Impact of LCP proteins on cell wall biosynthesis in *Corynebacterium glutamicum*

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Abstract

Proteins of the LCP (LytR, CpsA, Psr)-family were shown to inherit important roles in bacterial cell wall biosynthesis. However, their exact function in the formation of the complex cell wall structures of the corynebacteriales, including the prominent pathogens *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*, remains unclear. Here we analyzed the role of the LCP proteins LcpA and LcpB of *Corynebacterium glutamicum* both of which localize at regions of nascent cell wall biosynthesis. A strain lacking *lcpB* did not show any growth related or morphological phenotype under the tested conditions. In contrast, conditional silencing of the essential *lcpA* resulted in severe growth defects and drastic morphological changes. Compared to the wild-type, the cell wall of this mutant contained significantly less mycolic acids and a reduced amount of arabinogalactan. In particular rhamnose, a specific sugar component of the linker that connects arabinogalactan and peptidoglycan, was decreased. Complementation studies of the *lcpA*-silencing strain with several mutated and truncated LcpA variants suggested that both periplasmic domains are essential for function whereas the cytoplasmic N-terminal part is dispensable. Successful complementation experiments with proteins of *M. tuberculosis* and *C. diphtheriae* revealed a conserved function of LCP proteins in these species. Finally, pyrophosphatase activity of LcpA was shown in an *in vitro* assay. Taken together, our results suggest that LCP proteins are responsible for the transfer of arabinogalactan onto peptidoglycan in actinobacterial species and support a crucial function of the so far uncharacterized LytR_C domain, which is frequently found C-terminal of the LCP domain in this prokaryotic phylum.

Importance

About one third of the world's population is infected with *Mycobacterium tuberculosis* and multiple antibiotic resistances provoke the demand for novel antibiotics. The special cell wall architecture of corynebacteriales is critical for treatments because it is either a direct target or a barrier that the drug has to cross. Here we present the analysis of LcpA and LcpB of the closely related *Corynebacterium glutamicum*, the first of which is an essential protein involved in cell wall biogenesis. Our work provides a comprehensive characterization of the impact of LCP proteins on cell wall biogenesis in this medically and biotechnologically important class of bacteria. Special focus is set on the two periplasmic LcpA domains and their contribution to the physiological function.

Introduction

The LCP protein family was named after its first three described representatives LytR (1), CpsA (2), and Psr (3) and comprises a class of transmembrane proteins sharing a characteristic extracellular domain, the LCP domain (InterPro IPR004474 (4)). A phylogenetic analysis revealed that LCP proteins are present in all Gram-positive bacteria, whereas they are not found in most Gram-negative bacteria (5). Often, bacterial genomes encode several (up to eleven) LCP proteins which all follow a common structural organization consisting of a short N-terminal cytoplasmic domain, a transmembrane fragment with 1-3 transmembrane helices, and a putative extracellular part carrying the LCP domain (5). In LCP proteins of actinobacteria, the LCP domain is often accompanied by a LytR_C domain (IPR027381) of unknown function.

The LytR protein of *Bacillus subtilis* was originally thought to act as a cell wall-related attenuator because disruption of this gene led to an increased transcription of adjacent genes (1). However, recent results suggest that these observations were secondary effects due to severe alterations of cell wall biogenesis (6). Kawai et al. provided several pieces of evidence that LCP proteins are enzymes required for the transfer of anionic cell wall polymers from their membrane-linked precursors onto cell wall peptidoglycan (6). In *B. subtilis*, LCP proteins were isolated in a search for proteins interacting with MreB, a prokaryotic actin homolog (6). Another study showed that in *Staphylococcus aureus* LCP proteins are required for the transfer of capsular polysaccharides onto the glycan strands of peptidoglycan (7). A strain of this bacterium lacking all three LCP proteins was found to release type 5 capsular polysaccharides and wall teichoic acids into the medium (7, 8). A recent study of *Actinomyces oris* suggests that an LCP protein is involved in protein glycosylation, namely the transfer of glycan from a polyprenol lipid-linked glycan to GspA (glycosylated surface-linked protein A) prior to cell wall anchoring (9). Overall, these data

point to an involvement of LCP proteins in the transfer of saccharide compounds from a lipid–polyprenol–carrier to peptidoglycan or proteins.

Most investigations of LCP proteins published to date were performed in firmicutes. Furthermore, no study so far has addressed the function of the LytR_C domain that is commonly found in actinobacterial LCP proteins. One highly interesting order in this phylum is represented by the corynebacteriales, including prominent human pathogens such as *Mycobacterium tuberculosis* or *Corynebacterium diphtheriae*. The diseases caused by these bacteria can be difficult to treat, especially in the case of tuberculosis, because the bacteria harbor a cell wall with a very special architecture making them resistant against standard antibiotic treatments (10). In the cell wall of these species, the peptidoglycan is linked to arabinogalactan, which is itself esterified with mycolic acids forming a large mycolyl–arabinogalactan–peptidoglycan complex (11). Furthermore, lipomannan and lipoarabinomannan (LAM) are present (11). The mycolic acids are forming a second lipid bilayer which constitutes a diffusion barrier similar to the outer membrane of Gram-negative bacteria (11) (for a recent review on the structure and assembly of the mycobacterial cell wall, see (12)). As corynebacteria and mycobacteria have no teichoic acids within their cell wall and only mycobacteria harbor capsule-like structures (13), the function of LCP proteins in these species and their relation to cell wall biosynthesis remains unclear. A recent study revealed that the LCP protein CpsA of *Mycobacterium marinum* plays an important role in cell wall assembly and that it is essential for proliferation in macrophages and virulence in zebrafish (14). *Corynebacterium glutamicum* is a well-established model species for cell wall-related studies in the corynebacteriales because it shares the complex cell envelope organization with its pathogenic relatives (15-17). Furthermore, *C. glutamicum* is one of the main species used in biotechnological industry, especially for the production of amino acids (18). The transport of products across the complex cell wall can become an issue in the development of

biotechnological production strains. The genome of *C. glutamicum* encodes two LCP proteins one of which, cg3210, was recently found to be a target gene of IpsA, a regulator of cell wall biosynthesis (15). In this study we address the general importance of the two LCP proteins in *C. glutamicum* for cell wall biogenesis with a special focus on the impact of the LytR_C domain.

Materials and Methods

Bacterial strains, plasmids and growth conditions.

All bacterial strains and plasmids used in this study are listed in Table S1. The *C. glutamicum* type strain ATCC 13032 was used as wild-type. For growth experiments with the promoter exchange strains, as first preculture, 5 ml brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI, USA) supplemented with 100 mM sodium gluconate (for SilG-lcpA) were inoculated with a single colony from a fresh agar plate and incubated overnight, shaking at 30°C. The cells were washed once in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3) and used to inoculate a second preculture, consisting of 750 µl CGXII minimal medium (19) supplemented with 3,4-dihydroxybenzoate (30 mg/l) as iron chelator and glucose, sodium gluconate, *myo*-inositol, or a mixture thereof as carbon source, to an OD_{600} of 0.5. After 24 h cultivation at 30°C and 1200 rpm in a BioLector® system (m2p-labs, Baesweiler, Germany) in 48-well FlowerPlates 15 µl of this cultures were used to inoculate the main cultures in another flower plate containing the same medium as the second precultures. All cloning was performed in *Escherichia coli* DH5α cultivated at 37°C in lysogeny broth (LB,(20)) with 50 µg/ml kanamycin. *E. coli* BL21(DE3) was used for protein production.

Recombinant DNA work and construction of deletion mutants.

Routine methods such as PCR, DNA restriction and ligation were performed using standard protocols (20-22). The oligonucleotides used in this study were obtained from Eurofins Genomics (Ebersberg, Germany) and are listed in Table S2. Gibson assembly was performed according to (23). All plasmids were sequenced to exclude unwanted mutations (Eurofins Genomics (Ebersberg, Germany)). The Δ*lcpB* mutant of *C. glutamicum* and the strains with the exchanged promoter in front of LcpA were constructed *via* a two-step homologous recombination protocol

as described previously (24). For further details regarding plasmid and mutant construction, see Text S1.

Conditional gene silencing system for *C. glutamicum*

The elucidation of the function of essential genes is often challenging, as no deletion mutant can be obtained which allows the analysis of a phenotype and might give hints towards the function of the respective genes. For these cases, gene silencing systems can be used to study the effects of reduced levels of the respective genes. For other organisms there are well established systems such as the TetR based approach (25). For *C. glutamicum* this system appeared to be not efficient enough in earlier studies. Therefore we used two well characterized, native promoters of *C. glutamicum* for the silencing approach. The first was the promoter of the gluconate kinase GntK that is activated in the presence of gluconate (26). The second was the promoter of the inositol phosphate synthase Ino1 which is repressed in the presence of *myo*-inositol (15). Both promoters turned out to be very efficient in downregulating our gene of interest. From RNAseq-data we can estimate that about 2/3 of all *C. glutamicum* genes have a higher expression level than the normal LcpA expression level, and about 90% of all *C. glutamicum* genes have apparently a higher expression level than the silenced LcpA expression level. This suggests that the majority of *C. glutamicum* genes can potentially be silenced with the system described here. Besides studying essential genes in *C. glutamicum*, the promoters might also prove to be useful tools for metabolic engineering approaches.

Fluorescence microscopy and co-localization studies

For recording fluorescence microscopy with staining the cells were centrifuged and resuspended in PBS containing 100 ng/ml Hoechst 33342 and 300 ng/ml Nile red (Sigma-Aldrich, Munich, Germany). After 5-10 min incubation at room temperature, the cells were analyzed on agarose

pads using a Zeiss Axio Imager M2 microscope that was equipped with an AxioCam MRm camera and a Plan-Apochromat 100x, 1.40 Oil phase contrast oil-immersion objective. Cells with fluorescent proteins were imaged directly without any staining. For this purpose, a colony from a fresh agar plate was used to inoculate a preculture of 5 ml BHI with kanamycin which was cultivated shaking at 30°C overnight. On the following day 100 µl of this culture were used to inoculate a main culture consisting of 5 ml CGXII medium with 2% (w/v) glucose, kanamycin and 10 µM (Isopropyl β-D-1-thiogalactopyranoside) IPTG. Digital images were acquired and analyzed with AxioVision 4.8 software (Zeiss, Göttingen, Germany) 4 or 24 hours after inoculation of the main culture. Functionality of the Venus-lcpA fusion protein was proven by complementation studies with the SilG-lcpA-strain.

For co-localization experiments *C. glutamicum* ATCC 13032 carrying plasmids pAN6-*venus-lcpA* or pAN6-*eyfp-lcpB* were grown as described above. After 4 hours (OD_{600} = 1), aliquots of 200 µl were transferred for bioorthogonal labeling (27) into the wells of a microtiter plate and incubated 5 min at 30°C and 850 rpm. 1 µl of azido-D-alanine or D-alanine (1 M each) was added and the cells shaken for 3 min. The cells were harvested (5,000*g* , 25°C, 2 min), washed with PBS, and resuspended in 200 µl PBS. After addition of 1 µl of dibenzylcylcooctyne-PEG_4-ATTO425 (1 mM in DMSO, DBCO-PEG_4-Atto425; synthesized by Jena Bioscience, Jena, Germany) they were incubated at 30°C and 80 rpm for 20 min in the dark. Cells were harvested, washed three times with PBS containing 0.1% Tween 20 and resuspended in PBS. Cells were analyzed on agarose pads using a Zeiss Axio Imager M1 microscope equipped with an AxioCam HRm camera and a Plan-NEOFLUAR 100x, 1.30 Oil phase contrast oil-immersion objective. Digital images were analyzed with AxioVision 4.6 software (Zeiss, Göttingen, Germany).

