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## The molecular mechanism of bacterial lipoprotein modification—how, when and why?

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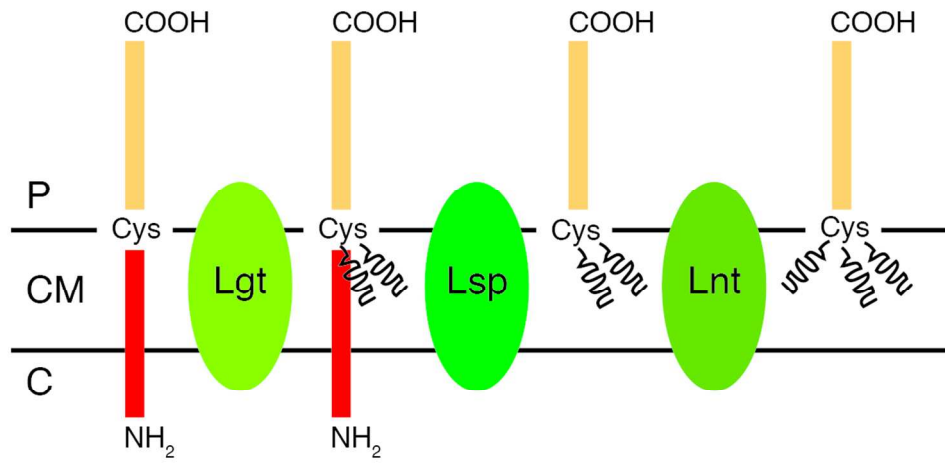
**The molecular mechanism of bacterial lipoprotein  
modification - how, when and why?**

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Review

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Peer Review

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3 Nienke Buddelmeijer

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7

### 8 *Abstract*

9 Posttranslational modification of proteins by lipidation is a common process in biological  
10 systems. Lipids provide protein stability, interaction with other membrane components, and in  
11 some cases, due to reversibility of the process, a mechanism for regulating protein  
12 localization and function. Bacterial lipoproteins possess fatty acids at their amino-termini that  
13 are derived from phospholipids and this lipid moiety anchors the proteins into the membrane.  
14 These lipids, as is the case for lipopolysaccharides and lipoteichoic acids, play an important  
15 role in signaling of the innate immune system through the interaction with Toll-like receptors.  
16 Over the past three years tremendous progress has been made in understanding the  
17 mechanism by which lipoproteins become lipidated. Advanced methodology in mass  
18 spectrometry, proteomics and genome-wide analyses allowed precise characterization of  
19 lipoprotein modifications and the identification of the enzymes catalyzing the reactions in  
20 diverse bacterial species. This review will highlight new findings on bacterial lipoprotein  
21 modification with focus on the reaction mechanisms and the role of lipoproteins in cell  
22 envelope homeostasis.

### 24 *Introduction*

1  
2  
3 25 Bacterial lipoproteins are exported proteins and often exposed on the bacterial cell surface  
4  
5 26 and include an important class of virulence factors expressed by many pathogenic species  
6  
7 27 (Kovacs-Simon *et al.*, 2010). Prolipoprotein is synthesized in the cytoplasm and translocated  
8  
9 28 via Sec or Tat secretion machineries (Inouye *et al.*, 1977, Hayashi & Wu, 1985, Gibbons *et*  
10  
11 29 *al.*, 2007, Siboo *et al.*, 2008, Shruthi *et al.*, 2010, Thompson *et al.*, 2010). Lipoprotein  
12  
13 30 modification occurs in the cytoplasmic (inner) membrane by the sequential action of three  
14  
15 31 membrane-bound enzymes (Fig. 1) (Wu *et al.*, 1982). Lipoproteins possess a conserved  
16  
17 32 sequence, so-called lipobox, which allows specific recognition by the modification  
18  
19 33 machinery. The invariant cysteine residue in the lipobox becomes lipidated and the first  
20  
21 34 amino acid after complete maturation of lipoprotein. Phosphatidylglycerol:prolipoprotein  
22  
23 35 diacylglyceryl transferase (Lgt) adds an *sn*-1,2-diacylglyceryl group from  
24  
25 36 phosphatidylglycerol (PG) to the sulfhydryl group of cysteine via a thioether bond.  
26  
27 37 Prolipoprotein signal peptidase (Lsp) cleaves the signal peptide thereby liberating the  $\alpha$ -  
28  
29 38 amino group of diacylglyceryl-cysteine. The third and last step is *N*-acylation of  
30  
31 39 diacylglyceride-cysteine by Apolipoprotein *N*-acyltransferase (Lnt), resulting in mature *N*-  
32  
33 40 acyl-*S*-diacylglyceryl-cysteine linked proteins. Lgt and Lsp are conserved in all bacterial  
34  
35 41 species, while Lnt has only been identified in proteobacteria and actinomycetes. Recent  
36  
37 42 findings suggest that a non-canonical lipidation pathway exist by the demonstration that  
38  
39 43 lipoproteins of firmicutes and terenicutes (that include mollicutes), whose genome lack a gene  
40  
41 44 encoding Lnt, exist in a triacylated form (Fig. 1) (Jan *et al.*, 1995, Kurokawa, 2009, Asanuma  
42  
43 45 *et al.*, 2010, Serebryakova *et al.*, 2011). Various *N*-terminal modifications were observed: *N*-  
44  
45 46 acyl-*S*-diacylglyceryl-cysteine, but also *N*-acyl-*S*-monoacyl-cysteine, *N*-acetyl-*S*-  
46  
47 47 diacylglyceryl-cysteine and *N*-peptidyl-*S*-diacylglyceryl-cysteine (Nakayama *et al.*, 2012).  
48  
49 48 Correct lipoprotein modification and consequently, the enzymes that carry them out, are  
50  
51 49 involved in a process essential for bacterial viability in proteobacteria and some  
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3 50 actinomycetes (Table 1). Understanding the molecular details of their catalytic mechanism  
4  
5 51 and function should provide detailed knowledge on bacterial envelope homeostasis and  
6  
7 52 facilitate the discovery of new antibacterial agents targeting this essential pathway. Most  
8  
9 53 enzymatic and functional studies have been performed with the lipoprotein modification  
10  
11 54 enzymes of *Escherichia coli* that are discussed in this review. It is stated in the text when  
12  
13  
14 55 enzymes from other bacterial species are described.  
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### 57 *Phospholipids are substrates for acyltransferases involved in lipoprotein modification*

20  
21 58 The lipid moiety of lipoproteins is derived from membrane phospholipids, linking the  
22  
23 59 lipoprotein modification pathway to phospholipid biogenesis (Lai *et al.*, 1980). Two different  
24  
25 60 systems exist in bacteria to transfer fatty acids from acyl-acyl carrier proteins (acyl-ACP), the  
26  
27 61 end products of the fatty acid synthase II pathway (FASII), to glycerol-3-phosphate. The  
28  
29 62 PlsX/Y/C is a widespread pathway while the PlsB/C system is found predominantly in  $\gamma$ -  
30  
31 63 proteobacteria, but both systems result in the synthesis of phosphatidic acid, the building  
32  
33 64 block of phospholipids. Phosphatidic acid is subsequently converted into the key intermediate  
34  
35 65 CDP-diacylglycerol. Two pathways then lead to the synthesis of phosphatidylethanolamine  
36  
37 66 (PE) (via phosphatidylserine) and phosphatidylglycerol (PG) and cardiolipin (CL), respectively  
38  
39 67 (for a recent review see (Parsons & Rock, 2013)). Movement of lipids, including PE and  
40  
41 68 lipopolysaccharides (LPS), across the cytoplasmic membrane is dependent on MsbA (Zhou *et*  
42  
43 69 *al.*, 1998, Doerrler & Raetz, 2002). The ABC transporter MsbA functions as a dimer in which  
44  
45 70 each monomer is composed of six transmembrane segments (TMS) and a nucleotide-binding  
46  
47 71 domain (Ward *et al.*, 2007). The dimer hydrolyses ATP and then undergoes large  
48  
49 72 conformational changes, which is thought to lead to the trans-bilayer movement of lipids  
50  
51 73 across the membrane (Doerrler *et al.*, 2001, Doerrler *et al.*, 2004, Doshi & van Veen, 2013).  
52  
53  
54 74 The main phospholipids of *E. coli* and *Salmonella enterica* serovar *Thyphimurium* are  
55  
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2  
3 75 phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL) and minor  
4  
5 76 phospholipids phosphatidylserine (PS) and phosphatidic acid (PA) (Ames, 1968). The fatty  
6  
7 77 acids of phospholipids of a typical *E. coli* strain contain about 45% palmitic acid (C16:0), 2%  
8  
9 78 myristic acid (C14:0), 35% palmitoleic acid (C16:1) and 18% *cis*-vaccenic acid (C18:1*cis*-11)  
10  
11 79 (Cronan & Rock, 1996). The main fatty acids of major lipoprotein Lpp (murein-lipoprotein or  
12  
13 Braun's lipoprotein) of *E. coli* are palmitic acid (53%), *cis*-vaccenic acid (20.7%), 9,10-  
14  
15 80 methylene-hexadecanoic acid (C17:0) (10.6%) and palmitoleic acid (9.4%) (Hantke & Braun,  
16  
17 81 1973), corresponding to the fatty acid moieties of phospholipids. Phospholipids that compose  
18  
19 82 membranes of *Staphylococcus aureus* include phosphatidylglycerol, cardiolipin, lysyl-  
20  
21 83 phosphatidylglycerol, and mono- and diglucosyl-diglyceride but not  
22  
23 84 phosphatidylethanolamine (White & Frerman, 1968, Fischer, 1994). Mass spectrometry data  
24  
25 85 has shown that lipoproteins in *S. aureus* are modified with C17:0 to C20:0 at the *sn*-1 position  
26  
27 86 of the diacylglyceryl group and with C15:0 at the *sn*-2 position. The  $\alpha$ -amino group of  
28  
29 87 lipidated diacylglyceryl-cysteine have fatty acids of varying lengths, from C15:0 to C20:0  
30  
31 88 (Kurokawa *et al.*, 2012). The lipid moiety of lipoproteins thus reflects the fatty acid  
32  
33 89 composition of phospholipids and varies among bacterial species.  
34  
35 90  
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#### 92 *Phosphatidylglycerol:prolipoprotein diacylglyceryl transferase (Lgt)*

