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1 **Role of the unique, non-essential phosphatidylglycerol::prolipoprotein**
2 **diacylglyceryl transferase (Lgt) in *Corynebacterium glutamicum***

3

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20 **Keywords:** lipoproteins; phosphatidylglycerol::prolipoprotein diacylglyceryl transferase;
21 *Corynebacterium glutamicum*; cell envelope.

22

22

23 **Abstract**

24 Bacterial lipoproteins are secreted proteins that are post-translationally lipidated.
25 Following synthesis, prelipoproteins are transported through the cytoplasmic
26 membrane via the Sec or Tat translocon. As they exit the transport machinery, they are
27 recognized by a phosphatidylglycerol::prolipoprotein diacylglyceryl transferase (Lgt),
28 which converts them to prolipoproteins by adding a diacylglyceryl group to the
29 sulfhydryl side chain of the invariant Cys₊₁ residue. Lipoprotein signal peptidase (LspA
30 or signal peptidase II) subsequently cleaves the signal peptide, liberating the α -amino
31 group of Cys₊₁ that can eventually be further modified. Here, we identified the *lgt* and
32 *lspA* genes from *C. glutamicum* and found that they are unique but not essential. We
33 found that Lgt is necessary for the acylation and membrane anchoring of two model
34 lipoproteins expressed in this species: MusE, a *C. glutamicum* maltose binding
35 lipoprotein, and LppX, a *Mycobacterium tuberculosis* lipoprotein. However, Lgt is not
36 required for these proteins signal peptide cleavage, nor for LppX glycosylation. Together,
37 these data show that in *C. glutamicum*, association of some lipoproteins with membranes
38 through the covalent attachment of a lipid moiety is not essential for further post-
39 translational modification.

40

40

41 **Introduction**

42

43 Bacterial lipoproteins are exported proteins that are post-translationally lipidated after
44 translocation through the cytoplasmic membrane. They are characterized by the presence
45 of a « lipobox » (L/AS/GA/C₊₁ based on *E. coli* lipoprotein sequences) at the extremity of
46 their signal sequence, in which the C₊₁ invariant cysteine is the only residue to be lipid-
47 modified and the first residue of the mature protein [1,2]. It is assumed that the main role
48 of lipoprotein modification is to anchor proteins to membranes, where they fulfill
49 essential roles in cell wall synthesis, outer membrane biogenesis and stability, nutrient
50 uptake, transmembrane signaling, adhesion, host immune response and virulence [3-6].

51 After synthesis in the cytoplasm, prelipoproteins are transported through the
52 cytoplasmic membrane via the Sec or Tat translocon. As they exit the translocon, they are
53 recognized by a phosphatidylglycerol:prolipoprotein diacylglyceryl transferase (Lgt),
54 which convert them to prolipoproteins by adding a diacylglyceryl group, derived from
55 phospholipid phosphatidylglycerol, to the sulfhydryl group of C₊₁. Lipoprotein signal
56 peptidase (LspA or signal peptidase II) subsequently cleaves the signal peptide, liberating
57 the α -amino group of C₊₁, which can be, in some organisms, further modified by an
58 apolipoprotein N-acyltransferase, Lnt [1,2,7].

59 The genes encoding Lgt and LspA are conserved in all bacteria, indicating that the first
60 two steps in lipoproteins biogenesis are ubiquitous. In contrast, the occurrence and
61 mechanism of the third step varies with species and envelope organization.

62 In diderm Gram-negative bacteria, such as *Escherichia coli* or *Salmonella enterica*, the
63 third step consists in N-acylation of the diacylglyceride C₊₁ by the apolipoprotein N-

64 acyltransferase (Lnt), resulting in mature triacylated proteins [8,9]. These mature
65 lipoproteins are then found mostly (~90%) in the outer membrane (OM), to which they
66 are targeted by the dedicated Lol (*localization of lipoproteins*) system [8]. Although Lnt-
67 dependent lipoproteins triacylation appears to be the rule for most Gram-negative
68 bacteria, few exceptions exist, in which *lnt* orthologues have not been found or appear not
69 to be functional [9-12]. For some time, *lgt*, *lspA*, *lnt*, and *lol* have been considered as
70 essential genes in all Gram-negative bacteria and thus attracted attention as new potential
71 targets for antibiotherapy [13-15]. However, a recent study showed that *lnt* is dispensable
72 in *Francisella tularensis*, *Neisseria gonorrhoeae*, *Acinetobacter baylyi* and *Acinetobacter*
73 *baumannii* and that its absence does not preclude targeting to the outer membrane [16,17].
74 Low-GC% Gram-positive bacteria, such as *Staphylococci* and Mollicutes, lack an outer
75 membrane (monoderms) and thus retain lipoproteins in the cytoplasmic membrane.
76 Although no *lnt* homologues have been identified in these bacteria, some of them
77 nevertheless produce triacylated lipoproteins [9,18,19], as well as lipoproteins carrying
78 other types of *N*-modifications (lyso, *N*-acetyl and peptidyl) [20]. In these organisms, the
79 third step of lipoproteins biogenesis is performed by other, yet uncharacterized, enzymes.
80 Interestingly, lipoproteins processing (di- versus tri-acylated) is regulated by
81 environmental conditions in *Staphylococcus aureus* [21]. In these low-GC% Gram-
82 positive bacteria, *lgt* and *lspA* are not essential. Generation of *lgt* mutants in
83 *Streptococcus pneumoniae*, *Streptococcus uberis*, *Bacillus subtilis* and *Listeria*
84 *monocytogenes* indicates that Lgt-dependent diacylation is not always a prerequisite for
85 cleavage of the signal sequence, either by LspA or other peptidases [22-25]. In most
86 cases, whether the signal sequence is cleaved or not, the non-acylated proteins produced
87 in *lgt* mutants appear to be released in the culture medium, consistent with the absence of

88 a membrane anchor [25-28].

89 Although less studied, the situation in GC-rich Gram-positive *Actinobacteria* appears to
90 be somehow intermediate between what is observed in Gram-negative bacteria and low-
91 GC% Gram-positive bacteria. This group comprises monoderm bacteria (e.g.
92 *Streptomyces*, the envelope of which is similar to low-GC Gram positive bacteria), as
93 well as *Corynebacteriales* (e.g. mycobacteria, corynebacteria, nocardia), whose members
94 possess an impermeable mycolic acid-containing outer membrane (mycomembrane). All
95 *Actinobacteria* examined so far possess, like Gram-negative bacteria, *Int* orthologue(s)
96 and produce triacylated lipoproteins [5, 29-32]. All *Streptomyces* species possess 2 *Int*
97 homologues [32] whereas the number of *lgt* genes varies (two in *Streptomyces coelicolor*
98 and *Streptomyces clavuligerus*; one in *Streptomyces scabies*) [32,33]. Both *S. coelicolor*
99 genes encode functional Lgt and have been individually deleted without affecting
100 lipoproteins biogenesis. However, a double mutant could not be obtained, suggesting that
101 Lgt activity is essential. In the same organism, *lsp* was also proposed to be essential [33].
102 Similarly, Δlgt and Δlsp mutants of *S. scabies* could be constructed but showed severe
103 growth and development defects [32]. In contrast, deletion of *S. scabies lnt1* or *lnt2*,
104 individually or in combination, had no effect on growth or development. The *lnt1* gene
105 was found to be necessary and sufficient for lipoprotein N-acylation, while the role of
106 *lnt2* remains unclear [32]. Finally, in *Streptomyces lividans*, a *lsp* mutant was barely
107 affected in growth but showed sporulation delay, activation of the stringent response and
108 blockage of the Sec translocase together with down-regulation of major extracytoplasmic
109 proteins [34].

110 In the didermic *Actinobacteria* (mycobacteria, corynebacteria and nocardia), *lgt*, *lsp* and
111 *lnt* have also been identified [5]. *lnt*-encoding genes were identified in *M. tuberculosis*,

112 *M. smegmatis*, *Mycobacterium bovis* BCG as well as *C. glutamicum*. In all cases, Lnt-
113 activity was dispensable for growth [29,30,31,35]. Although *M. tuberculosis* and *M.*
114 *bovis* both possess a second *lnt* gene, the catalytic cysteine residue is replaced by a serine
115 in these paralogues, suggesting that they are either inactive or function with a mechanism
116 different from the one proposed for *E.coli* Lnt.

117 *lspA* mutants of *M. tuberculosis* and *M. smegmatis* are also viable, but *M. tuberculosis*
118 *lspA* is severely affected in pathogenesis [36].

119 In contrast, results obtained with *lgt* mutants differ between species. The *lgt* gene is
120 essential in *M. tuberculosis* [37]. In *M. smegmatis*, 2 *lgt* paralogues were identified
121 (MSMEG_3222 and MSMEG_5408). Because MSMEG_5408 lacks important catalytic
122 residues, only MSMEG_3222 has been further studied. A Δ MSMEG_3222 mutant is
123 viable but shows growth defects [37]. Surprisingly, lipoproteins are massively released in
124 the culture supernatant of this mutant, despite the presence of the mycomembrane. This
125 may result from alterations of the mycobacterial cell envelope. However, the observation
126 that lipoproteins mutated at the conserved C₊₁ (i.e. non acylated), are also released in the
127 culture medium when produced in wild-type strain of *M. smegmatis* or *C. glutamicum*
128 [30,38] rules out the above hypothesis and the molecular mechanism underlying this
129 release remains obscure.