RT-qPCR

For RT-qPCR *C. glutamicum* wild-type and the promoter mutants were in principle cultivated as described above, but the cultivation volumes were increased to 20 ml (precultures) or 50 ml (main cultures). The cells were cultivated in baffled shake flasks (100 ml and 500 ml volume) at 30°C and 130 rpm. 25 ml cells of the main cultures were harvested by centrifugation (4,120*g*, 10 min and 4°C) at an OD_{600} of 5 using precooled 50 ml falcon tubes filled with 25g crushed ice. The cell pellets were subsequently frozen in liquid nitrogen and stored at -70°C. The preparation of total RNA was performed using the RNeasy Kit from Qiagen (Hilden, Germany) with DNA on column digest. To remove any residual DNA, an additional DNase treatment was performed as following: 5-10 µg RNA from the Kit-purification were mixed with 5 µl RDD-buffer and 8 µl (20 U) DNase (Qiagen, Hilden, Germany) in a total volume of 50 µl, incubated at 30°C for 30 min followed by 70°C for 10 min to inactivate the DNase. Afterwards the RNA was purified by phenol-chloroform extraction and resuspended in 15 µl RNase-free H_2O. RNA integrity was assessed using gel electrophoresis. For reverse transcription, 1 µg RNA in a total volume of 10 µl was mixed with 1 µl Random Primers (0.5 µg/µl, Life Technologies, Darmstadt, Germany) and incubated at 70°C for 10 min. The samples were immediately cooled on ice and then mixed with the following solutions: 4 µl First Strand Buffer (5x), 2 µl DTT (0.1 M), 1 µl dNTPs (10 mM each), 2 µl Superscript III reverse transcriptase (200 U/µl, Life Technologies, Darmstadt, Germany). For each RNA sample, a No Amplification Control (NAC) was prepared containing all above mentioned substances except for the reverse transcriptase. The samples were incubated at 42°C for 50 min followed by incubation at 70°C for 15 min to inactivate the reverse transcriptase. RNA was removed from the samples by addition of 1 µl Ribonuclease H (5 U/µl, NEB, Frankfurt, Germany) and incubation at 37°C for 20 min. The samples were mixed with 179 µl H_2O to reach a concentration of about 5 ng/µl cDNA for the qPCR. qPCR was performed in a

196 qTOWER real time PCR cycler (Analytic Jena, Jena, Germany) using the *innu*MIX qPCR
197 MasterMix SyGreen (Analytic Jena, Jena, Germany). The 20 µl reactions contained 10 µl
198 MasterMix, 1 µM of each primer and 2 µl cDNA. Measurements were performed with three
199 biological and two technical replicates each. For further details regarding quality controls,
200 referencing and the PCR-protocol please refer to Text S1.

201 **Transmission Electron Microscopy**

202 Bacteria were fixed with 3% (v/v) glutaraldehyde (Agar scientific, Wetzlar, Germany) in PBS for
203 at least 4 h, washed in 0.1 M Soerensen's phosphate buffer (Merck, Darmstadt, Germany), and
204 post-fixed in 1% OsO_4 in 17% sucrose buffer. After fixation, bacteria were embedded in 2.5%
205 agarose (Sigma, Steinheim, Germany) and then rinsed in 17% sucrose buffer and deionized water
206 and dehydrated by an ethanol series (30%, 50%, 70%, 90%, 100%) for 10 min each and the last
207 step thrice. The dehydrated specimens were incubated in propylene oxide (Serva, Heidelberg,
208 Germany) for 30 min, in a mixture of Epon resin (Sigma) and propylene oxide (1:1) for 1 h and
209 finally in pure Epon for 1 h. Samples were embedded in pure Epon. Epon polymerization was
210 performed at 37°C for 12 h and then at 80°C for 48 h. Ultrathin sections (70-100 nm) were cut
211 with a diamond knife and picked up on Cu/Rh grids. Negative staining by uranyl acetate and lead
212 citrate (all EMS, Munich, Germany) was performed to enhance TEM contrast. The specimens
213 were viewed using a PhilipsEM400T electron microscope, operated at 60 kV.

214 **Scanning Electron Microscopy**

215 Bacteria were fixed with 3% (v/v) glutaraldehyde (Agar scientific, Wetzlar, Germany) in PBS for
216 at least 4 h, washed in 0.1 M Soerensen's phosphate buffer (Merck, Darmstadt, Germany) for 15
217 min and dehydrated by incubating consecutively in ascending acetone series (30%, 50%, 70%,
218 90%, 100%) for 10 min each and the last step thrice. The samples were critical-point-dried in

liquid CO_2, and then sputter-coated (Sputter Coater EM SCD500, Leica, Wetzlar, Germany) with a 10 nm gold/palladium layer. Samples were analyzed using an environmental scanning electron microscope (ESEM XL 30 FEG, FEI, Philips, Eindhoven, Netherlands) with 10 kV acceleration voltage in a high vacuum environment.

Cell wall preparation

Cell walls were essentially prepared as described previously (28). In brief, cells were cultivated as described for the isolation of material from culture supernatants (text S1) and harvested at an OD_{600} of 15-20, frozen and lyophilized. **Crude cell walls** were prepared as following. Cells were mixed with glass beads in distilled water and disrupted using a Fast Prep 24 (MP Biomedicals, Eschwege, Germany). Cell disruption was controlled by phase contrast microscopy. Crude cell walls were obtained by centrifugation at 48,000*g* for 20 min at 4°C.

The preparation of **SDS-cell walls** was performed similar as described previously (28, 29). Crude cell walls were resuspended in double distilled water and added dropwise to the same volume of a stirring, boiling 8% (w/v) SDS-solution. After boiling for further 30 min the solution was led cool down to room temperature and centrifuged at 48,000*g* for 20 min at 20-25°C. To get rid of any residual SDS, the sediment was washed five times with double distilled water. Cell walls were further purified by protease treatment.

To remove covalently bound protein, 1 g of SDS cell walls were resuspended in 10 mM Tris-HCl pH 7.5 ad 10 ml. Proteinase K (final concentration 200 µg/ml) and NaN_3 (final concentration 0.2 mM) were added and the mixture was incubated with shaking (100-130 rpm) overnight at 37°C. **SDS-ProtK-cell walls** were obtained by centrifugation (48,000*g* for 20 min), washed three times with double distilled water and lyophilized.

Analysis of cell wall components

The extraction of **arabinogalactan** was essentially performed as following (30): 50 mg SDS-ProtK-cell walls were mixed in a centrifuge tube (16x100 mm) with Teflon cap using a small magnetic stir bar and 5 ml of 50 mM H_2SO_4 and left stirring for four days at 37°C and 850 rpm. Afterwards, the suspension was transferred to 15 ml falcon tubes and centrifuged for 15 min at 3,000*g* and 25°C. The supernatant was transferred to a new tube, mixed with a few granules of $Ba(OH)_2$ and incubated for 1 h at room temperature. After 15 min centrifugation at 3,000*g* and 25°C the supernatant was transferred to a new tube and dried in a Speed-Vac (Savant, Farmingdale, USA) (→**arabinogalactan**). The pellet from the first centrifugation was washed two times with double distilled water and subsequently lyophilized (→ **arabinogalactan extracted cell walls**).

For sugar analysis arabinogalactan was hydrolyzed with 2 M HCl for 3 h at 100°C (31) and analyzed by TLC. A standard containing free sugars was treated in the same way. Hydrolysates of 0.2 mg SDS-ProtK-cell walls and 20 nmols/sugar (standard) were applied per spot. TLC was carried out on Silica 60 plates (Merck, Darmstadt, Germany) according to (32). In brief: plates were run in acetonitrile:water 85:15 (v/v) twice and developed with diphenylamine/aniline. Spots were quantified with a ChemiDoc analyzer (BioRad, Munich, Germany). To elucidate the efficiency of the arabinogalactan extraction, arabinogalactan extracted cell walls were hydrolyzed (2 M HCl, 16 h, 100°C) and the hydrolysate analyzed by TLC.

Preparation and detection of mycolic acid methyl esters

Preparation and detection of mycolic acid methyl esters was essentially performed as described previously (33). In brief, SDS-ProtK-cell walls were subjected to acidic methanolysis: 10 mg of SDS-ProtK-cell walls were incubated with 600 µl methanol:toluene:sulfuric acid 30:15:1 (v/v/v)

for 16 h at 50°C. After cooling to room temperature, 400 µl of petroleum ether (60-80°C) was added and shaken vigorously. After phase separation, mycolic acid methyl esters were released into the petroleum ether phase, dried under a stream of nitrogen and analyzed by TLC. This was performed on silica 60 plates (Merck, Darmstadt, Germany) in toluene:acetone 97:3 (v/v) and developed with phosphomolybdic acid (3% in isopropanol). Ammonium vapor was used to destain the yellow background.

Pyrophosphatase-assay

For the pyrophosphatase activity assay, *E. coli* BL21(DE3) was transformed with pET-TEV-lcpAΔTM, pET-TEV-lcpAΔTM-D88A, pET-TEV-lcpAΔTM-R138A, pET-TEV-lcpAΔTMR257A and pET-TEV-cgtR10 (negative control). A colony from a fresh agar plate was used to inoculate a preculture of 50 ml LB medium with kanamycin and cultivated overnight at 37°C and 120 rpm. 500 ml of LB medium with kanamycin were inoculated with 10 ml from the preculture and cultivated at 37 °C and 100 rpm until an OD_{600} of ~0.5 was reached. Protein production as induced with 100 µM IPTG and the cells were further cultivated at 16°C until the next morning. The pellet from 250 ml culture was resuspended in TNI20 buffer (100 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM imidazole) with cOmplete™, Mini, EDTA-free protease inhibitor (Roche, Basel, Switzerland) and cell were broken by passing three times through a french press. The cell extract was centrifuged and the supernatant was used for Ni-NTA affinity chromatography. The protein was eluted with TNI400 (TNI20 with 400 mM imidazole). The buffer was exchanged to GF-buffer (20 mM Tris-HCl pH 8.0, 200 mM NaCl) using PD10 columns (GE lifesciences, Munich, Germany) and the proteins were concentrated to at least 5 mg/ml with Amicon Ultra Centrifugal filters (Merck Millipore, Cork, Ireland). Gel filtration was performed at a flow rate of 0.5 ml/min in GF-buffer using a Superdex 200 Increase 10/300

column, connected to an Äkta Pure25 system. For the gel filtration under reducing conditions, 5 mM 1,4-Dithiothreitol (DTT) were added to the protein sample which was then incubated for 1h on ice, prior to the gel filtration using GF-buffer also supplemented with 1 mM DTT. The molecular weight of the protein was estimated by comparison with standard proteins of known molecular weight.

For the pyrophosphatase assay, 0.18 mg protein were mixed with 1 mM geranyl-pyrophosphate (GPP) and either 10 mM $MgCl_2$ or 5 mM EDTA in 20 mM Tris-HCl pH 8.0 (100 µl final volume). The reactions were incubated overnight at 30°C. Controls lacking either protein or substrate or both were also included. For analysis, the samples were diluted 1:5 with water and filtered through Amicon ultra centrifugal filters to remove the protein.

LC-MS analysis was performed using an ultra-high-performance LC (uHPLC) 1290 Infinity System coupled to a 6130 quadrupole LC-MS system (Agilent, SantaClara, CA, USA). LC separation was carried out with a Kinetex 1.7u C_{18} 100-Å-pore-size column (50 mm by 2.1 mm [internal diameter]; Phenomenex, Torrance, CA, USA) at 50°C. For elution, 0.1% acetic acid (solvent A) and acetonitrile supplemented with 0.1% acetic acid (solvent B) were applied as the mobile phases at a flow rate of 0.5 mL/min. A gradient was used, where solvent B was increased stepwise: minute 0 to 6: 10% to 30%, minute 6 to 7: 30% to 50%, minute 7 to 8: 50% to 100% and minute 8 to 8.5: 100% to 10%. The mass spectrometer was operated in negative electrospray ionization (ESI) mode, and data acquisition was performed in selected-ion-monitoring mode with m/z = 233 corresponding to $[M-H]^-$ of geranyl-monophosphate. An authentic geranyl-monophosphate standard was obtained from Sigma Aldrich (Munich, Germany).

Results

Domain structure and conservation of *C. glutamicum* LCP proteins.

The genome of *C. glutamicum* strain ATCC 13032 contains two genes encoding LCP proteins comprising different domain organization. In the following, these proteins are designated as LcpA (cg0847/NCgl0708) and LcpB (cg3210/ NCgl2802).