43 Modification of prolipoprotein by Lgt is the first step in lipoprotein modification (Tokunaga  
44  
45 94 *et al.*, 1982). Upon membrane insertion of prolipoprotein by Sec or Tat, Lgt transfers the  
46  
47 95 diacylglyceryl group from phosphatidylglycerol onto the sulfhydryl group of the invariant  
48  
49 96 cysteine in the lipobox resulting in a thioether-linked diacylglyceryl-prolipoprotein and a *sn*-  
50  
51 97 glycerol-1-phosphate by-product (Lai *et al.*, 1980, Sankaran & Wu, 1994). Signal sequences  
52  
53 98 of lipoproteins share common features including one or several positively charged residues at  
54  
55 99 the amino terminus followed by a hydrophobic stretch of about 20 amino acids that includes  
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1  
2  
3 100 the lipobox. Based on studies with Lpp, the consensus sequence of the lipobox was  
4  
5 101 determined as: (L)<sub>-3</sub>-(A/S)<sub>-2</sub>-(G/A)<sub>-1</sub>-C<sub>+1</sub>, in which the lipid-modified cysteine at position +1  
6  
7 102 becomes the first amino acid in the mature protein (Wu *et al.*, 1983, Wu, 1996, Madan Babu  
8  
9 103 & Sankaran, 2002, Babu *et al.*, 2006). This consensus sequence has been documented in the  
10  
11 104 PROSITE database under pattern entry PS00013/PDOC00013  
12  
13 105 (<http://www.expasy.ch/prosite/>). Lipobox motifs are similar in firmicutes but more variable in  
14  
15 106 spirochetes (Haake, 2000, Sutcliffe & Harrington, 2002), the -3 position in particular is more  
16  
17 107 loosely defined in the latter, where a leucine residue is often replaced by another hydrophobic  
18  
19 108 amino acid. A high percentage of ORFs in spirochetes encode lipoproteins, some of which are  
20  
21 109 highly abundant proteins (Haake, 2000). For Lpp it has also been demonstrated that residue  
22  
23 110 G14 in the signal peptide sequence is important for lipidation by Lgt, and that a signal  
24  
25 111 sequence shortened by several residues still leads to modified protein (Wu *et al.*, 1977, Yem  
26  
27 112 & Wu, 1977, Lin *et al.*, 1978, Lin *et al.*, 1980, Inouye *et al.*, 1984, Pollitt *et al.*, 1985). The  
28  
29 113 first biochemical characterization of Lgt was reported in the 1980-s using crude membrane  
30  
31 114 extracts. The optimal pH and reaction temperature for Lgt are pH 7.8 and 37°C, respectively  
32  
33 115 (Tokunaga *et al.*, 1984). Lgt is stable in membrane vesicles up to 65°C, the enzyme is active  
34  
35 116 in N-octyl-β-D-glucoside but many other detergents are not compatible with enzyme activity  
36  
37 117 (Tokunaga *et al.*, 1984, Sankaran & Wu, 1994). PG is specific as acyl donor since PE and CL  
38  
39 118 could not be used as substrate in the *in vitro* assay using a synthetic peptide, suggesting that  
40  
41 119 the polar headgroup of the phospholipid plays an important role in the Lgt reaction (Sankaran  
42  
43 120 & Wu, 1994). The gene encoding Lgt was identified by screening a collection of temperature  
44  
45 121 sensitive mutants of *S. enterica* for the accumulation of unmodified Lpp and was shown to be  
46  
47 122 located at the 5' end of *thyA* (Williams *et al.*, 1989, Bell-Pedersen *et al.*, 1991, Gan *et al.*,  
48  
49 123 1993). The enzyme is localized in the cytoplasmic membrane and spans the membrane by  
50  
51 124 seven transmembrane segments (Fig. 2.) (Pailler *et al.*, 2012). The majority of essential  
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3 125 residues are located in the membrane, however, it is currently unknown how Lgt interacts  
4  
5 126 with its substrates PG and prolipoprotein (Qi *et al.*, 1995, Sankaran *et al.*, 1997, Pailler *et al.*,  
6  
7 127 2012). Residues H103 and Y235 were identified as conserved by comparing four Lgt  
8  
9 128 sequences and were shown to be essential for Lgt activity (Sankaran *et al.*, 1997). These  
10  
11 129 residues are among twelve residues highly conserved in proteobacteria, firmicutes and  
12  
13 130 actinobacteria (Pailler *et al.*, 2012). A more recent analysis on conserved residues highlighted  
14  
15 131 the existence of a so-called Lgt signature motif; two amino acids in this sequence (N146 and  
16  
17 132 G154) were shown to be absolutely required for function (Pailler *et al.*, 2012). Together, these  
18  
19 133 data show that regions H103-G108 and G142-G154 and residues Y26 and Y235 are critical  
20  
21 134 for Lgt function. Initial kinetics studies were performed with crude membrane extracts but  
22  
23 135 further biochemical characterization of the *E. coli* enzyme turned out difficult due to  
24  
25 136 overproduction defects and instability of the purified enzyme (Sankaran *et al.*, 1997, Selvan  
26  
27 137 & Sankaran, 2008, Pailler *et al.*, 2012). Lgt has been purified recently from *Lactococcus*  
28  
29 138 *lactis* with which kinetic studies were performed (Banerjee & Sankaran, 2012), unfortunately,  
30  
31 139 assay conditions were not comparable with those described previously (Sankaran *et al.*, 1997,  
32  
33 140 Sundaram *et al.*, 2012), making it difficult to compare enzyme activities from different  
34  
35 141 bacterial species.  
36  
37 142 Lgt of *S. aureus* can complement a temperature sensitive mutant of *E. coli*, suggesting that  
38  
39 143 Lgt is functionally conserved among bacterial species (Qi *et al.*, 1995). Deletion mutants of  
40  
41 144 *lgt* in firmicutes often lead to shedding of lipoproteins into the culture supernatant in non-  
42  
43 145 lipidated precursor form with uncleaved signal peptide (Leskelä *et al.*, 1999) or processed by  
44  
45 146 an unidentified signal peptidase (Stoll *et al.*, 2005, Denham *et al.*, 2009) or Lsp (Baumgärtner  
46  
47 147 *et al.*, 2007, Henneke *et al.*, 2008). Two Lgt homologues were identified in *Streptomyces*  
48  
49 148 *coelicolor*, Lgt1 and Lgt2, which can both complement the *lgt* mutant of *Streptomyces*  
50  
51 149 *scabies*, suggesting that both enzymes are functional enzymes (Widdick *et al.*, 2011). A  
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3 150 double mutant could not be obtained suggesting that Lgt activity is essential in *S. coelicolor*  
4  
5 151 (Thompson *et al.*, 2010). Lipoproteins were purified from the complementing strains and the  
6  
7 152 composition of their N-terminal fatty acid was analyzed by mass spectrometry (Widdick *et*  
8  
9 153 *al.*, 2011). Several fatty acid acylated peptides were missing in these strains compared to wild  
10  
11 154 type *S. scabies*, suggesting that subtle differences in diacylglyceryl transferase activity might  
12  
13 155 exist. The gene encoding Lgt in *Mycobacterium tuberculosis* and two genes in  
14  
15 156 *Mycobacterium smegmatis* have recently been identified (Tschumi *et al.*, 2012). The  
16  
17 157 MSMEG\_3222 gene was shown to be responsible for the transfer of diacylglyceryl onto  
18  
19 158 lipoproteins, and although less conserved than MSMEG\_3222 the role of the second *lgt* gene  
20  
21 159 (MSMEG\_5408) in lipoprotein modification has not yet been addressed (Tschumi *et al.*,  
22  
23 160 2012). An *lgt* deletion mutant could not be obtained in *M. tuberculosis* suggesting that this  
24  
25 161 gene is essential (Sasseti *et al.*, 2003, Tschumi *et al.*, 2012). Strikingly, MSMEG\_3222 is not  
26  
27 162 essential in *M. smegmatis* and deletion did not completely abolish incorporation of radioactive  
28  
29 163 palmitate suggesting that either MSMEG\_5408 or another, as yet unknown, enzyme possesses  
30  
31 164 Lgt activity but is perhaps less efficient than MSMEG\_3222 (Tschumi *et al.*, 2012). Absence  
32  
33 165 of Lgt resulted in massive release of lipoproteins in the culture media but none of these  
34  
35 166 proteins contained a signal peptide, suggesting that they are processed by Lsp or other signal  
36  
37 167 peptidases (Tschumi *et al.*, 2012). No clear phenotype on cell wall biogenesis was observed.  
38  
39 168 Detailed biochemical analyses and comparison of kinetic parameters of various Lgt enzymes  
40  
41 169 will contribute to a better understanding of the mechanism of the reaction and are predicted to  
42  
43 170 highlight differences and similarities in enzyme activity. The thiol group of prolipoprotein  
44  
45 171 probably performs a nucleophilic attack on the carbonyl group of PG where Lgt provides the  
46  
47 172 catalytic residues to support transfer of diacylglyceryl onto prolipoprotein, and positions the  
48  
49 173 two substrates close together. PG is the sole acyl donor in the Lgt reaction, suggesting that the  
50  
51 174 glycerol-phosphate headgroup plays an important role in substrate recognition by Lgt.  
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175

176 *Lipoprotein signal peptidase (Lsp)*

177 Lsp (SPase II) is localized in the cytoplasmic membrane (Tokunaga *et al.*, 1984) and spans  
178 this membrane by four transmembrane segments with both the amino and carboxyl termini  
179 facing the cytoplasm (Munoa *et al.*, 1991) (Fig. 2). Five conserved regions were identified,  
180 i.e. region I (amino acids residues 23-27 in *E. coli* sequence), region II (residues 53-58),  
181 region III (residues 104-115) and region IV (residues 121-123) and region V (residues 137-  
182 141) (Munoa *et al.*, 1991, Tjalsma *et al.*, 1999). The role of these conserved regions in the  
183 peptidase reaction has not yet been addressed but probably plays a role in substrate  
184 recognition and/or binding. The active site of Lsp is composed of at least two aspartate  
185 residues (D114 and D141 in *E. coli*) (Tjalsma *et al.*, 1999). Modification of lipoproteins by  
186 addition of diacylglyceryl catalyzed by Lgt is a prerequisite for processing by Lsp in  
187 proteobacteria (Tokunaga *et al.*, 1982). The Lsp enzymes of actinomycetes have an N-  
188 terminal extension, truncations of which led to loss of Lsp function, either due to inactivation  
189 or instability of the enzyme (Thompson *et al.*, 2010). Biochemical studies with Lsp were  
190 performed in the early 1980-s and showed that Lsp is temperature resistant in isolated  
191 membranes, but unlike Lnt still active at 80°C (Hussain *et al.*, 1982). In detergent-solubilized  
192 membranes Lsp is active at a reaction temperature between 37°C-45°C with a pH optimum of  
193 7.9 (Tokunaga *et al.*, 1984). The enzyme does not require phospholipids and is active in non-  
194 ionic detergents.