130 Previously, we showed that lipoproteins are triacylated in *C. glutamicum*, an
131 environmental *Corynebacteriales* used for the production of amino acids in the food
132 industry and a model organism for the study of mycobacteria cell wall biogenesis. We
133 identified the non-essential gene *Cg-ppm2(=lnt)* as necessary for lipoprotein N-acylation
134 and subsequent glycosylation [30]. Here, we used two model lipoproteins to identify and
135 characterize the *lgt* and *lspA* genes in this organism. We found that none of these genes

136 are essential in this species and the resultant non-acylated lipoproteins were released in
137 the culture supernatant of the *lgt* strain, as observed in other high-GC Gram-positive
138 bacteria. In addition, we observed that the dependence of protein diacylation and/or LspA
139 for signal sequence cleavage differs with protein target. Finally, using LppX as a model,
140 we found that acylation is not required for glycosylation, a post-translational modification
141 frequently observed in *Corynebacteriales* lipoproteins.

142

143 **MATERIALS AND METHODS**

144

145 **Reagent and medium**

146 *C. glutamicum* was cultured in brain heart infusion (BHI) medium (Difco) or minimal
147 medium GCXII [39] at 30°C. Kanamycin (Km), chloramphenicol (Cm), tetracycline
148 (Tet), IPTG and sucrose (Suc) were added when required at final concentrations of 25 µg
149 ml⁻¹, 10 µg ml⁻¹, 5 µg ml⁻¹, 1mM and 10% (wt/vol), respectively. Transformation of *C.*
150 *glutamicum* by electroporation was performed as described by Bonamy *et al.* 1990 [40].

151

152 **Strains and plasmids**

153 ***Strains***

154 All *C. glutamicum* strains used in this study are derivative of the strain ATCC 13032
155 RES167, which is referred here as the wild-type (WT) [41]. *Escherichia coli* DH5α was
156 used for cloning steps.

157 ***Construction of the Δlgt and ΔlspA mutants.***

158 In order to delete *C. glutamicum lgt* (*Cg-lgt: NCgl2009*), we used the strategy described
159 by Schafer *et al.*, 1994 [42]. In brief, two DNA fragments overlapping the gene to be

160 deleted at its 5' and 3' extremities were amplified by PCR from *C. glutamicum* total
161 DNA by using the primers 2009-del1-XhoI/2009-del2-BamHI and 2009-del3-XhoI/2009-
162 del4-HindIII (see Table 1 for primers sequence) and cloned into the nonreplicative vector
163 pK18mobSac. The resulting plasmid (pK18mobsac Δ lgt) was sequenced and introduced
164 into *C. glutamicum* RES167 by electroporation. Transformants in which the construct
165 was integrated into the chromosome by single crossover were selected on BHI plates
166 containing Km. The second crossover event was selected by plating Km^r clones on BHI
167 plates containing sucrose. Km^s and Suc^r colonies were screened by PCR for the correct
168 deletion of the gene using combinations of primers localized upstream and downstream
169 of the NCgl2009 sequence. After verification of PCR products by sequencing, one strain
170 carrying the NCgl2009 deletion (Δ lgt) was selected for further studies. The same strategy
171 was used to construct the *C. glutamicum* *Als*pA mutant (*Cg-lspA*: NCgl2058), using
172 primers 2058-del1-BglII/2058-del2-XbaI and 2058-del3-XbaI/2058-del4-XhoI (Table 1).

173 **Plasmids**

174 pCg482-MusE-His (pCg482-AmyE-His) and pCg482-LppX-HA-His have been
175 previously described [30]. pCg482-LppX-C1L-HA-His was constructed using the PCR
176 overlap extension method [43]. Initial PCRs were performed using pCg482-LppX-HA-
177 His as a template and primers pairs DirLppX-C1L/482-AS or 482-S2/RevLppX-C1L
178 (Table 1). The 2 overlapping gene segments generated were then used as templates for
179 another PCR to create the full-length product which was inserted between the EcoRV and
180 XhoI sites of pCg482.

181 To construct pVWEx2-*Cg-lgt*, the NCgl2009 gene was amplified from *C. glutamicum*
182 ATCC13032 genomic DNA using primers DirNCgl2009-XbaI and RevNCgl2009-BamHI
183 (Table 1). The PCR product obtained (983 bp) was introduced between the XbaI and

184 BamHI sites of pVWEx2-*ppm1* [30]. *E. coli lgt* gene was amplified using plasmid
185 pCHAP7546 as template [44] and Dir*Ec-lgt*-XbaI and Rev*Ec-lgt*-BamHI as primers
186 (Table 1). The PCR product (921bp) was then introduced in pVWEx2-*ppm1* digested
187 with XbaI and BamHI, resulting in plasmid pVWEx2-*Ec-lgt*.

188 To construct pVWEx2-*MSMEG_3222* and pVWEx2-*MSMEG_3222*ΔCter, the
189 *MSMEG_3222* gene was amplified from *M. smegmatis* Mc²155 genomic DNA using
190 primers Dir*MSMEG_3222*-XbaI and either Rev*MSMEG_3222*-BamHI or Rev-
191 *MSMEG_3222*A288-BamHI (Table 1). The PCR products obtained (1890 bp and 908 bp
192 respectively) were then introduced between the XbaI and BamHI sites of pVWEx2-*ppm1*.
193 The same strategy was used to construct pVWEx2-*MSMEG_5408*, except that
194 Dir*MSMEG_5408*-XbaI and Rev*MSMEG_5408*-BamHI (Table 1) were use as primers.

195 The plasmid pVWEx2-*Cg-lspA* was constructed by amplifying *lspA* (*NCgl2058*) from *C.*
196 *glutamicum* 13032 genomic DNA with primers Dir*Cg-lspA*-XbaI and Rev*Cg-lspA*-
197 BamHI (Table 1) and inserting the resulting PCR fragment in the same sites of pVWEx2-
198 *ppm1*. All plasmids were sequenced by Eurofins genomics.

199 ***Cryo-Transmission Electron Microscopy (Cryo-TEM)***

200 A 4 μl aliquot of cells grown in BHI to stationary phase was adsorbed onto holey carbon-
201 coated grid (Lacey, Tedpella, USA), blotted with Whatman 1 filter paper and plunge-
202 frozen into liquid ethane at -180 °C using a vitrobot (FEI company, USA). Frozen grids
203 were transferred onto a CM200 FEG microscope (FEI, USA) using a Gatan 626 cryo-
204 holder (GATAN, USA). Electron micrographs were recorded at an accelerating voltage
205 of 200 KV using low-dose system (20 to 40 e⁻/Å²) and keeping the sample at -175°C.
206 Defocus values were -4 μm. Micrographs were recorded on 4K x 4K TemCam-F416
207 CMOS based camera (TVIPS, Germany).

208 ***Cell Fractionation***

209 Ten milliliters cultures of *C. glutamicum* were grown overnight in BHI medium at 30°C
210 with vigorous shaking (220 rpm). Approximately 2 ml culture aliquots (equivalent to
211 OD_{600nm}=10) were centrifuged, cell pellets were resuspended in 1 ml of 50 mM Tris-HCl
212 pH 7.0 with glass beads and vortexed for 15 min. Unbroken cells were discarded, and
213 total membranes were collected by centrifugation (230,000g, 30 min) in a Beckman TLA
214 100.3 rotor. Proteins in the culture supernatant were precipitated with 10% trichloroacetic
215 acid (TCA) at 4°C for 30 min. The precipitated proteins were recovered by centrifugation
216 (16,000g; 15 min) and washed with cold acetone. Protein samples were separated by
217 SDS-PAGE and analyzed by immunoblotting or Coomassie stain.

218 ***Proteins purification***

219 6His-tagged MusE (AmyE) and LppX were purified from the wild type *C. glutamicum*
220 membrane fraction as previously described [30].
221 For MusE purification from the culture supernatant, 300 ml of BHI was inoculated with
222 the *Cg-Δlgt/pCg482-MusE-His* strain and incubated at 30°C with vigorous shaking (220
223 rpm) overnight. Cells were removed by centrifugation, and culture supernatant were
224 concentrated using ultrafiltration units (Amicon YM 10 kDa) (1 ml min⁻¹ for 5 h at 4°C).
225 The resulting protein concentrate was dialyzed extensively against 25 mM phosphate
226 buffer pH 8.0 at 4°C and loaded on a Ni-NTA column (Quiagen Ni-NTA superflow)
227 previously equilibrated with 25 mM phosphate buffer pH 8.0 (buffer A). The column was
228 washed with ten column volume of buffer A containing 10 mM imidazole and His-tagged
229 proteins were eluted with buffer A containing 250 mM of imidazole. Elution fractions
230 were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining.
231 For LppX purification from culture supernatant, cells transformed with pCg482-LppX-

232 HA-His were grown as above. The culture supernatant was separated from the cells by
233 centrifugation and clarified by filtration through a 0.2- μ m filter. The filtrate was then
234 subjected to protein precipitation using 50 and 70% ammonium sulfate. The resulting
235 pellets were solubilized in a 25mM phosphate buffer and dialyzed extensively against 25
236 mM phosphate buffer pH 8.0 at 4°C. Further purification of LppX was performed as
237 previously described [30]. As LppX was detected both in the 50 and 70% ammonium
238 sulfate fractions, the purified proteins were pooled for mass spectrometry analyses.

239 ***Mass spectrometry analysis***

240 Protein bands were excised from the SDS-PAGE gel after Coomassie Blue staining. In-
241 gel protein digestion was performed in standard conditions. Briefly, protein bands were
242 extensively washed with acetonitrile (ACN) and 25 mM ammonium bicarbonate. For
243 MALDI peptide mass fingerprint (PMF), the cleaned bands were directly submitted to
244 trypsin digestion without other treatment. For nanoLC-MS/MS, cleaned bands were
245 treated with 10 mM dithiothreitol (DTT) at 56°C for 30 min. After DTT removal,
246 cysteine carbamidomethylation was performed at room temperature for 30 min by
247 addition of 55 mM iodoacetamide. After removal of the supernatant, the washing
248 procedure was repeated, gel slices were dried and submitted to enzymatic digestion. For
249 all gel bands, trypsin digestion was performed overnight at room temperature by addition
250 of 20 μ l of 10 ng/ μ l Porcine Gold Trypsin (Promega) diluted in 25mM ammonium
251 bicarbonate.

