MICROBIOLOGY

RESEARCH ARTICLE

Dautin *et al.*, *Microbiology* 2020;166:759–776

DOI 10.1099/mic.0.000937

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

Nathalie Dautin[1,*,†], Manuela Argentini[1‡], Niloofar Mohiman[1§], Cécile Labarre[1], David Cornu[1], Laila Sago[1], Mohamed Chami[2], Christiane Dietrich[1], Célia de Sousa d'Auria[1], Christine Houssin[1], Muriel Masi[1], Christophe Salmeron[1#] and Nicolas Bayan[1]

Abstract

Bacterial lipoproteins are secreted proteins that are post-translationally lipidated. Following synthesis, preprolipoproteins are transported through the cytoplasmic membrane via the Sec or Tat translocon. As they exit the transport machinery, they are recognized by a phosphatidylglycerol::prolipoprotein diacylglyceryl transferase (Lgt), which converts them to prolipoproteins by adding a diacylglyceryl group to the sulfhydryl side chain of the invariant Cys_{+1} residue. Lipoprotein signal peptidase (LspA or signal peptidase II) subsequently cleaves the signal peptide, liberating the α-amino group of Cys_{+1}, which can eventually be further modified. Here, we identified the *lgt* and *lspA* genes from *Corynebacterium glutamicum* and found that they are unique but not essential. We found that Lgt is necessary for the acylation and membrane anchoring of two model lipoproteins expressed in this species: MusE, a *C. glutamicum* maltose-binding lipoprotein, and LppX, a *Mycobacterium tuberculosis* lipoprotein. However, Lgt is not required for these proteins' signal peptide cleavage, or for LppX glycosylation. Taken together, these data show that in *C. glutamicum* the association of some lipoproteins with membranes through the covalent attachment of a lipid moiety is not essential for further post-translational modification.

INTRODUCTION

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

After synthesis in the cytoplasm, preprolipoproteins are transported through the cytoplasmic membrane via the Sec or Tat translocon. As they exit the translocon, they are recognized by a phosphatidylglycerol::prolipoprotein diacylglyceryl transferase (Lgt), which converts them to prolipoproteins by adding a diacylglyceryl group, derived from phospholipid phosphatidylglycerol, to the sulfhydryl group of C_{+1}. Lipoprotein signal peptidase (LspA or signal peptidase II) subsequently cleaves the signal peptide, liberating the α-amino

Received 17 November 2019; Accepted 05 May 2020; Published 03 June 2020

Author affiliations: [1]Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Univ. Paris-Sud, Université Paris-Saclay, 91198, Gif-sur-Yvette cedex, France; [2]CBioEM lab, Biozentrum, University of Basel, 4058 Basel, Switzerland.

***Correspondence:** Nathalie Dautin, dautin@ibpc.fr

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

Abbreviations: ACN, Acetonitrile; DTT, Dithiothreitol; FA, Formic Acid; Lgt, prolipoprotein diacylglyceryl transferase; Lnt, apolipoprotein N-acyltransferase; LspA, Lipoprotein signal peptidase; MALDI-TOF, Matrix Assisted Laser Desorption Ionization- Time of Flight; OM, Outer Membrane; PMF, Peptide Mass Fingerprinting; TFA, Trifluoroacetic Acid.

†Present address: Laboratoire de Biologie Physico-Chimique des Protéines Membranaires, CNRS, Institut de Biologie Physico-Chimique, F-75005 Paris, France

‡Present address: Sorbonne Université, INSERM, CNRS, Institut de la Vision, 17 rue Moreau, 75012 Paris, France

§Present address: Curakliniken, Erikslustvägen 22, 217 73 Malmö, Sweden

#Present address: Observatoire Océanologique de Banyuls Sur Mer, FR 3724-Laboratoire Arago - Sorbonne Université / CNRS, France.

Three supplementary figures are available with the online version of this article.

group of C_{+1}, which in some organisms can be further modified by an apolipoprotein *N*-acyltransferase, Lnt [1, 2, 7].

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

In diderm Gram-negative bacteria, such as *E. coli* or *Salmonella enterica*, the third step consists in N-acylation of the diacylglyceride $C_{+1}$by the apolipoprotein N-acyltransferase (Lnt), resulting in mature triacylated proteins [8, 9]. These mature lipoproteins are then mostly (~90%) found in the outer membrane (OM), to which they are targeted by the dedicated Lol (*l*ocalization *o*f *l*ipoproteins) system [8]. Although Lnt-dependent lipoprotein triacylation appears to be the rule for most Gram-negative bacteria, a 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 to be essential genes in all Gram-negative bacteria and thus have attracted attention as potential new 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 lipoprotein biogenesis is performed by other, yet uncharacterized, enzymes. Interestingly, lipoprotein 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 that of 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 two *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 lipoprotein 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, *Δlgt* and *Δlsp* 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*, an *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 *Mycobacterium tuberculosis*, *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG, as well as *Corynebacterium glutamicum*. In all cases, Lnt activity was dispensable for growth [29–31, 35]. Although *M. tuberculosis* and *M. bovis* both possess a second *lnt* gene, the catalytic cysteine residue is replaced by a serine in these paralogues, suggesting that they are either inactive or function with a different mechanism than that proposed for *E.coli* Lnt.

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

In contrast, the results obtained with *lgt* mutants differ between species. The *lgt* gene is essential *in M. tuberculosis* [37]. In *M. smegmatis,* two *lgt* paralogues were identified (*MSMEG_3222* and *MSMEG_5408*). Because MSMEG_5408 lacks important catalytic residues, only MSMEG_3222 has been studied further. A *ΔMSMEG_3222* mutant is viable but shows growth defects [37]. Surprisingly, lipoproteins are released massively in the culture supernatant of this mutant, despite the presence of the mycomembrane. This may result from alterations of the mycobacterial cell envelope. However, the observation that lipoproteins mutated at the conserved C_{+1} (i.e. non-acylated) are also released in the culture medium when produced in the wild-type (WT) strain of *M. smegmatis* or *C. glutamicum* [30, 38] rules out the above hypothesis and the molecular mechanism underlying this release remains obscure.

Previously, we showed that lipoproteins are triacylated in *C. glutamicum,* an environmental *Corynebacteriales* used for the production of amino acids in the food industry and a model organism for the study of mycobacteria cell wall biogenesis. We identified the non-essential gene *Cg-ppm2(=lnt)* as

Table 1. Oligonucleotides used in this study. Restriction sites used for cloning are indicated in italic

Name	Sequence (5′ to 3′)
2009-del1-XhoI	AAGTCATGAC*CTCGAG*AACAACG
2009-del2-BamHI	AGGGGACAGTT*GGATCC*CGG
2009-del3-XhoI	AGGGCATTG*CTCGAG*AAACG
2009-del4-HindIII	AGACGAGGCC*AAGCTT*GCCG
2058-del1-BglII	TGGA*AGATCT*TTGTATTGCAAGCGCTG
2058-del2-XbaI	CTAG*TCTAGA*GGTCACGTTGACCATC
2058-del3-XbaI	GATC*TCTAGA*GACGAGAAGGATGAGGCCTGA
2058-del4-XhoI	TATG*CTCGAG*GTGGAAATGCGGAAAC
DirLppX-C1L	GGCTATCAGGActTTCTTCGCCG
RevLppX-C1L	CGGCGAAGAAagTCCTGATAGCC
482-AS	GATATGATCATTTATTCTGC
482-S2	GCAGAATAAATGATCCGTCG
Dir*NCgl2009*-XbaI	GC*TCTAGA*CGTTGTTGTGGATGTCATGACTTTGG
Rev*NCgl2009*-BamHI	GC*GGATCC*CTATCGCGTTGAGGGCGTTTCTCC
Dir*Ec-lgt*-XbaI	GG*TCTAGA*GCGAAATAACAAGAAATTGTGG
Rev*Ec-lgt*-BamHI	CGC*GGATCC*TTAGGAAACGTGTTGCTGTGG
Dir*MSMEG_3222*-XbaI	GC*TCTAGA*GGTGTTCGGCGGAGGGTGTCAGCTTGACC
Rev*MSMEG_3222*-BamHI	GC*GGATCC*CTAGCGGTTGCGCCGCAACCACCGGCGC
Rev*MSMEG_3222*-A288-BamHI	GCGC*GGATCC*TTAGGCCTTGCCACGCAACGATTCCGG
Dir*MSMEG_5408*-XbaI	GC*TCTAGA*CGCGAAGGGCGTTACGTTCAGCAGG
Rev*MSMEG_5408*-BamHI	GC*GGATCC*TCATACCGGCACTTCCTCACTGCGGG
Dir*Cg-lspA*-XbaI	GC*TCTAGA*CCGCTCATGGGGAAAGATGGTCAACG
Rev*Cg-lspA*-BamHI	GC*GGATCC*TCAGGCCTCATCCTTCTCGTCAGTTGC

necessary for lipoprotein N-acylation and subsequent glycosylation [30]. Here, we used two model lipoproteins to identify and 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 on 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.

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^{-1}, 10 µg ml^{-1}, 5 µg ml^{-1}, 1 mM and 10% (wt/vol), respectively. Transformation of *C. glutamicum* by electroporation was performed as described by Bonamy *et al.* [40].