The gene *lcpA* is located in close vicinity to genes involved in cell wall biosynthesis such as *wbbL1* (cg0848, rhamnosyltransferase) or *rmlA2* (cg0849, GDP-mannose pyrophosphorylase) (Fig. 1A). This organization is conserved among corynebacteria and mycobacteria (Fig. 1A). The coding region of *lcpB* is flanked upstream by the gene cg3209 encoding a metal-dependent membrane protease, the amidotransferase GatA, *pheA*, encoding a prephenate dehydratase involved in the biosynthesis of aromatic amino acids, and *phoE*, encoding a phosphoglycerate mutase (Fig. S1A). The downstream region of *lcpB* contains several genes for uncharacterized hypothetical proteins and is not conserved.

LcpA consists of a short N-terminal cytoplasmic region followed by one transmembrane helix (Fig. 1B). Besides the characteristic LCP domain (IPR004474), it contains an additional uncharacterized C-terminal domain of unknown function (IPR027381, LytR_C domain) which is often found in combination with the LCP domain. LcpB does not contain a LytR_C domain but it has an unusually long cytoplasmic region of 118 amino acids that is very rich in proline, arginine, and glutamine, resulting in a positive net charge (Fig. 1B). This cytoplasmic region seems to be unique for just a few corynebacteria. The obvious differences in domain structure suggest that these two proteins have distinct functions in *C. glutamicum*. In *M. tuberculosis* the situation is even more complex as it encodes in total 4 genes with an LCP domain, three of which also have a

LytR_C domain (rv3484, rv3267, and rv0822) and one with just the LCP domain (rv3840) (Fig. S1B).

lcpA is an essential gene in *C. glutamicum*

Previous studies in different organisms revealed that deletion of LCP proteins—if possible—often leads to altered growth and/or morphology indicating a potential role in cell wall biogenesis. To elucidate whether a deletion of LCP proteins has an impact on *C. glutamicum*, deletion plasmids were constructed and gene deletion by double-crossover was performed. Deletion mutants were readily obtained for *lcpB*, whereas deletion as well as inactivation of *lcpA* failed in several different methodological attempts. Therefore, the *lcpA* gene likely encodes an essential protein in *C. glutamicum*.

Development of a conditional gene silencing system for *C. glutamicum*

To enable the conditional downregulation of *lcpA* a gene silencing system for application in *C. glutamicum* was developed. The native *lcp* promoter was exchanged to i), the promoter of the gluconate kinase (*gntK*, cg2732) (26), which is repressed in the absence of gluconate in the medium and ii), the promoter of the inositol phosphate synthase (*ino1*, cg3323) (15), which can be downregulated by the addition of *myo*-inositol. The relative expression levels of *lcpA* were measured in the resulting strains to determine the regulatory strength and the dynamic range of the two systems. The expression level of *lcpA* was very similar in the wild-type cultivated on either glucose or gluconate (Fig. 1C). Cultivation of the SilG-lcpA (**Sil**encing strain with **G**luconate switch for ***lcpA***) strain on glucose led to a nearly 8-fold decrease in *lcpA* expression compared to the wild-type on glucose. When the same strain was cultivated on gluconate, the expression of *lcpA* was about 10-fold increased compared to the level in the wild-type on glucose. This results in a dynamic range of this system of about 80-fold. For the SilI-lcpA strain

(**Sil**encing strain with **I**nositol switch for ***lcpA***) similar results were obtained (Fig. 1C). Here, even a mixture of glucose and *myo*-inositol (1% (w/v) each) was sufficient for an 8-fold down regulation of *lcpA*.

Characterization of strains with decreased LCP protein levels

After proving the functionality of the gene silencing system, the SilG-lcpA strain was used for investigating the effects of decreased LcpA levels. First of all, the growth of the silencing strain was compared with the wild-type under different conditions. In CGXII medium with gluconate SilG-lcpA grew comparable to the wild-type (growth rates 0.61±0.01 h^{-1} and 0.61±0.01 h^{-1}) (Fig. 1D). When gluconate was exchanged with glucose as carbon source, a significantly reduced *lcpA* expression was achieved, resulting in a strongly decreased growth rate and a lower final backscatter (growth rates 0.28±0.02 h^{-1} for SilG-lcpA and 0.58±0.02 h^{-1} for the wild-type) (Fig. 1D). Colony counting revealed a decreased viability of SilG-lcpA compared to the wild-type (Fig. S2). No growth defect of the Δ*lcpB* mutant was observed on glucose minimal medium (Fig. 1E). However, when *lcpB* was deleted in the SilG-lcpA strain, an even more pronounced growth defect was observed (growth rate 0.30±0.01 h^{-1} for SilG-lcpA vs. 0.26±0.01 h^{-1} for SilG-lcpA Δ*lcpB*) (Fig. 1E). These data suggest non-identical but possibly partially overlapping functions of LcpA and LcpB.

Furthermore, the cell morphology was analyzed by fluorescence microscopy. Whereas SilG-lcpA displayed wild-type morphology on gluconate-containing medium, it showed severely altered cell morphology after cultivation on glucose (Fig. 2A). The cells seemed to be swelling, losing their coryneform shape and formed larger cell clusters. The morphology was further studied by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Fig. 2B and C). In the SEM pictures several bubbles were visible on the surface of the SilG-lcpA strain which

were absent in the wild-type sample. Furthermore, it was apparent on several pictures that numerous cells were losing large pieces of their cell envelope. The TEM pictures also showed the more irregular cell shape of the mutant and the dissociation of cell wall material from the bacterial surface. Furthermore, fringe-like structures are visible on the surface of wild-type cells possibly representing mycolic acids or arabinogalactan, which appeared to be much shorter in the mutant (Fig. 2C, lower panel, black arrows).

LCP proteins of *C. glutamicum* are localized at regions of nascent cell wall biosynthesis

LCP proteins are known to contain at least one transmembrane helix suggesting their location in the cytoplasmic membrane. In order to assess their localization within the cytoplasmic membrane N-terminal translational fusions of LcpA and LcpB to fluorescent proteins (Venus or EYFP) were constructed and analyzed by fluorescence microscopy. Interestingly, both proteins were located in the cell membrane at regions of strong cell wall biosynthesis, such as the poles and the division plane (Fig. 3A). This was confirmed by labelling of newly synthesized peptidoglycan using azido-D-alanine and DBCO-PEG_4-Atto425 (Fig. 3B). As control, this finding was compared to a homogenously distributed membrane protein, which only shows a slightly stronger fluorescence signal at the division plane due to the doubled amount of membrane in this region (Fig. S3). In contrast, LcpA and LcpB were clearly enriched at cell poles and septa. Furthermore, the functionality of the Venus-LcpA fusion protein was tested by complementation studies in SilG-lcpA. The fusion protein complemented equally well as the non-labelled variant (Fig. S4).

Impact of reduced *lcpA* expression on *C. glutamicum* cell wall composition

As LcpA seemed to be involved in cell wall formation, we next analyzed putative changes of the different cell wall components, in order to gain further information regarding the function of this protein. Cell walls were isolated and analyzed as described in material and methods.

The two strains did not reveal any significant differences with respect to the peptidoglycan crosslinking and amidation (data not shown). We next isolated specifically the arabinogalactan component of the cell wall (Fig. 4A). Quantification of the isolated sugar components revealed that the arabinogalactan isolated from the SilG-lcpA mutant was significantly reduced in rhamnose, but not in arabinose and galactose, thereby suggesting that LcpA likely acts in the enzymatic step linking the rhamnose linker of the nascent arabinogalactan to peptidoglycan. We also analyzed how much arabinogalactan remained in the extracted cell walls. Indeed, a large proportion of the arabinogalactan was not extracted and a clear difference between wild-type and the SilG-lcpA mutant was observed. The mutant cell walls contained significantly less arabinogalactan compared to wild-type (Fig. 4B), indicating that the mutant cell walls had an overall decreased amount of arabinogalactan within their cell envelope.

We next analyzed the mycolic acid contents of wild-type and mutant cells after liberating them as mycolic acid methyl esters (MAME) from purified cell walls. Consistently, we observed a reduced amount of mycolic acids in the SilG-lcpA mutant (Fig. 4C-D and S5). A reduced amount of mycolic acids is likely a direct consequence of the reduced arabinogalactan content in the SilG-lcpA mutant. In turn, the stronger decrease of mycolic acids compared to arabinogalactan could be a reason for the observed different extraction behavior of arabinogalactan in SilG-lcpA, in other words, arabinogalactan extraction was more complete in the mutant background. However, the remaining arabinogalactan in the wild-type and mutant background had similar ratios between galactose, arabinose and rhamnose (Fig. 4B). By comparison of the residual sugar content of arabinogalactan-extracted cell walls about 60 % of arabinogalactan remained in extracted cell walls of the wild-type, whereas it was only 50% in SilG-lcpA (Fig. 4B).

Analysis of material from the culture supernatant

Other bacteria lacking LCP proteins have been described to release capsule material (34) or teichoic acids (8) into the supernatant. Furthermore, the electron microscopy pictures suggested impaired cell wall integrity of SilG-lcpA (Fig. 2). Therefore, solid material from the supernatant of the wild-type and SilG-lcpA was isolated by centrifugation. Given that the supernatant of the mutant culture contained much more material compared to the wild-type (Fig. S1C) it was only possible to collect enough material from this culture for further analysis. Protein analysis by SDS-PAGE and MALDI-TOF revealed two major bands (Fig. S1C) containing two trehalose corynomycolyl transferases (Cg0413 and Cg3182) (Table S3) which are associated with the outer membrane. Lipid analysis of the material showed the presence of mycolic acid and fatty acid methyl esters (Fig. 4C and S5). In the arabinogalactan analysis, rhamnose, arabinose and glucose (used as carbon source and possibly derived from trehalose-mycolates) as well as another so far unidentified sugar were found (Fig. S6). However, in contrast to the whole cell analysis, galactose was absent (Fig. S6). Therefore, the material extracted from the culture supernatant clearly differed from material of whole cells which would indicate a high amount of lysed cells.

Complementation studies with plasmid-encoded *lcpA* and *lcpB*

Complementation experiments with plasmid encoded *lcpA* under control of an IPTG-inducible promoter were performed to confirm that the observed phenotype is in fact a result of the silenced *lcpA* gene expression. Full complementation was already achieved by basal expression from the leaky P_{tac} (Fig. 5A, blue curve). Strong induction (100 µM IPTG) led to a growth rate lower than that of SilG-lcpA and a decreased final backscatter (data not shown). Interestingly, the effect of the silenced *lcpA* could be partially complemented by overexpressing *lcpB*, but only to a certain extent (Fig. S1D). Higher expression levels led to severe growth effects (Fig. S1D). In contrast,

the SilG-lcpA Δ*lcpB* strain could be fully complemented by plasmid-encoded LcpA. Under the tested conditions, LcpB was not required for normal growth as long as sufficient amounts of LcpA were present (Fig. S1E).

Mutational analysis of conserved amino acid residues in the LCP domain of LcpA

The structural studies of Cps2A of *Streptococcus pneumoniae* with bound ligand and magnesium led to the identification of several amino acids that are likely crucial for enzymatic activity of the protein (6). Complementation studies in the SilG-lcpA strain with LcpA variants mutated at conserved residues were supposed to show whether the catalytic center of LcpA is similar to Cps2A. Three positions of LcpA that are potentially involved in binding the pyrophosphate head group of the substrate (R138 & R257) or coordinating the magnesium ion (D88) (corresponding to D234, R267 and R362 in Cps2A or D75, R106 and R198 in *B. subtilis* TagU) were chosen for mutation based on sequence comparisons with Cps2A (6) (Fig. S7). Remarkably, all three mutated variants failed to complement the growth defect of SilG-lcpA (Fig. 5A) and interestingly, growth of the three strains was even worse compared to the control strain with the empty plasmid suggesting a critical role of all three amino acids in LcpA function.

