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

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

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 lipoproteins by adding a diacylglyceryl group to the  
29 sulfhydryl side chain of the invariant Cys<sub>+1</sub> residue. Lipoprotein signal peptidase (LspA  
30 or signal peptidase II) subsequently cleaves the signal peptide, liberating the  $\alpha$ -amino  
31 group of Cys<sub>+1</sub> 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<sub>+1</sub> based on *E. coli* lipoprotein sequences) at the extremity of  
46 their signal sequence, in which the C<sub>+1</sub> 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<sub>+1</sub>. Lipoprotein signal  
56 peptidase (LspA or signal peptidase II) subsequently cleaves the signal peptide, liberating  
57 the α-amino group of C<sub>+1</sub>, 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<sub>+1</sub> by the apolipoprotein N-

acyltransferase (Lnt), resulting in mature triacylated proteins [8,9]. These mature lipoproteins are then found mostly (~90%) in the outer membrane (OM), to which they are targeted by the dedicated Lol (*localization of lipoproteins*) system [8]. Although Lnt-dependent lipoproteins triacylation appears to be the rule for most Gram-negative bacteria, few exceptions exist, in which *lnt* orthologues have not been found or appear not to be functional [9-12]. For some time, *lgt*, *lspA*, *lnt*, and *lol* have been considered as essential genes in all Gram-negative bacteria and thus attracted attention as new potential targets for antibiotherapy [13-15]. However, a recent study showed that *lnt* is dispensable in *Francisella tularensis*, *Neisseria gonorrhoeae*, *Acinetobacter baylyi* and *Acinetobacter baumannii* and that its absence does not preclude targeting to the outer membrane [16,17].

Low-GC% Gram-positive bacteria, such as *Staphylococci* and Mollicutes, lack an outer membrane (monoderms) and thus retain lipoproteins in the cytoplasmic membrane. Although no *lnt* homologues have been identified in these bacteria, some of them nevertheless produce triacylated lipoproteins [9,18,19], as well as lipoproteins carrying other types of *N*-modifications (lyso, *N*-acetyl and peptidyl) [20]. In these organisms, the third step of lipoproteins biogenesis is performed by other, yet uncharacterized, enzymes. Interestingly, lipoproteins processing (di- versus tri-acylated) is regulated by environmental conditions in *Staphylococcus aureus* [21]. In these low-GC% Gram-positive bacteria, *lgt* and *lspA* are not essential. Generation of *lgt* mutants in *Streptococcus pneumoniae*, *Streptococcus uberis*, *Bacillus subtilis* and *Listeria monocytogenes* indicates that Lgt-dependent diacylation is not always a prerequisite for cleavage of the signal sequence, either by LspA or other peptidases [22-25]. In most cases, whether the signal sequence is cleaved or not, the non-acylated proteins produced in *lgt* mutants appear to be released in the culture medium, consistent with the absence of

a membrane anchor [25-28].

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

In the didermic *Actinobacteria* (mycobacteria, corynebacteria and nocardia), *lgt*, *lsp* and *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  $\Delta$ 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<sub>+1</sub> (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

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

## MATERIALS AND METHODS

### Reagent and medium

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

### Strains and plasmids

#### *Strains*

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

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

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

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

### **Plasmids**

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

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

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

To construct pVWEx2-*MSMEG\_3222* and pVWEx2-*MSMEG\_3222*ΔCter, the *MSMEG\_3222* gene was amplified from *M. smegmatis* Mc<sup>2</sup>155 genomic DNA using primers Dir*MSMEG\_3222*-XbaI and either Rev*MSMEG\_3222*-BamHI or Rev-*MSMEG\_3222*A288-BamHI (Table 1). The PCR products obtained (1890 bp and 908 bp respectively) were then introduced between the XbaI and BamHI sites of pVWEx2-*ppmI*. The same strategy was used to construct pVWEx2-*MSMEG\_5408*, except that Dir*MSMEG\_5408*-XbaI and Rev*MSMEG\_5408*-BamHI (Table 1) were use as primers.