Strains and plasmids

Strains

All of the *C. glutamicum* strains used in this study are derivative of the strain ATCC 13032 RES167, which is referred to here as the wild-type (WT) [41]. *E. 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.* [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

primer sequences) and cloned into the nonreplicative vector pK18mobSac. The resulting plasmid (pK18mobsac*Δlgt*) 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^r clones on BHI plates containing sucrose. $Km^s$and Suc^r 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 (*Δlgt*) was selected for further study. The same strategy was used to construct the *C. glutamicum ΔlspA 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 described previously [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 two 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 the primers Dir*NCgl2009*-XbaI and Rev*NCgl2009*-BamHI (Table 1). The PCR product obtained (983 bp) was introduced between the XbaI and BamHI sites of pVWEx2-*ppm1* [30]. The *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 (921 bp) was then introduced in pVWEx2-*ppm1* 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^2155 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 and 908 bp, respectively) were then introduced between the XbaI and BamHI sites of pVWEx2-*ppm1*. The same strategy was used to construct pVWEx2-*MSMEG_5408,* except that Dir*MSMEG_5408*-XbaI and Rev*MSMEG_5408*-BamHI (Table 1) were used 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 into the same sites of pVWEx2-*ppm1*. 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 a low-dose system (20 to 40 $e^-/Å^2$) and keeping the sample at −175 °C. Defocus values were −4 µm. Micrographs were recorded on a 4×4K TemCam-F416 CMOS-based camera (TVIPS, Germany).

Cell fractionation

Ten-millilitre cultures of *C. glutamicum* were grown overnight in BHI medium at 30 °C with vigorous shaking (220 r.p.m.). Approximately 2 ml culture aliquots (equivalent to OD_{650nm}=10) were centrifuged, cell pellets were resuspended in 1 ml of 50 mM Tris/HCl pH 7.0 with glass beads and vortexed for 15 min. Unbroken cells were discarded, and total membranes were collected by centrifugation (230000 ***g***, 30 min) in a Beckman TLA 100.3 rotor. Proteins in the culture supernatant were precipitated with 10% trichloroacetic acid (TCA) at 4 °C for 30 min. The precipitated proteins were recovered by centrifugation (16000 ***g***; 15 min) and washed with cold acetone. Protein samples were separated by SDS-PAGE and analysed by immunoblotting or Coomassie stain.

Proteins purification

6His-tagged MusE (AmyE) and LppX were purified from the WT *C. glutamicum* membrane fraction as described previously [30].

For MusE purification from the culture supernatant, 300 ml of BHI was inoculated with the *Cg-Δlgt*/pCg482-MusE-His strain and incubated at 30 °C with vigorous shaking (220 r.p.m.) overnight. Cells were removed by centrifugation, and culture supernatants were concentrated using ultrafiltration units (Amicon YM 10 kDa) (1 ml min^{-1} for 5 h at 4 °C). The resulting protein concentrate was dialyzed extensively against 25 mM phosphate buffer pH 8.0 at 4 °C and loaded on a Ni-NTA column (Quiagen Ni-NTA superflow) previously equilibrated with 25 mM phosphate buffer pH 8.0 (buffer A). The column was washed with ten column volume of buffer A containing 10 mM imidazole and His-tagged proteins were eluted with buffer A containing 250 mM of imidazole. Elution fractions were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining.

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 -µm filter. The filtrate was then subjected to protein precipitation using 50 and 70% ammonium sulfate. The resulting pellets were solubilized in a 25 mM phosphate buffer and dialyzed extensively against 25 mM phosphate buffer pH 8.0 at 4 °C. Further purification of LppX was performed as described previously [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 washed extensively with acetonitrile (ACN) and 25 mM ammonium bicarbonate. For Matrix Assisted Laser Desorption Ionization (MALDI) peptide mass fingerprint (PMF), the cleaned bands were directly submitted to trypsin digestion without other treatment. For nanoLC-MS/MS (Liquid Chromatrography-Mass Spectrometry), 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 the addition of 55 mM iodoacetamide. After removal of the supernatant, the washing procedure was repeated and gel slices were dried and submitted to enzymatic digestion. For all gel bands, trypsin digestion was performed overnight at room temperature by the addition of 20 µl of 10 ng µl^{-1} porcine gold trypsin (Promega) diluted in 25 mM 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 µl of 1% dodecyl maltoside (Anatrace, *n*-dodecyl-*β*-D-maltopyranoside DDM, Sol-Grade) was added and the solution was mixed (1500 r.p.m.) for 1 h at room temperature; 35 µl of a 2:1 chloroform :methanol solution was added and the solution was mixed as before. Peptide extracts from the aqueous and organic phases (0.5 µl) were quickly spotted onto a 0.5 µl droplet of 2,5-dihydroxybenzoic acid [Sigma-Aldrich; 20 mg ml^{-1}, 50% ACN, 0.1% trifluoroacetic acid (TFA)]. Peptide mixtures were analysed in the positive reflectron mode with the Voyager-DE STR MALDI-TOF mass spectrometer (Absciex) and ~500 mass spectra were averaged per spot. Mass spectrometry analyses were performed with Data Explorer software (Absciex). MALDI PMF spectra were calibrated internally with tryptic peptides of *m/z* 3451.65 ($MusE_{334-365}$) and 4045.89 ($MusE_{55-91}$) for MusE and *m/z* 3164.99 ($LppX_{200-224}$) and 3296.34 ($LppX_{114-146}$) for LppX.

Data analyses were performed with Mascot (Matrix Science, London, UK) and Peptide Mass (https://web.expasy.org/pepide_mass/) search engines on MusE and LppX protein sequences with a mass accuracy of <50 p.p.m.

Tryptic peptides for nanoLC-MS/MS analysis were extracted first by the addition of 20 µl of 50% ACN and 0.1% formic acid (FA) and second by addition of 20 µl of 100% ACN. 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 NanoRSLC system (Thermo Scientific). Briefly, peptides were desalted on a C18 reverse phase pre-column (C18 Acclaim Pepmap100, 3 µm, 100 Å, 75 µm i.d., 2 cm length) using a loading buffer containing H2O/ACN/TFA (98%/2%/0.05%) at 5 µl min^{-1} and were then eluted at a flow rate of 300 nl min^{-1} from the reverse phase analytical C18 column (C18 Acclaim Pepmap100, 2 µm, 100 Å, 75 µm 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_2O/FA (100%/0,1%). NanoLC-MS/MS experiments were conducted using the data-dependent acquisition method by selecting the 20 most intense precursors for CID fragmentation with the Q1 quadrupole set at low resolution for better sensitivity and with a collision energy ramp (±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 p.p.m. and 0.05 Da, respectively. Only peptides with Mascot ions scores higher than the identity threshold (30) at <1% 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*, and *M. smegmatis* MSMEG_3222 and MSMEG_5408 Lgt sequences did not identify any additional paralogues 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, 34 kDa protein in which the residues defined as the 'Lgt signature' in *E. coli* Lgt (Y26, N126, G154, Y235) are all conserved (Fig. S1, available in the online version of this article) [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) only possesses 25 additional residues compared with *E. coli* Lgt, suggesting that it lacks this additional domain (Fig. S1).

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 that of 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_{tac} promoter. The Cg-*Δlgt* strain, when transformed with pVWEX2-*Cg-lgt*, did indeed grow as efficiently as the WT in the presence of IPTG (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 WT strain (Fig. 1b). Sensitivity to antibiotics and detergents was not increased in the *Δlgt* strain compared to the WT (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 the *C. glutamicum* cell envelope.

The AmyE/MusE lipoprotein is not membrane-associated in the *C. glutamicum Δlgt* 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_{+1} to leucine, the protein is not membrane-associated, but released in the culture medium [30]. Identical results were obtained for two C_{+1}-mutated *M. smegmatis* lipoproteins [38]. In addition, Tschumi *et al.* [37] reported that deletion of *M. smegmatis lgt* causes the release of lipoproteins in the extracellular medium. Taken together, these results suggest that in *Actinobacteria* the absence of S-acylation leads to lipoprotein release in the extracellular space.

We thus reasoned that if NCgl2009 indeed possesses 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. The Cg-*Δlgt* strain did indeed appear to secrete more proteins than the WT 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 fractions of the WT strain, whereas a shorter form, that may represent a processed form of the secreted protein, was found in the culture supernatant (Fig. 2b). In contrast, MusE was detected in the supernatant of the *Δlgt* strain, mostly in an unprocessed form. 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, WT 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-$_{6His}$ was purified by affinity chromatography from the membrane fraction of the WT and Δ*lnt* 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 Δ*lgt* mutant. In that case, a tryptic peptide of $[M+H]^+$=4870.3147 is expected (IASISMASMLAAASLVA **C**$^{+1}$SGSTDEEGDVYFLNF KPEQDVAYQEIAK). However, no peak corresponding to this mass was observed in any of the strains 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 *Δlgt* strain (Fig. 3, lower panel). The *m/z* 3282.50 peak matches to the N-terminal unmodified MusE1-29 peptide (C^{+1}SGSTDEEGDVYFLNFKPEQDVAYQEIAK, expected $[M+H]^+$=3282,48; mass accuracy=7 p.p.m.) and the *m/z* 3358.50 peak corresponds to the same peptide harbouring a beta-mercaptoethanol adduct (expected Δmass=76), most probably linked by a disulfide bond to the Cys_{+1}. In order to confirm that MusE is not acylated in the *Δlgt* strain, MALDI PMF mass spectra from WT, *Δlnt* and *Δlgt* 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 WT strain and the diacylated peptide was observed in the *Δlnt* strain (Fig. 3b). However, no N-terminal acylated MusE peptide was detected in the *Δlgt* strain (Fig. 3b). Altogether, these results show that MusE, a bona fide triacylated, membrane-associated lipoprotein, is not acylated in the *Δlgt* strain, indicating that this gene is necessary for the initial step of lipoprotein triacylation and hence encodes the *C. glutamicum* phosphatidylglycerol::prolipoprotein diacylglyceryl 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.