252 To detect acylated peptides in the MALDI PMF spectra, peptide, extraction was
253 performed according to the protocol described by Ujihara *et al.* [45] with minor
254 modifications: 20 μ l of 1% dodecyl maltoside (Anatrace, n-Dodecyl- β -D-
255 Maltopyranoside DDM, Sol-Grade) was added and the solution was mixed (1500 rpm)

256 for 1 hour at room temperature; 35 μl of a 2:1 chloroform:methanol solution was added
257 and the solution mixed as before. Peptide extracts from the aqueous and organic phases
258 (0.5 μl) were quickly spotted onto a 0.5 μl droplet of 2,5-dihydroxybenzoic acid (Sigma-
259 Aldrich; 20 mg ml⁻¹, 50% ACN, 0.1% trifluoroacetic acid (TFA)). Peptide mixtures were
260 analyzed in positive reflectron mode with the Voyager-DE STRMALDI-TOF mass
261 spectrometer (Absciex) and \sim 500 mass spectra were averaged per spot. Mass
262 spectrometry analyses were performed with the Data Explorer software (Absciex).
263 MALDI PMF spectra were internally calibrated with tryptic peptides of m/z 3451.65
264 (MusE₃₃₄₋₃₆₅) and 4045.89 (MusE₅₅₋₉₁) for MusE and m/z 3164.99 (LppX₂₀₀₋₂₂₄) and
265 3296.34 (LppX₁₁₄₋₁₄₆) for LppX.

266 Data analyses were performed with Mascot (Matrix science, London, UK) and Peptide
267 Mass (https://web.expasy.org/peptide_mass/) search engines on MusE and LppX protein
268 sequences with mass accuracy < 50 ppm.

269 Tryptic peptides for nanoLC-MS/MS analysis were extracted first by addition of 20 μl of
270 50% ACN and 0.1% formic acid (FA) and second by addition of 20 μl of 100%
271 acetonitrile. Tryptic peptides extracted were vacuum dried and resuspended in 5% ACN
272 and 0.1% TFA prior to nanoLC-MS/MS mass spectrometry analysis. NanoLC-MS/MS
273 analyses were performed with the triple-TOF 4600 mass spectrometer (Absciex,
274 Framingham, MA, USA) coupled to the Nano-RSLC system (Thermo scientific). Briefly,
275 peptides were desalted on a C18 reverse phase pre-column (C18 Acclaim Pepmap100, 3
276 μm , 100 \AA , 75 μm i.d., 2 cm length) using a loading buffer containing H₂O/ACN/TFA
277 (98%/2%/0.05%) at 5 $\mu\text{l min}^{-1}$ and were then eluted at a flow rate of 300nl/min from the
278 reverse phase analytical C18 column (C18 Acclaim Pepmap100, 2 μm , 100 \AA , 75 μm i.d.,
279 50 cm length) using a 5-35% solvent B gradient for 40 min. Solvent B was ACN/FA

280 (100%/0,1%) and solvent A was H₂O/FA (100%/0,1%). NanoLC-MS/MS experiments
281 were conducted in Data Dependent acquisition method by selecting the 20 most intense
282 precursors for CID fragmentation with Q1 quadrupole set at low resolution for better
283 sensitivity and with a collision energy ramp ($\pm 15\%$) set to 35 V. Raw data were
284 processed using MS Data Converter software (Absciex) and protein identification was
285 performed using the Mascot search engine against the LppX sequence with
286 carbamidomethylation of cysteines set as fixed modification. Oxidation of methionine
287 and glycosylation of serine and threonine were set as variable modifications. Peptide and
288 fragment tolerance were set at 25 ppm and 0.05 Da respectively. Only peptides with
289 Mascot ions scores higher than the identity threshold (30) at less than 1% of false positive
290 discovery rate are considered.

291 All the illustrations of mass spectrometry spectra were made using the OriginPro 8.5.1
292 software using the raw data extracted as ASCII files from analysis software (Absciex
293 Data Explorer and MS Data Converter).

294

295 **RESULTS**

296 ***Identification and inactivation of the C. glutamicum lgt gene***

297 A single open reading frame (NCgl2009) was previously annotated as a putative
298 phosphatidylglycerol::prolipoprotein diacylglyceryl transferase (Lgt) in the *C.*
299 *glutamicum* ATCC13032 sequenced genome [46,47]. An additional BLASTp search
300 using *M. tuberculosis*, *E. coli*, *M. smegmatis* MSMEG_3222 and MSMEG_5408 Lgt
301 sequences did not identify any additional paralogs of the *lgt* gene in the genome of *C.*
302 *glutamicum* 13032. Thus, similar to most Gram-positive bacteria, but in contrast to *M.*
303 *smegmatis* and *S. coelicolor*, *C. glutamicum* ATCC13032 appears to possess only a single

304 copy of the *lgt* gene.

305 *NCgl2009* encodes a 316-aa long, 34kDa protein in which the residues defined as the
306 “Lgt-signature” in *E. coli* Lgt (Y26, N126, G154, Y235) are all conserved (Suppl. Fig. 1)
307 [44]. The protein has 23,6% and 36,8% identity to *E. coli* and *M. tuberculosis* Lgt
308 (Rv1614), respectively. Previously, Rezwan *et al* proposed that *Actinomycetales* Lgts
309 would be characterized by an additional C-terminal domain of unknown function [5].
310 Although *M. tuberculosis* and *M. smegmatis* Lgt (Rv1614: 468aa and MSMEG_3222:
311 616aa) are indeed much longer than *E. coli* Lgt (291aa), *NCgl2009* (316aa) possesses
312 only 25 additional residues compared with *E. coli* Lgt, suggesting it lacks this additional
313 domain (Suppl. Fig. 1).

314 In order to investigate the role of *NCgl2009* in lipoprotein biogenesis, we inactivated
315 the corresponding gene by constructing an in-frame deletion mutant. When grown on
316 solid rich medium (BHI), the colony morphology of the mutant (*Cg-Δlgt*) was identical to
317 the parental strain (not shown). When grown in liquid BHI medium at 30°C, the mutant
318 showed slight growth delay and decreased final optical density compared to the parental
319 strain (Fig. 1A). To ensure that the observed growth retardation was caused by the
320 absence of *NCgl2009*, the gene was cloned in the pVWEX2 *E. coli*/*C. glutamicum*
321 shuttle vector [48] under the control of a *P_{tac}* promoter. Indeed, the *Cg-Δlgt* strain, when
322 transformed with pVWEX2-*Cg-lgt* grew, in the presence of IPTG, as efficiently as the
323 wild-type (Fig. 1A).

324 Deletion of *lgt* in *M. smegmatis* caused loss of acid-fastness and was proposed to cause
325 cell lysis [37]. To determine whether deletion of *NCgl2009* caused major alterations of *C.*
326 *glutamicum* envelope, different tests were performed. Cryo-electromicroscopy
327 examination of the mutant strain did not indicate differences in size or cell morphology,

328 nor envelope alterations compared with the wild-type strain (Fig. 1B). Sensitivity to
329 antibiotics and detergents was not increased in the Δlgt strain compared to the wild-type
330 (not shown), and the quantity and ratio of trehalose dicorynomycolate versus trehalose
331 monocorynomycolate in the envelope of the two strains were identical (Fig. 1C). Thus,
332 deletion of *Cg-lgt* does not appear to cause any obvious alteration of *C. glutamicum* cell
333 envelope.

334

335 ***The AmyE/MusE lipoprotein is not membrane associated in the C. glutamicum Δlgt***
336 ***mutant.***

337 Previously, we used two model lipoproteins as tools to identify and characterize the Lnt
338 activity of *C. glutamicum*. One of them, NCgl2375, was initially annotated as a maltose-
339 binding protein and/or putative amylase, and thus named AmyE [30]. Its role in maltose
340 uptake was later established and its name changed to MusE (Maltose-uptake system
341 protein E) [49]. We previously demonstrated that MusE is a *bona fide*, triacylated,
342 membrane-associated lipoprotein, which is diacylated in the absence of *lnt* in *C.*
343 *glutamicum*. We also observed that when MusE is non-acylated due to a mutation of the
344 conserved C₊₁ to leucine, the protein is not membrane associated but released in the
345 culture medium [30]. Identical results were obtained for two C₊₁ mutated *M. smegmatis*
346 lipoproteins [38]. In addition, Tschumi *et al.* (2012) [37] reported that deletion of *M.*
347 *smegmatis lgt* causes the release of lipoproteins in the extracellular medium. Altogether,
348 these results suggest that, in *Actinobacteria*, the absence of S-acylation leads to
349 lipoproteins release in the extracellular space.

350 We thus reasoned that if NCgl2009 indeed possess phosphatidylglycerol::prolipoprotein
351 diacylglyceryl transferase (Lgt) activity, all lipoproteins, including MusE, would lack

352 acylation in the *Cg-Δlgt* strain and would be found in the culture filtrate. To test this
353 hypothesis, membrane fractions and culture supernatants were prepared and compared by
354 Coomassie-stained SDS-PAGE. Indeed, the *Cg-Δlgt* strain appears to secrete more
355 proteins compared with the wild-type or complemented strains (Fig. 2A). To confirm that
356 our model protein, MusE was also secreted in the *Cg-Δlgt* strain, the same samples were
357 subjected to immunoblotting using MusE-specific antibodies. As previously observed,
358 MusE was found in the membrane fraction of the wild-type strain, whereas a shorter form
359 was found in the culture supernatant (Fig. 2B). In contrast, MusE was detected in the
360 supernatant of the *Δlgt* strain. MusE membrane localization was restored when *NCgl2009*
361 was expressed *in trans* in the *Δlgt* strain. This result indicates that *Cg-lgt* is necessary for
362 MusE membrane anchoring.