195 Initially two enzymatic activities were shown to be involved in the proteolytic cleavage of  
196 lipoproteins, Lsp that cleaves off the signal peptide of diacylglyceryl-proLpp and a signal  
197 peptide peptidase responsible for the degradation of signal peptide (SppA or Protease IV)  
198 (Suzuki *et al.*, 1978, Novak & Dev, 1988). SppA is an intramembrane protease (Pacaud,  
199 1982, Pacaud, 1982) utilizing a Ser/Lys dyad for catalysis (Wang *et al.*, 2008). It adopts a

1  
2  
3 200 tetrameric structure with a hydrophobic interior providing access to the catalytic site (Ichihara  
4  
5 201 *et al.*, 1986, Kim *et al.*, 2008) and cleaves its substrate in the hydrophobic region (Novak &  
6  
7 202 Dev, 1988). Recently, Saito *et al.* (Saito *et al.*, 2011) provided evidence that peptidase RseP  
8  
9 203 (YaeL in *E. coli*), which belongs to the zinc-metalloprotease group (S2P) of the  
10  
11 204 intramembrane-cleaving proteases (I-CLiPs) family of proteases (Urban, 2009), is responsible  
12  
13 205 for cleavage of signal peptides including those obtained after cleavage by Lsp. RseP is not the  
14  
15 206 exclusive signal peptide peptidase since some signal peptides were cleaved in the absence of  
16  
17 207 RseP and SppA. The sole natural substrate of RseP is the anti- $\sigma^E$  factor RseA that upon cell  
18  
19 208 envelope stress is cleaved by DegS and further processed by RseP resulting in activation of  $\sigma^E$   
20  
21 209 and the expression of stress-induced genes (Alba *et al.*, 2002). RseP has four transmembrane  
22  
23 210 segments with its N- and C-termini facing the periplasm (Kanehara *et al.*, 2001). The  
24  
25 211 periplasmic PDZ domain is specific for regulated processing of RseA after truncation by  
26  
27 212 DegS. Removal of the PDZ domain led to intramembrane cleavage of RseA (Hizukuri *et al.*,  
28  
29 213 2014), suggesting that this domain is not required for processing of lipoprotein signal  
30  
31 214 peptides.  
32  
33 215 Lsp proteins from firmicutes can complement an *lsp* temperature sensitive mutant of *E. coli*,  
34  
35 216 suggesting that the enzymes are functionally conserved as seen for Lgt (Pragai *et al.*, 1997),  
36  
37 217 however kinetic analyses have not yet been performed to determine specificity and  
38  
39 218 mechanism of the reaction. Lsp does not process prolipoprotein without S-diacylglyceryl  
40  
41 219 modification in proteobacteria, indicating that the enzyme specifically recognizes the fatty  
42  
43 220 acid acylated cysteine. Processing of Lpp occurs both in *E. coli* and *Bacillus subtilis* but at  
44  
45 221 different rates (Hayashi *et al.*, 1985). Apo-Lpp accumulates in *B. subtilis* probably due to  
46  
47 222 inefficient N-acylation or N-acetylation of apoLpp (Kurokawa *et al.*, 2012). Furthermore, the  
48  
49 223 two organisms differ in their susceptibility to globomycin as shown by the processing of  
50  
51 224 penicillinase (Hayashi & Wu, 1983). *Myxococcus xanthus* possesses four *lsp* genes, two of  
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3 225 which are part of the antibiotic TA biosynthetic cluster (Xiao & Wall, 2014). The Lsp  
4  
5 226 enzymes can complement an *lsp* depletion strain of *E. coli* only in the absence of Lpp  
6  
7 227 suggesting that Lsp-s of *M. xanthus* are less efficient than Lsp of *E. coli*. Lipoproteins of  
8  
9 228 *Streptococcus uberis* are processed by Eep, a homologue of enhanced expression of  
10  
11 229 pheromone from *Enterococcus faecalis* (An *et al.*, 1999), in the absence of Lsp in late-  
12  
13 230 stationary-phase cells (Denham *et al.*, 2009).

14  
15  
16 231 Structural information is not available for any of the lipoprotein modification enzymes,  
17  
18 232 including Lsp. Other bacterial signal peptidases, such as signal peptidase I (leader peptidase)  
19  
20 233 and type IV pilin signal peptidase (prepilin peptidase), share their membrane-embedded  
21  
22 234 characteristics and the hydrophobic nature of their substrates. The catalytic mechanism  
23  
24 235 (Paetzel, 2014) and the Ser/Lys active site (Wang *et al.*, 2008) of leader peptidase have been  
25  
26 236 reported. A soluble version of the enzyme, lacking its two N-terminal transmembrane  
27  
28 237 segments, is active *in vitro* (Kuo *et al.*, 1993), although slightly less compared to full-length  
29  
30 238 protein and it requires detergents or phospholipids for its activity (Tschantz *et al.*, 1995).  
31  
32 239 Several crystal structures of the soluble form have been solved (Kim *et al.*, 2008) and a recent  
33  
34 240 NMR structure suggests that the signal peptide remains in the bilayer in an unstructured  
35  
36 241 conformation and that the active site of leader peptidase functions at the membrane surface  
37  
38 242 (De Bona *et al.*, 2012) or well within the lipid bilayer (Paetzel, 2014). The membrane is  
39  
40 243 probably important in stabilizing the signal peptide while under reconstituted conditions the  
41  
42 244 detergents or phospholipids would serve an analogous role. Several inhibitors have been  
43  
44 245 identified including penem-type inhibitors (Allsop *et al.*, 1996) and lipopeptides (Paetzel *et*  
45  
46 246 *al.*, 2002). Differences in specificity between leader peptidases from different species may be  
47  
48 247 explained by structural variations in the active site (Auclair *et al.*, 2012). Prepilin peptidase is,  
49  
50 248 like Lsp, an aspartic protease that belongs to the class of aspartyl protease I-CLiPs (Erez *et*  
51  
52 249 *al.*, 2009) and is involved in processing of pre-pilin proteins (LaPointe & Taylor, 2000). I-  
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3 250 CLiPs are involved in intramembrane proteolysis liberating membrane-tethered domains of  
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5 251 membrane proteins that can function as regulatory molecules, and are found in many different  
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7 252 organisms (Erez *et al.*, 2009). Prepilin peptidase is an integral membrane protein and  
8  
9 253 processing of prepilin has been postulated to occur at the cytoplasmic side of the membrane,  
10  
11 254 allowing the subsequent addition of methyl groups onto phenylalanine (Strom & Lory, 1987).  
12  
13 255 A topology model was proposed based on data obtained with reporter fusion proteins and  
14  
15 256 topology prediction programs; prepilin peptidases have eight transmembrane segments with  
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17 257 their N- and C-termini are located in the periplasm, and the catalytic Asp dyad is facing the  
18  
19 258 cytoplasm (LaPointe & Taylor, 2000). The crystal structure of aspartyl protease FlaK, has  
20  
21 259 recently been solved (Hu *et al.*, 2011). The two active site Asp residues are located on top of  
22  
23 260 two  $\alpha$ -helices, housed in a hydrophilic cavity in the membrane and facing the cytoplasm,  
24  
25 261 similar to the structure of presenilin (Hu *et al.*, 2011, Lu *et al.*, 2014). Lsp probably shares  
26  
27 262 structural features with both leader peptidase and aspartyl proteases. It interacts with  
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29 263 diacylglyceryl-prolipoprotein as leader peptidase with signal peptides of secreted proteins in  
30  
31 264 such a way that the diacylglyceryl-cysteine is accessible to the Asp dyad active site as in  
32  
33 265 aspartyl proteases, with the only difference that the active site is facing the periplasm.  
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#### 267 *Globomycin – a specific inhibitor of Lsp*

268 Globomycin was first identified in 1978, as an antibiotic produced by various *Streptomyces*  
269 strains with spheroplast-forming (global-shape) activity on *E. coli* (Inukai *et al.*, 1978, Inukai  
270 *et al.*, 1978). It specifically inhibits proteobacteria such as *E. coli* (MIC 0.4  $\mu\text{g/ml}$  for *E. coli*  
271 B) but not *Pseudomonas aeruginosa*. It is also less active against firmicutes (for example,  
272 MIC >100  $\mu\text{g/ml}$  for *S. aureus* and *B. subtilis*) (Inukai *et al.*, 1978). Globomycin is a  
273 lipophilic cyclic peptide with molecular formula  $\text{C}_{32}\text{H}_{57}\text{N}_5\text{O}_2$  and a molecular weight of 655  
274 (Nakajima *et al.*, 1978) (Fig. 3). Indications that the target of globomycin is Lsp were



