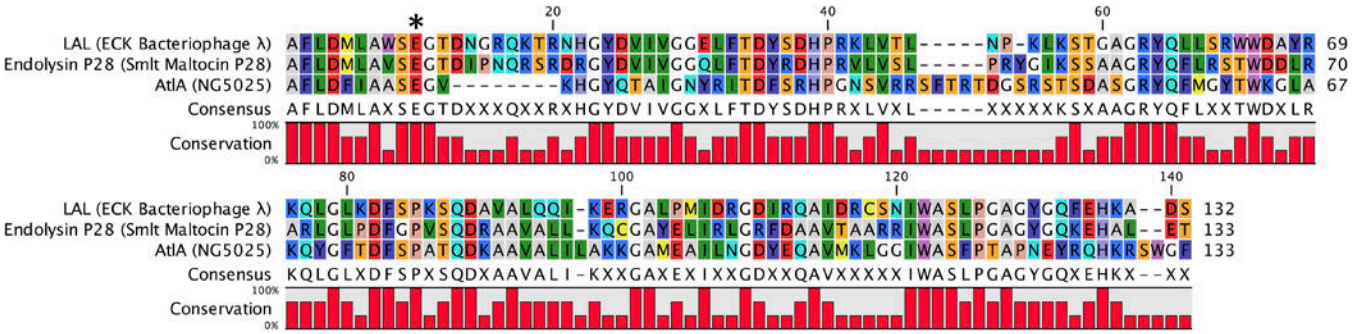

Figure 10.

Multiple-sequence alignment displaying the LT domain of Gram-negative Family 4 LTs. The asterisk denotes the position of the catalytic residue. A color version of this figure is available at www.tandfonline.com/ibmg.

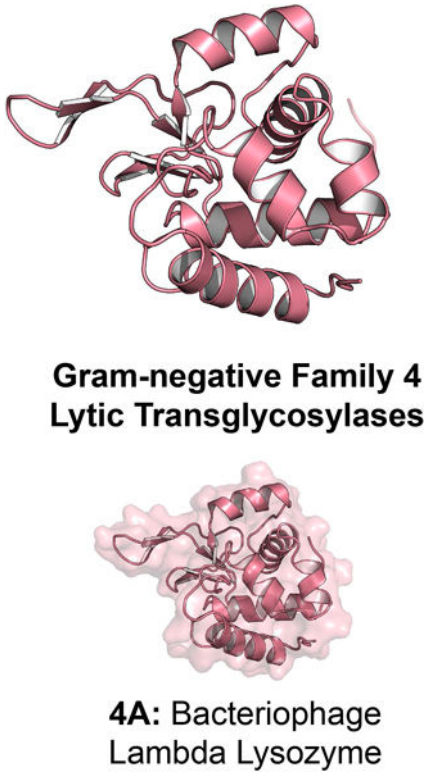

Figure 11.

X-ray structure of the Gram-negative Family 4 LT bacteriophage endolysin λ; the Phage_Lysozyme domain (Pfam: PF00959) comprises most of the solved structure. The ribbon representation of the apo LT crystal structure is displayed with a transparent surface representation. A color version of this figure is available at www.tandfonline.com/ibmg.