363

364 ***Cg-lgt is necessary for MusE acylation but not for MusE signal peptide cleavage***

365 To assess the role of *Cg-lgt* in MusE acylation, wild-type and *Δlgt* strains were
366 transformed with plasmid pCg482-MusE-His, which expressed a His-tagged version of
367 MusE under the control of its own promoter [30]. MusE-_{6His} was purified by affinity
368 chromatography from the membrane fraction of the wild-type and *Δlnt* strain and from
369 the supernatant of the *Δlgt* strain and subjected to MALDI peptide mass fingerprinting
370 (PMF) analyses after in-gel trypsin digestion (Fig. 3). If Lgt-mediated acylation is
371 necessary for the subsequent cleavage of MusE signal peptide, one would expect that
372 such processing is abolished in the *Δlgt* mutant. In that case, a tryptic peptide of
373 $[M+H]^+=4870.3147$ is expected (IASISMASMLAAASLVAC⁺¹SGSTDEEGDVYFLNF
374 KPEQDVAYQEIAK). However, no peak corresponding to this mass was observed in
375 any of the strain tested (Fig. 3). Instead, comparative analyses of the PMF MALDI MusE

376 mass spectra reveal that two peaks of m/z 3282.50 and 3358.50, are specifically detected
377 in the Δlgt strain (Fig. 3, lower panel). The m/z 3282.50 peak matches to the N-terminal
378 unmodified MusE₁₋₂₉ peptide (C^{+1} SGSTDEEGDVYFLNFKPEQDVAYQEIAK,
379 expected $[M+H]^+=3282,48$; mass accuracy=7ppm) and the m/z 3358.50 peak corresponds
380 to the same peptide harboring a beta-mercaptoethanol adduct (expected $\Delta_{mass}=76$) most
381 probably linked by a disulfide bond to the Cys₊₁. In order to confirm that MusE is not
382 acylated in the Δlgt strain, MALDI PMF mass spectra from wild-type, Δlnt and Δlgt were
383 compared in the 3300-4200 m/z region where N-terminal mono ($m/z=3520.52$), di
384 ($m/z=3858.92$) and triacylated ($m/z=4097.26$) MusE peptides are expected. As anticipated,
385 the triacylated peptide was detected in the wild-type strain and the diacylated peptide
386 observed in the Δlnt strain (Fig. 3B). However, no N-terminal acylated MusE peptide was
387 detected in the Δlgt strain (Fig. 3B). Altogether, these results show that MusE, a *bona*
388 *fide* triacylated, membrane-associated lipoprotein, is not acylated in the Δlgt strain,
389 indicating that this gene is necessary for the initial step of lipoprotein triacylation and
390 hence encodes the *C. glutamicum* phosphatidylglycerol::prolipoprotein diacylglyceryl
391 transferase (Lgt). Importantly, these results also show that the absence of Lgt-mediated S-
392 acylation does not affect the cleavage of MusE signal sequence, but prevents subsequent
393 *lnt*-dependent N-acylation.

394

395 ***E. coli* and *M. smegmatis* Lgt are active in *C. glutamicum*.**

396 Whereas an *E. coli* *lnt* conditional mutant could not be complemented by *in trans*
397 expression of *C. glutamicum* or *M. tuberculosis* *lnt* [31, 50], the *C. glutamicum* *lnt* strain
398 could be complemented by *E. coli* or *M. tuberculosis* *lnt* when expressed *in trans* [30].
399 This suggests that some significant differences exist between *Corynebacteriales* and

400 Gram-negative bacteria lipoprotein biogenesis pathways. We hence tested whether *E. coli*
401 *lgt* was able to complement *C. glutamicum* Δ *lgt* and thus restore MusE membrane
402 association. We cloned *E. coli* *lgt* gene in the shuttle vector pVWEx2 under the control of
403 the *P_{tac}* promoter and the resulting plasmid, pVWEx2-*Ec-lgt* was used to transform Cg-
404 Δ *lgt*. MusE localization, used as a reporter for protein acylation, were tested by western
405 blot on membrane and supernatant fractions. As observed for Cg- Δ *lgt* + pVWEx2-Cg-*lgt*,
406 *E. coli* *lgt* fully restore MusE membrane association in the presence of 1mM IPTG (Fig.
407 4). Then, we tested whether the Cg- Δ *lgt* strain could be complemented by *M. smegmatis*
408 *lgt* orthologues. The two *M. smegmatis* *lgt* genes (*MSMEG_3222* and *MSMEG_5408*)
409 were cloned and their activity tested as above. Whereas *MSMEG_3222* was able to
410 complement Cg- Δ *lgt*, *MSMEG-5408* was not, consistent with the mutation, in this protein,
411 of 2 residues found to be essential for Lgt activity in *E. coli* (Y26, N126 according to
412 *E. coli* Lgt numbering) (Suppl. Fig. 1)[37]. Since *MSMEG_3222*, which possesses a C-
413 terminal extension, was active in *C. glutamicum*, we then tested the role of its C-terminal
414 extension. To do so, a plasmid encoding a *MSMEG_3222* mutant lacking the 327 C-
415 terminal residues, pVWEX₂-*MSMEG_3222* Δ Cter (Δ 288-616) was constructed and
416 transformed in Cg- Δ *lgt*. As seen in Fig. 4, the deleted mutant was as efficient as the full-
417 length *MSMEG_3222* to complement Cg- Δ *lgt*. Thus, the Lgt C-terminal domain, which
418 is only present in some *Actinobacteria*, is not necessary for activity of *MSMEG_3222*, at
419 least in *C. glutamicum*. However, we cannot entirely exclude the possibility that this
420 domain plays a more subtle effect on Lgt activity, not detected in these experiments.

421 Finally, we tested the ability of these different *lgt* to complement the *E. coli* *lgt* depleted
422 mutant PAP9403 [44]. The *lgt* gene is essential in *E. coli*. The PAP9403 mutant, in which
423 the *lgt* gene is replaced by a Km^R cassette, is only viable when a copy of the *E. coli* *lgt*

424 (*Ec-lgt*) gene is expressed *in trans*. In this strain, *Ec-lgt* is present on a plasmid
425 (pCHAP9231) and expressed under the control of the *P_{ara}* promoter [44]. PAP9403 thus
426 grows in the presence of 0.2% L-arabinose, whereas 0.1% D-fucose abolishes growth
427 [44]. PAP9403 was hence transformed with the pVWEx2 derivatives encoding Cg-Lgt,
428 MSMEG_3222, MSMEG_3222ΔCter or MSMEG_5408 and its growth tested in the
429 presence of 0,1% D-fucose and 1mM IPTG. Whereas PAP9403/pVWEx2-*Ec lgt* was able
430 to grow in these conditions, the plasmids encoding Cg-Lgt, MSMEG_3222,
431 MSMEG_3222ΔCter or MSMEG_5408 did not restore growth (not shown). Thus,
432 similarly to what has been observed for Lnt, *E. coli* Lgt is active in *C. glutamicum*
433 whereas *C. glutamicum* and *M. smegmatis* enzymes are not active in *E. coli*, confirming a
434 putative difference between Gram-negative and *Actinobacteria* lipoprotein biogenesis
435 pathway.

436

437 ***Cg-lgt is not necessary for growth on maltose***

438 The gene encoding MusE is part of a cluster encoding an ABC-transport system
439 involved in maltose uptake [49]. In this system, MusE is annotated as a substrate binding
440 protein, the role of which would be to bind maltose in the periplasmic space before
441 delivering it to the inner membrane ABC-transporter. In a strain disrupted in the *musE*
442 gene, uptake of maltose is abolished and growth with maltose as a sole source of carbon
443 is not possible [49].

444 Because the lack of acylation severely affects MusE localization, we wondered whether
445 this change in localization would affect maltose uptake. We thus follow the growth of
446 the Cg-Δ*lgt* strain in minimum medium supplemented with either maltose or glucose. As
447 previously observed in rich medium, the growth of Cg-Δ*lgt* was impaired compared to

448 the wild-type strain. However, the effect was much more pronounced in media with
449 restricted carbon source than in complex media (compare Fig. 5A and Fig. 1A). Still, the
450 Cg-*Δlgt* mutant growth rate was identical whether the minimal medium was
451 supplemented with glucose or maltose as unique carbon source, indicating that maltose
452 uptake is not affected in the Cg-*Δlgt* strain and suggesting that non-acylated MusE is able
453 to sustain its function in maltose transport, despite its release in the extracellular medium.
454 Indeed, MusE was also completely secreted when the Cg-*Δlgt* mutant was grown in
455 minimum medium (Fig. 5B).

456

457 ***Effect of lgt deletion on LppX biogenesis***

458 LppX is a mycobacterial lipoprotein involved in the transport of phenolic glycolipids
459 DIMs (phthiocerol dimycocerosates) to the membrane and is necessary for *M.*
460 *tuberculosis* virulence [51]. In *M. tuberculosis* and *M. bovis* BCG, LppX is associated to
461 the membrane and cell wall but also found in the culture supernatant [52-54]. LppX was
462 identified as a Concanavalin A-binding protein in a glycoproteomic study, and thus
463 proposed to be mannosylated [55].