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

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

A 4 µl aliquot of cells grown in BHI to stationary phase was adsorbed onto holey carbon-coated grid (Lacey, Tedpella, USA), blotted with Whatman 1 filter paper and plunge-frozen into liquid ethane at -180 °C using a vitrobot (FEI company, USA). Frozen grids were transferred onto a CM200 FEG microscope (FEI, USA) using a Gatan 626 cryo-holder (GATAN, USA). Electron micrographs were recorded at an accelerating voltage of 200 KV using low-dose system (20 to 40 e<sup>-</sup>/Å<sup>2</sup>) and keeping the sample at -175°C. Defocus values were -4 µm. Micrographs were recorded on 4K x 4K TemCam-F416 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<sub>600nm</sub>=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<sup>-1</sup> 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-

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

### ***Mass spectrometry analysis***

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

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

for 1 hour at room temperature; 35  $\mu$ l of a 2:1 chloroform:methanol solution was added and the solution mixed as before. Peptide extracts from the aqueous and organic phases (0.5  $\mu$ l) were quickly spotted onto a 0.5  $\mu$ l droplet of 2,5-dihydroxybenzoic acid (Sigma-Aldrich; 20 mg ml<sup>-1</sup>, 50% ACN, 0.1% trifluoacetic acid (TFA)). Peptide mixtures were analyzed in positive reflectron mode with the Voyager-DE STRMALDI-TOF mass spectrometer (Absciex) and ~500 mass spectra were averaged per spot. Mass spectrometry analyses were performed with the Data Explorer software (Absciex). MALDI PMF spectra were internally calibrated with tryptic peptides of  $m/z$  3451.65 (MusE<sub>334-365</sub>) and 4045.89 (MusE<sub>55-91</sub>) for MusE and  $m/z$  3164.99 (LppX<sub>200-224</sub>) and 3296.34 (LppX<sub>114-146</sub>) for LppX.

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

Tryptic peptides for nanoLC-MS/MS analysis were extracted first by addition of 20  $\mu$ l of 50% ACN and 0.1% formic acid (FA) and second by addition of 20  $\mu$ l of 100% acetonitrile. Tryptic peptides extracted were vacuum dried and resuspended in 5% ACN and 0.1% TFA prior to nanoLC-MS/MS mass spectrometry analysis. NanoLC-MS/MS analyses were performed with the triple-TOF 4600 mass spectrometer (Absciex, Framingham, MA, USA) coupled to the Nano-RSLC system (Thermo scientific). Briefly, peptides were desalted on a C18 reverse phase pre-column (C18 Acclaim Pepmap100, 3  $\mu$ m, 100 Å, 75  $\mu$ m i.d., 2 cm length) using a loading buffer containing H<sub>2</sub>O/ACN/TFA (98%/2%/0.05%) at 5  $\mu$ l min<sup>-1</sup> and were then eluted at a flow rate of 300nl/min from the reverse phase analytical C18 column (C18 Acclaim Pepmap100, 2  $\mu$ m, 100 Å, 75  $\mu$ m i.d., 50 cm length) using a 5-35% solvent B gradient for 40 min. Solvent B was ACN/FA

(100%/0,1%) and solvent A was H<sub>2</sub>O/FA (100%/0,1%). NanoLC-MS/MS experiments were conducted in Data Dependent acquisition method by selecting the 20 most intense precursors for CID fragmentation with Q1 quadrupole set at low resolution for better sensitivity and with a collision energy ramp ( $\pm 15\%$ ) set to 35 V. Raw data were processed using MS Data Converter software (Absciex) and protein identification was performed using the Mascot search engine against the LppX sequence with carbamidomethylation of cysteines set as fixed modification. Oxidation of methionine and glycosylation of serine and threonine were set as variable modifications. Peptide and fragment tolerance were set at 25 ppm and 0.05 Da respectively. Only peptides with Mascot ions scores higher than the identity threshold (30) at less than 1% of false positive discovery rate are considered.

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

## RESULTS

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

A single open reading frame (NCgl2009) was previously annotated as a putative phosphatidylglycerol::prolipoprotein diacylglyceryl transferase (Lgt) in the *C. glutamicum* ATCC13032 sequenced genome [46,47]. An additional BLASTp search using *M. tuberculosis*, *E. coli*, *M. smegmatis* MSMEG\_3222 and MSMEG\_5408 Lgt sequences did not identify any additional paralogs of the *lgt* gene in the genome of *C. glutamicum* 13032. Thus, similar to most Gram-positive bacteria, but in contrast to *M. smegmatis* and *S. coelicolor*, *C. glutamicum* ATCC13032 appears to possess only a single

copy of the *lgt* gene.

*NCgl2009* encodes a 316-aa long, 34kDa protein in which the residues defined as the “Lgt-signature” in *E. coli* Lgt (Y26, N126, G154, Y235) are all conserved (Suppl. Fig. 1) [44]. The protein has 23,6% and 36,8% identity to *E. coli* and *M. tuberculosis* Lgt (Rv1614), respectively. Previously, Rezwan *et al* proposed that *Actinomycetales* Lgts would be characterized by an additional C-terminal domain of unknown function [5]. Although *M. tuberculosis* and *M. smegmatis* Lgt (Rv1614: 468aa and MSMEG\_3222: 616aa) are indeed much longer than *E. coli* Lgt (291aa), *NCgl2009* (316aa) possesses only 25 additional residues compared with *E. coli* Lgt, suggesting it lacks this additional domain (Suppl. Fig. 1).