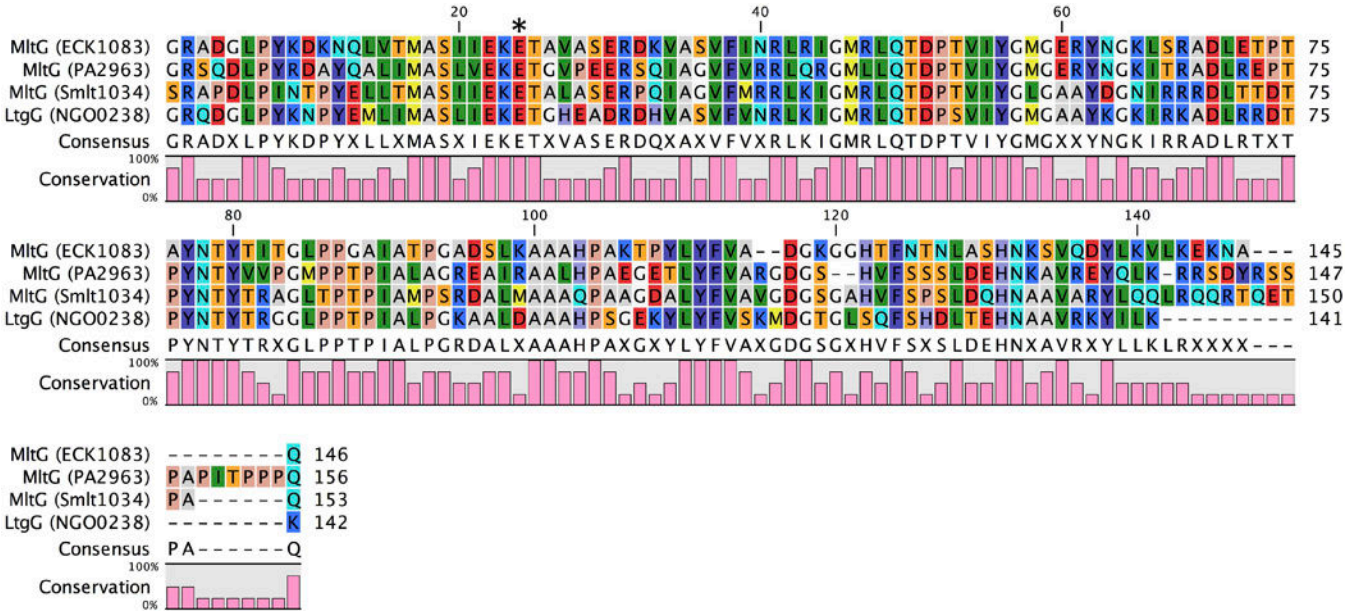

Figure 12.

Multiple-sequence alignment displaying the LT domain of Gram-negative Family 5 LTs. The asterisk denotes the position of the catalytic residue. A color version of this figure is available at www.tandfonline.com/ibmg.