464 The non-pathogenic model bacteria *M. smegmatis* or *C. glutamicum* do not produce
465 DIMs nor encode LppX homologues. However, when the gene encoding Mt-LppX is
466 introduced in these species, the protein is correctly expressed and found to be triacylated
467 and glycosylated. Whereas only a small fraction of monoglycosylated Mt-LppX was
468 detected in *M. smegmatis* [31], mono- and di-glycosylated forms were detected in *M.*
469 *bovis* BCG [29] and mono-, di-, tri- and tetra-glycosylated forms were identified in *C.*
470 *glutamicum* [30]. In *C. glutamicum*, Mt-LppX glycosylation was shown to be dependent
471 on the presence of the apolipoprotein N-acyltransferase (Lnt; NCgl1424)[30]. In contrast,

472 lipoprotein glycosylation is not dependent on Lnt in *M. smegmatis*, *M. bovis* or *S.*
473 *coelicolor* [29, 56]. However, the 2 latter species each possess 2 Lnt homologues and, in
474 both cases, only one has been tested for a possible implication in glycosylation [29, 56].

475 In order to investigate the role of *Cg-lgt* in LppX biogenesis, wild-type and Δ *lgt* strains
476 of *C. glutamicum* were transformed with pCg482-LppX-HA-His, a shuttle vector in
477 which expression of Mt-LppX_{HA-His} is under the control of the *musE* promoter [30].
478 Membrane and secreted proteins were prepared from the transformed strains and
479 analyzed by SDS-PAGE followed by immunoblotting using a monoclonal anti-HA-
480 peroxidase conjugate. As previously observed, LppX was found both in the membrane
481 and secreted fractions in the wild-type strain ([30] Fig. 6A). In contrast, LppX was not
482 detected in the membrane fraction of *Cg-Δlgt* but found in the supernatant of this strain.
483 As observed for MusE, the membrane localization of LppX was restored when the *Cg-*
484 Δ *lgt* strain was complemented with either *Cg-lgt*, *Ec-lgt*, *MSMEG_3222* or
485 *MSMEG_3222ΔCter*, but not with *MSMEG_5408* (Fig. 6A), confirming that, contrary to
486 *MSMEG_5408*, *E.coli* Lgt, *MSMEG_3222* and *MSMEG_3222ΔCter* are active in *C.*
487 *glutamicum*. To test whether LppX secretion was a direct consequence of the lack of
488 acylation, we expressed, in the wild-type strain, a LppX mutant in which the conserved
489 C₊₁ from the lipobox was changed to a leucine. As shown in Figure 6B, LppX-C1L is
490 only detected in the supernatant of the wild-type strain, confirming that non-acylated
491 lipoproteins are released in the culture supernatant of *Corynebacteriales* even when *lgt* is
492 present.

493

494 ***Cg-Lgt is essential for acylation of LppX but dispensable for its glycosylation***

495 As expected from the activity of *Cg-Lgt* as a phosphatidylglycerol::prolipoprotein

496 diacylglyceryl transferase, LppX was not acylated in the *Cg-Δlgt* strain. Indeed, when the
497 protein was purified by affinity chromatography from the supernatant fraction of the *Cg-*
498 *Δlgt* strain and subjected to MALDI peptide mass fingerprinting, only peaks
499 corresponding to non-acylated LppX₁₋₂₉ peptides could be detected (Suppl. Fig. 2). This
500 result, together with confirming the role of *Cg-lgt*, also indicates that, as for MusE,
501 acylation is not required for signal peptide cleavage. Interestingly, among the detected
502 peaks, some corresponded to glycosylated forms of LppX₁₋₂₉ (Suppl. Fig. 2). This
503 prompted us to examine the influence of *Cg-lgt* on LppX glycosylation. Previously, we
504 showed that LppX is modified with hexoses (*m/z* 162) when expressed in *C. glutamicum*
505 and that this modification (either mono, di, or tetraglycosylation) occurs on LppX₆₋₂₉ [30].
506 We hence purified LppX from the supernatant fraction of the *Cg-Δlgt* strain and
507 performed nano-LC-MS/MS analysis after trypsin digestion. As previously found for
508 LppX expressed in wild-type *C. glutamicum*, LppX₆₋₂₉ was found in mono, di, or
509 tetraglycosylated forms in the *Cg-Δlgt* strain (Suppl. Fig. 3), indicating that Lgt-
510 dependent S-acylation is not a prerequisite for glycosylation

511

512 ***Cleavage of LppX and MusE signal peptide by LspA***

513 A single gene, *NCgl2058*, was annotated as a putative lipoprotein signal peptidase in the
514 *C. glutamicum* ATCC13032 genome and was deleted [46,47]. The *Cg-ΔlspA* strain
515 obtained showed a very slight growth delay in rich medium compared to the parental
516 strain, but reach similar final optical density. The growth delay was complemented when
517 the strain was transformed with pVWEX2-*Cg-lspA*, independently of the presence of
518 IPTG (Fig. 7A).

519 MusE expression and signal peptide processing was then evaluated in *Cg-ΔlspA*. As

520 shown in Fig. 2 and Fig. 7B, MusE migration in *Cg-Δlgt* or *Cg-ΔlspA* strain is similar to
521 the one observed in the wild-type strain, which suggests that the signal peptide cleavage
522 still occurs in these strains. This indicates that, for MusE, signal peptide processing can
523 occur independently of LspA or S-acylation, which is consistent with the mass
524 measurement of MusE secreted by *Cg-Δlgt* (Fig. 3) and the fact that a non-acylated
525 MusE-C1L mutant, when expressed in the wild-type strain, is cleaved upstream of the L₊₁
526 residue [30].

527

528 The *Cg-ΔlspA* strain was then transformed with pCg482-LppX_{HA-His} and LppX
529 localization was analyzed as before. In *Cg-ΔlspA*, LppX behaves as in *Cg-Δlgt*: the
530 protein is mostly localized in the culture supernatant and resolves at a higher mass than in
531 the wild-type strain. When the *Cg-ΔlspA* strain was complemented with *Cg-lspA*, LppX
532 recovered its initial migration behavior (Fig. 7B). This result is similar to what has been
533 observed by Tschumi *et al.*, (2012) in *M. smegmatis* [31], where the migration behavior
534 of LppX is similar in *Ms-ΔlspA* and *Ms-Δlgt* mutants. These authors concluded from this
535 observation that signal peptide cleavage is abolished in the *Ms-Δlgt* strain [31]. However,
536 our mass spectrometry analysis clearly showed that the LppX₁₋₂₉ signal peptide is cleaved
537 in this strain.

538

538

539 **DISCUSSION**

540 *Lgt essentiality*

541 The genes necessary for lipoprotein modification and trafficking (i.e. *lgt*, *lspA*, *lnt*, *lol*),
542 are essential in some but not all Gram-negative bacteria. The essentiality of this pathway
543 was attributed to its role in targeting several vital lipoproteins to the outer membrane
544 where they are necessary to the biogenesis of the cell envelope (BamD, LolB, LptE,
545 LpoA, LpoB) [8,58,59].

546 *Corynebacteriales* are diderm bacteria possessing an atypical outer membrane called the
547 “mycomembrane” which is functionally similar to the outer membrane from Gram-
548 negative bacteria [60,61]. However, very little is known about the biogenesis and
549 trafficking of lipoproteins in these bacteria, despite their important role in pathogenesis
550 and physiology [3,6,36,60].

551 Here, we identified *C. glutamicum lgt* gene and found that a mutant lacking this gene is
552 viable. Although essential in *M. tuberculosis*, this gene can also be deleted in *M.*
553 *smegmatis* [37].

554 Importantly, this is not the first report showing that a gene involved in cell envelope
555 biogenesis is essential in *M. tuberculosis* whereas it can be deleted from *M. smegmatis* or
556 *C. glutamicum* [61-63]. This probably reflects major physiological differences between
557 pathogenic, slow-growing *M. tuberculosis* and the non-pathogenic, fast-growing *M.*
558 *smegmatis* or *C. glutamicum*. Alternatively, the function of some essential lipoproteins
559 might be strictly dependent on acylation in *M. tuberculosis* but not in *M. smegmatis* or *C.*
560 *glutamicum*. Several lipoproteins have indeed been proposed to be essential in *M.*
561 *tuberculosis* [64]. One of these, LpqW, is implicated in the biogenesis of PIM

562 (phosphatidylinositol mannosides) and LM/LAM (lipomannan/lipoarabinomannan). The
563 *lpqW* gene could be inactivated in both *M. smegmatis* and *C. glutamicum* and the mutants
564 obtained were severely affected in growth and unstable, frequently acquiring suppressor
565 mutations elsewhere in the genome [60,65]. LpqB, a lipoprotein involved in the
566 modulation of the MtrAB two-component system, was predicted as being essential in *M.*
567 *tuberculosis*, but could be interrupted in *M. smegmatis* resulting in pleiotropic effects [66].
568 As the defects observed in the *lpqB* and *lpqW* mutant strains were more pronounced than
569 those observed for the *lgt* strains, these lipoproteins probably retain at least part of their
570 function when non-acylated. Several other examples of lipoproteins displaying activity
571 when non lipid-modified have been reported in other genera: the non-acylated form of the
572 essential *B. subtilis* lipoprotein PrsA, a foldase involved in protein secretion, is still active
573 [25]. The MutA lipoprotein, involved in Mn²⁺ active transport, is necessary for *S. uberis*
574 growth in milk. However, growth of a *S. uberis* Δ *lgt* mutant is not affected in milk
575 despite MutA being mislocalized [24]. Finally, the lipoprotein OpuAC, which is
576 necessary for glycine betaine transport in *B. subtilis*, is also active when the conserved
577 C₊₁ cysteine is mutated to alanine [66].