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

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

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

***The AmyE/MusE lipoprotein is not membrane associated in the C. glutamicum  $\Delta lgt$  mutant.***

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

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

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

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

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

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

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

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

Gram-negative bacteria lipoprotein biogenesis pathways. We hence tested whether *E. coli* *lgt* was able to complement *C. glutamicum*  $\Delta$ *lgt* and thus restore MusE membrane association. We cloned *E. coli* *lgt* gene in the shuttle vector pVWEx2 under the control of the *P<sub>tac</sub>* promoter and the resulting plasmid, pVWEx2-*Ec-lgt* was used to transform Cg- $\Delta$ *lgt*. MusE localization, used as a reporter for protein acylation, were tested by western blot on membrane and supernatant fractions. As observed for Cg- $\Delta$ *lgt* + pVWEx2-Cg-*lgt*, *E. coli* *lgt* fully restore MusE membrane association in the presence of 1mM IPTG (Fig. 4). Then, we tested whether the Cg- $\Delta$ *lgt* strain could be complemented by *M. smegmatis* *lgt* orthologues. The two *M. smegmatis* *lgt* genes (*MSMEG\_3222* and *MSMEG\_5408*) were cloned and their activity tested as above. Whereas *MSMEG\_3222* was able to complement Cg- $\Delta$ *lgt*, *MSMEG\_5408* was not, consistent with the mutation, in this protein, of 2 residues found to be essential for Lgt activity in *E. coli* (Y26, N126 according to *E. coli* Lgt numbering) (Suppl. Fig. 1)[37]. Since *MSMEG\_3222*, which possesses a C-terminal extension, was active in *C. glutamicum*, we then tested the role of its C-terminal extension. To do so, a plasmid encoding a *MSMEG\_3222* mutant lacking the 327 C-terminal residues, pVWEX<sub>2</sub>-*MSMEG\_3222* $\Delta$ Cter ( $\Delta$ 288-616) was constructed and transformed in Cg- $\Delta$ *lgt*. As seen in Fig. 4, the deleted mutant was as efficient as the full-length *MSMEG\_3222* to complement Cg- $\Delta$ *lgt*. Thus, the Lgt C-terminal domain, which is only present in some *Actinobacteria*, is not necessary for activity of *MSMEG\_3222*, at least in *C. glutamicum*. However, we cannot entirely exclude the possibility that this domain plays a more subtle effect on Lgt activity, not detected in these experiments.

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

(*Ec-lgt*) gene is expressed *in trans*. In this strain, *Ec-lgt* is present on a plasmid (pCHAP9231) and expressed under the control of the *P<sub>ara</sub>* promoter [44]. PAP9403 thus grows in the presence of 0.2% L-arabinose, whereas 0.1% D-fucose abolishes growth [44]. PAP9403 was hence transformed with the pVWEx2 derivatives encoding Cg-Lgt, MSMEG\_3222, MSMEG\_3222ΔCter or MSMEG\_5408 and its growth tested in the presence of 0.1% D-fucose and 1mM IPTG. Whereas PAP9403/pVWEx2-*Ec lgt* was able to grow in these conditions, the plasmids encoding Cg-Lgt, MSMEG\_3222, MSMEG\_3222ΔCter or MSMEG\_5408 did not restore growth (not shown). Thus, similarly to what has been observed for Lnt, *E. coli* Lgt is active in *C. glutamicum* whereas *C. glutamicum* and *M. smegmatis* enzymes are not active in *E. coli*, confirming a putative difference between Gram-negative and *Actinobacteria* lipoprotein biogenesis pathway.