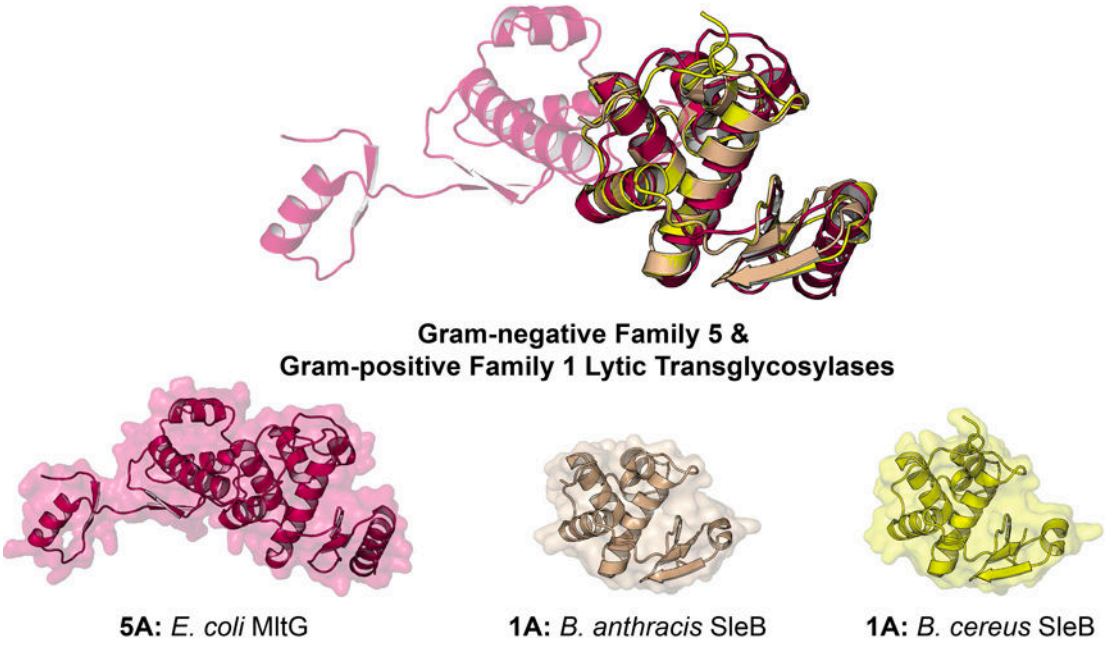

Figure 13.

X-ray structure alignment of Gram-negative Family 5 and Gram-positive Family 1 LTs, displaying the similar folds of the YceG domain (Pfam: PF02618) of Gram-negative Family 5 and the PG_Hydrolase_2 domain (Pfam: PF07486) of Gram-positive Family 1. Notably, the amino-acid sequence between the two domains is not similar. The ribbon representation of each apo LT crystal structure is displayed with a transparent surface representation. A color version of this figure is available at www.tandfonline.com/ibmg.

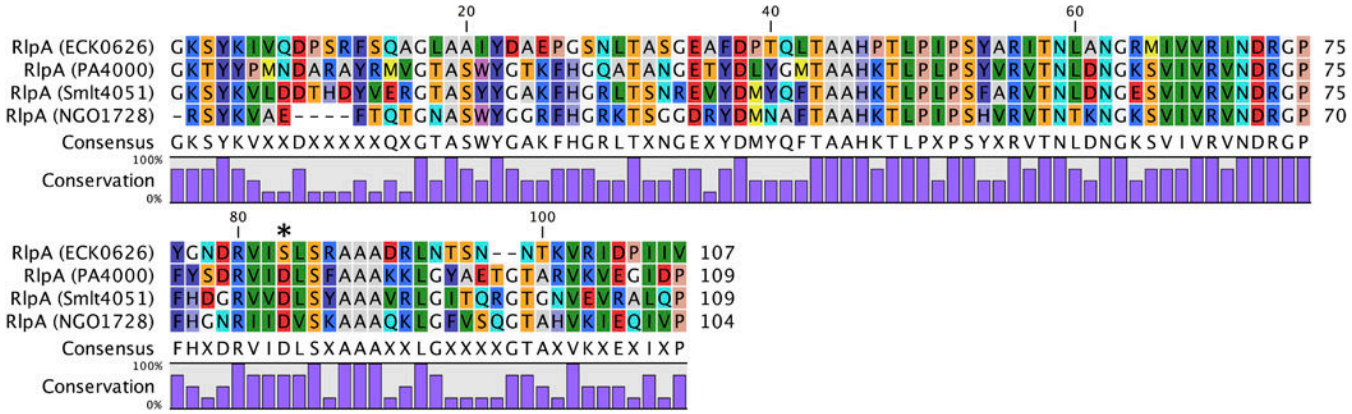

Figure 14.

Multiple-sequence alignment displaying the LT domain of Gram-negative Family 6 LTs. The asterisk denotes the position of the catalytic residue. A color version of this figure is available at www.tandfonline.com/ibmg.