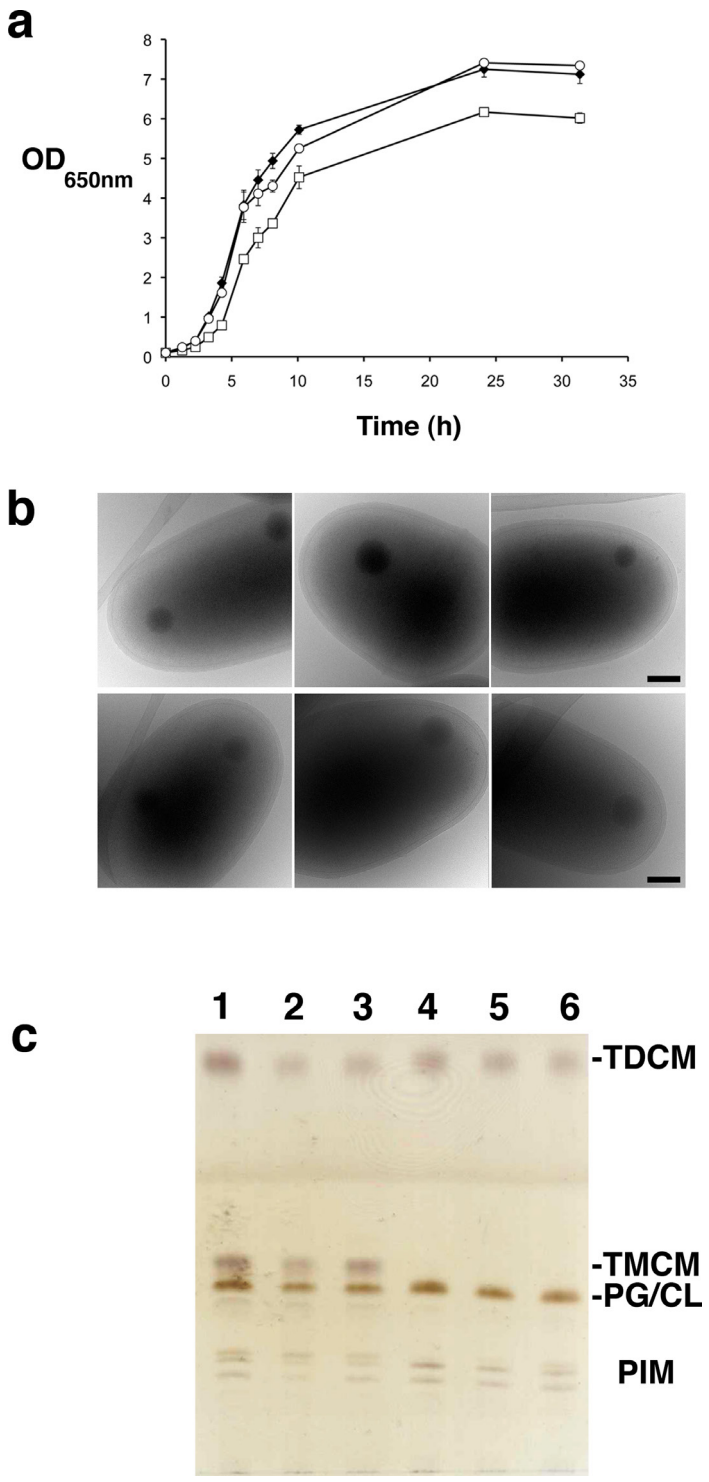

Fig. 1. Characterization of *C. glutamicum Δlgt*. (a) *C. glutamicum* WT (circles) and Δ*lgt* (squares) were grown in BHI medium at 30 °C. Cg-Δ*lgt*+pVWEx2-*Cg-lgt* (diamonds) was grown at 30 °C in BHI supplemented with 1 mM IPTG. Shown is a representative experiment of strains grown in triplicate, where errors bars indicate standard deviation from the mean. (b) Cryo-TEM of three selected bacteria from the WT *C. glutamicum* strain (top) and the Cg-Δ*lgt* strain (bottom). Scale bar: 200 nm. (c) TLC analysis of total lipids extracted from whole *C. glutamicum* WT (lanes 1 and 4), Δ*lgt* (lanes 2 and 5) or Δ*lgt*+ pVWEx2-*Cg-lgt* (lanes 3 and 6) in the exponential (lanes 1–3) or stationary (lanes 4–6) phase of growth. TLC plates were developed in the solvent system $CHCl_3/CH_3OH/H_2O$ (65/25/4 by volume) and revealed by immersion in 10% H_2SO_4 in ethanol followed by heating. PG, phosphatidylglycerol; CL, cardiolipin; PIM, phosphatidylinositol mannosides; TMCM, trehalose monocorynomycolates; TDCM, trehalose dicorynomycolates.

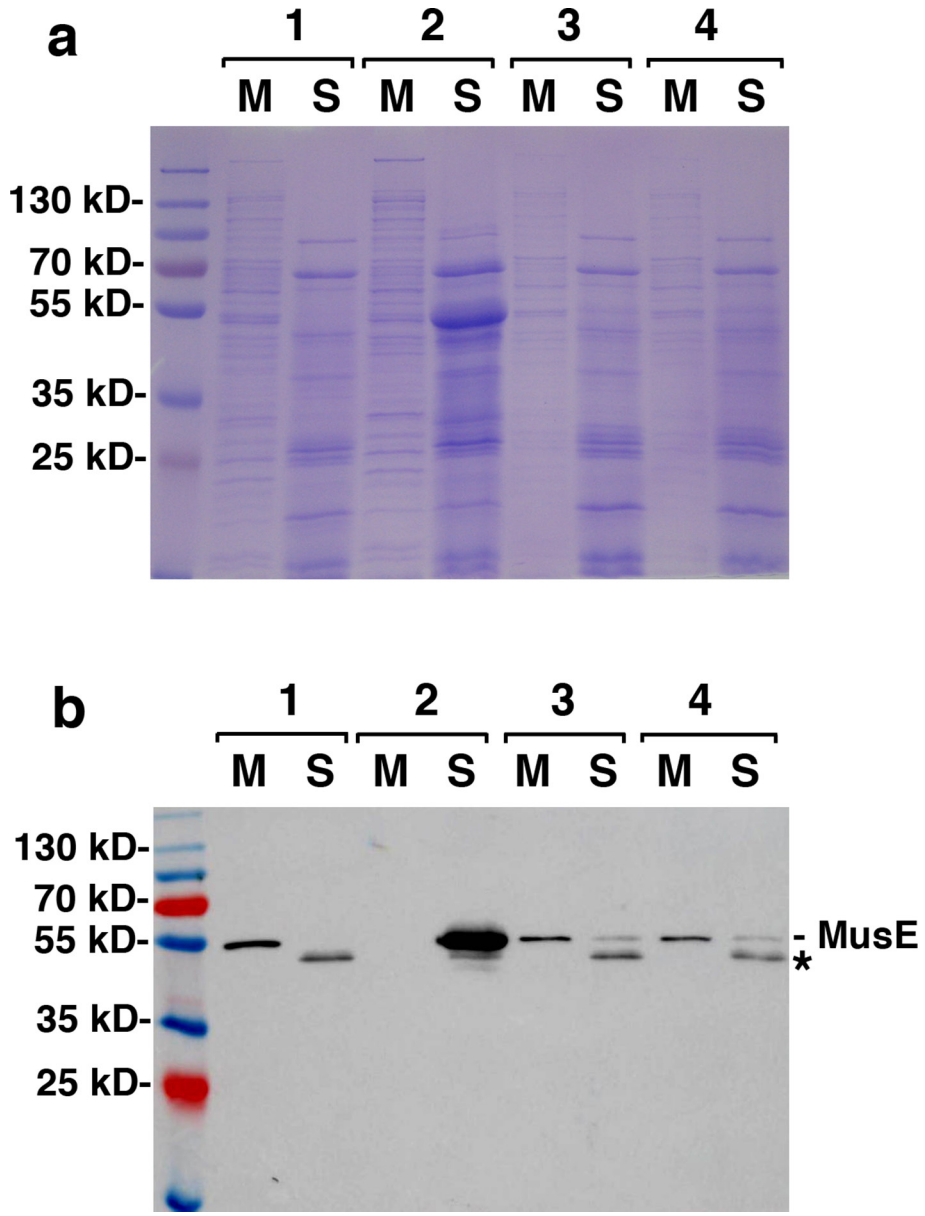

Fig. 2. MusE is secreted in *C. glutamicum* Δ*lgt*. Membrane (M) and secreted (S) proteins corresponding to an OD_{650nm} of 10 were prepared for WT strain (1), Cg-*Δlgt* (2) and Cg-*Δlgt* + pVWEX2-*Cg-lgt* grown in the absence (3) or presence of 1 mM IPTG (4), and analysed by SDS-PAGE followed by coloration with Coomassie blue (a) or immunoblotting using polyclonal anti-MusE antibodies (b). The band labelled with an asterisk corresponds to the previously observed shorter form of MusE [30].