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

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

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

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

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

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

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

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

In order to investigate the role of *Cg-lgt* in LppX biogenesis, wild-type and  $\Delta lgt$  strains of *C. glutamicum* were transformed with pCg482-LppX-HA-His, a shuttle vector in which expression of Mt-LppX<sub>HA-His</sub> is under the control of the *musE* promoter [30]. Membrane and secreted proteins were prepared from the transformed strains and analyzed by SDS-PAGE followed by immunoblotting using a monoclonal anti-HA-peroxidase conjugate. As previously observed, LppX was found both in the membrane and secreted fractions in the wild-type strain ([30] Fig. 6A). In contrast, LppX was not detected in the membrane fraction of *Cg-Δlgt* but found in the supernatant of this strain. As observed for MusE, the membrane localization of LppX was restored when the *Cg-Δlgt* strain was complemented with either *Cg-lgt*, *Ec-lgt*, *MSMEG\_3222* or *MSMEG\_3222ΔCter*, but not with *MSMEG\_5408* (Fig. 6A), confirming that, contrary to *MSMEG\_5408*, *E.coli* Lgt, *MSMEG\_3222* and *MSMEG\_3222ΔCter* are active in *C. glutamicum*. To test whether LppX secretion was a direct consequence of the lack of acylation, we expressed, in the wild-type strain, a LppX mutant in which the conserved C<sub>+1</sub> from the lipobox was changed to a leucine. As shown in Figure 6B, LppX-C1L is only detected in the supernatant of the wild-type strain, confirming that non-acylated lipoproteins are released in the culture supernatant of *Corynebacteriales* even when *lgt* is present.

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

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

diacylglyceryl transferase, LppX was not acylated in the *Cg-Δlgt* strain. Indeed, when the protein was purified by affinity chromatography from the supernatant fraction of the *Cg-Δlgt* strain and subjected to MALDI peptide mass fingerprinting, only peaks corresponding to non-acylated LppX<sub>1-29</sub> peptides could be detected (Suppl. Fig. 2). This result, together with confirming the role of *Cg-lgt*, also indicates that, as for MusE, acylation is not required for signal peptide cleavage. Interestingly, among the detected peaks, some corresponded to glycosylated forms of LppX<sub>1-29</sub> (Suppl. Fig. 2). This prompted us to examine the influence of *Cg-lgt* on LppX glycosylation. Previously, we showed that LppX is modified with hexoses (*m/z* 162) when expressed in *C. glutamicum* and that this modification (either mono, di, or tetraglycosylation) occurs on LppX<sub>6-29</sub> [30]. We hence purified LppX from the supernatant fraction of the *Cg-Δlgt* strain and performed nano-LC-MS/MS analysis after trypsin digestion. As previously found for LppX expressed in wild-type *C. glutamicum*, LppX<sub>6-29</sub> was found in mono, di, or tetraglycosylated forms in the *Cg-Δlgt* strain (Suppl. Fig. 3), indicating that Lgt-dependent S-acylation is not a prerequisite for glycosylation

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

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

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

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

The *Cg-ΔlspA* strain was then transformed with pCg482-LppX<sub>HA-His</sub> and LppX localization was analyzed as before. In *Cg-ΔlspA*, LppX behaves as in *Cg-Δlgt*: the protein is mostly localized in the culture supernatant and resolves at a higher mass than in the wild-type strain. When the *Cg-ΔlspA* strain was complemented with *Cg-lspA*, LppX recovered its initial migration behavior (Fig. 7B). This result is similar to what has been observed by Tschumi *et al.*, (2012) in *M. smegmatis* [31], where the migration behavior of LppX is similar in *Ms-ΔlspA* and *Ms-Δlgt* mutants. These authors concluded from this observation that signal peptide cleavage is abolished in the *Ms-Δlgt* strain [31]. However, our mass spectrometry analysis clearly showed that the LppX<sub>1-29</sub> signal peptide is cleaved in this strain.

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

(phosphatidylinositol mannosides) and LM/LAM (lipomannan/lipoarabinomannan). The *lpqW* gene could be inactivated in both *M. smegmatis* and *C. glutamicum* and the mutants obtained were severely affected in growth and unstable, frequently acquiring suppressor mutations elsewhere in the genome [60,65]. LpqB, a lipoprotein involved in the modulation of the MtrAB two-component system, was predicted as being essential in *M. tuberculosis*, but could be interrupted in *M. smegmatis* resulting in pleiotropic effects [66]. As the defects observed in the *lpqB* and *lpqW* mutant strains were more pronounced than those observed for the *lgt* strains, these lipoproteins probably retain at least part of their function when non-acylated. Several other examples of lipoproteins displaying activity when non lipid-modified have been reported in other genera: the non-acylated form of the essential *B. subtilis* lipoprotein PrsA, a foldase involved in protein secretion, is still active [25]. The MutA lipoprotein, involved in  $Mn^{2+}$  active transport, is necessary for *S. uberis* growth in milk. However, growth of a *S. uberis*  $\Delta lgt$  mutant is not affected in milk despite MutA being mislocalized [24]. Finally, the lipoprotein OpuAC, which is necessary for glycine betaine transport in *B. subtilis*, is also active when the conserved C<sub>+1</sub> cysteine is mutated to alanine [66].