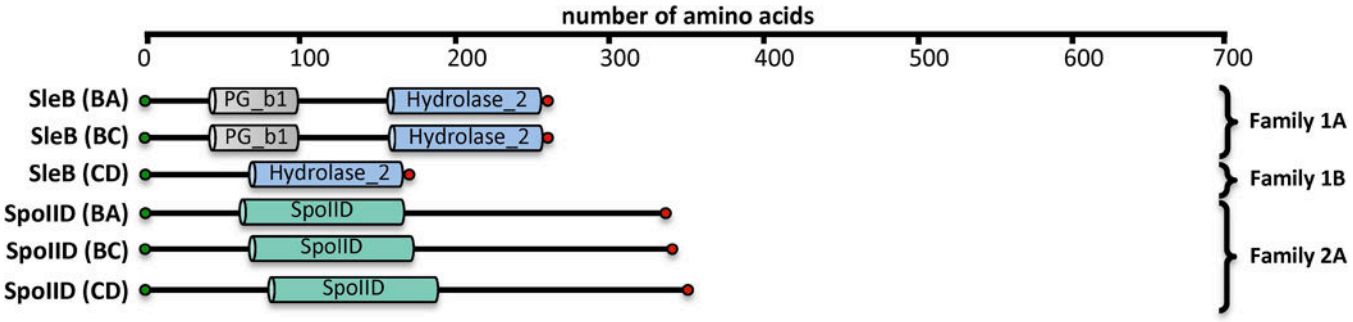

Figure 15.

Domain architecture of Gram-positive LTs. Domains were assigned based on consensus analysis of Pfam Database and NCBI conserved-domain database. Protein names and those of the organisms are given for *B. anthracis* (BA), *B. cereus* (BC) and *C. difficile* (CD). Locus tags are not given. Notably, it is not known if the SleB homologue of *C. difficile*, which lacks the N-terminal PG_binding_1 (PG_b1) domain, is an LT, therefore it is not included. A color version of this figure is available at www.tandfonline.com/ibmg.

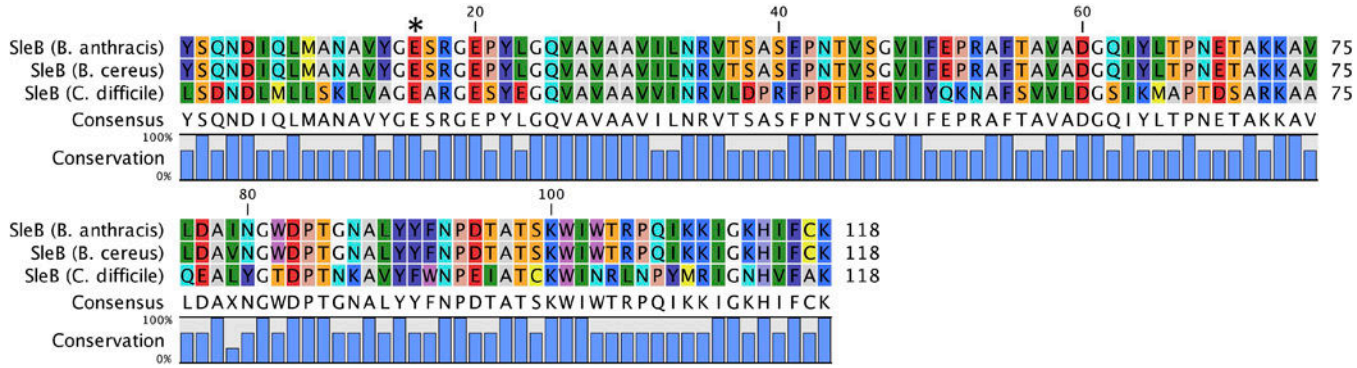

Figure 16.

Multiple-sequence alignment displaying the LT domain of Gram-positive Family 1 LTs. The sequence of a *C. difficile* SleB homologue is included for comparison, although it is not known whether this protein is an LT. The asterisk denotes the position of the catalytic residue. A color version of this figure is available at www.tandfonline.com/ibmg.