The C-terminal region of LcpA is important for its function in cell wall biogenesis

To study the importance of different domains of LcpA for functionality, several truncated variants were cloned and tested for their ability to complement the phenotype of SilG-lcpA. The first variant (amino acids 24-518), lacking the N-terminal cytoplasmic part of the protein (Fig. 5B) fully complemented the SilG-lcpA phenotype, suggesting that the N-terminal part of the protein is not strictly required for function. In contrast, variants with truncations lacking 41, 130, or 232 amino acids of the C-terminal region were also tested and all of them lost the ability to complement the SilG-lcpA phenotype (Fig. 5B). This indicates the importance of the outer C-

terminal part containing the LytR_C domain for LcpA function in *C. glutamicum.* Supported by the high conservation of a C-terminal motif (CXN), these amino acids suggest being crucial for LcpA function (Fig. S7).

Complementation of SilG-lcpA with *C. diphtheriae* and *M. tuberculosis* LCP-proteins

In the following, complementation studies with proteins of *M. tuberculosis* and *C. diphtheriae* were conducted to test these for a similar function in cell wall biosynthesis, which would make them promising drug targets. Three proteins (CDC7B0634, rv3267 and rv3484) were chosen by homology searches for analysis, cloned into a *C. glutamicum* compatible plasmid and expressed in the SilG-lcpA strain (Fig. 5C). Different amounts of IPTG were tested to obtain optimal complementation. The *C. diphtheriae* protein already fully complemented the SilG-lcpA phenotype by leaky expression without any additional induction. In contrast, both mycobacterial proteins only partially complemented after induction with 100 µM IPTG, of which Rv3267 performed significantly better than Rv3484 (Fig. 5C).

Pyrophosphatase activity and oligomerization of LcpA

To test whether LcpA also exhibits pyrophosphatase activity similar to TagT of *Bacillus subtilis* (6), the following proteins were analyzed: LcpAΔTM (LcpA lacking the transmembrane helix) and three variants mutated at the same residues that led to loss of complementation ability (D88A, R138A, R257A). Gel filtration of LcpAΔTM suggested the formation of dimers under oxidizing conditions (theoretical molecular weight of monomer: 55.05 kDa; molecular weight according to gel filtration: ~104 kDa) and the formation of presumably tetramers under reducing conditions (~244 kDa) (Fig. S8). Because the native substrate of LcpA is presently unknown, geranyl pyrophosphate (GPP) was chosen as artificial substrate for the assay. To test whether the conversion to geranyl monophosphate (GMP) is magnesium dependent, the reaction was carried

493 out either with addition of $MgCl_2$ or in the presence of EDTA. All LcpA protein variants showed
494 significant amounts of pyrophosphatase activity, which was always higher in the presence of
495 EDTA compared to the addition of $MgCl_2$ (Fig. 5D). This means that in contrast to other LCP
496 proteins the pyrophosphatase activity of LcpA does not seems to be magnesium-dependent. The
497 pyrophosphatase activity measured with the control protein (CgtR10) was within the range of the
498 no-protein sample, excluding unspecific activity resulting from the protein purification
499 procedure.

500 **Domain distribution of LCP and LytR_C in bacteria**

501 The complementation studies with mutated and truncated LcpA revealed that both domains are
502 important for function. A more detailed analysis of the distribution of the two domains among
503 bacteria showed that 56% of the LCP domains known to date are found in firmicutes and 35% in
504 actinobacteria (Fig. 6A) (Data from Interpro, July 18th, 2016, 15838 domain sequences in total).
505 The distribution of the LytR_C domain differs significantly: 74% of the LytR_C domains belong
506 to actinobacteria and only 5% to firmicutes (Fig. 6B) (5614 proteins in total). Most (84%) of the
507 latter are found in members of the genus *Clostridium*. This analysis of sequence data suggests a
508 distinct function of the LytR_C domain in actinobacteria. The relative distribution of both
509 domains among actinobacteria is comparable. Therefore, both domains are equally important for
510 different members of this group.
511 The LCP and the LytR_C domains occur both separately and in combination. Among all LCP
512 domain containing proteins the majority (~76%) contains only an LCP domain whereas 19%
513 contain an additional LytR_C domain (5% in combination with other domains). In contrast, 53%
514 of the LytR_C domains are found in association with an LCP domain, whereas 44% contained
515 only the LytR_C domain. LytR_C solos are also present in *C. glutamicum* (Cg3032) and *M.*

tuberculosis (Rv0431 and Rv2700) but so far there is only one study reporting Rv0431 being involved in vesiculogenesis (Fig. S1B) (35).

Discussion

Relevance of LCP proteins in *C. glutamicum*

LCP-proteins are omnipresent in Gram-positive bacteria and appear to have an important function in cell wall biogenesis. However, recent studies suggest functional differences depending on the particular cell wall structure of the respective bacterial group. Here we present a physiological and biochemical analysis of two LCP proteins of *C. glutamicum*. Whereas a mutant lacking *lcpB* exhibited a wild-type-like phenotype, deletion of *lcpA* failed and silencing of *lcpA* expression resulted in a strong effect on cell growth and morphology. One LCP protein of *M. marinum* (MMAR_1274, rv3267 in *M. tuberculosis*) has also been described as essential but not been studied further to date (14). In contrast, a *M. marinum cpsA* mutant (MMAR_4966, rv3484) displayed negative effects on cell wall biosynthesis and growth leading to the hypothesis that CpsA might be responsible for the ligation of arabinogalactan to peptidoglycan (14). On sequence level LcpA is more similar to MMAR_1274 than to CpsA favoring a function comparable to the uncharacterized essential protein. This was further supported by the better complementation of SilG-lcpA by expressing rv3267 compared to rv3484 (Fig. 5C). *B. subtilis* survives a deletion of two out of its three LCP proteins but a triple deletion strain is not viable proposing an essential but redundant function of these three proteins in transfer of anionic cell wall polymers onto peptidoglycan (6).

Both LCP proteins localize at sites of active cell wall biosynthesis

Both LCP proteins of *C. glutamicum* are highly enriched in regions of nascent cell wall biosynthesis which has also been demonstrated for *S. pneumoniae* harboring LCP proteins

539 involved in capsule formation (34). In *B. subtilis*, LCP proteins were found to interact with MreB,
540 a cytoskeleton protein which is probably responsible for their localization (6). The localization of
541 the LCP proteins in *C. glutamicum* suggest that, analogous to MreB/TagTUV in *B. subtilis*, they
542 might be part of the elongasome which is required for polar growth and be spatially organized by
543 DivIVA (17, 36-38). However, given that *C. glutamicum* lacks MreB and that the cytoplasmic
544 part of LcpA is dispensable for function, the localization likely appears within the membrane or
545 in the periplasm and additional proteins would be required to connect it to the elongasome.
546 However, the identification of putative interaction partners of LcpA by co-purification failed so
547 far (data not shown).

548 **Both the LCP and the LytR_C domains of LcpA are essential for function**

549 With the exception of *M. marinum* CpsA (14) and *Anabaena* sp. strain PCC 7120 All0187 (39),
550 most studies of LCP proteins published to date are dealing with those proteins containing only the
551 LCP and not the LytR_C domain (6-9, 40). As described above, in actinobacteria the two
552 domains frequently exist in combination within one protein and the question arises, whether both
553 domains are required for LcpA function. Our complementation studies with truncated and
554 mutated LcpA-variants revealed not only the importance of the conserved aspartate and arginine
555 residues located in the LCP domain but also suggest an important function for the LytR_C
556 domain.

557 There are currently two possible explanations for the necessity of two domains in LcpA: i) LcpA
558 catalyzes one single reaction for which both domains are strictly required, or ii) LcpA catalyzes
559 at least two separate reactions which are catalyzed by either one of the domains. Two arguments
560 supporting the latter case are: i) both domains also quite frequently occur without the other one
561 (6, 35) and ii) although both domains appeared to be important for LcpA function, mutations in

562 the LCP domain were more detrimental to the growth of *C. glutamicum* than the absence of the
563 LytR_C domain (Fig. 5A and B). Maybe a toxic intermediate produced by the LytR_C domain
564 was accumulating, which is further processed by the LCP domain under normal circumstances.
565 The oligomerization of LcpA observed here is a unique feature among LCP proteins
566 characterized so far; ΔTM-Cps2A, e.g. was reported to be monomeric (6). Considering the fact
567 that Cps2A lacks a LytR_C domain it is possible that this domain is involved in dimerization or
568 oligomerization. Given that LcpA forms dimers or tetramers, the effects of mutated LcpA
569 expression (Fig. 5A) could be explained by a titration of functional LcpA by mutated, non-
570 functional LcpA proteins through the formation of non-functional heterodimers. This would
571 result in a further reduction of the low amounts of active LcpA (Fig. 5A) and consequently
572 further impaired growth. In contrast, the LytR_C truncated variants were possibly no longer able
573 to form dimers and just present as separate inactive proteins (Fig. 5B).
574 Interestingly, although the LcpA variants with mutations in the LCP domain failed to
575 complement, the pyrophosphatase-activity measured with the purified proteins was not affected.
576 One reason for this could be that the artificial substrate GPP was used whose catalysis might not
577 be influenced by the mutations of the protein. Furthermore, the measured pyrophosphatase
578 activity might differ from that of TagT (6) by being catalyzed by a different, magnesium
579 independent mechanism and possibly by being located within the LytR_C and not within the LCP
580 domain. Of course, further studies are required to test these hypotheses.

581 **Possible functions of LcpA in cell wall biogenesis**

582 Known or proposed steps of cell wall biogenesis in *C. glutamicum* that are relevant to discuss the
583 possible function of LCP proteins in this species are summarized in Figure 7. Similar
584 mechanisms of mycobacteria have been reviewed recently (12). It was proposed by Wang et al.,

585 (14) that the LCP protein CpsA of *M. marinum* is responsible for the transfer of arabinogalactan
586 onto peptidoglycan. This hypothesis is based on data showing a reduced arabinogalactan content
587 of the cell wall in the mutant (14).
588 As stated above, the mycobacterial proteins with the highest similarity to LcpA are not
589 *M. marinum* CpsA (*M. tuberculosis* Rv3484), but Rv3267 (47% identity with LcpA) and
590 MMAR_1274 (46% identity), which have been reported as being essential (14, 41). In contrast,
591 CpsA (33% identity), Rv3484 (33% identity), and the third *M. tuberculosis* LCP protein with
592 both an LCP and a LytR_C domain, Rv0822c (26% identity) (Fig. S1B) can be deleted (14, 41),
593 which points toward distinct, non-redundant functions of CpsA and Rv3484 on one side and
594 MMAR_1274 and Rv3267 on the other side. So far nothing is known about MMAR_4858 (27%
595 identity), the homolog of Rv0822c, but it is presumably also not essential.
596 In our studies the silencing of *lcpA* lead to a significant reduction of the arabinogalactan content
597 in the cell wall. These data, indeed, support the hypothesis, that LcpA is responsible for the
598 transfer of arabinogalactan onto peptidoglycan, which represents a central missing link in the
599 mechanistic understanding of cell wall biosynthesis in corynebacteria and mycobacteria. This
600 mechanism is furthermore corroborated by the observed pyrophosphatase activity and the
601 significant reduction of rhamnose in the SilG-lcpA mutant. As a third piece of evidence, this
602 strain also released a considerable amount of cell wall material into the supernatant during
603 cultivation. Altogether, these data support a model where LcpA is involved in the covalent
604 attachment of arabinogalactan to peptidoglycan in *C. glutamicum*.
605 Considering the domain structure of LcpA and the differences in functionality of LcpA and
606 LcpB, further potential functions need to be considered in future studies. For *A. oris* it has been
607 shown that an LCP protein (containing only the LCP domain) is involved in a protein
608 glycosylation pathway also including a sortase which couples certain cell-surface proteins to

peptidoglycan (9). Here the LCP protein is responsible for the transfer of a glycan polymer from its lipid-linked precursor onto the protein before it is attached to peptidoglycan. However, although *C. glutamicum* is predicted to contain a sortase (cg3251), no sortase substrate could be identified so far (42). Therefore, given also that the *A. oris* protein lacks the LytR_C-domain, a similar function is rather unlikely for LcpA.