### ***Interspecies complementation***

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

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

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

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

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

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

*In vitro* characterization of Lgt enzymes would also be useful to understand the function of the C-terminal extension found in some, but not all Lgts. Indeed, in addition to *M. tuberculosis* Lgt and MSMG\_3222 (469 and 612 amino-acids long respectively), other mycobacterial Lgt, such as Lgt from *Mycobacterium chubuense* (Mycch\_2541: 986aa), *Mycobacterium rhodesiae* (MycrhN\_5205: 774aa), *Mycobacterium intracellulare* (OCU\_30050:546aa), *Mycobacterium marinum* (MMAR\_2416: 714aa) and *Mycobacterium kansasii* (MKAN\_27935: 676aa) are predicted to be much longer than the 291 aa-long *E. coli*. This is not a generality however, as *Mycobacterium abscessus* and *Mycobacterium leprae* Lgts are respectively 343aa (MAB\_2642c) and 330aa (ML1274), which indicates that the presence of the C-terminal extension is not a strict requirement for Lgt function in *Mycobacterium*. Other *Corynebacteriales*, outside the *Mycobacterium* genus, also possess a C-terminal extension (*Gordonia bronchialis* (Gbro\_2945: 585aa) or *Nocardia farcinica* (NFA\_18630: 491aa)), but it appears to be completely absent in other genus (*Corynebacterium*, *Rhodococcus*). This C-terminal domain is extremely variable in size amongst Lgts and its sequence, which is rich in glutamate, aspartate and alanine residues, is not conserved or homologous to any domain of known function. Here, we found that this extension was not necessary for MSMEG\_3222 to restore LppX and MusE membrane localization in Cg-*Algt*, indicating that it is not required for activity in *C. glutamicum*, which is consistent with the absence,

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

### ***Signal peptide cleavage***

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

### ***Glycosylation***

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

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

## **AUTHOR STATEMENTS**

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

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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	AAGTCATGACCT <i>CGAGAACAACG</i>
2009-del2-BamHI	AGGGGACAGTT <i>TGGATCCCGG</i>
2009-del3-XhoI	AGGGCATTGCT <i>CGAGAAACG</i>
2009-del4-HindIII	AGACGAGGCCA <i>AGCTTGCCG</i>
2058-del1-BglII	TGGA <i>AGATC</i> TTTGTATTGCAAGCGCTG
2058-del2-XbaI	CTAGTCTAGAGGTCACGTTGACCATC
2058-del3-XbaI	GATCTCTAGAGACGAGAAGGATGAGGCCTGA
2058-del4-XhoI	TATGCTCGAGGTGGAAATGCGGAAAC
DirLppX-C1L	GGCTATCAGGA <i>cTTCTTCGCCG</i>
RevLppX-C1L	CGGCGAAGAA <i>gTCCTGATAGCC</i>
482-AS	GATATGATCATTTATTCTGC
482-S2	GCAGAATAAATGATCCGTCG
DirNCgl2009-XbaI	GCTCTAGACGTTGTTGTGGATGTCATGACTTTGG
RevNCgl2009-BamHI	GCGGATCCCTATCGCGTTGAGGGCGTTTCTCC
DirEc-lgt-XbaI	GGTCTAGAGCGAAATAACAAGAAATTGTGG
RevEc-lgt-BamHI	CGCGGATCCTTAGGAAACGTGTTGCTGTGG
DirMSMEG_3222-XbaI	GCTCTAGAGGTGTTTCGGCGGAGGGTGTGAGCTTGACC
RevMSMEG_3222-BamHI	GCGGATCCCTAGCGGTTGCGCCGCAACCAACCGGCGC
RevMSMEG_3222-A288-BamHI	GCGCGGATCCTTAGGCCTTGCCACGCAACGATTCCGG
DirMSMEG_5408-XbaI	GCTCTAGACGCGAAGGGCGTTACGTTGAGCAGG
RevMSMEG_5408-BamHI	GCGGATCCTCATACCGGCACTTCCTCACTGCGGG
DirCg-lspA-XbaI	GCTCTAGACCGCTCATGGGGAAAGATGGTCAACG
RevCg-lspA-BamHI	GCGGATCCTCAGGCCTCATCCTTCTCGTCAGTTGC