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3 275 reported in the last paper in a series of articles on globomycin discovery and its  
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5 276 characterization by Mamoru Arai's group (Inukai *et al.*, 1978). It was later shown that  
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7 277 diacylglyceryl-proLpp that accumulated in the cytoplasmic membrane in cells grown in the  
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9 278 presence of globomycin (Hussain *et al.*, 1980) could be converted into mature protein *in vitro*  
10  
11 279 when cell envelope fractions were incubated under optimal conditions. The gene encoding  
12  
13 280 Lsp was identified in *E. coli* by selecting clones from the Carbon-Clarke collection for  
14  
15 281 globomycin resistance, or by screening for complementation of a strain that is temperature  
16  
17 282 sensitive in the processing of diacylglyceryl-proLpp (Tokunaga *et al.*, 1983, Yamagata *et al.*,  
18  
19 283 1983). Kinetics studies showed that globomycin inhibits Lsp in a non-competitive manner  
20  
21 284 with a  $K_i$  of 36 nM, and a  $K_m$  value of 6  $\mu$ M was determined for diacylglyceryl-prolipoprotein  
22  
23 285 substrate (Dev *et al.*, 1985). Globomycin is mycobactericidal at elevated concentrations but  
24  
25 286 killing is independent of Lsp in *M. tuberculosis*, suggesting an alternative pathway of  
26  
27 287 inhibition in this bacterium (Banaiee *et al.*, 2007). Globomycin analogues have been  
28  
29 288 described that are more potent against proteobacteria and exhibit antibacterial activity against  
30  
31 289 firmicutes (Kiho *et al.*, 2003, Kiho *et al.*, 2004). A long alkyl side chain, the lipophilic part of  
32  
33 290 the molecule, increases activity and also shows moderate activity against *S. aureus*. The  
34  
35 291 hydroxyl group of L-Ser is essential but not that of L-allo-Thr (Kiho *et al.*, 2004). The lipid  
36  
37 292 moiety of globomycin is probably required to anchor the antibiotic into the membrane to  
38  
39 293 allow correct positioning of the peptidic part for interaction with the enzyme, but also seems  
40  
41 294 to play a direct role in globomycin-enzyme interaction. Differences in membrane composition  
42  
43 295 and/or membrane topology of Lsp might explain differences in antibacterial activities of  
44  
45 296 globomycin and its analogues on Lsp of different bacterial species. Another antibiotic, TA or  
46  
47 297 myxovirescin isolated from *Myxococcus xantus*, has been shown to target Lsp (Xiao *et al.*,  
48  
49 298 2012). Antibiotic TA is a cyclic macrolactam lactone that is active against proteobacteria and  
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51 299 some firmicutes (Rosenberg *et al.*, 1973).  
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301 *Apolipoprotein N-acyltransferase (Lnt)*

302 The membrane localization of Lnt of *E. coli* was demonstrated by Gupta and Wu in 1991  
303 (Gupta & Wu, 1991), in the same study it was demonstrated that Lnt has a broad pH optimum  
304 (pH 6.5-7.5) and that it requires the presence of detergents for its activity *in vitro*. In 2005 the  
305 membrane topology of Lnt and the phenotype of an *lnt* depletion mutant was reported  
306 (Robichon *et al.*, 2005). Several essential residues in Lnt were identified that are mainly  
307 located in the catalytic periplasmic domain and include the catalytic triad E-K-C (Vidal-  
308 Ingigliardi *et al.*, 2007). It was shown that growth of the *lnt* depletion mutant could be  
309 restored by Lnt homologues of proteobacteria, but not actinobacteria (Vidal-Ingigliardi *et al.*,  
310 2007). The reaction catalyzed by Lnt involves two steps. In the first step, the *sn*-1 of PE reacts  
311 with the active site cysteine of Lnt resulting in the formation of a thioester-acyl enzyme  
312 intermediate and a lysophospholipid by-product (Jackowski & Rock, 1986, Buddelmeijer &  
313 Young, 2010, Hillmann *et al.*, 2011). In the second step, the acyl group is transferred to  
314 apolipoprotein resulting in triacylated, mature lipoprotein (Buddelmeijer & Young, 2010).  
315 Kinetics studies showed that Lnt follows a ping-pong mechanism whereby lyso-phospholipid  
316 produced in the first reaction is released before the second substrate, apolipoprotein, is bound  
317 and modified. With PE and a synthetic diacylated peptide (FSL-1) a  $K_m$  value of 8.0  $\mu\text{M}$  and  
318 a  $V_{\max}$  value of  $350 \mu\text{mol}_{\text{FSL-1}} \text{min}^{-1} \mu\text{mol}^{-1}_{\text{Lnt}}$  were determined, corresponding to a specific  
319 activity of  $5.6 \mu\text{mol}_{\text{FSL-1}} \text{min}^{-1} \text{mg}^{-1}_{\text{Lnt}}$ . Interestingly, similar kinetics were obtained with PagP  
320 that catalyzes the transfer of an acyl group from PE to lipid A (Bishop *et al.*, 2000). Although  
321 acyltransferases Lnt and PagP are involved in completely different pathways, their substrates  
322 are highly abundant;  $5 \times 10^5$  molecules of Lpp and  $2 \times 10^6$  molecules of lipid A per cell  
323 (Braun & Rehn, 1969, Raetz, 1986). Furthermore, similar  $K_m$  values have been reported for



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3 324 Lsp and Lnt of *E. coli* for diacylglyceryl-proLpp and apo-lipopeptide, respectively (Dev *et al.*,  
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5 325 1985, Hillmann *et al.*, 2011).  
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7 326 Lnt is highly efficient in the second step of the reaction but less in the first step:  $k_{cat}/K_m$  of 4.8  
8  
9 327  $\times 10^3$  for PE versus  $7.3 \times 10^5$  for FSL-1. Based on the kinetic data, the number of Lnt  
10  
11 328 molecules per cell was estimated between 100 and 200, indicating that the *N*-acyltransferase  
12  
13 329 reaction is fast and efficient since lipoproteins are very abundant. PE is the preferred substrate  
14  
15 330 with an optimal fatty acid chain length of C16-18, more specifically palmitate or oleate with a  
16  
17 331 saturated acyl chain in position *sn*-1 and a non-saturated acyl chain at *sn*-2 (Jackowski &  
18  
19 332 Rock, 1986, Hillmann *et al.*, 2011). Phospholipids with small polar headgroups can also serve  
20  
21 333 as acyl donor (Gupta *et al.*, 1991, Hillmann *et al.*, 2011). PagP and LpxA, which is involved  
22  
23 334 in the first step of lipopolysaccharide biosynthesis, possess a carbon-ruler that makes these  
24  
25 335 enzymes highly specific for the chain length of the acyl donor substrate (Wyckoff *et al.*, 1998,  
26  
27 336 Ahn *et al.*, 2004, Williams & Raetz, 2007). It is unlikely that Lnt has carbon ruler capacity  
28  
29 337 since the acyl chain length and composition of the phospholipid substrate are variable. On the  
30  
31 338 other hand, the *S*-diacylglyceryl moiety of the apolipoprotein substrate probably determines  
32  
33 339 substrate specificity due to variations in lipid composition depending on the bacterial species.  
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35 340 The gene encoding Lnt was first identified in *S. enterica* by screening the same library as  
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37 341 described for *lgt* for the accumulation of apo-Lpp (Gupta *et al.*, 1993). The mutation causing  
38  
39 342 the temperature sensitive defect in Lnt was a substitution of glutamate 435 to lysine; a similar  
40  
41 343 mutation in *E. coli* did not result in a temperature sensitive phenotype (Robichon *et al.*, 2005).  
42  
43 344 Lnt activity in Actinomycetes was first reported in *M. smegmatis* (Tschumi *et al.*, 2009). A  
44  
45 345 strain of *M. smegmatis* deleted for *lnt* was viable and resulted in the accumulation of the  
46  
47 346 apolipoprotein form of two model lipoproteins LppX and LprF, directly demonstrating its *N*-  
48  
49 347 acyltransferase activity (Tschumi *et al.*, 2009, Brülle *et al.*, 2010). Lnt of mycobacteria  
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51 348 transfers either palmitate or tuberculostearic acid (in *M. bovis*) from phospholipids onto  
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3 349 apolipoprotein (Brülle *et al.*, 2013). Phospholipids of mycobacteria possess mainly  
4  
5 350 octadecanoic acid and tuberculostearic acid at the *sn*-1 position and palmitate at the *sn*-2  
6  
7 351 position (Okuyama *et al.*, 1967), suggesting that Lnt has different substrate specificity in  
8  
9 352 these species. Lnt of *M. smegmatis* was unable to complement a conditional *lnt* null mutant of  
10  
11 353 *E. coli*, suggesting that indeed these enzymes have different substrate specificity.  
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13 354 Furthermore, the enzyme was correctly inserted into the cytoplasmic membrane and shown to  
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15 355 have a similar membrane topology as Lnt of *E. coli* (Baulard *et al.*, 2003). Among the  
16  
17 356 essential residues in Lnt of *E. coli*, W237 and Y388 are not conserved in Lnt of *M. smegmatis*  
18  
19 357 (Vidal-Ingigliardi *et al.*, 2007, Tschumi *et al.*, 2009). These residues were shown to be  
20  
21 358 involved in the second step of the reaction, *N*-acylation of apolipoprotein, suggesting they  
22  
23 359 might play a role in substrate specificity (Buddelmeijer & Young, 2010). The *lnt* gene in *M.*  
24  
25 360 *smegmatis* (MSMEG\_3860 or ppm2) is located adjacent to *ppm1* encoding polyprenol  
26  
27 361 monophosphomannose (Ppm) synthase that is involved in the transfer of mannose from GDP-  
28  
29 362 mannose to polyprenol phosphate, an important step in the biosynthesis of mannose-  
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31 363 containing lipoglycans (Gurcha *et al.*, 2002). Ppm synthase is essential for viability in *M.*  
32  
33 364 *smegmatis* (Rana *et al.*, 2012).