578 ***Interspecies complementation***

579 Lgt is responsible for the transfer of a diacylglyceryl moiety from phosphatidylglycerol
580 (PG) to the side chain of the conserved C₊₁ cysteine residue found in the consensus
581 “lipobox”, which, after cleavage of the signal peptide, becomes the first residue of the
582 mature protein. Whereas *C. glutamicum*, *M. smegmatis*, and *E. coli* Lgt could all
583 complement Cg- Δ *lgt*, only *E. coli* Lgt could restore growth of an *E. coli* conditional
584 mutant. In contrast, *S. aureus* Lgt was active in *E. coli* [67]. Although we cannot exclude
585 that in the conditions used in our study, *C. glutamicum* and *M. smegmatis* Lgts are not

586 properly expressed in *E. coli*, these results perfectly parallel those obtained with the third
587 enzyme of the lipoprotein biogenesis pathway, Lnt.

588 Indeed, *C. glutamicum* and *M. smegmatis* *lnt* mutants could be complemented with *M.*
589 *tuberculosis* *lnt* [30] whereas an *E. coli* *lnt* conditional mutant could only be
590 complemented by *lnt* from *Proteobacteria*, but not from *Actinobacteria* [31, 68]. This
591 discrepancy could be due to differences in substrates specificity between enzymes from
592 *Actinobacteria* and *Proteobacteria*. Indeed, the most common fatty acids of *E. coli*
593 (palmitic (C16:0), palmitoleic (C16:1), and cis-vaccenic (C18:1_{cis}-11) acids), are found
594 modifying *E. coli* Lpp [2]. In contrast, *Mycobacterium* lipoproteins have been shown to
595 be S-acylated by diacylglycerol residues carrying C16 and C19:0 fatty acids, and N-
596 acylated by C16 or C19 fatty acids [29, 31]. In the case of *C. glutamicum*, C16/C18
597 diacylglycerol and C16 fatty acids were found to modify lipoproteins [30].

598 Tschumi *et al.* (2009) [31] proposed that failure of mycobacterial *lnt* to complement *E.*
599 *coli* could be due to the incapacity of the enzyme to recognize lipoproteins S-acylated
600 with diacylglycerol moiety carrying only “small” (C16) fatty acids. However, this cannot
601 explain why Lnt from *Streptomyces* would not complement *E. coli* as in this gender, most
602 lipoprotein S-modifying fatty acids are C15 or C16 [32]. Variations could also come from
603 the specificity of each enzyme for the phospholipid substrates used as fatty acid donors
604 for N-acylation. Indeed, *E. coli* *Lnt* activity *in vitro* is strongly affected by the
605 phospholipid headgroup and acyl chain composition [69]. It is thus tempting to speculate
606 that, as for Lnt, Lgt of different species differ in substrate specificity. Interestingly, it was
607 recently shown that a gene conserved exclusively in *Actinobacteria* is responsible for the
608 production of alanylated-diacylglycerol (DAG) and alanylated-phosphatidylglycerol in *C.*
609 *glutamicum*. *E. coli*, in contrast, does not harbor any aminoacyl-phosphatidylglycerol

610 synthase activity [70]. Whether this modified DAG could be used preferentially as
611 substrate by actinobacterial Lgts is not known. To address these questions of substrate
612 specificity, further *in vitro* biochemical, enzymatic and structural characterization will be
613 necessary to precisely characterize these enzymes.

614 ***Role of Lgt C-terminal extension***

615 *In vitro* characterization of Lgt enzymes would also be useful to understand the function
616 of the C-terminal extension found in some, but not all Lgts. Indeed, in addition to *M.*
617 *tuberculosis* Lgt and MSMG_3222 (469 and 612 amino-acids long respectively), other
618 mycobacterial Lgt, such as Lgt from *Mycobacterium chubuense* (Mycch_2541: 986aa),
619 *Mycobacterium rhodesiae* (MycrhN_5205: 774aa), *Mycobacterium intracellulare*
620 (OCU_30050:546aa), *Mycobacterium marinum* (MMAR_2416: 714aa) and
621 *Mycobacterium kansasii* (MKAN_27935: 676aa) are predicted to be much longer than
622 the 291 aa-long *E. coli*. This is not a generality however, as *Mycobacterium abscessus*
623 and *Mycobacterium leprae* Lgts are respectively 343aa (MAB_2642c) and 330aa
624 (ML1274), which indicates that the presence of the C-terminal extension is not a strict
625 requirement for Lgt function in *Mycobacterium*. Other *Corynebacteriales*, outside the
626 *Mycobacterium* genus, also possess a C-terminal extension (*Gordonia bronchialis*
627 (Gbro_2945: 585aa) or *Nocardia farcinica* (NFA_18630: 491aa)), but it appears to be
628 completely absent in other genus (*Corynebacterium*, *Rhodococcus*). This C-terminal
629 domain is extremely variable in size amongst Lgts and its sequence, which is rich in
630 glutamate, aspartate and alanine residues, is not conserved or homologous to any domain
631 of known function. Here, we found that this extension was not necessary for
632 MSMEG_3222 to restore LppX and MusE membrane localization in Cg-*Algt*, indicating
633 that it is not required for activity in *C. glutamicum*, which is consistent with the absence,

634 in this genus, of “long” Lgt. Whether this domain is necessary for activity in
635 *Mycobacterium* has not been tested. It is thus still possible that this domain, without
636 being strictly necessary for function, has an effect on the enzyme activity.

637 ***Signal peptide cleavage***

638 LspA cleaves lipoprotein signal peptide after translocation across the cytoplasmic
639 membrane. The S-acylation of lipoprotein is not always necessary for lipoprotein signal
640 peptide cleavage, which can also, in some cases, occur in the absence of LspA [22, 24, 25,
641 37, 71]. Here, we did not observe any differences in migration whether the *C. glutamicum*
642 lipoprotein MusE was expressed in the wild-type, Δlgt or $\Delta lspA$ strain, indicating that
643 signal sequence processing occurs independently of acylation or LspA. This is consistent
644 with previous results showing that a non-lipomodified MusE-C1L mutant is processed
645 and secreted in the wild-type strain [30]. Results obtained for LppX were less clear. The
646 protein migrates in SDS-PAGE gels, at the same position whether expressed in *Cg- Δlgt*
647 or *Cg- $\Delta lspA$* and resolved at a higher mass compared with the protein expressed in the
648 wild-type strain. Identical observations were made when LppX was expressed in *M.*
649 *smegmatis lgt* or *lsp* mutants [37]. The slower migration could be explained by the
650 presence of an uncleaved signal peptide, which would suggest that LspA cannot cleave
651 LppX when it is not acylated. However, according to mass spectrometry analysis, LppX
652 purified from the culture supernatant of *Cg- Δlgt* lacked its signal peptide. The reason for
653 LppX slower migration, when expressed from *Cg- Δlgt* is hence still unknown.

654 ***Glycosylation***

655 Numerous lipoproteins have been found to be glycosylated in mycobacteria and
656 *Streptomyces* [2]. In *Streptomyces*, the enzyme responsible for this modification is Pmt
657 (protein *O*-mannosyltransferase), a membrane protein that transfers a mannose from the

658 sugar donor polyprenyl-monophosphomannose (PPM) to the protein target [72].
659 Topology studies suggest that Pmt catalytic site is located in the periplasm and that
660 protein modification occurs after export of the target through the cytoplasmic membrane.
661 PPM is formed on the cytoplasmic side of the inner membrane by the addition of
662 mannose to polyprenyl-monophosphate by Ppm1, before being “flipped” across the
663 cytoplasmic membrane [72]. Interestingly, in *M. tuberculosis*, the *ppm1* gene encodes
664 both Lnt and Ppm1 activities [58]. In *M. smegmatis* and *C. glutamicum*, the two enzymes
665 are encoded by two genes of a single operon, but Ms-Lnt and Ms-Ppm1 interact with
666 each other [57]. It was thus proposed that Ppm1 association with the membrane protein
667 Lnt allows this soluble protein to remain close enough from the membrane to perform its
668 transferase activity. In a previous study, we found that LppX was nor N-acylated, nor
669 glycosylated in a *Cg-lnt* mutant. It was not clear however if the absence of glycosylation
670 was due to either 1) the lack of association of Ppm1 with the membrane in the absence of
671 Lnt, and hence the absence of PPM synthesis; or 2) the lack of N-acylation, which could
672 abolish lipoprotein glycosylation. Here, we unambiguously showed that in the *Cg-Δlgt*
673 mutant, LppX is not N or S-acylated, but is still glycosylated. Hence, acylation is not a
674 prerequisite for lipoprotein glycosylation in *C. glutamicum*. The sequence of event
675 leading to lipoprotein acylation and glycosylation in the wild-type context still remains to
676 be determined.

677

678 **AUTHOR STATEMENTS**

679 The author(s) declare that there are no conflicts of interest.

680

681

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686

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691

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- 948

948

949 **Table 1:** Oligonucleotides used in this study. Restriction sites used for cloning are

950 indicated in italic.