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## FIGURES LEGENDS

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

## **Figure 2: MusE is secreted in *C. glutamicum* $\Delta lgt$ .**

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

**Figure 3: MALDI PMFs of MusE proteins purified from *C. glutamicum* WT,  $\Delta lnt$  and  $\Delta lgt$  strains.**

The whole MALDI PMF mass spectra of MusE proteins are shown ( $m/z$  800-5000) and detected monoisotopic  $[M+H]^+$  peaks are indicated. Inserts highlight the region of the spectra with the N-terminal MusE peptide. The  $m/z$  3451.65 and the  $m/z$  4045.89 peaks match to the MusE<sub>334-365</sub> and MusE<sub>55-91</sub> tryptic peptides respectively. They are detected in all spectra. The  $m/z$  4097.26 peak is detected only in the WT strain and corresponds to the tri-acylated N-terminal MusE<sub>1-29</sub> peptide while the  $m/z$  3858.92 peak is detected only in the  $\Delta lnt$  strain and corresponds to the di-acylated N-terminal MusE<sub>1-29</sub> peptide [30]. The  $m/z$  3282.50 and 3358.50 peaks are specific of the  $\Delta lgt$  MusE spectrum. Both peaks correspond to the unacylated N-terminal MusE<sub>1-29</sub> peptide. The  $m/z$  3282.50 corresponds to the standard MusE<sub>1-29</sub> peptide and the  $m/z$  3358.50 correspond to a beta-mercaptoethanol adduct of the same MusE<sub>1-29</sub> peptide (Delta mass=76). In the  $\Delta lgt$  strain, no peaks corresponding to mono, di or tri-acylated MusE<sub>1-29</sub> peptide are observed.

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

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

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

**Figure 6: Acylation is necessary for LppX membrane localization.** **(A)** Membrane (M) and secreted (S) proteins corresponding to an OD<sub>600nm</sub> of 10 were prepared from WT strain transformed with pCg482-LppX-HA-His, Cg- $\Delta lgt$  + pCg482-LppX-HA-His, pCg482-LppX-HA-His + pVWEx2-Cg-lgt or Cg- $\Delta lgt$  + pCg482-LppX-HA-His + pVWEx2-*Ec-lgt* (upper panel) Cg- $\Delta lgt$  + pCg482-LppX-HA-His + pVWEx2-*MSMEGM\_3222*, Cg- $\Delta lgt$  + pCg482-LppX-HA-His + pVWEx2-*MSMEGM\_3222- $\Delta Cter$*  or Cg- $\Delta lgt$  + pCg482-LppX-HA-His + pVWEx2-*MSMEGM\_5408* (lower panel) grown in the absence or in the presence of 1mM IPTG, and analyzed by immunoblotting using anti-HA peroxidase conjugate (Roche). **(B)** The same experiments were performed on wild-type strain transformed with pCg482-LppX-HA-His; Cg- $\Delta lgt$  + pCg482-LppX-HA-His and Cg- $\Delta lgt$  + pCg482-LppX-C1L-HA-His + pVWEx2-Cg-lgt

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

1025 (Roche) to detect LppX (C).