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 Δlgt* and thus restore MusE membrane association. We cloned he *E. coli lgt* gene in the shuttle vector pVWEx2 under the control of the P_{tac} promoter and the resulting plasmid, pVWEx2-*Ec-lgt*, was used to transform Cg-*Δlgt*. MusE localization, used as a reporter for protein acylation, were tested by Western blot on membrane and supernatant fractions. As observed for Cg-*Δlgt*+pVWEx2-*Cg-lgt*, *E.coli lgt* fully restored MusE membrane association in the presence of 1 mM IPTG (Fig. 4). We then tested whether the Cg-*Δlgt* 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 was tested as above. Whereas

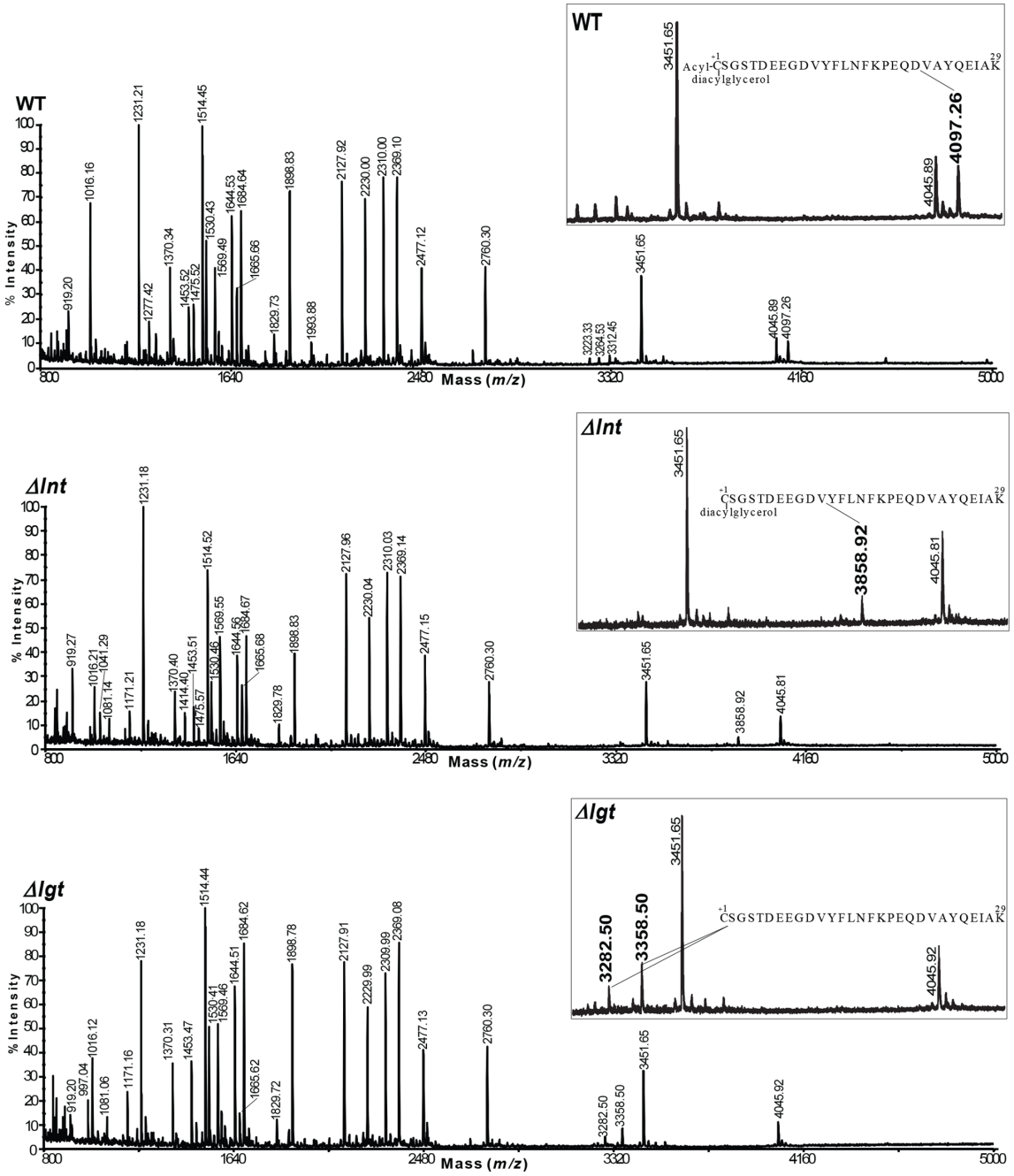

Fig. 3. MALDI PMFs of MusE proteins purified from *C. glutamicum* WT, Δ*lnt* and Δ*lgt* strains. The whole MALDI PMF mass spectra of MusE proteins are shown (*m/z* 800–5000) and detected monoisotopic $[M+H]^{+1}$ peaks are indicated. Inserts highlight the region of the spectra with the N-terminal MusE peptide. The *m/z* 3451.65 and the *m/z* 4045.89 peaks match to the $MusE_{334-365}$ and $MusE_{55-91}$ tryptic peptides respectively. They are detected in all spectra. The *m/z* 4097.26 peak is only detected in the WT strain and corresponds to the tri-acylated N-terminal $MusE_{1-29}$ peptide, while the *m/z* 3858.92 peak is only detected in the Δ*lnt* strain and corresponds to the di-acylated N-terminal $MusE_{1-29}$ peptide [30]. The *m/z* 3282.50 and 3358.50 peaks are specific of the Δ*lgt* MusE spectrum. Both peaks correspond to the unacylated N-terminal $MusE_{1-29}$ peptide. The *m/z* 3282.50 corresponds to the standard $MusE_{1-29}$ peptide and the *m/z* 3358.50 corresponds to a beta-mercaptoethanol adduct of the same $MusE_{1-29}$ peptide (delta mass=76). In the Δ*lgt* strain, no peaks corresponding to mono-, di- or tri-acylated $MusE_{1-29}$ peptides are observed.

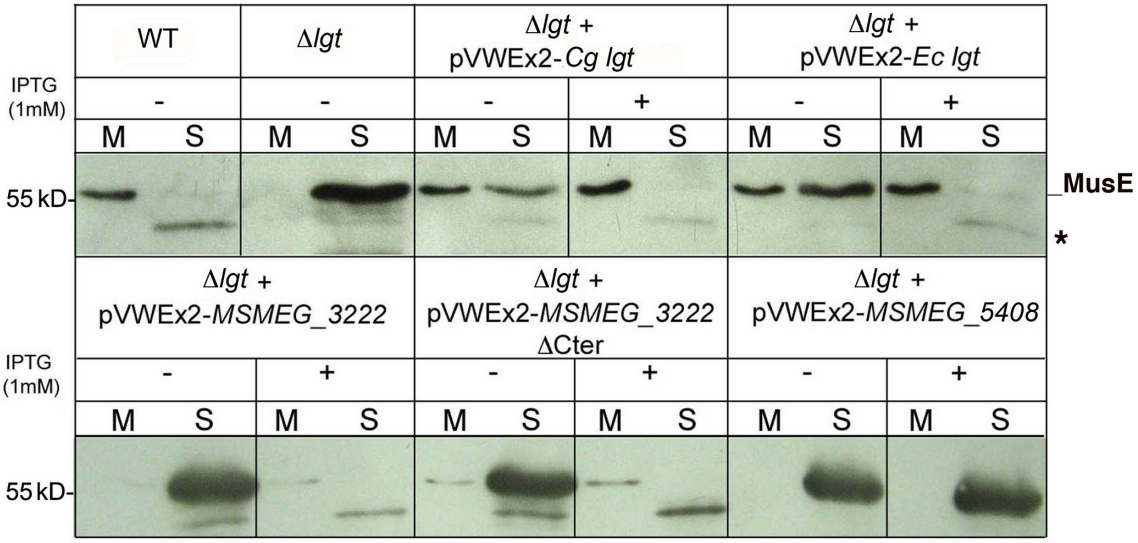

Fig. 4. Role of Cg-*lgt* homologues in MusE localization. Cells transformed with the indicated plasmids were grown overnight in BHI at 30 °C in the presence or absence of IPTG 1 mM. Membrane (M) and secreted (S) fractions were prepared and analysed by SDS-PAGE followed by immunoblotting using polyclonal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

MSMEG_3222 was able to complement Cg-*Δlgt*, *MSMEG-5408* was not, consistent with the mutation, in this protein, of two residues found to be essential for Lgt activity in *E. coli* (Y26 and N126 according to *E.coli* Lgt numbering) (Fig. S1) [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$_2$-*MSMEG_3222*ΔCter (Δ288–616) was constructed and transformed in Cg-*Δlgt*. As seen in Fig. 4, the deleted mutant was as efficient as the full-length MSMEG_3222 in complementing Cg-*Δlgt*. 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 has a more subtle effect on Lgt activity that was 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^R 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_{ara} 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 was tested in the presence of 0,1% D-fucose and 1 mM 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 the Gram-negative and *Actinobacteria* lipoprotein biogenesis pathways.