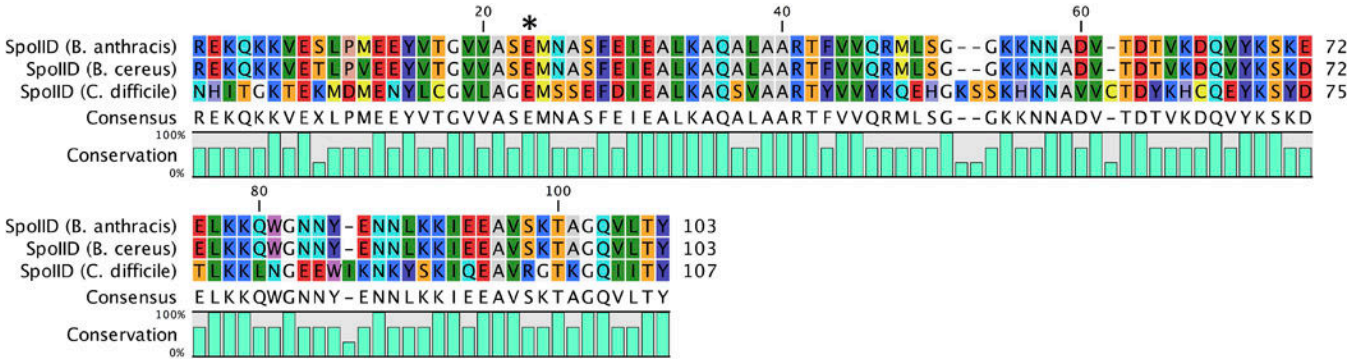

Figure 17.

Multiple-sequence alignment displaying the LT domain of Gram-positive Family 2 LTs. The asterisk denotes the position of the catalytic residue. A color version of this figure is available at www.tandfonline.com/ibmg.

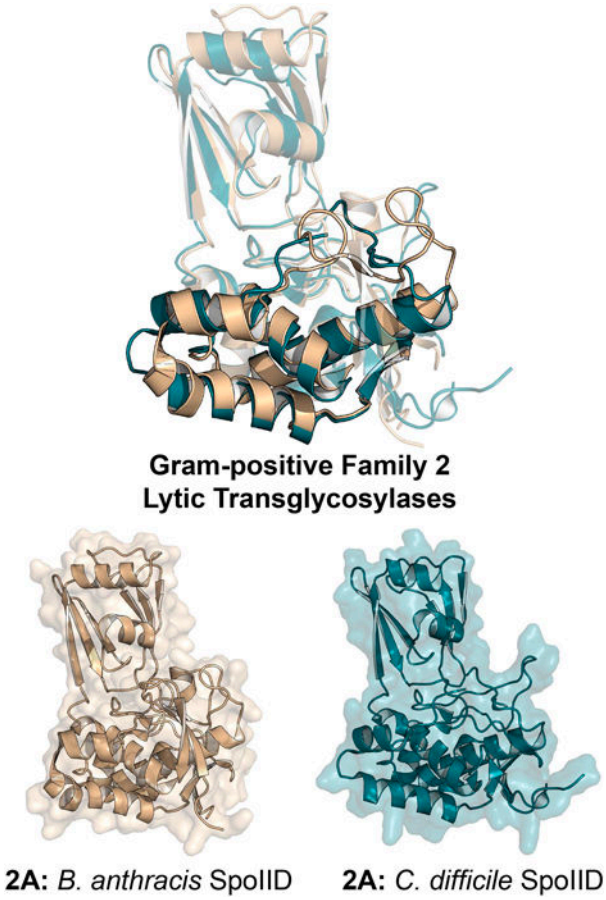

Figure 18.

X-ray structure alignment of Gram-positive Family 2 LTs, displaying the conservation of the SpoIID domain (Pfam: PF08486). The ribbon representation of each apo LT crystal structure is displayed below with a transparent surface representation. A color version of this figure is available at www.tandfonline.com/ibmg.