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37 365 In *M. tuberculosis ppm1* (Rv2051c) encodes a two-domain protein, in which the N-terminal  
38  
39 366 domain is Lnt and the C-terminal domain Ppm synthase. The Lnt domain of Ppm1 enhances  
40  
41 367 Ppm synthase activity (Gurcha *et al.*, 2002). In mycobacteria where Lnt and Ppm synthase are  
42  
43 368 encoded by two genes, the proteins were shown to interact (Baulard *et al.*, 2003). In both  
44  
45 369 cases Lnt is thought to stabilize the Ppm synthase (Gurcha *et al.*, 2002, Baulard *et al.*, 2003).  
46  
47 370 A second *lnt*-like gene could be identified in *M. tuberculosis* (Rv2261c and Rv2262c) and *M.*  
48  
49 371 *bovis* (BCG\_2279c), but the active site cysteine residue is replaced by serine (Brülle *et al.*,  
50  
51 372 2013). The role of these enzymes in lipoprotein modification is currently unknown.  
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3 374 Lipoproteins in Mycobacteria and Streptomyces are often, in addition to being acylated,  
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5 375 glycosylated (Espitia & Mancilla, 1989, Herrmann *et al.*, 1996, Gonzalez-Zamorano *et al.*,  
6  
7 376 2009, Sartain & Belisle, 2009, Wehmeier *et al.*, 2009, Brülle *et al.*, 2010). Recent findings  
8  
9 377 demonstrated a direct correlation between *N*-acylation and glycosylation of lipoproteins in  
10  
11 378 *Corynebacterium glutamicum* (Mohiman *et al.*, 2012). Lnt (Cg-Ppm2) of *C. glutamicum*  
12  
13 379 catalyzes *N*-acylation of apolipoproteins by addition of a palmitoyl moiety similar to *M.*  
14  
15 380 *smegmatis* (Tschumi *et al.*, 2009, Mohiman *et al.*, 2012). Lipid modifications in *C.*  
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17 381 *glutamicum* included a diacylglyceryl moiety (C16:0, C18:1) and a *N*-palmitoyl group  
18  
19 382 (C16:0) similar to lipoproteins of *E. coli* (Mohiman *et al.*, 2012). *N*-acylation of lipoproteins  
20  
21 383 was not affected in a Cg-*ppm1* mutant but proteins were no longer *O*-glycosylated with  
22  
23 384 hexose units, indicating that Ppm synthase is required for glycosylation of lipoproteins in *C.*  
24  
25 385 *glutamicum*. In a Cg-*ppm2* mutant lipoproteins are only diacylated and not glycosylated  
26  
27 386 (Mohiman *et al.*, 2012). A Cg-*ppm1* deletion mutant does not synthesize lipoglycans,  
28  
29 387 furthermore, their biosynthesis is also affected in the absence of Cg-*ppm2* (Lnt), probably  
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31 388 because Ppm synthase is less stable under these conditions (Gibson *et al.*, 2003).  
32  
33 389 Interestingly, Ppm1 of *M. tuberculosis* is able to complement both *N*-acylation and  
34  
35 390 glycosylation defects in *C. glutamicum* (Mohiman *et al.*, 2012) and to restore *N*-acylation in  
36  
37 391 *M. smegmatis* (Tschumi *et al.*, 2009). The Lnt domain of Ppm1 alone does not restore *N*-  
38  
39 392 acylation but does restore glycosylation in *C. glutamicum*. This strengthens the interpretation  
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41 393 that the activities of Lnt and Ppm synthase are tightly linked and illustrate that these enzymes  
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43 394 operate in a common pathway implicated in envelope biosynthesis.  
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52 396 All Streptomyces species have two *lnt* genes (Widdick *et al.*, 2011). In *S. scabies*, Lnt1 is  
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54 397 responsible for *N*-acylation of lipoproteins since lipoproteins are diacylated in *lnt1* and  
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56 398 *lnt1lnt2* mutants. In the absence of Lnt2, however, both di- and triacylated proteins were  
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3 399 detected, suggesting a role for Lnt2 in efficient *N*-acylation of lipoproteins (Widdick *et al.*,  
4  
5 400 2011). Since these bacteria undergo different developmental stages, gene expression and  
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7 401 activity of lipoprotein modifying enzymes might be tightly regulated depending on  
8  
9 402 environmental conditions. In this respect it is interesting to note that the degree of fatty acid  
10  
11 403 acylation varies greatly in firmicutes depending on growth conditions. For example,  
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13 404 diacylated lipoproteins accumulated in *S. aureus* during late-exponential growth at low pH  
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15 405 (Kurokawa *et al.*, 2012). New protein synthesis is required for both accumulation of  
16  
17 406 diacylated proteins and *N*-acylation of these proteins during pH upshift. These data suggest  
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19 407 that regulation of *lnt* expression or pH-mediated enzyme activity control *N*-acylation of  
20  
21 408 lipoproteins. Assuming that phospholipids are also substrate for *N*-acylation in firmicutes,  
22  
23 409 Lnt-like enzymes of these species probably have different specificities for the acyl-donor  
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25 410 substrate than those of proteobacteria.  
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32 412 After completion of the Lnt reaction, the lysophosphatidylethanolamine, or 2-acyl-  
33  
34 413 glycerophosphoethanolamine (2-acyl-GPE) by-product can either be degraded by  
35  
36 414 lysophospholipase PldB (Hsu *et al.*, 1991) or recycled in the synthesis of phospholipid via the  
37  
38 415 acyl-acyl carrier protein synthetase/2-acylglycerophosphoethanolamine acyltransferase  
39  
40 416 pathway (Cooper *et al.*, 1989, Jackowski *et al.*, 1994). 2-acyl-GPE first needs to be  
41  
42 417 translocated to the inner leaflet of the cytoplasmic membrane, a process catalyzed by LplT,  
43  
44 418 before it can be used as a substrate for PL synthesis (Harvat *et al.*, 2005). The Aas enzyme is  
45  
46 419 a cytoplasmic membrane protein (Cooper *et al.*, 1989) that contains both enzymatic activities  
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48 420 with acyltransferase located at the N-terminal end and acyl ACP synthetase located at the C-  
49  
50 421 terminal end (Jackowski *et al.*, 1994). In the first step of the reaction acyl-ACP synthetase  
51  
52 422 adds saturated fatty acids onto ACP and in the second step fatty acid is transferred to the *sn*-1  
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54 423 position of 2-acyl-GPE by the acyltransferase resulting in the formation of new PE.  
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3 424 Alternatives for removal of 2-acyl-GPE are the production of PE and GPE from two  
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5 425 molecules of GPE (Homma & Nojima, 1982), and another phospholipase that degrades 2-  
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7 426 acyl-GPE (Doi & Nojima, 1975).  
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#### 11 428 *Sorting of lipoproteins to the outer membrane*

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13 429 The majority of lipoproteins reside in the inner leaflet of the outer membrane (Tokuda *et al.*,  
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15 430 2007), although recent examples of surface-exposed lipoproteins have been reported (see  
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17 431 below). They reach this cellular location by a specific lipoprotein outer membrane  
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19 432 localization pathway (Lol) that is composed of five proteins: a cytoplasmic ATP-ase LolD,  
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21 433 two cytoplasmic membrane proteins LolC and LolE, a periplasmic chaperone LolA and an  
22  
23 434 outer membrane receptor LolB (Fig. 4). Sorting and translocation of lipoproteins by Lol is  
24  
25 435 dependent on the lipoprotein-sorting signal determined by specific residues located C-  
26  
27 436 terminally to the diacylglyceryl-cysteine. The Lol pathway has been described in great detail  
28  
29 437 in several excellent reviews (Tokuda *et al.*, 2014, Zuckert, 2014) and will only be briefly  
30  
31 438 discussed in light of functionality with enzymes involved in lipoprotein modification. *N*-  
32  
33 439 acylation of lipoproteins by Lnt is essential for the Lol-dependent release of lipoproteins from  
34  
35 440 the cytoplasmic membrane (Fukuda *et al.*, 2002), however, overexpression of *lolCDE* can  
36  
37 441 compensate for lack of *N*-acylation, in the absence of Lpp, and results in the release of  
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39 442 diacylated lipoproteins from the membrane and correct outer membrane localization (Narita  
40  
41 443 & Tokuda, 2011). However, the efficiency of outer membrane targeting is dependent on the  
42  
43 444 nature of the lipoprotein (Narita & Tokuda, 2011). Model lipoproteins containing an outer  
44  
45 445 membrane-sorting signal are not localized in the outer membrane, neither released from the  
46  
47 446 cytoplasmic membrane in absence of Lnt. This suggests that the *N*-acyl moiety of lipoproteins  
48  
49 447 plays an important role in lipoprotein translocation through the periplasm. Mature lipoprotein  
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51 448 first interacts with LolE, is then transferred to LolC, and upon ATP hydrolysis by LolD, the  
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3 449 interaction between LolC and LolA leads to charging of LolA with lipoprotein (Okuda &  
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5 450 Tokuda, 2009). The interaction between LolA and lipoprotein is weaker than the interaction  
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7 451 between LolB and lipoprotein, driving transfer from LolA to LolB and preventing back-  
8  
9 452 transfer to LolCE (Taniguchi *et al.*, 2005). It is unknown how lipoproteins interact with the  
10  
11 453 ABC transporter LolCDE and by which mechanism lipoproteins are released from the  
12  
13 454 cytoplasmic membrane. Crystal structures of LolA and soluble LolB, lacking its lipid-anchor,  
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15  
16 455 have been solved (Takeda *et al.*, 2003). The calculated size of the cavity is predicted to  
17  
18 456 accommodate one but is not large enough for three fatty acid chains. Structure-function  
19  
20 457 analyses of LolA of *P. aeruginosa* showed that one large hydrophobic surface patch is likely  
21  
22 458 to be involved in binding of acyl chains (Remans *et al.*, 2010). Decreasing its hydrophobic  
23  
24 459 nature abolished LolA-dependent release of lipoproteins from the cytoplasmic membrane  
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26  
27 460 (Remans *et al.*, 2010). The fatty acid acyl moiety of lipoprotein, together with its sorting  
28  
29 461 signal, clearly plays a role in substrate recognition by the Lol machinery. LolC and LolE  
30  
31 462 share a similar membrane topology and their periplasmic domains share sequence identity  
32  
33 463 with each other as well as with LolB (Okuda & Tokuda, 2009). LolA interacts specifically  
34  
35 464 with LolC and not with LolE. Differences in binding affinity between LolE and LolC for  
36  
37 465 lipoprotein, similar as described for LolA and LolB, may explain the molecular mechanism of  
38  
39 466 transfer. Characterization of the lipid-protein interaction by structural- and biophysical studies  
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41 467 is likely to provide more insight into the mechanism of Lol-dependent targeting of  
42  
43 468 lipoproteins to the outer membrane.