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 followed the growth of the Cg-*Δlgt* strain in minimal medium supplemented with either maltose or glucose. As previously observed in rich medium, the growth of Cg-*Δlgt* was impaired compared to the WT strain. However, the effect was much more pronounced in media with a restricted carbon source than in complex media (compare Figs 5a and 1a). Still, the Cg-*Δlgt* mutant growth rate was identical whether the minimal medium was supplemented with glucose or maltose as a 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

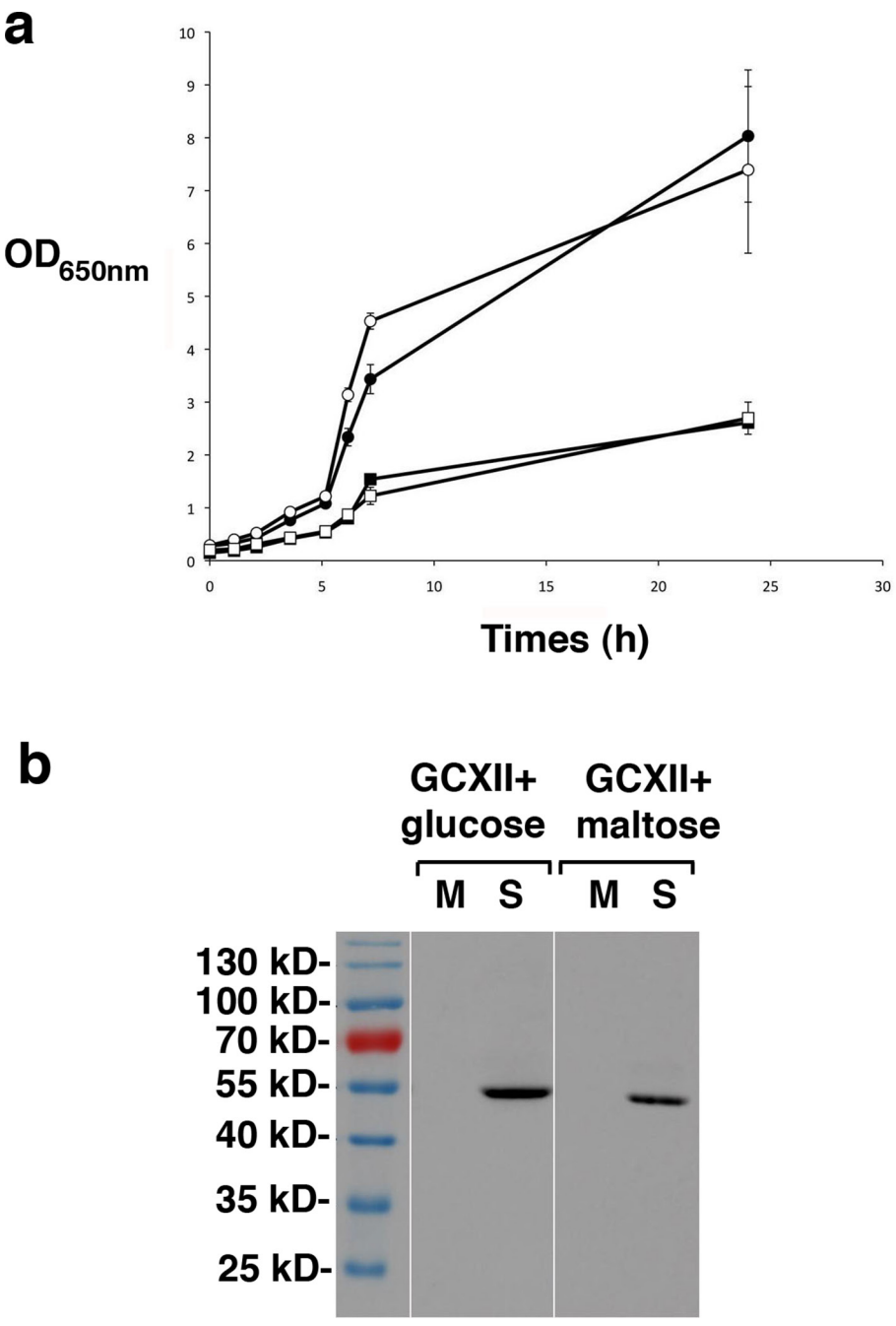

Fig. 5. MusE acylation is not necessary for growth on maltose. (a) Precultures of *C. glutamicum* WT (circle) and *Δlgt* (squares) were grown overnight at 30 °C in CGXII minimal medium with glucose. The next day, cells were washed several times with CGXII containing no carbon source and used to inoculate fresh CGXII with either maltose (open symbols) or glucose (filled symbols) as a 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 Δ*lgt* strain grown in GCXII were prepared and analysed by SDS-PAGE followed by immunoblotting using polyclonal anti-MusE antibodies.

when the Cg-*Δlgt* mutant was grown in minimal 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 with 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 is thus proposed to be mannosylated [55].

The non-pathogenic model bacteria *M. smegmatis* or *C. glutamicum* do not produce DIMs or 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 latter two species each possess two Lnt homologues and, in both cases, only one has been tested for a possible role in glycosylation [29, 56].

In order to investigate the role of *Cg-lgt* in LppX biogenesis, WT and Δ*lgt* strains of *C. glutamicum* were transformed with pCg482-LppX-HA-His, a shuttle vector in which expression of Mt-LppX-$_{\text{HA-His}}$ is under the control of the *musE* promoter [30]. Membrane and secreted proteins were prepared from the transformed strains and analysed by SDS-PAGE followed by immunoblotting using a monoclonal anti-HA peroxidase conjugate. As previously observed, LppX was found in both the membrane and secreted fractions in the WT strain ([30] Fig. 6a). In contrast, LppX was not detected in the membrane fraction of Cg-Δ*lgt* but was 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, in contrast 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 WT strain, a LppX mutant in which the conserved C_{+1} from the lipobox was changed to a leucine. As shown in Fig. 6b, LppX-C1L is only detected in the supernatant of the WT 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 PMF, only peaks corresponding to non-acylated $LppX_{1\text{-}29}$ peptides could be detected (Fig. S2). This result, in addition to 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 $LppX_{1\text{-}29}$ (Fig. S2). 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 $LppX_{6\text{-}29}$ [30]. We hence purified LppX from the supernatant fraction of the *Cg-Δlgt* strain and performed nanoLC-MS/MS analysis after trypsin digestion. As previously found for LppX expressed in WT *C. glutamicum*, $LppX_{6\text{-}29}$ was found in mono-, di-, or tetra-glycosylated forms in the Cg-Δ*lgt* strain (Fig. S3), 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 deleted [46, 47]. The Cg-*ΔlspA* strain obtained showed a very slight growth delay in rich medium compared to the parental strain, but reached a 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 Figs 2 and 7b, MusE migration in *Cg-Δlgt* or *Cg-ΔlspA* strain is similar to that observed in the WT 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 WT strain, is cleaved upstream of the L_{+1} residue [30].

The Cg-*ΔlspA* strain was then transformed with pCg482-$LppX_{\text{HA-His}}$ and LppX localization was analysed 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 WT strain. When the Cg-*ΔlspA* strain was complemented with *Cg-lspA*, LppX recovered its initial migration behaviour (Fig. 7b). This result is similar to what has been observed by Tschumi *et al.*) in *M. smegmatis* [31], where the migration behaviour 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