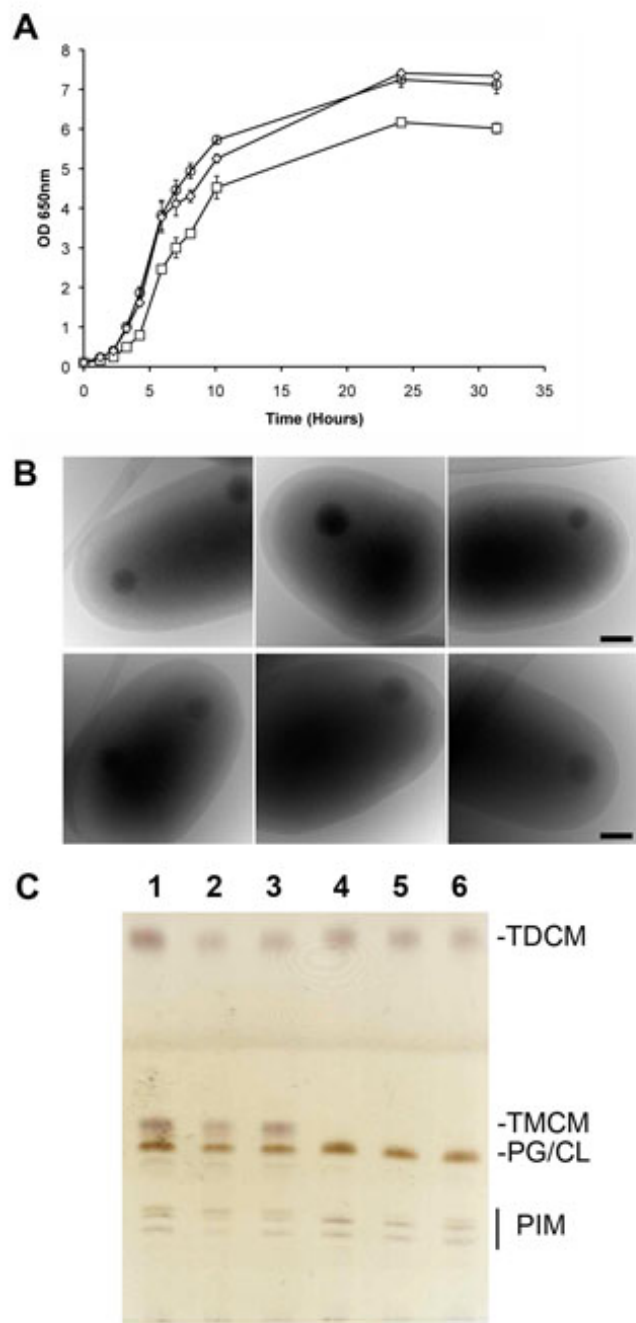


Figure 1

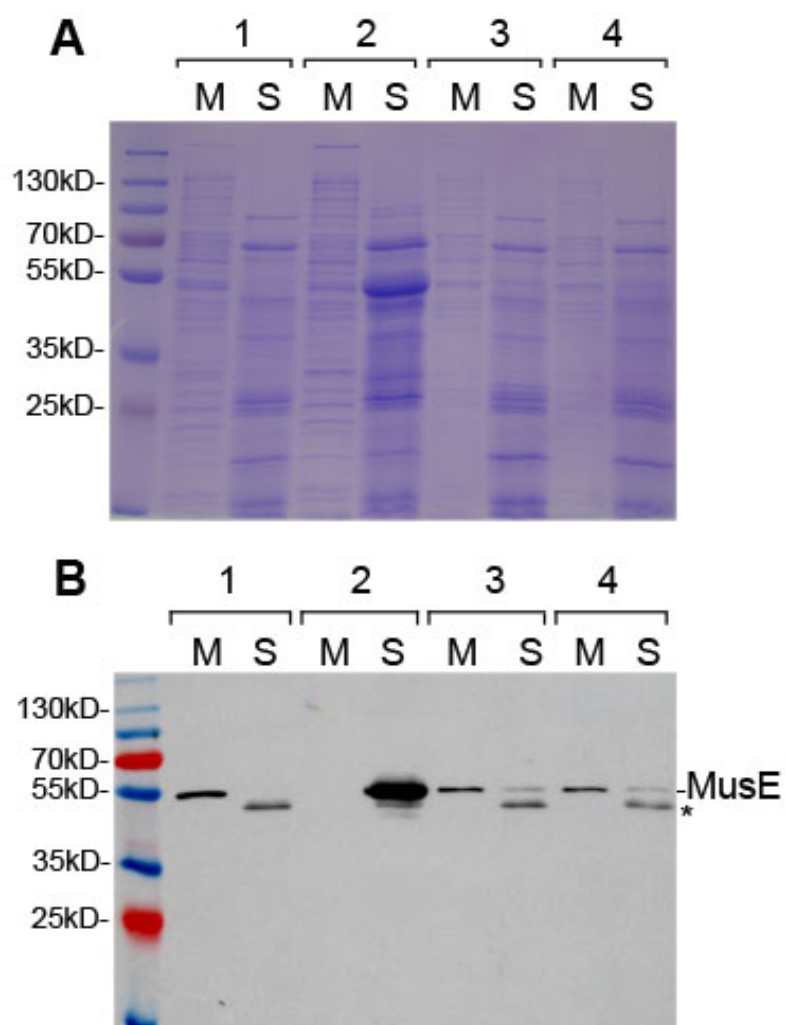


Figure 2