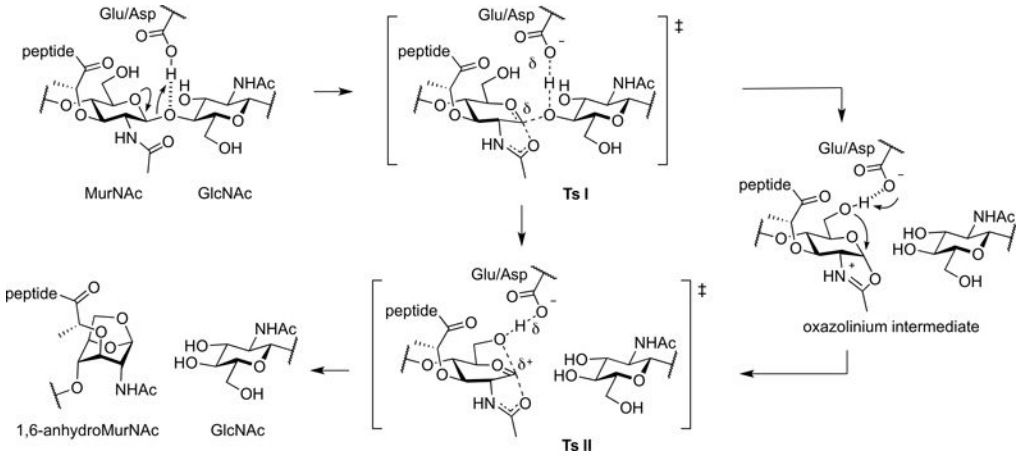

Figure 19.
Proposed LT reaction mechanisms.

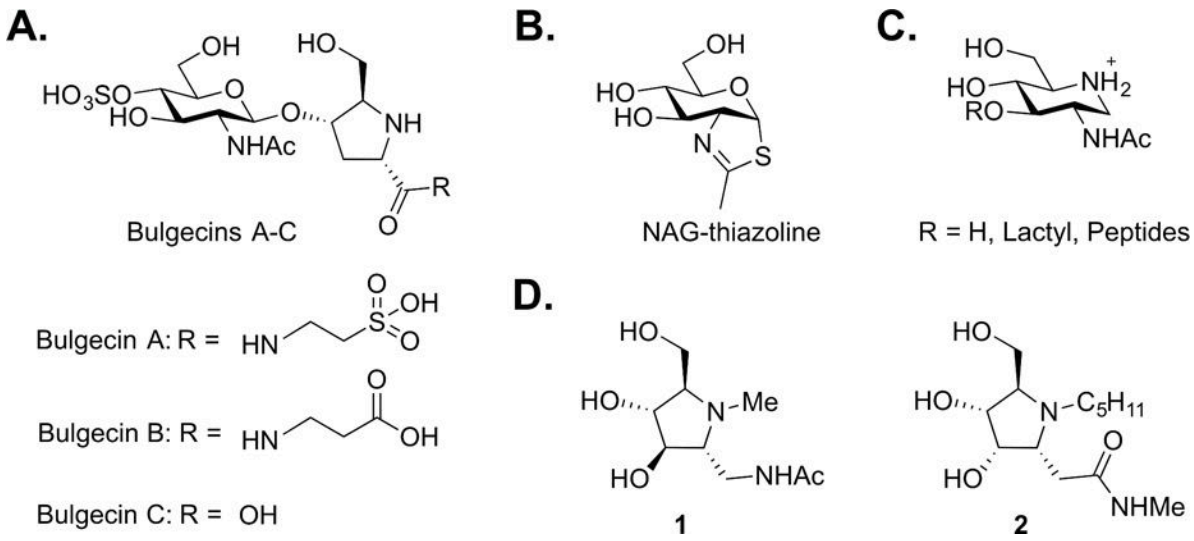

Figure 20.

LT inhibitors: (A) the natural products bulgecin A-C, (B) NAG-thiazoline, (C) iminosaccharides, and (D) iminocyclitols.

Table 1

Summary of the Gram-negative Family 1 LTs.