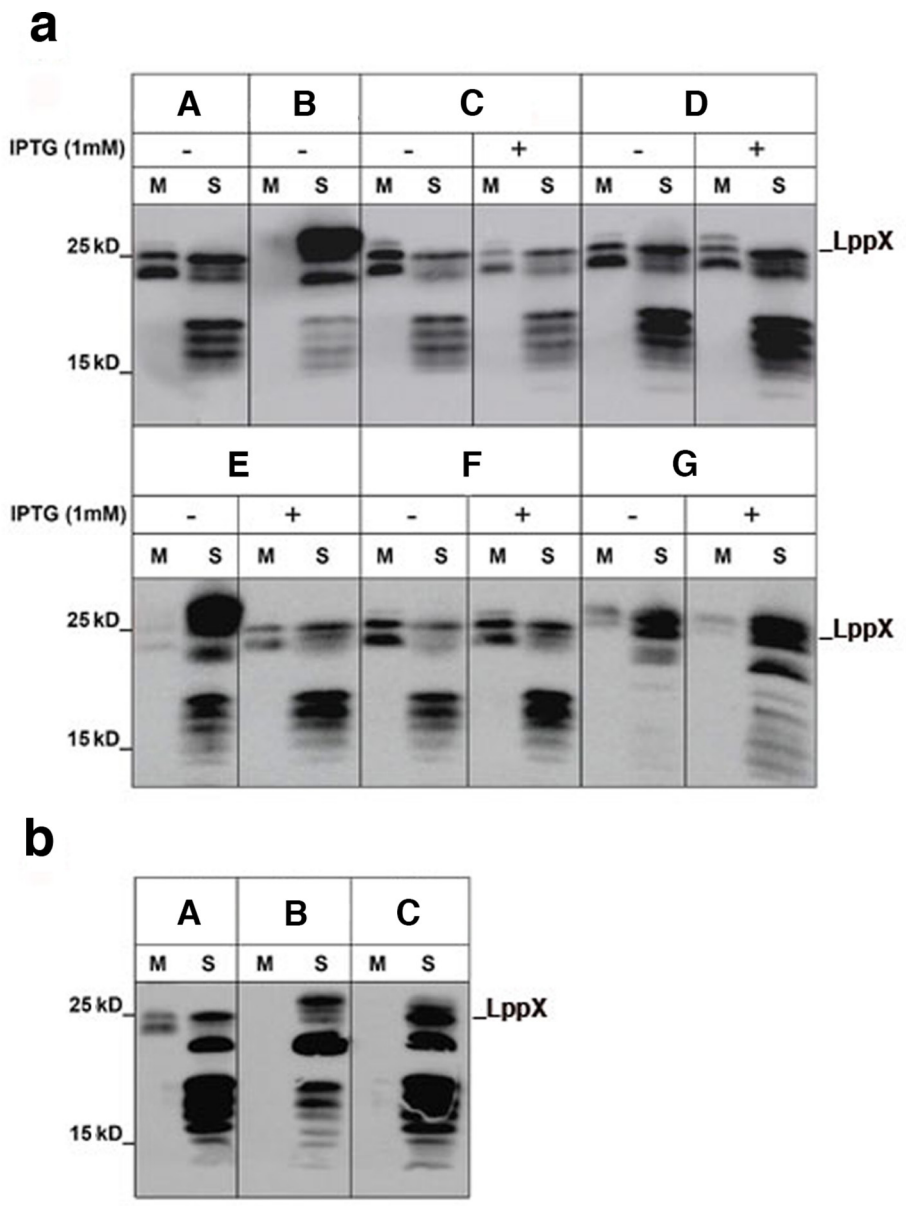

Fig. 6. Acylation is necessary for LppX membrane localization. (a) Membrane (M) and secreted (S) proteins corresponding to an OD_{650nm} of 10 were prepared from WT strain transformed with pCg482-LppX-HA-His (A), Cg-Δ*lgt*+pCg482-LppX-HA-His (B), pCg482-LppX-HA-His+pVWEx2-*Cg-lgt* (C) or Cg-Δ*lgt*+pCg482-LppX-HA-His+pVWEx2-*Ec-lgt* (D), or Cg-Δ*lgt*+pCg482-LppX-HA-His+pVWEx2-*MSMEGM_3222* (E), Cg-Δ*lgt*+pCg482-LppX-HA-His+pVWEx2-*MSMEGM_3222-ΔCter* (F) or Cg-Δ*lgt*+pCg482-LppX-HA-His+pVWEx2-*MSMEGM_5408* (G), grown in the absence or in the presence of 1 mM IPTG, and analysed by immunoblotting using anti-HA peroxidase conjugate (Roche). (b) The same experiments were performed on WT strain transformed with pCg482-LppX-HA-His (A), Cg-Δ*lgt*+pCg482-LppX-HA-His (B) and Cg-Δ*lgt*+pCg482-LppX-C1L-HA-His+pVWEx2-*Cg-lgt* (C).

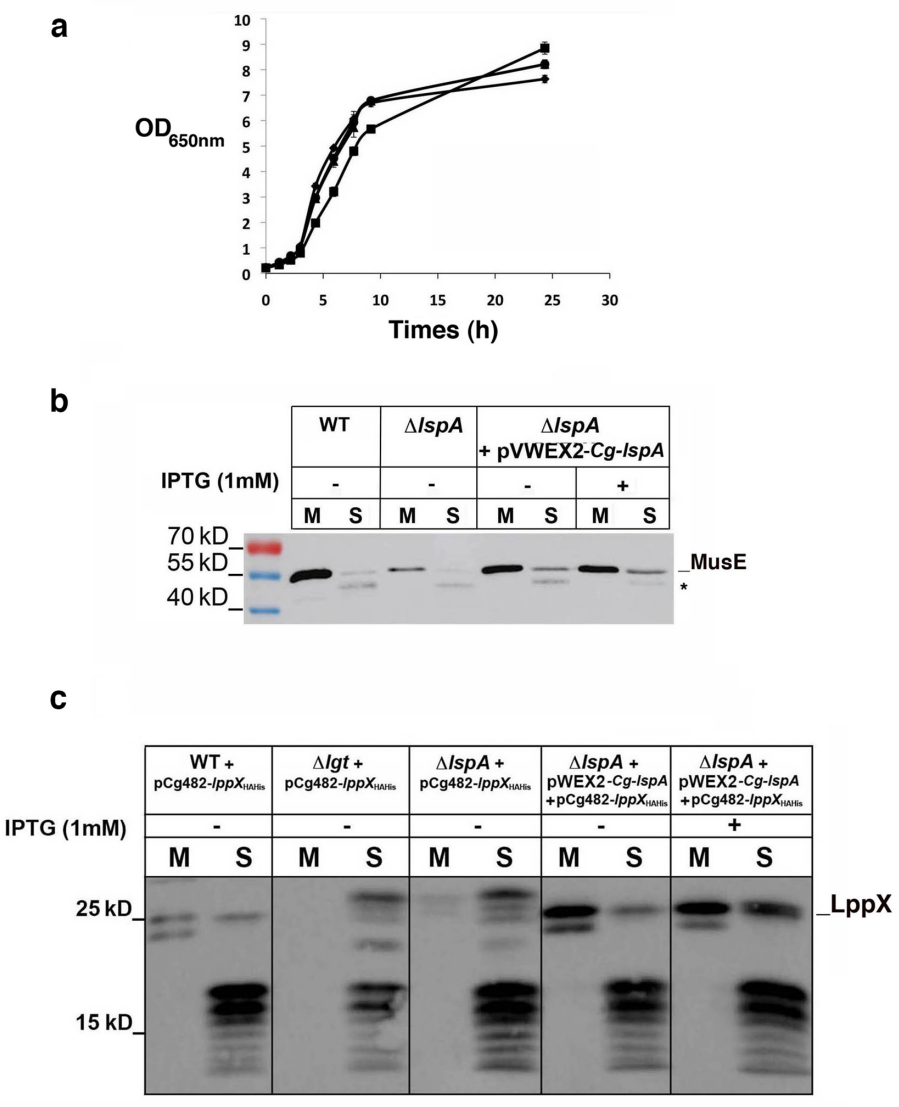

Fig. 7. Characterization of *C. glutamicum ΔlspA*. (a) WT *C. glutamicum* (diamonds) and *ΔlspA* (squares) were grown in BHI medium at 30 °C. *ΔlspA*+pVWEx2-*Cg-lspA* was grown at 30 °C in BHI with (crosses) or without (triangles) 1 mM IPTG. (b) Cells were grown overnight in BHI at 30 °C in the presence or absence of IPTG 1 mM. Membrane (M) and secreted (S) fractions from cells corresponding to an OD_{650nm} 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 (Roche) to detect LppX (c).

showed that the $LppX_{1-29}$ signal peptide is cleaved in this strain.

DISCUSSION

Lgt essentiality

The genes necessary for lipoprotein modification and trafficking (i.e. *lgt*, *lspA*, *lnt*, *lol*), are essential in some but not all Gram-negative bacteria. The essentiality of this pathway was attributed to its role in targeting several vital lipoproteins to the outer membrane, where they are necessary for the biogenesis of the cell envelope (BamD, LolB, LptE, LpoA, LpoB) [8, 57, 58].

Corynebacteriales are diderm bacteria possessing an atypical outer membrane called the 'mycomembrane', which is functionally similar to the outer membrane from Gram-negative bacteria [59, 60]. However, very little is known about the biogenesis and trafficking of lipoproteins in these bacteria, despite their important role in pathogenesis and physiology [3, 6, 36, 59].

Here, we identified *C. glutamicum lgt* gene and found that a mutant lacking this gene is viable. Although essential in

M. tuberculosis, this gene can also be deleted in *M. smegmatis* [37].

Importantly, this is not the first report showing that a gene involved in cell envelope biogenesis is essential in *M. tuberculosis*, whereas it can be deleted from *M. smegmatis* or *C. glutamicum* [60–62]. This probably reflects major physiological differences between pathogenic, slow-growing *M. tuberculosis* and the non-pathogenic, fast-growing *M. smegmatis* or *C. glutamicum*. Alternatively, the function of some essential lipoproteins might be strictly dependent on acylation in *M. tuberculosis* but not in *M. smegmatis* or *C. glutamicum*. Several lipoproteins have indeed been proposed to be essential in *M. tuberculosis* [63]. 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 [59, 64]. LpqB, a lipoprotein involved in the modulation of the MtrAB two-component system, was predicted to be essential in *M. tuberculosis*, but could be interrupted in *M. smegmatis*, resulting in pleiotropic effects [65]. 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, which is involved in Mn^{2+} active transport, is necessary for *S. uberis* growth in milk. However, the growth of a *S. uberis Δlgt* 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_{+1} cysteine is mutated to alanine [65].

Interspecies complementation

Lgt is responsible for the transfer of a diacylglyceryl moiety from phosphatidylglycerol (PG) to the side chain of the conserved C_{+1} 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-*Δlgt*, only *E. coli* Lgt could restore the growth of an *E. coli* conditional mutant. In contrast, *S. aureus* Lgt was active in *E. coli* [66]. 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, 67]. This discrepancy could be due to differences in substrate 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*cis*-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.* [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 gender, 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 [68]. 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,* by contrast, does not harbour any aminoacyl-phosphatidylglycerol synthase activity [69]. Whether this modified DAG could be used preferentially as a 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 characterize these enzymes precisely.