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47 469 Inhibitors of the Lol machinery were identified in a chemical genomic approach (Pathania *et*  
48  
49 470 *al.*, 2009) which utilized a combination of three *in vitro* assays (Ito *et al.*, 2007). Treatment  
50  
51 471 of cells with MAC13243 led to the accumulation of Lpp in the cytoplasmic membrane. It  
52  
53 472 directly interacts with LolA (Pathania *et al.*, 2009). Further characterization of this compound  
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55 473 led to the finding that a degradation product is the active species (Barker *et al.*, 2013). This  
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3 474 thiourea compound is an analog of A22, an inhibitor of bacterial actin MreB (Barker *et al.*,  
4  
5 475 2013) and seems to inhibit bacterial growth in a non-specific fashion.  
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7 476 The Lol machinery is not fully conserved in proteobacteria, LolB is only found in  $\beta$ - and  $\gamma$ -  
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9  
10 477 proteobacteria and also LolCE are often missing (Okuda & Tokuda, 2011). Proteomics  
11  
12 478 analyses showed that lipoproteins reside in the outer membrane of diderm actinomycetes but  
13  
14 479 the sorting mechanism is completely unknown.  
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19 481 *Lipoproteins involved in key processes of envelope biosynthesis*

20  
21 482 The most abundant lipoprotein in  $\gamma$ -proteobacteria is Lpp that is covalently linked to murein  
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23 483 (peptidoglycan) and the first protein shown to be lipidated (Braun & Rehn, 1969). Lpp is also  
24  
25 484 the most abundant protein in the cell and plays an important role in homeostasis of the cell  
26  
27 485 envelope. A recent study identified a  $\sigma^E$ -regulated small RNA, MicL, which specifically  
28  
29 486 targets *lpp* (Guo *et al.*, 2014). The mechanism of regulation allows decrease of Lpp  
30  
31 487 translation and acceleration of degradation of the stable *lpp* mRNA.  $\sigma^E$  up-regulates proteins  
32  
33 488 that facilitate assembly of outer membrane proteins and transport of LPS under stress  
34  
35 489 conditions. At the same time it down-regulates OMP synthesis by up-regulation of sRNAs  
36  
37 490 such as MicL (Guo *et al.*, 2014). A strain deleted for *lpp* is very sensitive to EDTA and  
38  
39 491 cationic dyes and somewhat sensitive to detergents and it also leaks periplasmic proteins into  
40  
41 492 the extracellular space (Hirota *et al.*, 1977, Yem & Wu, 1978). One-third of Lpp is covalently  
42  
43 493 linked to peptidoglycan, while two-third is in the cell envelope but not anchored to the cell  
44  
45 494 wall, the so-called free form (Inouye *et al.*, 1972). There is a dynamic equilibrium between  
46  
47 495 the two forms and the protein is homogeneously distributed over the cell (Hiemstra *et al.*,  
48  
49 496 1986, Hiemstra *et al.*, 1987). The enzymes responsible for cross-linking Lpp to peptidoglycan  
50  
51 497 were identified in 2008 (Magnet *et al.*, 2008). YbiS was shown to catalyze the covalent  
52  
53 498 anchoring of Lpp to peptidoglycan, but two other enzymes ErfK and YcfS could also perform  
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2  
3 499 this reaction. These enzymes belong to the family of L,D-transpeptidases found in firmicutes  
4  
5 500 (Mainardi *et al.*, 2005, Magnet *et al.*, 2007). YbiS is expected to cleave the peptide bond  
6  
7 501 between meso-DAP and D-Ala and to link meso-DAP to the L-Lys residue in Lpp (Magnet *et*  
8  
9 502 *al.*, 2008). Mis-localization of Lpp in the cytoplasmic membrane is lethal when cross-linked  
10  
11 503 to the peptidoglycan (Yakushi *et al.*, 1997). The highly abundant Lpp homologue in *P.*  
12  
13 504 *aeruginosa*, OprI exist in a free form and is not covalently cross-linked to peptidoglycan  
14  
15 505 (Mizuno & Kageyama, 1979, Cornelis *et al.*, 1989). When produced in *E. coli* it is exposed on  
16  
17 506 the surface of cells (Cornelis *et al.*, 1996). Interestingly, the free form of *E. coli* Lpp is also  
18  
19 507 exposed on the cell surface (Cowles *et al.*, 2011), although detection of Lpp by immunogold  
20  
21 508 labeling was only successful upon permeabilization of the outer membrane with TRIS-EDTA  
22  
23 509 (Hiemstra *et al.*, 1986). Lpp is an elongated trimer composed of long  $\alpha$ -helical domains that  
24  
25 510 adopt a coiled coil, which reflects its repetitive characteristics (Braun & Bosch, 1972, Choi *et*  
26  
27 511 *al.*, 1986). The last amino acids of the C-terminus form a hydrophobic capping motif that is  
28  
29 512 important for binding to PG and possibly involved in the recognition of lipoprotein by ligase  
30  
31 513 YbiS (Zhang & Wu, 1992, Shu *et al.*, 2000). It is thus likely that Lpp has a dual orientation,  
32  
33 514 similar as described for peptidoglycan-associated Lipoprotein P6 of *Haemophilus influenza*  
34  
35 515 (Michel *et al.*, 2013), although the correlation between surface-exposed orientation and  
36  
37 516 biological function is not yet known.

38  
39 517 Surface exposed lipoproteins are abundant in *Borrelia*, which lacks LPS in the outer  
40  
41 518 membrane (for a recent review see (Zuckert, 2014)). The N-terminal membrane-tethering  
42  
43 519 peptide keeps lipoproteins of *Borrelia burgdorferi* in a translocation compatible state that  
44  
45 520 allow them to be secreted to the cell surface probably via a flippase complex. Several other  
46  
47 521 examples of surface-exposed lipoproteins have been reported in proteobacteria, including  
48  
49 522 proteins that are translocated via type II secretion machinery (PulA) (Pugsley *et al.*, 1986) or  
50  
51 523 via an autotransporter mechanism (NalP and SphB1) (Coutte *et al.*, 2003, van Ulsen *et al.*,  
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3 524 2003). In case of NalP of *Neisseria meningitidis*, it was demonstrated that lipidation is  
4  
5 525 required for efficient release of surface-exposed proteins by delaying its own release from the  
6  
7 526 cell surface through autocatalytic processing (Roussel-Jazede *et al.*, 2013). Similarly, non-  
8  
9 527 lipidated SphB1 is efficiently secreted but is unable to cleave its substrate filamentous  
10  
11 528 haemagglutinin FHA (Coutte *et al.*, 2003), which is important for virulence of *Bordetella*  
12  
13 529 *pertussis* (Coutte *et al.*, 2003).

14  
15  
16 530 The Lpt pathway transports LPS from the cytoplasmic membrane to the cell surface (Ruiz *et*  
17  
18 531 *al.*, 2009). LptE is an essential OM lipoprotein that stabilizes the LptD OM channel  
19  
20 532 responsible for correct translocation of LPS (Bos *et al.*, 2004, Chng *et al.*, 2010). Recent  
21  
22 533 studies showed that LptE forms a plug inside the  $\beta$ -barrel structure of LptD (Freinkman *et al.*,  
23  
24 534 2011) and that it changes the biophysical state of LPS to allow transport and OM insertion  
25  
26 535 (Malojcic *et al.*, 2014). The N-terminal lipid anchor is not required for function since a variant  
27  
28 536 of LptE lacking the signal sequence including the cysteine complements a *lptE* deletion strain  
29  
30 537 and is able to form a complex with LptD (Chng *et al.*, 2010). LptD is inserted into the OM by  
31  
32 538 the  $\beta$ -barrel assembly machine (Bam complex) (Chimalakonda *et al.*, 2011). Four lipoproteins  
33  
34 539 (BamB-E) are part of the Bam complex, of which BamD is essential for the assembly and/or  
35  
36 540 insertion of proteins in the OM (Malinverni *et al.*, 2006). BamC is surface exposed (Webb *et*  
37  
38 541 *al.*, 2012). How the lipid moiety is orientated in the outer membrane is still an open question.

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41 542 Biosynthesis of peptidoglycan is catalyzed by several membrane-bound enzymes, among  
42  
43 543 which the bifunctional penicillin-binding proteins PBP1A and PBP1B. These enzymes each  
44  
45 544 require an outer membrane lipoprotein, LpoA and LpoB, respectively, for their function  
46  
47 545 (Paradis-Bleau *et al.*, 2010, Typas *et al.*, 2010). The Lpo proteins bind specifically to their  
48  
49 546 cognate PBP and stimulate transpeptidase activity. LpoB with an inner membrane retention  
50  
51 547 signal is functional as long as the flexible N-terminal domain is present (Typas *et al.*, 2010). It  
52  
53 548 is currently unknown whether non-lipidated LpoA is functional.

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3 5494  
5 550 *The role of lipoprotein modification in virulence*

6  
7 551 The role of individual lipoproteins on virulence of pathogenic bacteria has been studied (for a  
8  
9 552 review see (Kovacs-Simon *et al.*, 2010)). Signaling of the host immune system by  
10  
11 553 lipoproteins and lipopeptides occurs via Toll-like receptor 2, either via TLR2-1 in case of  
12  
13 554 diacylated proteins or via TLR2-6 heterodimers in case of triacylated proteins. The lipid  
14  
15 555 moiety plays an important role in the interaction with these receptors (Jin *et al.*, 2007, Kang *et*  
16  
17 556 *al.*, 2009). In recent years the effect of lipoprotein modification on virulence has been  
18  
19 557 addressed using *lgt* and *lsp* deletion mutants of pathogenic bacteria and depending on the  
20  
21 558 pathogen and the infection model used, various effects on virulence were obtained.