Name	Sequence (5' to 3')
2009-del1-XhoI	AAGTCATGACCTCGAGAACAACG
2009-del2-BamHI	AGGGGACAGTTGGATCCCGG
2009-del3-XhoI	AGGGCATTGCTCGAGAAACG
2009-del4-HindIII	AGACGAGGCCAAGCTTGCCG
2058-del1-BglII	TGGAAGATCCTTGTATTGCAAGCGCTG
2058-del2-XbaI	CTAGTCTAGAGGTCACGTTGACCATC
2058-del3-XbaI	GATCTCTAGAGACGAGAAGGATGAGGCCTGA
2058-del4-XhoI	TATGCTCGAGGTGGAAATGCGGAAAC
DirLppX-C1L	GGCTATCAGGA ^c tTTCTTCGCCG
RevLppX-C1L	CGGCGAAGAA ^a gTCCTGATAGCC
482-AS	GATATGATCATTTATTCTGC
482-S2	GCAGAATAAATGATCCGTCG
DirNCgl2009-XbaI	GCTCTAGACGTTGTTGTGGATGTCATGACTTTGG
RevNCgl2009-BamHI	GCGGATCCCTATCGCGTTGAGGGCGTTTCTCC
DirEc-igt-XbaI	GGTCTAGAGCGAAATAACAAGAAATTGTGG
RevEc-igt-BamHI	CGCGGATCCTTAGGAAACGTGTTGCTGTGG
DirMSMEG_3222-XbaI	GCTCTAGAGGTGTTTCGGCGGAGGGTGTGAGCTTGACC
RevMSMEG_3222-BamHI	GCGGATCCCTAGCGGTTGCGCCGCAACCACCGGCGC
RevMSMEG_3222-A288-BamHI	GCGCGGATCCTTAGGCCTTGCCACGCAACGATTCCGG
DirMSMEG_5408-XbaI	GCTCTAGACGCGAAGGGCGTTACGTTTCAGCAGG
RevMSMEG_5408-BamHI	GCGGATCCTCATACCGGCACTTCCTCACTGCGGG
DirCg-lspA-XbaI	GCTCTAGACCGCTCATGGGGAAAGATGGTCAACG
RevCg-lspA-BamHI	GCGGATCCTCAGGCCTCATCCTTCTCGTCAGTTGC

951

952

952

953 **FIGURES LEGENDS**

954 **Figure 1: Characterization of *C. glutamicum* Δlgt .** (A) *C. glutamicum* WT (circles)
955 and Δlgt (squares) were grown in BHI medium at 30°C. *Cg- Δlgt + pVWEx2-*Cg-lgt**
956 (diamonds) was grown at 30°C in BHI supplemented with 1mM IPTG. Shown is a
957 representative experiment of strains grown in triplicate, where errors bars indicate
958 standard deviation from the mean. (B) Cryo-TEM of 3 selected bacteria from the wild-
959 type *C. glutamicum* strain (top) and *Cg- Δlgt* strain (bottom). Scale bar: 200nm. (C) TLC
960 analysis of total lipids extracted from whole *C. glutamicum* WT (lanes 1 and 4), Δlgt
961 (lanes 2 and 5) or *Δlgt + pVWEx2-*Cg-lgt** (lanes 3 and 6) in exponential (lanes 1-3) or
962 sstationary (lanes 4-6) phase of growth. TLC plate were developed in the solvent system
963 $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ 65/25/4 by volume and revealed by immersion in 10% H_2SO_4 in
964 ethanol followed by heating. PG: phosphatidylglycerol; CL: cardiolipin; PIM:
965 phosphatidylinositol mannosides; TMCM, trehalose monocorynomycolates; TDCM,
966 trehalose dicorynomycolates.

967

968 **Figure 2: MusE is secreted in *C. glutamicum* Δlgt .**

969 Membrane (M) and secreted (S) proteins corresponding to an $\text{OD}_{600\text{nm}}$ of 10 were
970 prepared for WT strain (1), *Cg- Δlgt* (2), *Cg- Δlgt + pVWEX2-*Cg-lgt** grown in absence (3)
971 or in the presence of 1mM IPTG (4), and analyzed by SDS-PAGE followed by coloration
972 with Coomassie blue (A) or immunoblotting using polyclonal anti-MusE antibodies (B).
973 The band labeled with an asterisk corresponds to the previously observed shorter form of
974 MusE [30].

975

976 **Figure 3: MALDI PMFs of MusE proteins purified from *C. glutamicum* WT, ΔInt**
977 **and Δlgt strains.**

978 The whole MALDI PMF mass spectra of MusE proteins are shown (m/z 800-5000) and
979 detected monoisotopic $[M+H]^+$ peaks are indicated. Inserts highlight the region of the
980 spectra with the N-terminal MusE peptide. The m/z 3451.65 and the m/z 4045.89 peaks
981 match to the MusE₃₃₄₋₃₆₅ and MusE₅₅₋₉₁ tryptic peptides respectively. They are detected in
982 all spectra. The m/z 4097.26 peak is detected only in the WT strain and corresponds to the
983 tri-acylated N-terminal MusE₁₋₂₉ peptide while the m/z 3858.92 peak is detected only in
984 the ΔInt strain and corresponds to the di-acylated N-terminal MusE₁₋₂₉ peptide [30]. The
985 m/z 3282.50 and 3358.50 peaks are specific of the Δlgt MusE spectrum. Both peaks
986 correspond to the unacylated N-terminal MusE₁₋₂₉ peptide. The m/z 3282.50 corresponds
987 to the standard MusE₁₋₂₉ peptide and the m/z 3358.50 correspond to a beta-
988 mercaptoethanol adduct of the same MusE₁₋₂₉ peptide (Delta mass=76). In the Δlgt strain,
989 no peaks corresponding to mono, di or tri-acylated MusE₁₋₂₉ peptide are observed.

990
991 **Figure 4: Role of *Cg-lgt* homologues in MusE localization.** Cells transformed with the
992 indicated plasmids were grown overnight in BHI at 30°C in the presence or absence of
993 IPTG 1mM. Membrane (M) and secreted (S) fractions were prepared and analyzed by
994 SDS-PAGE followed by immunoblotting using polyclonal anti-MusE antibodies.

995

996 **Figure 5: MusE acylation is not necessary for growth on maltose. (A)** Preculture of *C.*
997 *glutamicum* WT (circle) and Δlgt (squares) were grown overnight at 30°C in CGXII
998 minimal medium with glucose. The next day, cells were washed several times with
999 CGXII containing no carbon source and used to inoculate fresh CGXII with either
1000 maltose (open symbols) or glucose (filled symbols) as unique carbon source. Shown is a

1001 representative experiment of strains grown in triplicate, where errors bars indicate
1002 standard deviation from the mean. **(B)** Membrane (M) and secreted (S) fractions of the
1003 Δlgt strain grown in GCXII were prepared and analyzed by SDS-PAGE followed by
1004 immunoblotting using polyclonal anti-MusE antibodies.

1005

1006 **Figure 6: Acylation is necessary for LppX membrane localization.** **(A)** Membrane
1007 (M) and secreted (S) proteins corresponding to an OD_{600nm} of 10 were prepared from WT
1008 strain transformed with pCg482-LppX-HA-His, Cg- Δlgt + pCg482-LppX-HA-His,
1009 pCg482-LppX-HA-His + pVWEx2-Cg-lgt or Cg- Δlgt + pCg482-LppX-HA-His +
1010 pVWEx2-*Ec-lgt* (upper panel) Cg- Δlgt + pCg482-LppX-HA-His + pVWEx2-
1011 *MSMEGM_3222*, Cg- Δlgt + pCg482-LppX-HA-His + pVWEx2-*MSMEGM_3222- $\Delta Cter$*
1012 or Cg- Δlgt + pCg482-LppX-HA-His + pVWEx2-*MSMEGM_5408* (lower panel) grown
1013 in the absence or in the presence of 1mM IPTG, and analyzed by immunoblotting using
1014 anti-HA peroxidase conjugate (Roche). **(B)** The same experiments were performed on
1015 wild-type strain transformed with pCg482-LppX-HA-His; Cg- Δlgt + pCg482-LppX-HA-
1016 His and Cg- Δlgt + pCg482-LppX-C1L-HA-His + pVWEx2-Cg-lgt

1017

1018 **Figure 7: Characterization of *C. glutamicum* $\Delta lspA$.** **(A)** WT *C. glutamicum*
1019 (diamonds) and $\Delta lspA$ (squares) were grown in BHI medium at 30°C. $\Delta lspA$ + pVWEX2-
1020 *Cg-lspA* was grown at 30°C in BHI with (crosses) or without (triangles) 1mM IPTG; **(B)**
1021 Cells were grown overnight in BHI at 30°C in presence or absence of IPTG 1mM.
1022 Membrane (M) and secreted (S) fractions from cells corresponding to an OD_{600nm} of 10
1023 were prepared and analyzed by SDS-PAGE followed by immunoblotting using either
1024 polyclonal anti-MusE antibodies **(B)** or monoclonal anti-HA peroxidase conjugate

1025 (Roche) to detect LppX (C).