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 genus *Mycobacterium*, 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 genera (*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-*Δlgt*, 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, 70]. Here, we did not observe any differences in migration whether the *C. glutamicum* lipoprotein MusE was expressed in the WT, Δ*lgt* or Δ*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 WT strain [30]. The results obtained for LppX were less clear. The protein migrates in SDS-PAGE gels at the same position, whether expressed in Cg-*Δlgt* or Cg-*ΔlspA*, and is resolved at a higher mass compared with the protein expressed in the WT 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-Δlgt* lacked its signal peptide. The reason for LppX's slower migration when expressed from Cg-Δ*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 [71]. Topology studies suggest that the 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 [71]. Interestingly, in *M. tuberculosis*, the *ppm1* gene encodes both Lnt and Ppm1 activities [57]. 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 [72]. It was thus proposed that the association of Ppm1 with the membrane protein Lnt allows this soluble protein to remain close enough to the membrane to perform its transferase activity. In a previous study, we found that LppX was neither N-acylated nor glycosylated in a Cg-*lnt* mutant. It was not clear, however, if the absence of glycosylation was due to either (i) the lack of association of Ppm1 with the membrane in the absence of Lnt, and hence the absence of PPM synthesis, or (ii) 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 events leading to lipoprotein acylation and glycosylation in the WT context still remains to be determined.

Funding information
This work was funded by CNRS and University of Paris-Sud. N. M. was the recipient of a private grant from Arastoo Company, Tehran, Iran. N. D. was the recipient of a post-doctoral fellowship from the Foundation for Medical Research (FRM).

Acknowledgements
We are grateful to Nienke Buddelmeijer (Institut Pasteur, Paris) for providing plasmid pCHAP7546 and strain PAP9403. We also thank Marc Millot for his precious help for constructing the Cg-Δ*Lgt* mutant.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Zückert WR. Secretion of bacterial lipoproteins: through the cytoplasmic membrane, the periplasm and beyond. *Biochim Biophys Acta* 2014;1843:1509–1516.
2. Buddelmeijer N. The molecular mechanism of bacterial lipoprotein modification--how, when and why? *FEMS Microbiol Rev* 2015;39:246–261.
3. Kovacs-Simon A, Titball RW, Michell SL. Lipoproteins of bacterial pathogens. *Infect Immun* 2011;79:548–561.
4. Reffuveille F, Leneveu C, Chevalier S, Auffray Y, Rincé A. Lipoproteins of *Enterococcus faecalis*: bioinformatic identification, expression analysis and relation to virulence. *Microbiology* 2011;157:3001–3013.
5. Rezwan M, Grau T, Tschumi A, Sander P. Lipoprotein synthesis in mycobacteria. *Microbiology* 2007;153:652–658.
6. Sutcliffe IC, Harrington DJ. Lipoproteins of *Mycobacterium tuberculosis*: an abundant and functionally diverse class of cell envelope components. *FEMS Microbiol Rev* 2004;28:645–659.
7. Szewczyk J, Collet J-F. The journey of lipoproteins through the cell: one birthplace, multiple destinations. *Adv Microb Physiol* 2016;69:1–50.
8. Okuda S, Tokuda H. Lipoprotein sorting in bacteria. *Annu Rev Microbiol* 2011;65:239–259.
9. Nakayama H, Kurokawa K, Lee BL. Lipoproteins in bacteria: structures and biosynthetic pathways. *Febs J* 2012;279:4247–4268.
10. Foster J, Ganatra M, Kamal I, Ware J, Makarova K *et al*. The *Wolbachia* genome of *Brugia malayi*: endosymbiont evolution within a human pathogenic nematode. *PLoS Biol* 2005;3:e121.
11. Shigenobu S, Watanabe H, Hattori M, Sakaki Y, Ishikawa H. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera sp*. APS. *Nature* 2000;407:81–86.
12. Weyer KA, Schaefer W, Lottspeich F, Michel H. Cytochrome subunit of the photosynthetic reaction center from *Rhodopseudomonas viridis* is a lipoprotein. *Biochemistry* 1987;26:2909–2914.
13. Inukai M, Takeuchi M, Shimizu K, Arai M. Mechanism of action of globomycin. *J Antibiot* 1978;31:1203–1205.
14. McLeod SM, Fleming PR, MacCormack K, McLaughlin RE, Whiteaker JD *et al*. Small-Molecule inhibitors of gram-negative lipoprotein trafficking discovered by phenotypic screening. *J Bacteriol* 2015;197:1075–1082.

15. Xiao Y, Gerth K, Müller R, Wall D. Myxobacterium-produced antibiotic TA (myxovirescin) inhibits type II signal peptidase. *Antimicrob Agents Chemother* 2012;56:2014–2021.
16. LoVullo ED, Wright LF, Isabella V, Huntley JF, Pavelka MS. Revisiting the Gram-negative lipoprotein paradigm. *J Bacteriol* 2015;197:1705–1715.
17. Gwin CM, Prakash N, Christian Belisario J, Haider L, Rosen ML *et al*. The apolipoprotein N-acyl transferase Lnt is dispensable for growth in Acinetobacter species. *Microbiology* 2018;164:1547–1556.
18. Asanuma M, Kurokawa K, Ichikawa R, Ryu K-H, Chae J-H *et al*. Structural evidence of α-aminoacylated lipoproteins of *Staphylococcus aureus*. *Febs J* 2011;278:716–728.
19. Serebryakova MV, Demina IA, Galyamina MA, Kondratov IG, Ladygina VG *et al*. The Acylation State of Surface Lipoproteins of Mollicute *Acholeplasma laidlawii*. *J Biol Chem* 2011;286:22769–22776.
20. Kurokawa K, Ryu K-H, Ichikawa R, Masuda A, Kim M-S *et al*. Novel bacterial lipoprotein structures conserved in low-GC content gram-positive bacteria are recognized by Toll-like receptor 2. *J Biol Chem* 2012;287:13170–13181.
21. Kurokawa K, Kim M-S, Ichikawa R, Ryu K-H, Dohmae N *et al*. Environment-mediated accumulation of diacyl lipoproteins over their triacyl counterparts in *Staphylococcus aureus*. *J Bacteriol* 2012;194:3299–3306.
22. Baumgärtner M, Kärst U, Gerstel B, Loessner M, Wehland J *et al*. Inactivation of *lgt* allows systematic characterization of lipoproteins from *Listeria monocytogenes*. *J Bacteriol* 2007;189:313–324.
23. Chimalapati S, Cohen JM, Camberlein E, MacDonald N, Durmort C *et al*. Effects of deletion of the *Streptococcus pneumoniae* lipoprotein diacylglyceryl transferase gene *lgt* on ABC transporter function and on growth *in vivo*. *PLoS One* 2012;7:e41393.
24. Denham EL, Ward PN, Leigh JA. In the absence of Lgt, lipoproteins are shed from *Streptococcus uberis* independently of Lsp. *Microbiology* 2009;155:134–141.
25. Leskelä S, Wahlström E, Kontinen VP, Sarvas M. Lipid modification of prelipoproteins is dispensable for growth but essential for efficient protein secretion in *Bacillus subtilis*: characterization of the *lgt* gene. *Mol Microbiol* 1999;31:1075–1085.
26. Hutchings MI, Palmer T, Harrington DJ, Sutcliffe IC. Lipoprotein biogenesis in Gram-positive bacteria: knowing when to hold'em, knowing when to fold'em. *Trends Microbiol* 2009;17:13–21.
27. Pribyl T, Moche M, Dreisbach A, Bijlsma JJE, Saleh M *et al*. Influence of impaired lipoprotein biogenesis on surface and exoproteome of *Streptococcus pneumoniae*. *J Proteome Res* 2014;13:650–667.
28. Saleh M, Bartual SG, Abdullah MR, Jensch I, Asmat TM *et al*. Molecular architecture of *Streptococcus pneumoniae* surface thioredoxin-fold lipoproteins crucial for extracellular oxidative stress resistance and maintenance of virulence. *EMBO Mol. Med* 2013;5:1852–1870.
29. Brülle JK, Tschumi A, Sander P. Lipoproteins of slow-growing mycobacteria carry three fatty acids and are N-acylated by apolipoprotein *N*-acyltransferase BCG_2070c. *BMC Microbiol* 2013;13:223.
30. Mohiman N, Argentini M, Batt SM, Cornu D, Masi M *et al*. The PPM operon is essential for acylation and glycosylation of lipoproteins in *Corynebacterium glutamicum*. *PLoS One* 2012;7:e46225.
31. Tschumi A, Nai C, Auchli Y, Hunziker P, Gehrig P *et al*. Identification of apolipoprotein N-acyltransferase (Lnt) in mycobacteria. *J Biol Chem* 2009;284:27146–27156.
32. Widdick DA, Hicks MG, Thompson BJ, Tschumi A, Chandra G *et al*. Dissecting the complete lipoprotein biogenesis pathway in *Streptomyces scabies*. *Mol Microbiol* 2011;80:1395–1412.
33. Thompson BJ, Widdick DA, Hicks MG, Chandra G, Sutcliffe IC *et al*. Investigating lipoprotein biogenesis and function in the model Gram-positive bacterium *Streptomyces coelicolor*. *Mol Microbiol* 2010;77:943–957.
34. Gullón S, Arranz EIG, Mellado RP. Transcriptional characterisation of the negative effect exerted by a deficiency in type II signal peptidase on extracellular protein secretion in *Streptomyces lividans*. *Appl Microbiol Biotechnol* 2013;97:10069–10080.
35. Zhang YJ, Ioerger TR, Huttenhower C, Long JE, Sassetti CM *et al*. Global assessment of genomic regions required for growth in *Mycobacterium tuberculosis*. *PLoS Pathog* 2012;8:e1002946.
36. Sander P, Rezwan M, Walker B, Rampini SK, Kroppenstedt RM *et al*. Lipoprotein processing is required for virulence of *Mycobacterium tuberculosis*†. *Mol Microbiol* 2004;52:1543–1552.
37. Tschumi A, Grau T, Albrecht D, Rezwan M, Antelmann H *et al*. Functional analyses of mycobacterial lipoprotein diacylglyceryl transferase and comparative secretome analysis of a mycobacterial *lgt* mutant. *J Bacteriol* 2012;194:3938–3949.
38. Feltcher ME, Gibbons HS, Ligon LS, Braunstein M. Protein export by the mycobacterial SecA2 system is determined by the preprotein mature domain. *J Bacteriol* 2013;195:672–681.
39. Eggeling L, Reyes OEggeling L, Botts M (editors). *Handbook of Corynebacterium Glutamicum*. Boca Raton, FL: CRC Press, Inc., Taylor Francis Group; 2005. pp. 535–566.
40. Bonamy C, Guyonvarch A, Reyes O, David F, Leblon G. Interspecies electro-transformation in Corynebacteria. *FEMS Microbiol Lett* 1990;66:263–269.
41. Dusch N, Pühler A, Kalinowski Jörn. Expression of the *Corynebacterium glutamicum* panD gene encoding l-Aspartate-α-decarboxylase leads to pantothenate overproduction in *Escherichia coli*. *Appl Environ Microbiol* 1999;65:1530–1539.
42. Schäfer A, Tauch A, Jäger W, Kalinowski J, Thierbach G *et al*. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* 1994;145:69–73.
43. Heckman KL, Pease LR. Gene splicing and mutagenesis by PCR-driven overlap extension. *Nat Protoc* 2007;2:924–932.
44. Pailler J, Aucher W, Pires M, Buddelmeijer N. Phosphatidylglycero l::prolipoprotein diacylglyceryl transferase (Lgt) of *Escherichia coli* has seven transmembrane segments, and its essential residus are embedded in the membrane. *J Bacteriol* 2012;194:2142–2151.
45. Ujihara T, Sakurai I, Mizusawa N, Wada H. A method for analyzing lipid-modified proteins with mass spectrometry. *Anal Biochem* 2008;374:429–431.
46. Ikeda M, Nakagawa S. The *Corynebacterium glutamicum* genome: features and impacts on biotechnological processes. *Appl Microbiol Biotechnol* 2003;62:99–109.
47. Kalinowski J, Bathe B, Bartels D, Bischoff N, Bott M *et al*. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of l-aspartate-derived amino acids and vitamins. *J Biotechnol* 2003;104:5–25.
48. Gibson KJC, Eggeling L, Maughan WN, Krumbach K, Gurcha SS *et al*. Disruption of Cg-Ppm1, a polyprenyl monophosphomannose synthase, and the generation of lipoglycanless mutants in Corynebacterium glutamicum. *J Biol Chem* 2003;278:40842–40850.
49. Henrich A, Kuhlmann N, Eck AW, Krämer R, Seibold GM. Maltose uptake by the novel ABC transport system MusEFGK2I causes increased expression of *ptsG* in *Corynebacterium glutamicum*. *J Bacteriol* 2013;195:2573–2584.
50. Vidal-Ingigliardi D, Lewenza S, Buddelmeijer N. Identification of essential residues in apolipoprotein N-acyl transferase, a member of the CN hydrolase family. *J Bacteriol* 2007;189:4456–4464.
51. Sulzenbacher G, Canaan S, Bordat Y, Neyrolles O, Stadthagen G *et al*. LppX is a lipoprotein required for the translocation of phthiocerol dimycocerosates to the surface of *Mycobacterium tuberculosis*. *Embo J* 2006;25:1436–1444.
52. Lefèvre P, Denis O, De Wit L, Tanghe A, Vandenbussche P *et al*. Cloning of the gene encoding a 22-kilodalton cell surface antigen of *Mycobacterium* bovisBCG and analysis of its potential for DNA vaccination against tuberculosis. *Infect Immun* 2000;68:1040–1047.