22  
23 559 For example, impaired colonization of the nasopharynx and a decrease in virulence in  
24  
25 560 pneumonia and septicemia mouse models was reported for *Streptococcus pneumoniae*  
26  
27 561 deleted for *lgt* (Petit *et al.*, 2001, Chimalapati *et al.*, 2012). TLR-2 signaling is dependent on  
28  
29 562 pneumococcal lipoproteins and NF- $\kappa$ B and cytokine release were reduced in a  $\Delta$ *lgt* strain  
30  
31 563 (Tomlinson *et al.*, 2014). Intracellular survival of *Listeria monocytogenes* was affected  
32  
33 564 (Baumgärtner *et al.*, 2007) and the  $\Delta$ *lgt* strain was attenuated in a mouse model (Machata *et*  
34  
35 565 *al.*, 2008). Lipoproteins are released into the culture supernatant of this strain (Baumgärtner *et*  
36  
37 566 *al.*, 2007). Inactivation of *lsp* in *L. monocytogenes* led to a severe growth defect in  
38  
39 567 macrophages and a reduced capacity of phagosomal escape leading to attenuated virulence  
40  
41 568 (Reglier-Poupet *et al.*, 2003). Expression of the *lsp* gene was strongly induced inside  
42  
43 569 phagosomes probably caused by environmental conditions similar to pH-regulation of N-  
44  
45 570 acylation in *S. aureus* (Reglier-Poupet *et al.*, 2003, Kurokawa *et al.*, 2012). Inefficient  
46  
47 571 germination of spores of a *lgt* deletion strain of *Bacillus anthracis* led to attenuation of  
48  
49 572 virulence (Okugawa *et al.*, 2012). Lipoproteins of an *lsp* deletion mutant of *M. tuberculosis*  
50  
51 573 are not processed and the strain was attenuated in an animal model of tuberculosis (Sander *et*  
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3 574 *al.*, 2004, Rampini *et al.*, 2008). Reduced innate immune activation was observed with a  $\Delta lgt$   
4  
5 575 strain of *Streptococcus suis* (Wichgers Schreur *et al.*, 2011), but no difference in virulence  
6  
7 576 was observed between a wild type strain and a *lsp* deletion mutant in an infection model in  
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9  
10 577 piglets (De Greeff *et al.*, 2003). Deletion of either *lgt* or *lsp* in *Streptococcus sanguinis* has  
11  
12 578 only a slight effect on endocarditis virulence (Das *et al.*, 2009). No significant effect on  
13  
14 579 virulence was observed in *Streptococcus equi* (Hamilton *et al.*, 2006, Das *et al.*, 2009).  
15  
16 580 Escape of immune recognition caused lethal infections by a  $\Delta lgt$  strain of *S. aureus* but a  $\Delta lsp$   
17  
18 581 strain was attenuated (Bubeck-Wardenburg *et al.*, 2006). Enhanced lethality was shown in a  
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20  
21 582 mouse model for neonatal sepsis by  $\Delta lgt$  of *Streptococcus agalactiae* (Henneke *et al.*, 2008).  
22  
23 583 Stimulation of the immune system via TLR2 during early stages of infection is important for  
24  
25 584 elimination of pathogens. The examples described here clearly show that both the lipid  
26  
27 585 moiety and the functional characteristics of lipoproteins determine virulence capacity.  
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### 31 587 *Protein lipidation in other microbes*

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33  
34 588 Lipoproteins are predicted in Archaea, but orthologous genes for lipoprotein modifying  
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36 589 enzymes could not be identified in their genomes. Recent findings in *Haloferax volcanii*  
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38 590 show that the Tat-dependent secretion of predicted lipoproteins depends on the conserved  
39  
40 591 cysteine in the putative lipobox (Gimenez *et al.*, 2007, Storf *et al.*, 2010). Future studies will  
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42 592 provide direct evidence for lipidation of these proteins and will demonstrate whether  
43  
44 593 phospho-, glyco- or phospho-glyco-lipids that constitute archaeal membranes, and are  
45  
46 594 substantially different from their bacterial counterparts, are the source of modification  
47  
48 595 (Villanueva *et al.*, 2014).

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52 596 In eukaryotes, including yeast, various types of protein lipidation exist including *S*-  
53  
54 597 palmitoylation, *N*-myristoylation and *S*-prenylation (Aicart-Ramos *et al.*, 2011). In the  
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56 598 process of *S*-palmitoylation, palmitate is transferred onto a cysteine residue catalyzed by  
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3 599 protein palmitate transferases (PAT) containing DHHC (Asp-His-His-Cys) cysteine rich  
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5 600 domains that results in the formation of a thioester-linked palmitoylated protein (Dietrich &  
6  
7 601 Ungermann, 2004). The PAT enzymes are polytopic integral membrane proteins located in  
8  
9 602 the ER or Golgi. The active site is facing the cytoplasm where acyl-CoA functions as the acyl  
10  
11 603 donor. Multiple DHHC enzymes have been identified in *Saccharomyces cerevisiae* but their  
12  
13 604 substrate specificity is still poorly understood. Recent work on the Erf2-Erf4 PAT complex in  
14  
15 605 *S. cerevisiae* showed that it catalyzes a two-step reaction in which Erf2 undergoes auto-  
16  
17 606 acylation (Mitchell *et al.*, 2010). Erf2 transfers the palmitate group onto the substrate and Erf4  
18  
19 607 is required for stabilization of Erf2 and for the formation of the Erf2-palmitate intermediate  
20  
21 608 (Mitchell *et al.*, 2012). S-palmitoylation is a reversible process due to the action of acyl-  
22  
23 609 protein thioesterases. Together these two processes allow for regulated control of membrane-  
24  
25 610 associated proteins, such as small GTPase Ras, the function of which depends on their lipid  
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27 611 anchor.

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31 612 Several examples have been described of bacterial effector proteins that contain eukaryotic  
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33 613 lipidation motifs and that are modified by lipids upon entry into host cells. A farnesyl or  
34  
35 614 geranylgeranyl isoprenyl lipid moiety is added to cysteine residue of the conserved sequence  
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37 615 leading to enhanced membrane affinity and localization of effector proteins to membranes of  
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39 616 host cells (Ivanov *et al.*, 2010).

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#### 43 618 *Concluding remarks*

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47 619 Lipoproteins are highly abundant in bacteria, and require therefore efficient maturation. The  
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49 620 acyltransferases and signal peptidase involved in this pathway are not abundant, estimated to  
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51 621 only a few hundred molecules per cell. It is very likely that these enzymes exist in a complex  
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53 622 to guarantee complete and efficient lipoprotein modification. The existence of a lipoprotein  
54  
55 623 modification complex was already suggested in 1982 (Tokunaga *et al.*, 1982). This presumed  
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3 624 complex might co-localize and function together with other essential protein complexes  
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5 625 including the Sec and Tat secretion machineries. In this respect it is intriguing to understand  
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7 626 how the lipoprotein modification machinery recognizes non-folded- and fully folded  
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9 627 substrates that are translocated via Sec and Tat, respectively. The signal peptide of both types  
10  
11 628 of lipoprotein substrates including the early mature region of the precursor protein is thought  
12  
13 629 to exist in a flexible conformation inside the translocon. Lateral opening of the Sec channel  
14  
15 630 can be envisaged for insertion of lipoproteins into the lipid bilayer (Chatzi *et al.*, 2014),  
16  
17 631 whether lateral gating of the Tat complex occurs is currently unknown (Patel *et al.*, 2014).  
18  
19 632 Once inserted into the membrane, Lgt binds the signal peptide and the first few amino acids  
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21 633 of the mature protein and catalyzes the first step in the lipidation pathway.  
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23 634 Several examples clearly demonstrate that some essential lipoproteins are perfectly functional  
24  
25 635 without their lipid anchor what raises the following questions: what is the role of the lipid  
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27 636 moiety, and how does the degree of lipidation influence protein function? Unlike membrane  
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29 637 proteins, lipoproteins are expected to be more mobile within lipid bilayers giving them more  
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31 638 flexibility, although this has not been experimentally tested. For lipoproteins in monoderm  
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33 639 bacteria it has been postulated that the N-terminal lipid anchor keeps the protein close to the  
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35 640 membrane while the extended functional domain participates in various biological processes.  
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37 641 Many lipoproteins in these organisms fulfill equivalent functions as periplasmic proteins in  
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39 642 diderm bacteria. Accessory proteins such as LptE and LpoA/B interact strongly with their  
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41 643 partner proteins, for which the lipid part is not essential, although the process in which they  
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43 644 are involved might be more efficient when these proteins are present in lipidated form. In the  
44  
45 645 case of lipoproteins that themselves fulfill specific functions and not in complex with other  
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47 646 proteins, such as surface-exposed proteases or targeting of proteins to the OM, the lipid  
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49 647 anchor becomes essential. The lipoprotein modification pathway seems to be tightly regulated  
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51 648 since a lipoprotein can exist in both di- or triacylated form in one bacterial species, depending  
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3 649 on growth conditions. The fact that multiple copies of genes encoding modification enzymes  
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5 650 exist in bacteria that undergo various developmental stages also suggest that regulation of  
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7 651 their expression is environmentally controlled.  
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10 652 The lipoprotein modification pathway represents an attractive target for antibiotic  
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12 653 development. It is essential for the viability of all proteobacteria examined to date, which  
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14 654 include many important human pathogens. Furthermore, Lsp and Lnt are membrane-bound  
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16 655 with catalytic domains facing the bacterial periplasm. This topology makes at least two steps  
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18 656 of lipoprotein biogenesis accessible to small molecules that will be capable of inhibiting  
19  
20 657 enzymes without a need to traverse the relatively impermeable cytoplasmic membrane or risk  
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22 658 elimination by the action of efflux pumps. For future studies on lipoprotein modification and  
23  
24 659 function it is important to include proteomic analysis of membrane-bound and secreted  
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26 660 lipoproteins and to determine the lipidation state and lipid composition by mass spectrometry  
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28 661 (Cain *et al.*, 2014). These approaches not only allow the identification and characterization of  
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30 662 specific lipoproteins that act as virulence factors but also demonstrate a correlation between  
31  
32 663 lipidation and the stage of infection. Structural and mechanistic properties of enzymes  
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34 664 involved in lipidation of proteins in various organisms will highlight similarities and  
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36 665 differences and will provide ways of finding new species specific antibacterial agents.  
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43  
44  
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46  
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48  
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3 671 Figure legends  
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7 **673 Figure 1. Lipoprotein modification pathway in bacteria**  
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10 674 Lipoprotein modification occurs in the cytoplasmic membrane (CM) by the sequential action  
11  
12 675 of three membrane-bound enzymes Lgt, Lsp and Lnt. Prolipoprotein is inserted into the  
13  
14 676 membrane via the Sec or Tat secretion machinery through recognition of its signal peptide  
15  
16 677 (SP). This sequence also contains the lipobox (LB) specific for bacterial lipoproteins. Lgt  
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18 678 catalyzes the transfer of a diacylglyceryl group from phosphatidylglycerol (PG) onto  
19  
20 679 prolipoprotein resulting in diacylglyceryl-prolipoprotein. Cleavage of the signal peptide by  
21  
22 680 Lsp liberates the  $\alpha$ -amino group of diacylglyceryl-cysteine that then becomes acylated by Lnt  
23  
24 681 resulting in mature triacylated lipoprotein. Lnt preferentially uses phosphatidylethanolamine  
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26 682 (PE) as acyl donor. Alternative N-acyl modifications have been observed in lipoproteins from  
27  
28 683 firmicutes and mollicutes.  
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34 **685 Figure 2. Membrane topology of modification enzymes**  
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36 686 Schematic representation of membrane topology and essential residues (in red) in Lgt, Lsp  
37  
38 687 and Lnt. Numbering of amino acids correspond to sequences of *E. coli* proteins.  
39  
40 688 C=cytoplasm, CM=cytoplasmic membrane, P=periplasm. Catalytic site residues are shown in  
41  
42 689 yellow. Conserved domains are indicated as white boxes. See text for details.  
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47 **691 Figure 3. Structure of globomycin**  
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49  
50 692 Globomycin is a lipophilic cyclic peptide. Derivatives have been synthesized and analyzed for  
51  
52 693 their activity against a variety of bacteria, in particular the length of the lipophilic part (A),  
53  
54 694 and the role of the hydroxyl groups of threonine (B) and serine (C) have been addressed.  
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3 696 **Figure 4. Lol machinery in proteobacteria**