While this manuscript was in revision, two further studies addressing the function of LCP proteins in the corynebacteriales were published (43, 44) which provide further evidence that LCP proteins are responsible for the ligation of arabinogalactan to peptidoglycan. However, these studies did not address a potential additional function of the C-terminal domain. Further studies are required to investigate whether arabinogalactan-peptidoglycan ligation is the only function of Lcp proteins in corynebacteriales and to clarify the roles of LcpB and the C-terminal domain of LcpA in this context.

Conclusion

The results presented in this study clearly show that LcpA has a very important role in cell wall biogenesis and integrity, presumably being responsible for the ligation of arabinogalactan to peptidoglycan, while both domains seem to be essential for function. Since the enzymes involved in the ligation of arabinogalactan to peptidoglycan were so far unknown, our data represent an important contribution to the mechanistic understanding of this missing link in the corynebacterial cell wall biosynthesis. Our data emphasize this class of proteins as a potential drug target highly interesting for further investigation. Furthermore, the *C. glutamicum* SilG-lcpA strain offers an ideal platform strain for further functional and physiological studies of LCP proteins of corynebacteriales since we have shown that cross-species complementation with mycobacterial proteins is possible and functionality of protein variants can easily be tested by

growth studies. Altogether, our data underline the importance of LCP proteins in cell wall biogenesis in this medically and biotechnologically important class of bacteria and suggest an important role of the conserved C-terminal domain for functionality.

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References

1. **Lazarevic V, Margot P, Soldo B, Karamata D.** 1992. Sequencing and analysis of the *Bacillus subtilis lytRABC* divergon: a regulatory unit encompassing the structural genes of the *N*-acetylmuramoyl-L-alanine amidase and its modifier. J Gen Microbiol **138:**1949-1961.

2. **Cieslewicz MJ, Kasper DL, Wang Y, Wessels MR.** 2001. Functional analysis in type Ia group B *Streptococcus* of a cluster of genes involved in extracellular polysaccharide production by diverse species of Streptococci. J Biol Chem **276:**139-146.

3. **Ligozzi M, Pittaluga F, Fontana R.** 1993. Identification of a genetic element (*psr*) which negatively controls expression of *Enterococcus hirae* penicillin-binding protein 5. J Bacteriol **175:**2046-2051.

4. **Mitchell A, Chang HY, Daugherty L, Fraser M, Hunter S, Lopez R, McAnulla C, McMenamin C, Nuka G, Pesseat S, Sangrador-Vegas A, Scheremetjew M, Rato C, Yong SY, Bateman A, Punta M, Attwood TK, Sigrist CJ, Redaschi N, Rivoire C, Xenarios I, Kahn D, Guyot D, Bork P, Letunic I, Gough J, Oates M, Haft D, Huang H, Natale DA, Wu CH, Orengo C, Sillitoe I, Mi H, Thomas PD, Finn RD.** 2015. The InterPro protein families database: the classification resource after 15 years. Nucleic Acids Res **43:**D213-221.

5. **Hübscher J, Lüthy L, Berger-Bächi B, Stutzmann Meier P.** 2008. Phylogenetic distribution and membrane topology of the LytR-CpsA-Psr protein family. BMC Genomics **9:**617 doi:610.1186/1471-2164-1189-1617.

6. **Kawai Y, Marles-Wright J, Cleverley RM, Emmins R, Ishikawa S, Kuwano M, Heinz N, Bui NK, Hoyland CN, Ogasawara N, Lewis RJ, Vollmer W, Daniel RA,**

Errington J. 2011. A widespread family of bacterial cell wall assembly proteins. EMBO J **30:**4931-4941.

7. **Chan YG, Kim HK, Schneewind O, Missiakas D.** 2014. The capsular polysaccharide of *Staphylococcus aureus* is attached to peptidoglycan by the LytR-CpsA-Psr (LCP) family of enzymes. J Biol Chem **289:**15680-15690.

8. **Chan YG, Frankel MB, Dengler V, Schneewind O, Missiakas D.** 2013. *Staphylococcus aureus* mutants lacking the LytR-CpsA-Psr family of enzymes release cell wall teichoic acids into the extracellular medium. J Bacteriol **195:**4650-4659.

9. **Wu C, Huang IH, Chang C, Reardon-Robinson ME, Das A, Ton-That H.** 2014. Lethality of sortase depletion in *Actinomyces oris* caused by excessive membrane accumulation of a surface glycoprotein. Mol Microbiol **94:**1227-1241.

10. **Mishra AK, Driessen NN, Appelmelk BJ, Besra GS.** 2011. Lipoarabinomannan and related glycoconjugates: structure, biogenesis and role in *Mycobacterium tuberculosis* physiology and host-pathogen interaction. FEMS Microbiol Rev **35:**1126-1157.

11. **Eggeling L, Besra GS, Alderwick L.** 2008. Structure and synthesis of the cell wall, p 267-294. *In* Burkovski A (ed), Corynebacteria: genomics and molecular biology. Caister Academic Press, Norfolk, UK.

12. **Jankute M, Cox JA, Harrison J, Besra GS.** 2015. Assembly of the mycobacterial cell wall. Annu Rev Microbiol **69:**405-423.

13. **Daffe M, Crick DC, Jackson M.** 2014. Genetics of capsular polysaccharides and cell envelope (glyco)lipids. Microbiol Spectr **2:**MGM2-0021-2013 doi: 0010.1128/microbiolspec.MGM0022-0021-2013.

14. **Wang Q, Zhu L, Jones V, Wang C, Hua Y, Shi X, Feng X, Jackson M, Niu C, Gao Q.** 2015. CpsA, a LytR-CpsA-Psr family protein in *Mycobacterium marinum*, is required for cell wall integrity and virulence. Infect Immun **83:**2844-2854.

15. **Baumgart M, Luder K, Grover S, Gätgens C, Besra GS, Frunzke J.** 2013. IpsA, a novel LacI-type regulator, is required for inositol-derived lipid formation in *Corynebacteria* and *Mycobacteria*. BMC Biol **11:**122 doi:110.1186/1741-7007-1111-1122.

16. **Seidel M, Alderwick LJ, Birch HL, Sahm H, Eggeling L, Besra GS.** 2007. Identification of a novel arabinofuranosyltransferase AftB involved in a terminal step of cell wall arabinan biosynthesis in Corynebacterianeae, such as *Corynebacterium glutamicum* and *Mycobacterium tuberculosis*. J Biol Chem **282:**14729-14740.

17. **Donovan C, Bramkamp M.** 2014. Cell division in *Corynebacterineae*. Front Microbiol **5:**132.

18. **Eggeling L, Bott M.** 2015. A giant market and a powerful metabolism: L-lysine provided by *Corynebacterium glutamicum*. Appl Microbiol Biotechnol **99:**3387-3394.

19. **Keilhauer C, Eggeling L, Sahm H.** 1993. Isoleucine synthesis in *Corynebacterium glutamicum* - Molecular analysis of the *IlvB-IlvN-IlvC* operon. J Bacteriol **175:**5595-5603.

20. **Sambrook J, Russell DW.** 2001. Molecular cloning: A laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, NY.

21. **Hanahan D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol **166:**557-580.

22. **van der Rest ME, Lange C, Molenaar D.** 1999. A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. Appl Microbiol Biotechnol **52:**541-545.

23. **Gibson DG.** 2011. Enzymatic assembly of overlapping DNA fragments. Methods Enzymol **498:**349-361.

24. **Niebisch A, Bott M.** 2001. Molecular analysis of the cytochrome bc_1-aa_3 branch of the *Corynebacterium glutamicum* respiratory chain containing an unusual diheme cytochrome c_1. Arch Microbiol **175:**282-294.

25. **Lutz R, Bujard H.** 1997. Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I_1-I_2 regulatory elements. Nucleic Acids Res **25:**1203-1210.

26. **Frunzke J, Engels V, Hasenbein S, Gätgens C, Bott M.** 2008. Co-ordinated regulation of gluconate catabolism and glucose uptake in *Corynebacterium glutamicum* by two functionally equivalent transcriptional regulators, GntR1 and GntR2. Mol Microbiol **67:**305-322.

27. **Siegrist MS, Whiteside S, Jewett JC, Aditham A, Cava F, Bertozzi CR.** 2013. D-Amino acid chemical reporters reveal peptidoglycan dynamics of an intracellular pathogen. ACS Chem Biol **8:**500-505.

28. **Braun V, Rehn K.** 1969. Chemical characterization, spatial distribution and function of a lipoprotein (murein-lipoprotein) of the *E. coli* cell wall. The specific effect of trypsin on the membrane structure. Eur J Biochem **10:**426-438.

29. **Glauner B, Holtje JV, Schwarz U.** 1988. The composition of the murein of *Escherichia coli*. J Biol Chem **263:**10088-10095.

30. **Draper P.** 1971. The walls of *Mycobacterium lepraemurium*: chemistry and ultrastructure. J Gen Microbiol **69:**313-324.

31. **Schubert K, Reiml D, Accolas JP, Fiedler F.** 1993. A novel type of *meso*-diaminopimelic acid-based peptidoglycan and novel poly(erythritol phosphate) teichoic acids in cell walls of two coryneform isolates from the surface flora of French cooked cheeses. Arch Microbiol **160:**222-228.

32. **Gauch R, Leuenberger U, Baumgartner E.** 1979. Quantitative determination of mono-, di- and trisaccharides by thin-layer chromatography. J Chromatogr **174:**195-200.

33. **Minnikin DE, Alshamaony L, Goodfellow M.** 1975. Differentiation of *Mycobacterium*, *Nocardia*, and related taxa by thin-layer chromatographic analysis of whole organism methanolysates. J Gen Microbiol **88:**200-204.

34. **Eberhardt A, Hoyland CN, Vollmer D, Bisle S, Cleverley RM, Johnsborg O, Havarstein LS, Lewis RJ, Vollmer W.** 2012. Attachment of capsular polysaccharide to the cell wall in *Streptococcus pneumoniae*. Microb Drug Resist **18:**240-255.

35. **Rath P, Huang C, Wang T, Wang T, Li H, Prados-Rosales R, Elemento O, Casadevall A, Nathan CF.** 2013. Genetic regulation of vesiculogenesis and immunomodulation in *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA **110:**E4790-E4797.

36. **Letek M, Ordonez E, Vaquera J, Margolin W, Flardh K, Mateos LM, Gil JA.** 2008. DivIVA is required for polar growth in the MreB-lacking rod-shaped actinomycete *Corynebacterium glutamicum*. J Bacteriol **190:**3283-3292.

37. **Sieger B, Schubert K, Donovan C, Bramkamp M.** 2013. The lipid II flippase RodA determines morphology and growth in *Corynebacterium glutamicum*. Mol Microbiol **90:**966-982.

38. **Donovan C, Sieger B, Krämer R, Bramkamp M.** 2012. A synthetic *Escherichia coli* system identifies a conserved origin tethering factor in Actinobacteria. Mol Microbiol **84:**105-116.

39. **Mella-Herrera RA, Neunuebel MR, Golden JW.** 2011. *Anabaena* sp. strain PCC 7120 *conR* contains a LytR-CpsA-Psr domain, is developmentally regulated, and is essential for diazotrophic growth and heterocyst morphogenesis. Microbiology **157:**617-626.

40. **Rowe HM, Hanson BR, Runft DL, Lin Q, Firestine SM, Neely MN.** 2015. Modification of the CpsA protein reveals a role in alteration of the *Streptococcus agalactiae* cell envelope. Infect Immun **83:**1497-1506.

41. **Sassetti CM, Boyd DH, Rubin EJ.** 2003. Genes required for mycobacterial growth defined by high density mutagenesis. Mol Microbiol **48:**77-84.

42. **Boekhorst J, de Been MW, Kleerebezem M, Siezen RJ.** 2005. Genome-wide detection and analysis of cell wall-bound proteins with LPxTG-like sorting motifs. J Bacteriol **187:**4928-4934.

43. **Grzegorzewicz AE, De Sousa-d'Auria C, McNeil MR, Huc-Claustre E, Jones V, Petit C, Angala SK, Zemanova J, Wang Q, Belardinelli JM, Gao Q, Ishizaki Y, Mikusova K, Brennan PJ, Ronning DR, Chami M, Houssin C, Jackson M.** 2016. Assembling of the Mycobacterium tuberculosis Cell Wall Core. J Biol Chem doi:10.1074/jbc.M116.739227.