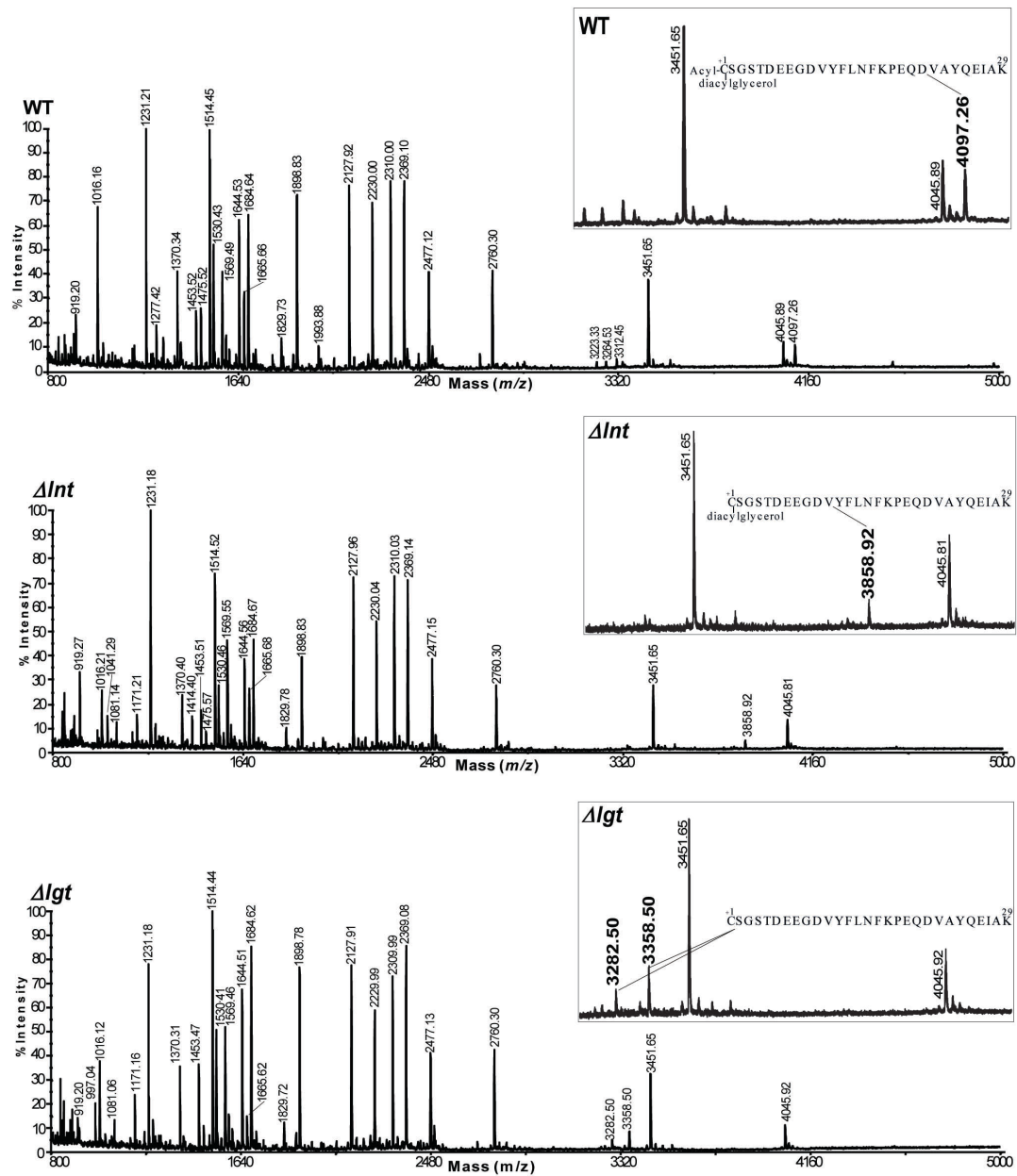


Fig.3

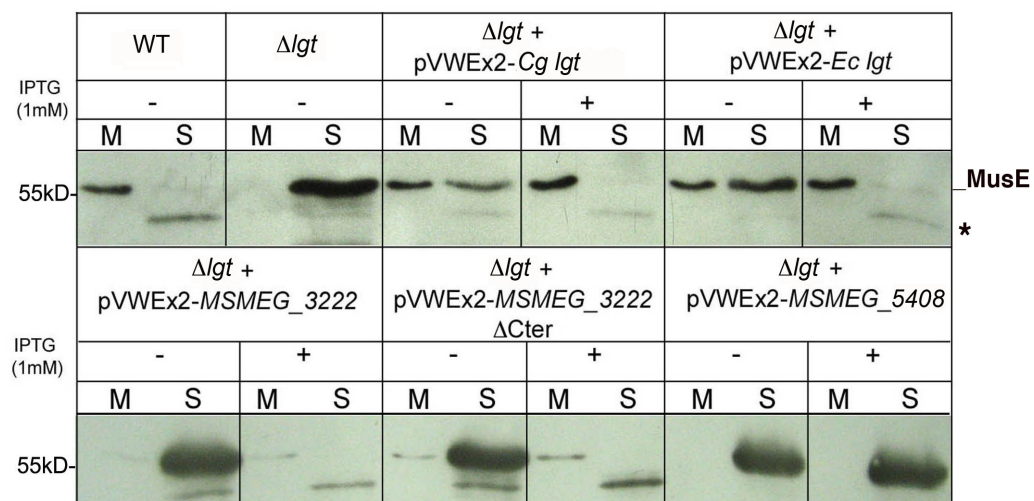


Figure 4