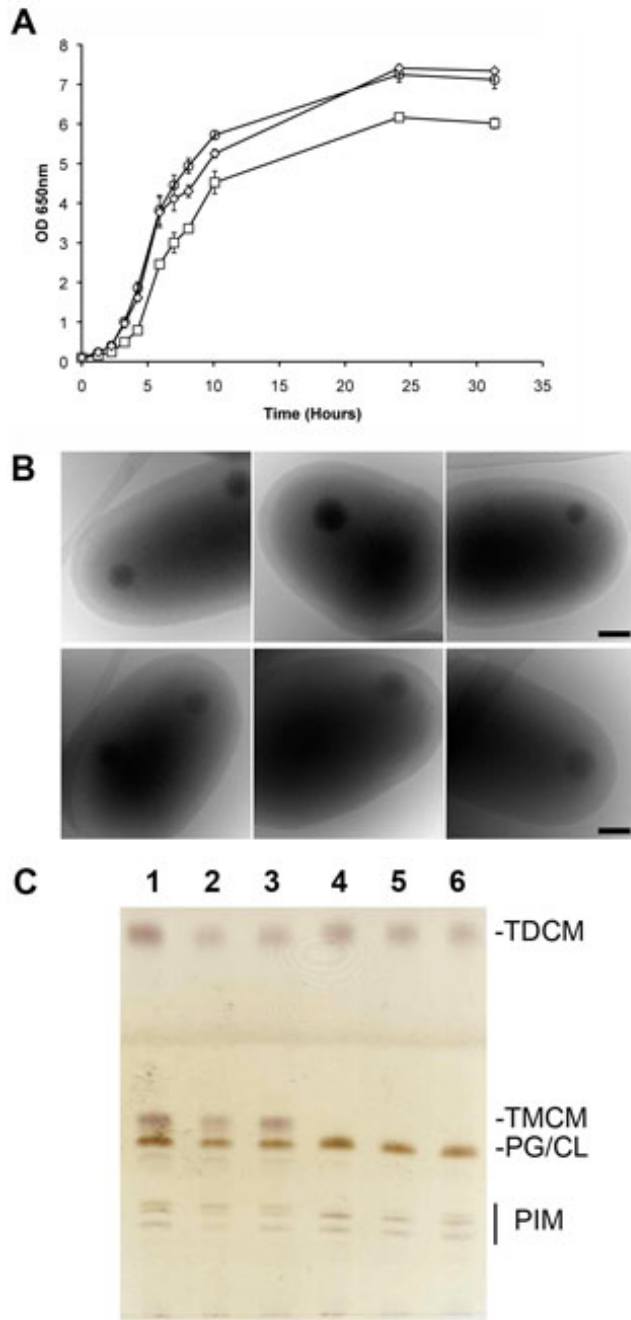


Figure 1

1026

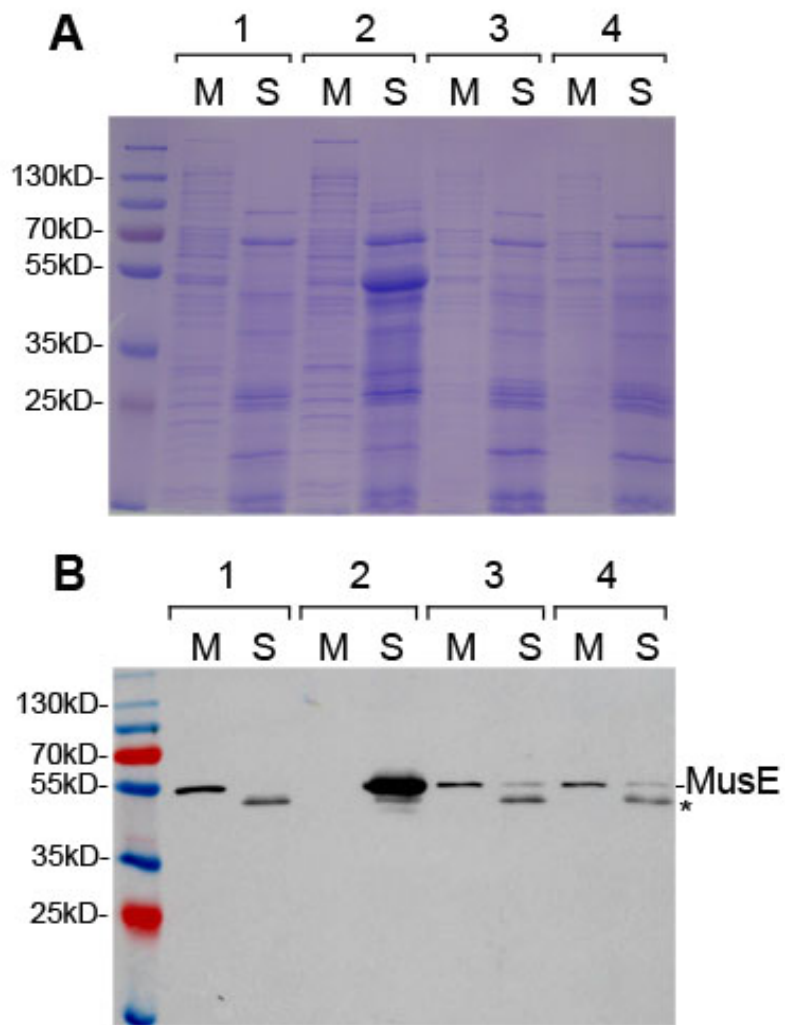
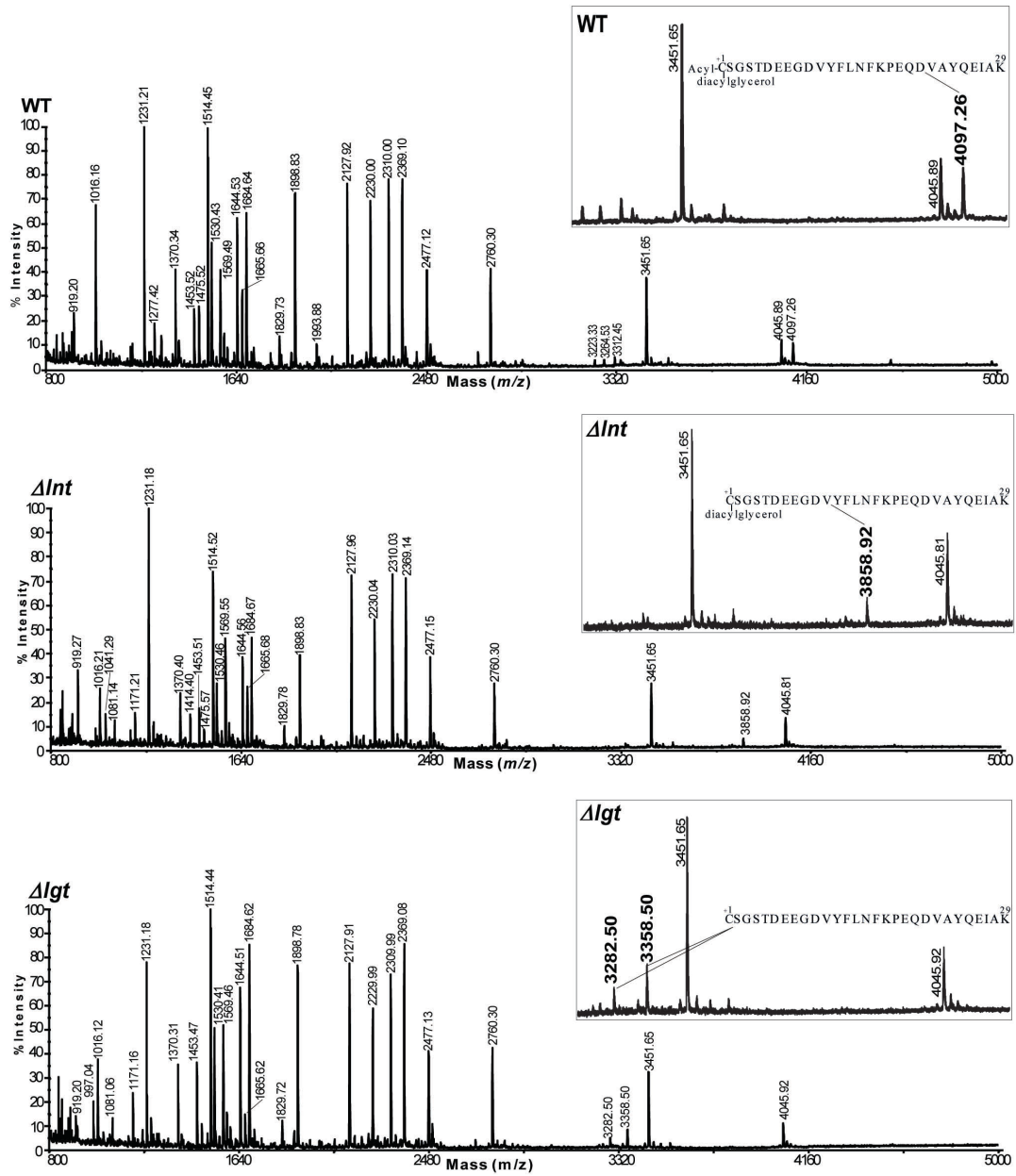


Figure 2

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1028



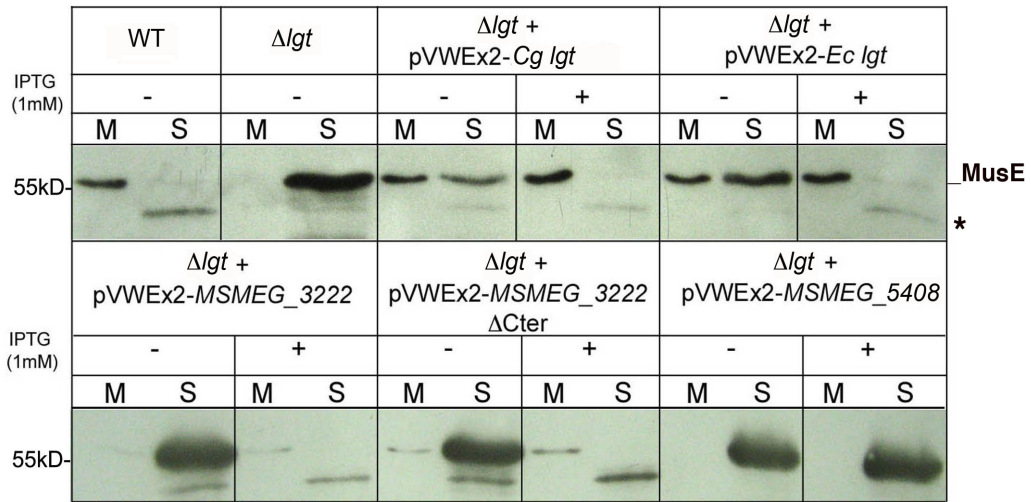


Figure 4