44. **Harrison J, Lloyd G, Joe M, Lowary TL, Reynolds E, Walters-Morgan H, Bhatt A, Lovering A, Besra GS, Alderwick LJ.** 2016. Lcp1 is a phosphotransferase responsible for ligating arabinogalactan to peptidoglycan in *Mycobacterium tuberculosis*. MBio **7:**e00972-00916. doi:00910.01128/mBio.00972-00916.

45. **Alm EJ, Huang KH, Price MN, Koche RP, Keller K, Dubchak IL, Arkin AP.** 2005. The MicrobesOnline Web site for comparative genomics. Genome Res **15:**1015-1022.

46. **Levefaudes M, Patin D, de Sousa-d'Auria C, Chami M, Blanot D, Herve M, Arthur M, Houssin C, Mengin-Lecreulx D.** 2015. Diaminopimelic acid amidation in Corynebacteriales: New insights into the role of LtsA in peptidoglycan modification. J Biol Chem **290:**13079-13094.

47. **Münch D, Roemer T, Lee SH, Engeser M, Sahl HG, Schneider T.** 2012. Identification and *in vitro* analysis of the GatD/MurT enzyme-complex catalyzing lipid II amidation in *Staphylococcus aureus*. PLoS Pathog **8:**e1002509 doi:1002510.1001371/journal.ppat.1002509.

48. **Figueiredo TA, Sobral RG, Ludovice AM, Almeida JM, Bui NK, Vollmer W, de Lencastre H, Tomasz A.** 2012. Identification of genetic determinants and enzymes involved with the amidation of glutamic acid residues in the peptidoglycan of *Staphylococcus aureus*. PLoS Pathog **8:**e1002508 doi:1002510.1001371/journal.ppat.1002508.

49. **Lavollay M, Arthur M, Fourgeaud M, Dubost L, Marie A, Riegel P, Gutmann L, Mainardi JL.** 2009. The beta-lactam-sensitive D,D-carboxypeptidase activity of Pbp4 controls the L,D and D,D transpeptidation pathways in *Corynebacterium jeikeium*. Mol Microbiol **74:**650-661.

50. **Lavollay M, Arthur M, Fourgeaud M, Dubost L, Marie A, Veziris N, Blanot D, Gutmann L, Mainardi JL.** 2008. The peptidoglycan of stationary-phase *Mycobacterium tuberculosi*s predominantly contains cross-links generated by L,D-transpeptidation. J Bacteriol **190:**4360-4366.

51. **Dubee V, Triboulet S, Mainardi JL, Etheve-Quelquejeu M, Gutmann L, Marie A, Dubost L, Hugonnet JE, Arthur M.** 2012. Inactivation of *Mycobacterium tuberculosis* L,D-transpeptidase Ldt_{Mt1} by carbapenems and cephalosporins. Antimicrob Agents Chemother **56:**4189-4195.

52. **Valbuena N, Letek M, Ordonez E, Ayala J, Daniel RA, Gil JA, Mateos LM.** 2007. Characterization of HMW-PBPs from the rod-shaped actinomycete *Corynebacterium glutamicum*: peptidoglycan synthesis in cells lacking actin-like cytoskeletal structures. Mol Microbiol **66:**643-657.

53. **Grover S, Alderwick LJ, Mishra AK, Krumbach K, Marienhagen J, Eggeling L, Bhatt A, Besra GS.** 2014. Benzothiazinones mediate killing of *Corynebacterineae* by blocking decaprenyl phosphate recycling involved in cell wall biosynthesis. J Biol Chem **289:**6177-6187.

54. **Alderwick LJ, Radmacher E, Seidel M, Gande R, Hitchen PG, Morris HR, Dell A, Sahm H, Eggeling L, Besra GS.** 2005. Deletion of Cg-*emb* in Corynebacterianeae leads to a novel truncated cell wall arabinogalactan, whereas inactivation of Cg-*ubiA* results in an arabinan-deficient mutant with a cell wall galactan core. J Biol Chem **280:**32362-32371.

55. **Dianiskova P, Kordulakova J, Skovierova H, Kaur D, Jackson M, Brennan PJ, Mikusova K.** 2011. Investigation of ABC transporter from mycobacterial arabinogalactan biosynthetic cluster. Gen Physiol Biophys **30:**239-250.

56. **Alderwick LJ, Dover LG, Seidel M, Gande R, Sahm H, Eggeling L, Besra GS.** 2006. Arabinan-deficient mutants of *Corynebacterium glutamicum* and the consequent flux in decaprenylmonophosphoryl-D-arabinose metabolism. Glycobiology **16:**1073-1081.

57. **Larrouy-Maumus G, Skovierova H, Dhouib R, Angala SK, Zuberogoitia S, Pham H, Villela AD, Mikusova K, Noguera A, Gilleron M, Valentinova L, Kordulakova J, Brennan PJ, Puzo G, Nigou J, Jackson M.** 2012. A small multidrug resistance-like transporter involved in the arabinosylation of arabinogalactan and lipoarabinomannan in mycobacteria. J Biol Chem **287:**39933-39941.

58. **Birch HL, Alderwick LJ, Rittmann D, Krumbach K, Etterich H, Grzegorzewicz A, McNeil MR, Eggeling L, Besra GS.** 2009. Identification of a terminal rhamnopyranosyltransferase (RptA) involved in *Corynebacterium glutamicum* cell wall biosynthesis. J Bacteriol **191:**4879-4887.

59. **Yagi T, Mahapatra S, Mikusova K, Crick DC, Brennan PJ.** 2003. Polymerization of mycobacterial arabinogalactan and ligation to peptidoglycan. J Biol Chem **278:**26497-26504.

60. **Hancock IC, Carman S, Besra GS, Brennan PJ, Waite E.** 2002. Ligation of arabinogalactan to peptidoglycan in the cell wall of *Mycobacterium smegmatis* requires concomitant synthesis of the two wall polymers. Microbiology **148:**3059-3067.

61. **Yamaryo-Botte Y, Rainczuk AK, Lea-Smith DJ, Brammananth R, van der Peet PL, Meikle P, Ralton JE, Rupasinghe TW, Williams SJ, Coppel RL, Crellin PK, McConville MJ.** 2015. Acetylation of trehalose mycolates is required for efficient MmpL-mediated membrane transport in Corynebacterineae. ACS Chem Biol **10:**734-746.

62. **Grzegorzewicz AE, Pham H, Gundi VA, Scherman MS, North EJ, Hess T, Jones V, Gruppo V, Born SE, Kordulakova J, Chavadi SS, Morisseau C, Lenaerts AJ, Lee RE, McNeil MR, Jackson M.** 2012. Inhibition of mycolic acid transport across the *Mycobacterium tuberculosis* plasma membrane. Nat Chem Biol **8:**334-341.

63. **Brand S, Niehaus K, Pühler A, Kalinowski J.** 2003. Identification and functional analysis of six mycolyltransferase genes of *Corynebacterium glutamicum* ATCC 13032: the genes *cop1*, *cmt1*, and *cmt2* can replace each other in the synthesis of trehalose dicorynomycolate, a component of the mycolic acid layer of the cell envelope. Arch Microbiol **180:**33-44.

Figure legends

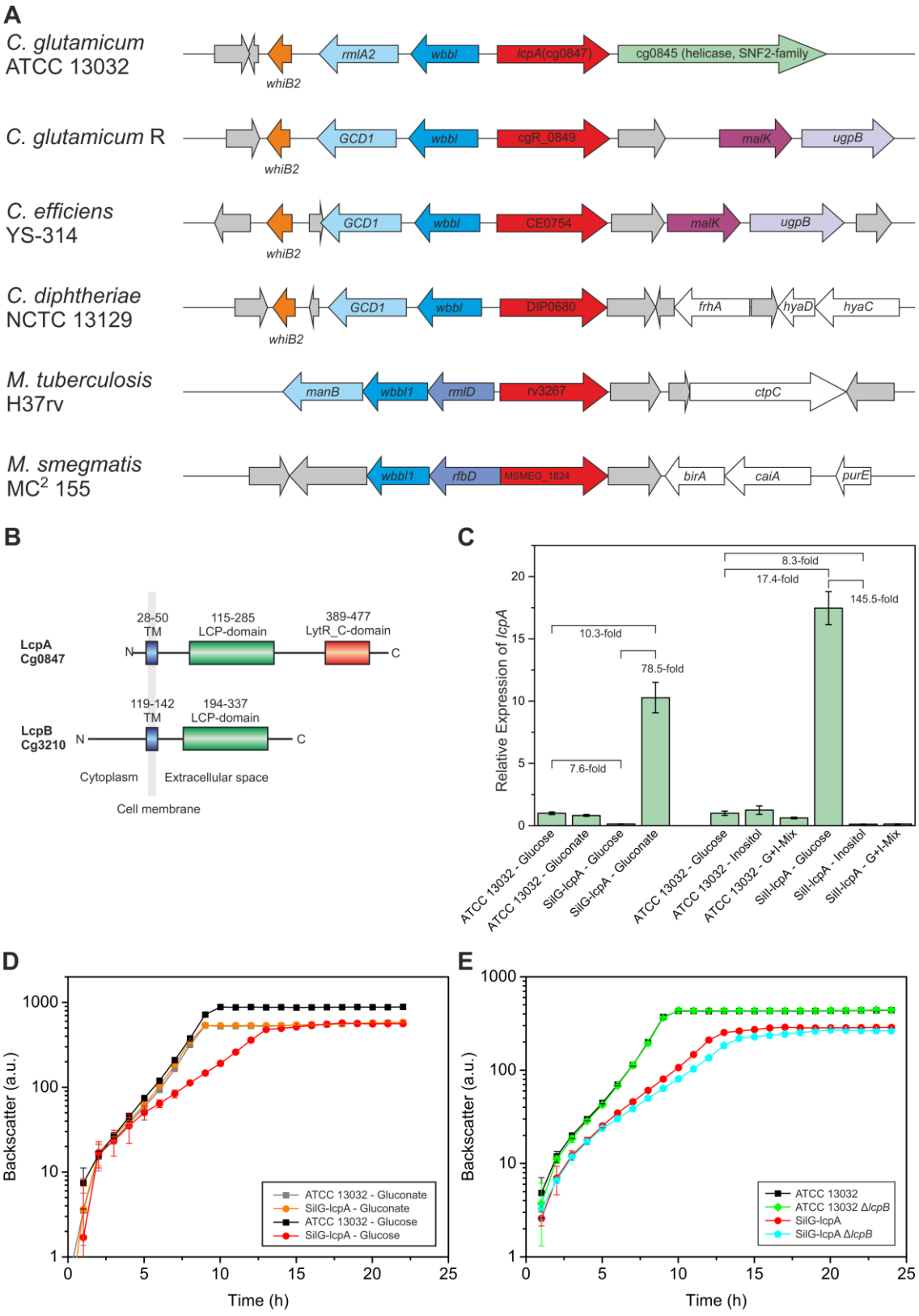
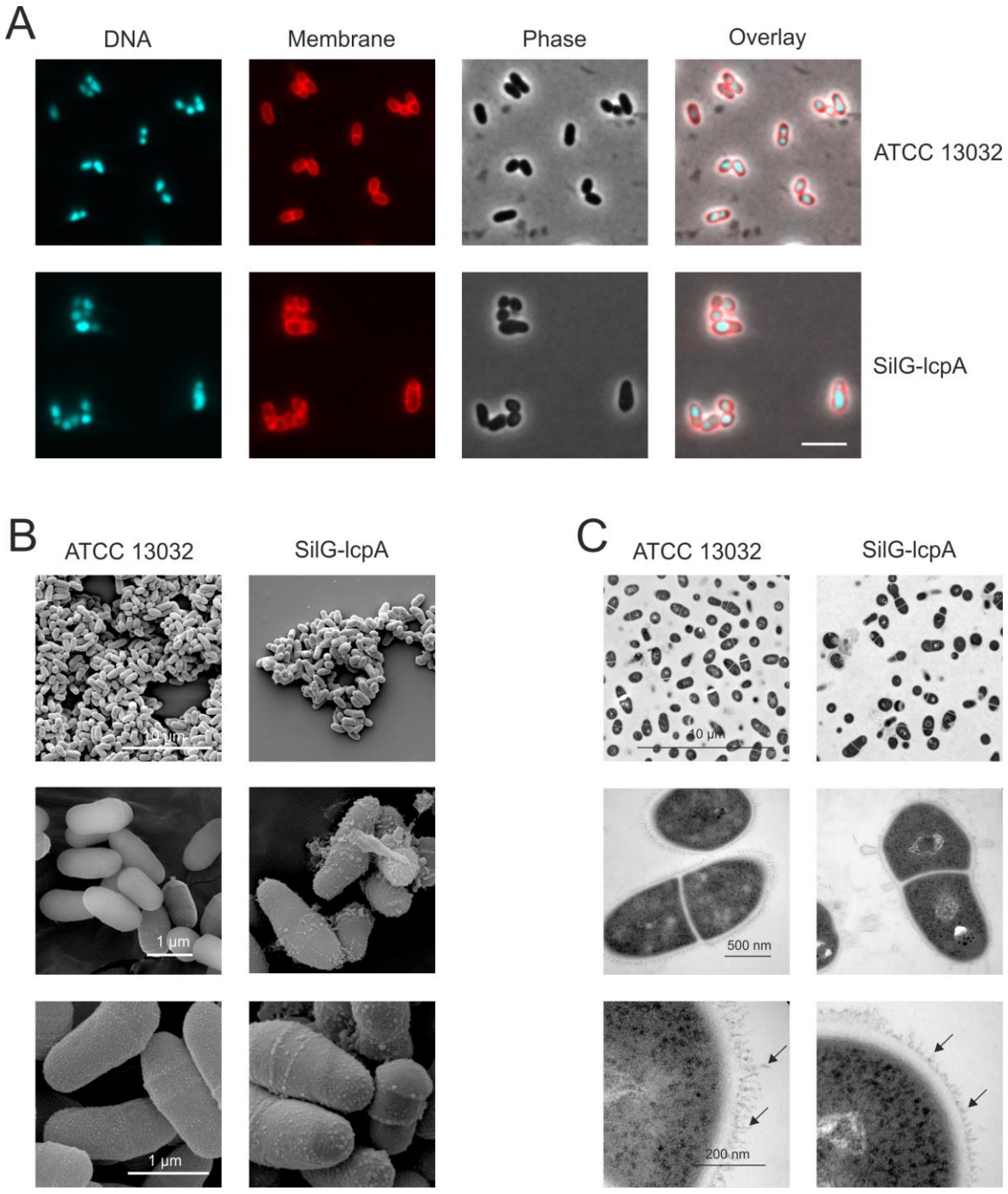