	Length (AA)	MW (Da)	Apo Structure	Complex Structure
Family 1A				
Slt70 (ECK4384)	645	73353.1	1QSA (van Asselt *et al.*, 1999b)	1QTE (1,6-Anhydromurotripeptide) (van Asselt *et al.*, 1999b) 1SLY (Bulgecin A) (Thunnissen *et al.*, 1995b)
Slt (PA3020)	642	73390.4		
Slt (Smlt4007)	661	73023.3		
LtgA (NGO2135)	616	67726.2		5MPQ (Bulgecin A) (Williams *et al.*, 2017)
Family 1B				
MltC (ECK2958)	359	40112.5	4C5F (Artola-Recolons *et al.*, 2014)	4CFO (Tetrasaccharide) (Artola-Recolons *et al.*, 2014) 4CFP (Tetrasaccharide) (Artola-Recolons *et al.*, 2014) 4CHX (Disaccharide pentapeptide) (Artola-Recolons *et al.*, 2014)
LtgB (NGO1033)	207	23374.9		
Family 1C				
MltE (ECK1181)	203	22226.5	2Y8P (Artola-Recolons *et al.*, 2011a) 3T36 (Fibriansah *et al.*, 2012)	4HJV (Bulgecin & Murodipeptide) (Fibriansah *et al.*, 2012) 4HJY (Catalytic Mutant, Chitopentaose) (Fibriansah *et al.*, 2012) 4HJZ (Catalytic Mutant, Chitopentaose) (Fibriansah *et al.*, 2012)
Family 1D				
MltD (ECK0211)	452	49417.4	1E0G (LysM domain) (Bateman & Bycroft, 2000)	
MltD (PA1812)	534	59734.9		
MltD1 (Smlt0994)	398	41874.6		
MltD2 (Smlt3434)	537	57751.6		
LtgE (NGO0608)	659	72159.9		
Family 1E				
MltF (ECK2556)	518	58302.1		
MltF (PA3764)	490	55226.5	4P11 5A5X (Dominguez-Gil *et al.*, 2016)	4OWD (Cysteine) 4OXV (Valine) 4OYV (Leucine) 4OZ9 (Isoleucine) 4P0G (Bulgecin A and Muropeptide) 5AA1 (NAG-anhNAM-pentapeptide) (Dominguez-Gil *et al.*, 2016) 5AA2 (NAM-pentapeptide) (Dominguez-Gil *et al.*, 2016) 5AA3 (tetrasaccharide and tetrapeptide) (Dominguez-Gil *et al.*, 2016)5AA4 (tetrapeptide) (Dominguez-Gil *et al.*, 2016)
MltF2 (PA2865)	476	53310.4		
Family 1F				
SltF				
Family 1G				

	Length (AA)	MW (Da)	Apo Structure	Complex Structure
EtgA (EPEC)	152	17026.5	4XP8 (Catalytic Mutant) (Burkinshaw *et al.*, 2015)	
LtgX (NG5004)	153	17106.9		
Family 1H				
GP144 (Bacteriophage ΦKZ)	260	28815.0	3BKH	3BKV (chitotetraose, (NAG)4) (Fokine *et al.*, 2015)

Table 2

Summary of the Gram-negative Family 2 LTs.

	Length (AA)	MW (Da)	Apo Structure	Complex Structure
Family 2A				
MltA (ECK2809)	365	40410.6	2AE0 (Apo) (van Straaten *et al.*, 2005) 2GAE (Apo) (Powell *et al.*, 2006) 2PIC (Catalytic Mutant) (van Straaten *et al.*, 2007) 2PJJ (Catalytic Mutant) (van Straaten *et al.*, 2007)	2PI8 (Chitohexaose) (van Straaten *et al.*, 2007)
MltA (PA1222)	385	41896.6		
MltA (Smlt0155)	388	41492.3		
LtgC (NGO2048)	441	47975.6	2G6G (Apo) (Powell *et al.*, 2006) 2G5D (Apo) (Powell *et al.*, 2006)	

Table 3

Summary of the Gram-negative Family 3 LTs.