53. Målen H, Berven FS, Fladmark KE, Wiker HG. Comprehensive analysis of exported proteins from *Mycobacterium tuberculosis* H37Rv. *Proteomics* 2007;7:1702–1718.
54. Mawuenyega KG, Forst CV, Dobos KM, Belisle JT, Chen J *et al*. *Mycobacterium tuberculosis* functional network analysis by global subcellular protein profiling. *Mol Biol Cell* 2005;16:396–404.
55. González-Zamorano M, Mendoza-Hernández G, Xolalpa W, Parada C, Vallecillo AJ *et al*. *Mycobacterium tuberculosis* glycoproteomics based on ConA-lectin affinity capture of mannosylated proteins. *J Proteome Res* 2009;8:721–733.
56. Córdova-Dávalos LE, Espitia C, González-Cerón G, Arreguín-Espinosa R, Soberón-Chávez G *et al*. Lnt1) is dispensable for protein O-mannosylation by *Streptomyces coelicolor*. *FEMS Microbiol. Lett* 2014;350:72–82.
57. Chimalakonda G, Ruiz N, Chng S-S, Garner RA, Kahne D *et al*. Lipoprotein LptE is required for the assembly of LptD by the -barrel assembly machine in the outer membrane of *Escherichia coli*. *Proc Natl Acad Sci USA* 2011;108:2492–2497.
58. Hagan CL, Silhavy TJ, Kahne D. β-Barrel membrane protein assembly by the Bam complex. *Annu Rev Biochem* 2011;80:189–210.
59. Rainczuk AK, Yamaryo-Botte Y, Brammananth R, Stinear TP, Seemann T *et al*. The lipoprotein LpqW is essential for the mannosylation of periplasmic glycolipids in corynebacteria. *J Biol Chem* 2012;287:42726–42738.
60. Amin AG, Goude R, Shi L, Zhang J, Chatterjee D *et al*. EmbA is an essential arabinosyltransferase in *Mycobacterium tuberculosis*. *Microbiology* 2008;154:240–248.
61. Pawelczyk J, Brzostek A, Kremer L, Dziadek B, Rumijowska-Galewicz A *et al*. AccD6, a key carboxyltransferase essential for mycolic acid synthesis in *Mycobacterium tuberculosis*, is dispensable in a nonpathogenic strain. *J Bacteriol* 2011;193:6960–6972.
62. Liu C-F, Tonini L, Malaga W, Beau M, Stella A *et al*. Bacterial protein-O-mannosylating enzyme is crucial for virulence of *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* 2013;110:6560–6565.
63. Sassetti CM, Rubin EJ. Genetic requirements for mycobacterial survival during infection. *Proc Natl Acad Sci U S A* 2003;100:12989–12994.
64. Kovacevic S, Anderson D, Morita YS, Patterson J, Haites R *et al*. Identification of a novel protein with a role in lipoarabinomannan biosynthesis in mycobacteria. *J Biol Chem* 2006;281:9011–9017.
65. Kempf B, Gade J, Bremer E. Lipoprotein from the osmoregulated ABC transport system OpuA of *Bacillus subtilis*: purification of the glycine betaine binding protein and characterization of a functional lipidless mutant. *J Bacteriol* 1997;179:6213–6220.
66. HY Q, Sankaran K, HC W. Structure-function relationship of bacterial prolipoprotein diacylglyceryl transferase: functionally significant conserved regions. *J Bacteriol* 1995;177:6820–6824.
67. Vidal-Ingigliardi D, Lewenza S, Buddelmeijer N. Identification of essential residues in apolipoprotein N-acyl transferase, a member of the CN hydrolase family. *J Bacteriol* 2007;189:4456–4464.
68. Hillmann F, Argentini M, Buddelmeijer N. Kinetics and phospholipid specificity of apolipoprotein N-acyltransferase. *J Biol Chem* 2011;286:27936–27946.
69. Smith AM, Harrison JS, Grube CD, Sheppe AEF, Sahara N *et al*. tRNA-dependent alanylation of diacylglycerol and phosphatidylglycerol in *Corynebacterium glutamicum*. *Mol Microbiol* 2015;98:681–693.
70. Tokunaga M, Tokunaga H, Wu HC. Post-translational modification and processing of *Escherichia coli* prolipoprotein *in vitro*. *Proc Natl Acad Sci USA* 1982;79:2255–2259.
71. Wehmeier S, Varghese AS, Gurcha SS, Tissot B, Panico M *et al*. Glycosylation of the phosphate binding protein, PstS, in *Streptomyces coelicolor* by a pathway that resembles protein O-mannosylation in eukaryotes. *Mol Microbiol* 2009;71:421–433.
72. Baulard AR, Gurcha SS, Engohang-Ndong J, Gouffi K, Locht C *et al*. *In vivo* interaction between the polyprenol phosphate mannose synthase Ppm1 and the integral membrane protein Ppm2 from *Mycobacterium smegmatis* revealed by a bacterial two-hybrid system. *J Biol Chem* 2003;278:2242–2248.

Edited by: C. Dahl and S. Gebhard