FIG 1 Overview of LCP proteins in *C. glutamicum* and their impact on cellular growth. (A) Phylogenetic conservation of *lcpA* among related species (red arrows). Neighboring conserved genes are colored accordingly. White indicates other, non-conserved proteins of known function and grey represents hypothetical or uncharacterized proteins. Data were taken from (45). The corresponding figure for *lcpB* is presented as Figure S1A. (B) Domain organization of LcpA and LcpB. (C) Silencing of *lcpA* expression. Given are relative expression levels of *lcpA* in different strains and on different media determined by RT-qPCR. G+I-Mix is a carbon source mixture of 1% (w/v) glucose and 1% (w/v) *myo*-inositol final concentration. The average and standard deviation of three biological replicates with two technical replicates each are presented. (D) Growth of the *lcpA*-silencing strain SilG-lcpA on glucose or gluconate as carbon source in comparison to the wild-type. Cells were cultivated in complex media with gluconate followed by two consecutive cultivations in CGXII minimal media with the indicated carbon source. The average and the standard deviation of three biological replicates of the second CGXII culture are presented. (E) Impact of *lcpA* silencing and/or *lcpB* deletion on growth of *C. glutamicum*. Same cultivation procedure as for D with glucose as carbon source.



879

880 **FIG 2** Microscopic pictures of *C. glutamicum* wild-type (ATCC 13032) and the *lcpA*-silencing
881 strain SilG-lcpA. **(**A) Fluorescence microscopy after the second cultivation (2x24 h) in CGXII
882 minimal medium with glucose as carbon source with staining of DNA (Hoechst 33342) and
883 membranes (Nile red). Scale bar 5 µm. (B) SEM and (C) TEM pictures at different magnification
884 (2,500x, 20,000x, 35,000x for SEM as well as 2,800x, 46,000x and 130,000x for TEM). Arrows
885 point towards fringe-like structures that are much shortened in the SilG-lcpA strain.

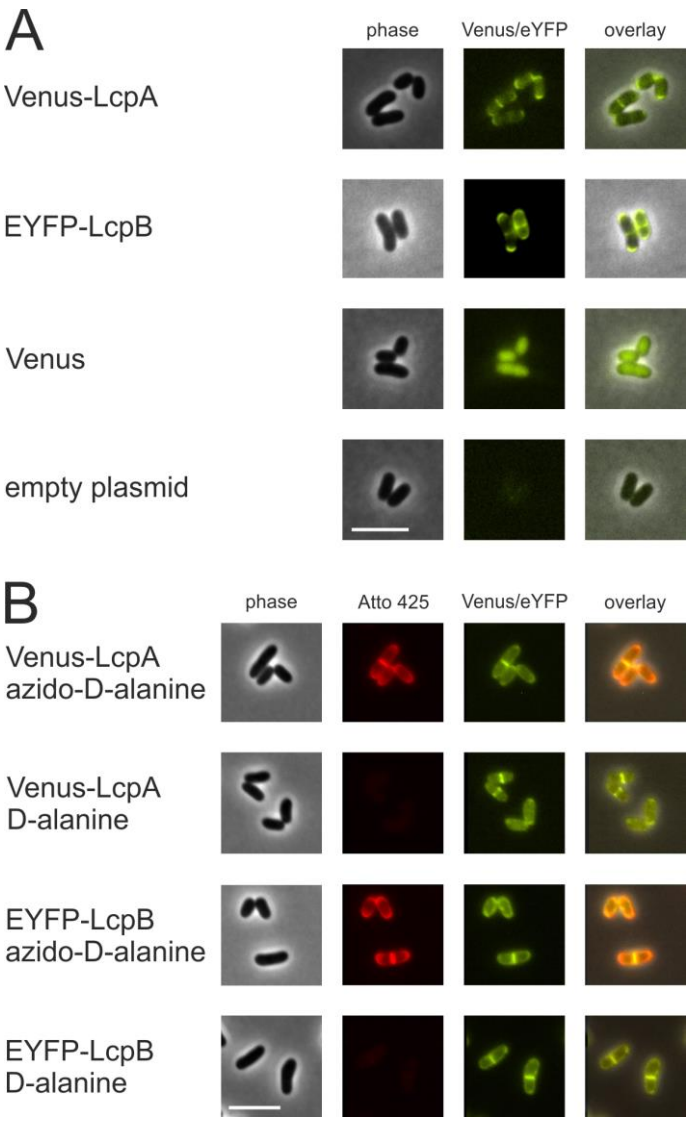


FIG 3 Co-localization of Venus-LcpA and EYFP-LcpB with newly synthesized peptidoglycan in *C. glutamicum* ATCC 13032. The cells carrying a plasmid encoding Venus N-terminally fused to LcpA (pAN6-venus-*lcpA*) or EYFP N-terminally fused to LcpB (pAN6-eyfp-*lcpB*) were cultivated in CGXII minimal medium with 2% (w/v) glucose as carbon source and 10 µM IPTG. (A) localization of Venus-LcpA and EYFP-LcpB. Strains with an empty plasmid and a plasmid encoding only Venus served as controls. (B) Co-localization of Venus-LcpA and EYFP-LcpB with newly synthesized peptidoglycan. After 4 hours of cultivation, newly synthesized peptidoglycan was visualized by bioorthogonal labeling (click chemistry with azido-D-alanine and DBCO-PEG_4-Atto425). Cells were either incubated for 3 min with azido-D-alanine or with D-alanine as control. For better visibility, fluorescence of Atto 425 (Ex 436 Em 484) is shown in red. Scale bars 5 µm.

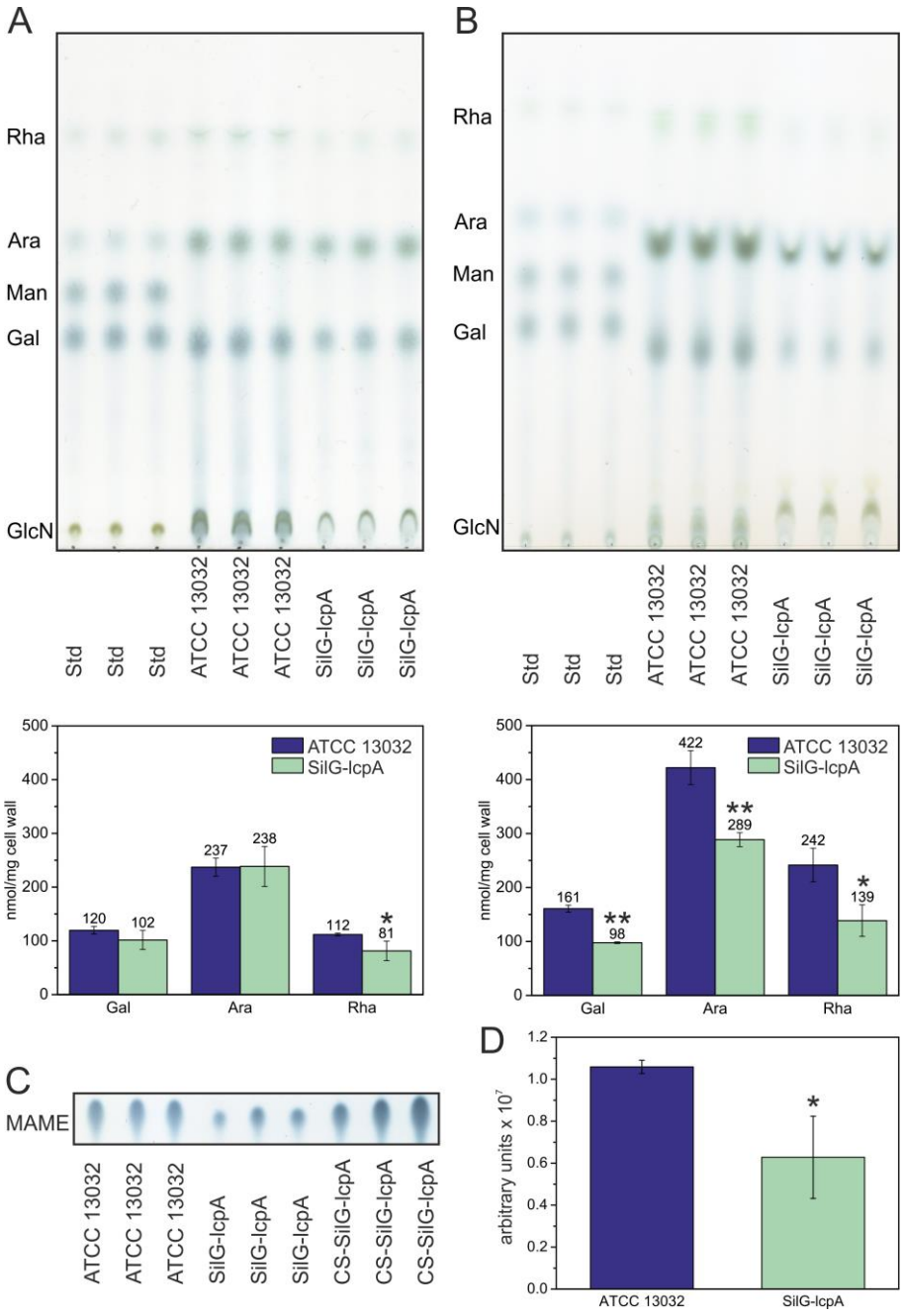


FIG 4 LcpA is presumably involved in linking arabinogalactan and peptidoglycan. (A) Sugar content in arabinogalactan and (B) remaining sugar content in SDS-ProtK-cell walls after extraction of arabinogalactan in nmol/mg cell wall (* $P < 0.05$ and ** $P < 0.01$; two-sided *t*-test compared to wild-type). (C) Mycolic acids were liberated as mycolic acid methyl esters (MAME) from purified cell walls by acidic methanolysis. TLC was run in toluene:acetone (97:3, v/v) and developed with phosphomolybdic acid. CS-SilG-lcpA, material from the culture supernatant of SilG-lcpA. Three technical replicates of one sample each are presented. The full picture is provided as Fig. S5. (D) Relative amounts of MAMEs per mg cell wall (* $P < 0.05$ two-sided *t*-test compared to wild-type).