	Length (AA)	MW (Da)	Apo Structure	Complex Structure
Family 3A				
MltB (ECK2696)	361	40255.7	1LTM (Slt35, Apo) (van Asselt *et al.*, 1998) 1QDR (Slt35, Apo) (van Asselt & Dijkstra, 1999) 1QUS (Slt35, Apo) (van Asselt *et al.*, 1999a)	1QDT (Slt35, Calcium) (van Asselt & Dijkstra, 1999) 1QUT (Slt35, NAG) (van Asselt *et al.*, 1999a) 1D0K (Slt35, GLCNAC-MURNAC-L-ALA-D-GLU) (van Asselt *et al.*, 2000) 1D0L (Slt35, Bulgecin A) (van Asselt *et al.*, 2000) 1D0M (Slt35, Bulgecin A & $(GLCNAC)_2$) (van Asselt *et al.*, 2000)
MltB (PA4444)	367	41404.1		
SltB1 (PA4001)	340	37835.6	4ANR (Apo) (Nikolaidis *et al.*, 2012)	
MltB1 (Smlt4052)	381	41821.6		
LtgD (NGO0626)	363	39821.2		
Family 3B				
SltB2 (PA1171)	398	42682.5		
SltB3 (PA3992)	448	47760.9	5ANZ (Apo) (Lee *et al.*, 2016b)	5AO7 (NAG-anhNAM-pentapeptide) (Lee *et al.*, 2016b) 5AO8 (NAG-NAM-pentapeptide) (Lee *et al.*, 2016b)
MltB2 (Smlt4650)	422	44588.4		

Table 4

Summary of the Gram-negative Family 4 LTs.

	Length (AA)	MW (Da)	Apo Structure	Complex Structure
Family 4A				
LAL (Bacteriophage λ)	154	17368.7	1AM7 (Evrard *et al.*, 1998)	3D3D (Chitohexasaccharide) (Leung *et al.*, 2001) 1D9U (Chitohexasaccharide) (Leung *et al.*, 2001)
Endolysin P28 (Maltocin P28)	161	17283.6		
AtlA (GGI)	181	20464.0		

Table 5

Summary of the Gram-negative Family 5 LTs.

	Length (AA)	MW (Da)	Apo Structure	Complex Structure
Family 5A				
MltG (ECK1083)	340	38247.4	2R1F (unpublished)	
MltG (PA2963)	349	39562.3		
MltG (Smlt1034)	353	38644.1		
LtgG (NGO2038)	331	36805.2		

Table 6

Summary of the Gram-negative Family 6 LTs.

	Length (AA)	MW (Da)	Apo Structure	Complex Structure
Family 6A				
RlpA (ECK0626)	362	37527.9		
RlpA (PA4000)	342	36482.6		
RlpA (Smlt4051)	422	43784.4		
RlpA (NGO1728)	228	24667.8		

Table 7

Summary of the Gram-positive Family 1 LTs.

	Length (AA)	MW (Da)	Apo Structure	Complex Structure
Family 1A				
SleB (*B. anthracis*)	253	27644.56	4FET(Jing *et al.*, 2012)	
SleB (*B. cereus*)	259	28247.20	4F55(Li *et al.*, 2012)	

Table 8

Summary of the Gram-positive Family 2 LTs.

	Length (AA)	MW (Da)	Apo Structure	Complex Structure
Family 2A				
SpoIID (*B. anthracis*)	339	37338.83	4RWR(Nocadello *et al.*, 2016)	
SpoIID (*B. cereus*)	344	37864.34		
SpoIID (*C. difficile*)	354	40005.60		5I1T (Triacetylchitotriose) (Nocadello *et al.*, 2016)