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5 697 The Lol machinery is involved in translocation of mature (triacylated) lipoproteins from the  
6  
7 698 cytoplasmic membrane (CM) to the outer membrane (OM). The ABC transporter component  
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9 699 of the machinery is composed of membrane proteins LolC and LolE and cytoplasmic ATPase  
10  
11 700 LolD. The periplasmic chaperone LolA binds the lipoprotein, upon interaction with LolC and  
12  
13 701 hydrolysis of ATP by LolD, and transfers it to receptor protein LolB. The lipoprotein is then  
14  
15 702 inserted into the outer membrane. Several lipoproteins are located on the cell surface;  
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17 703 however, the mechanism for their translocation and the orientation of the fatty acids in the  
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19 704 membrane is not yet understood.  
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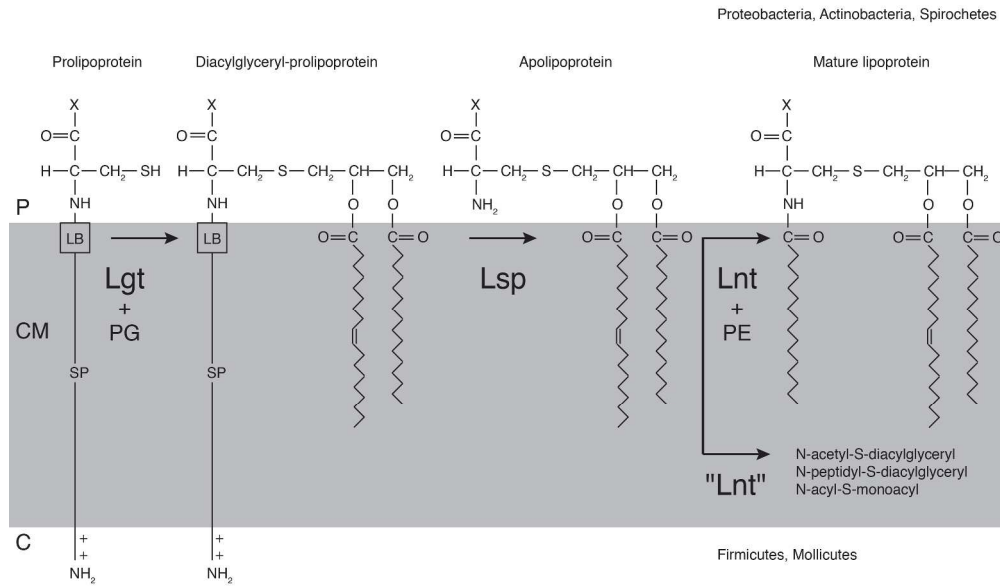
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Table 1. Essentiality of genes encoding lipoprotein modifying enzymes in diderm bacteria.

	<i>lgt</i>	<i>lsp</i>	<i>lnt</i>
<i>E. coli</i>	<i>lgt</i> ( <i>umpA</i> ) (Gan <i>et al.</i> , 1995)	<i>lsp</i> (Tokunaga <i>et al.</i> , 1983, Yamagata <i>et al.</i> , 1983)	<i>lnt</i> ( <i>cutE</i> ) (Gupta & Wu, 1991, Rogers <i>et al.</i> , 1991, Gupta <i>et al.</i> , 1993, Robichon <i>et al.</i> , 2005)
<i>S. enterica</i>	<i>lgt</i> (Gan <i>et al.</i> , 1993)		<i>lnt</i> (Gupta <i>et al.</i> , 1993)
<i>M. xantus</i>		<i>lsp1</i> , <i>lsp2</i> , <i>lsp3</i> , <i>lsp4</i> (Xiao & Wall, 2014)	
<i>M. smegmatis</i>	<i>lgt</i> (MSMEG_3222) (Tschumi <i>et al.</i> , 2012)		<i>lnt</i> (MSMEG_3860) ( <i>ppm2</i> ) (Tschumi <i>et al.</i> , 2009)
<i>M. tuberculosis</i>	<i>lgt</i> (Rv1614) (Sasseti <i>et al.</i> , 2003, Tschumi <i>et al.</i> , 2012)	<i>lsp</i> (Rv1539) (Sander <i>et al.</i> , 2004, Rampini <i>et al.</i> , 2008)	<i>ppm1</i> (Rv2051c) (Tschumi <i>et al.</i> , 2009)
<i>M. bovis</i>			<i>lnt</i> (BCG_2070c) (Brülle <i>et al.</i> , 2013)
<i>C. glutamicum</i>			<i>lnt</i> (NCgl1424) (Mohiman <i>et al.</i> , 2012)
<i>S. coelicolor</i>	<i>lgt1</i> (SCO2034), <i>lgt2</i> (SCO7822) (Thompson <i>et al.</i> , 2010)	<i>lsp</i> (SCO2074) (Thompson <i>et al.</i> , 2010)	<i>lnt1</i> (SCO1014), <i>lnt2</i> (SCO1336) (Widdick <i>et al.</i> , 2011, Cordova-Davalos <i>et al.</i> , 2014)
<i>S. scabies</i>	<i>lgt</i> (SCAB68531) (Widdick <i>et al.</i> , 2011)	<i>lsp</i> (SCAB68121) (Widdick <i>et al.</i> , 2011)	<i>lnt1</i> (SCAB83111), <i>lnt2</i> (SCAB76621) (Widdick <i>et al.</i> , 2011)
<i>S. lividans</i>		<i>lsp</i> (Gullon <i>et al.</i> , 2013)	

Essential genes are shown in red, non-essential genes are shown in blue. Table includes only published findings. See text for further details.

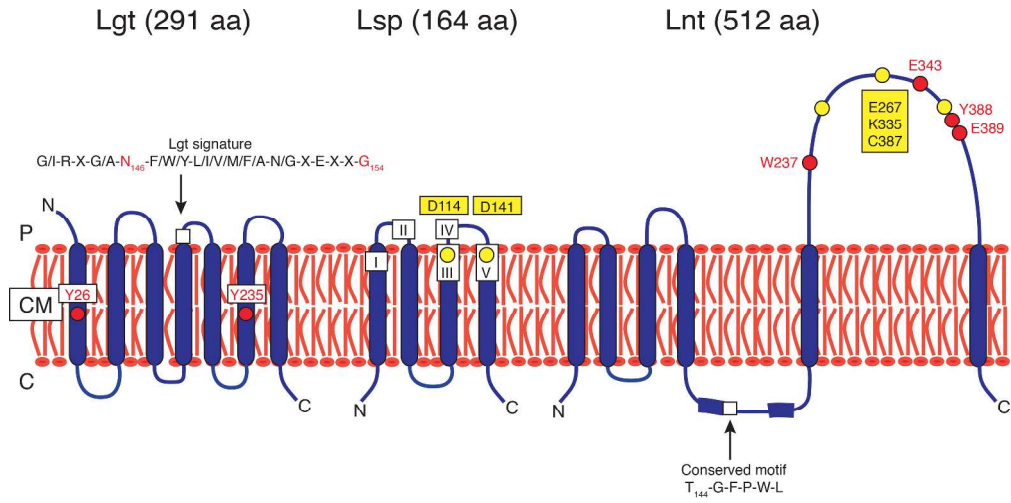
FIGURE 1



290x191mm (300 x 300 DPI)

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FIGURE 2



293x167mm (300 x 300 DPI)

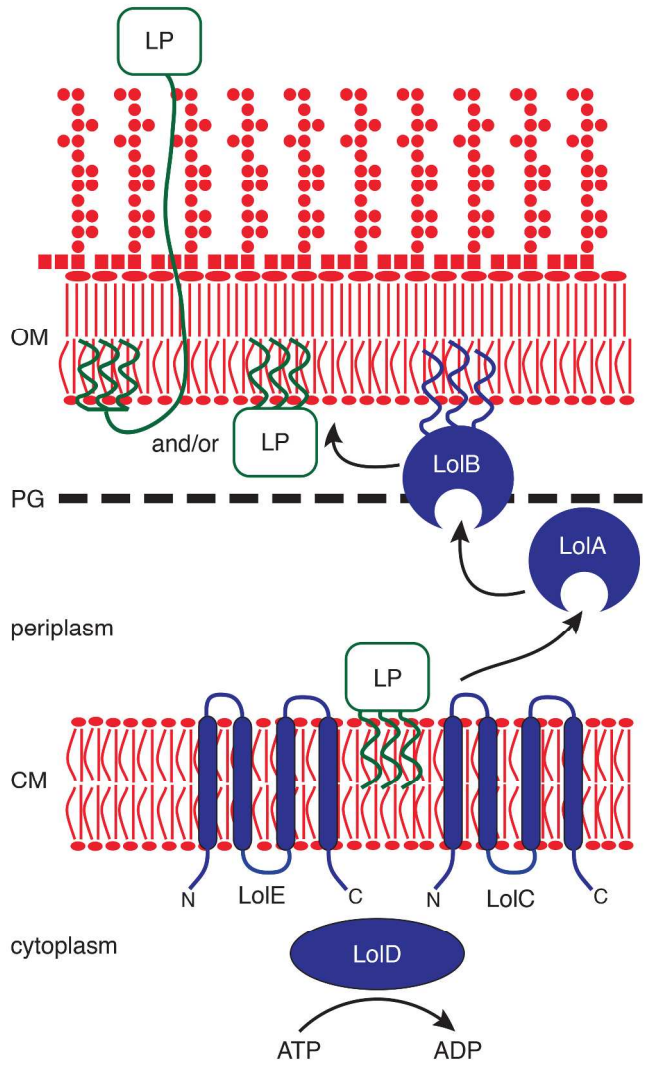
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FIGURE 4



180x320mm (300 x 300 DPI)