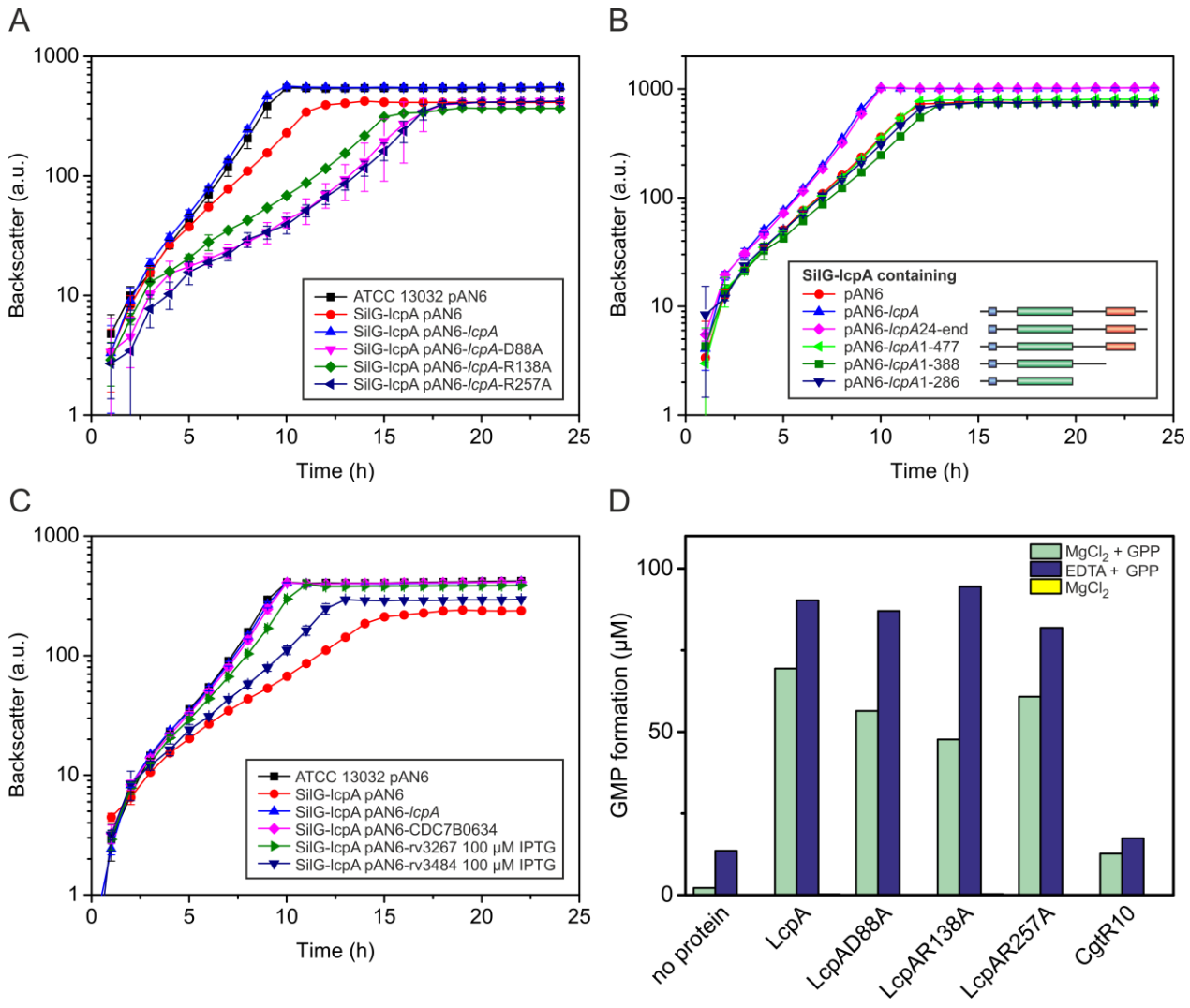
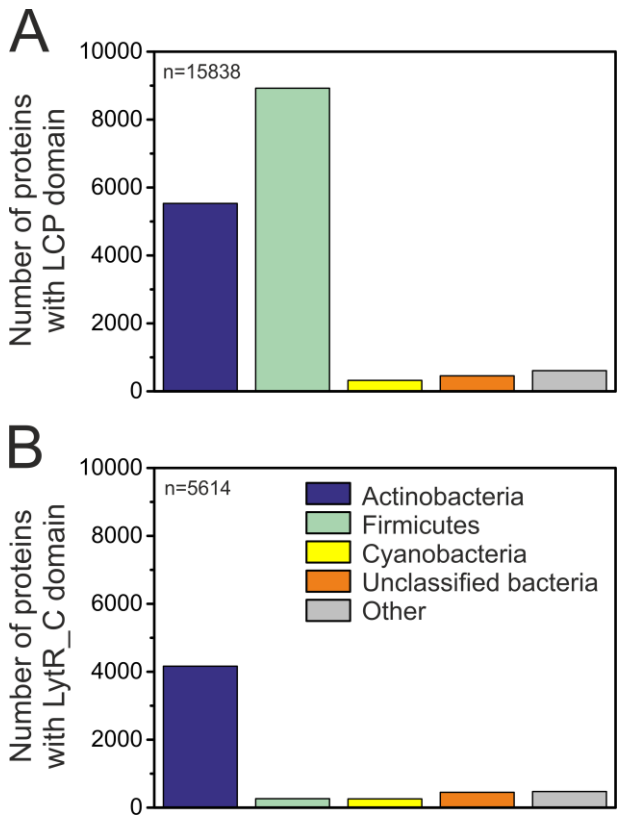


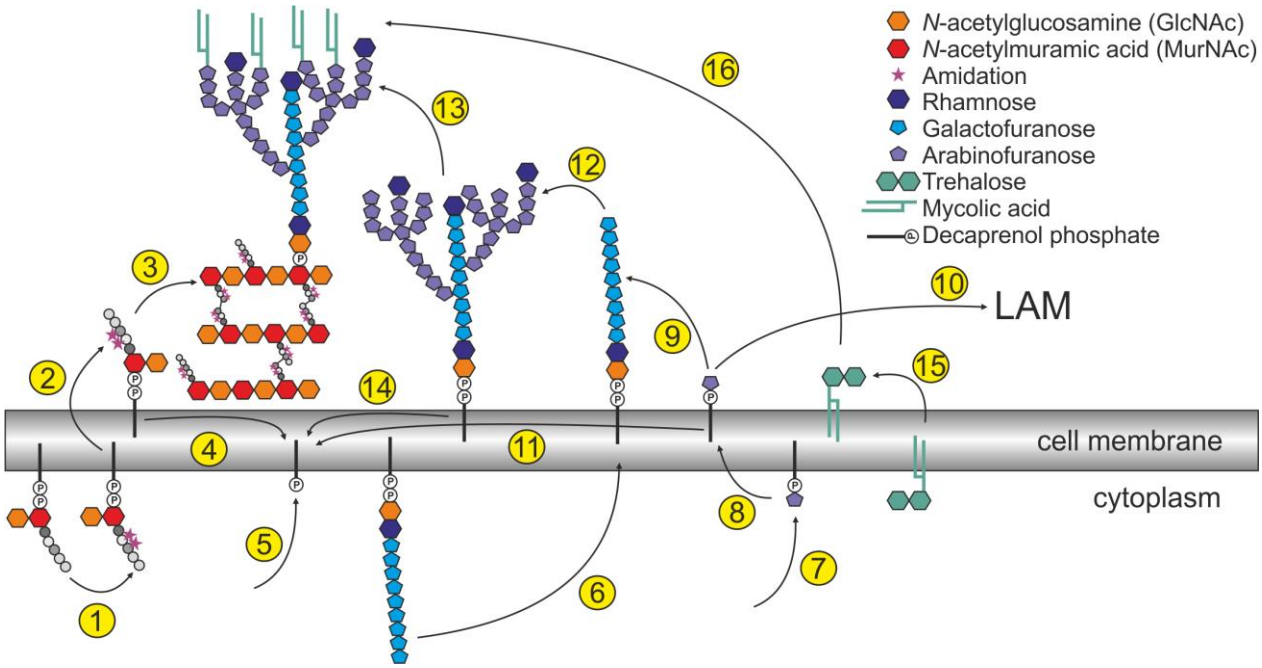
FIG 5 Complementation experiments and pyrophosphatase assay. (A) Complementation experiment of SilG-lcpA with LcpA variants carrying single mutations of conserved residues in the LCP-domain (residues highlighted in Fig. S7). (B) Complementation experiment with N- and C-terminally trimmed LcpA variants. (C) Complementation experiment with homologous proteins of the related species *C. diphtheriae* and *M. tuberculosis*. Unless indicated otherwise, the growth experiments were performed without induction by IPTG. The mycobacterial proteins were induced to achieve maximal complementation. (D) Pyrophosphatase assay with different mutated LcpAΔTM variants and CgtR10 as negative control. The proteins were purified from *E. coli* and incubated in 20 mM Tris-HCl pH 8.0 with the artificial substrate geranyl pyrophosphate (GPP, 1 mM) and either $MgCl_2$ or EDTA at 30°C overnight. The formation of geranyl monophosphate (GMP) was quantified by LC-MS. No activity was measurable in the absence of the substrate (yellow bars, not visible). One representative result of two independent protein purifications is presented.



925

926 **FIG 6** Domain distributions of the LCP (A) and the LytR_C (B) domains among bacteria.
927 Whereas the LCP domain is predominantly found in firmicutes, the LytR_C domain is most
928 widely spread among actinobacteria. Within firmicutes, 84% of the proteins with the LytR_C
929 domain belong to clostridia. Color coding is the same for both diagrams. Data obtained from
930 Interpro, July 18th, 2016. n represents the total number of the respective domains in the database
931 at the given date.

932



933
934 **FIG 7** Schematic summary of known or proposed important steps of cell wall biogenesis in
935 *C. glutamicum*. The peptidoglycan precursor Lipid II is composed of the carrier decaprenyl
936 phosphate, GlcNAc-MurNAc and a pentapeptide (L-Ala, D-Glu, mDAP (*meso* diaminopimelic
937 acid), D-Ala, D-Ala) (46). D-Glu and mDAP become amidated to a large extent in *C. glutamicum*
938 (→1). Recently it was shown that the enzyme LtsA is responsible for the amidation of mDAP
939 (46). The amidation of D-Glu is probably performed by Cg0299 and Cg0300, as shown for
940 homologous proteins of *Staphylococcus aureus* (47, 48). Subsequently, Lipid II is translocated
941 across the cell membrane (→2) where the peptidoglycan polymerization takes place. The
942 disaccharide-pentapetide is connected to the glycan chain by transglycosylation and
943 transpeptidation (→3), which is presumably performed by proteins of the penicillin-binding
944 protein family and L,D-transpeptidases (49-52). Several candidates can be identified by homology
945 searches (PBSs: Cg3313, Cg2375, Cg2199, Cg0336, Cg0060, Cg2649, Cg2478, Cg2987; L,D-
946 transpeptidases: Cg2720 (homologous to Rv0116c), Cg0650). After transfer of the disaccharide-
947 pentapeptide the carrier becomes recycled (→4). The decaprenyl phosphate is synthesized *de*
948 *novo* by UppS (cg2508) (→5) (53). Similar to the peptidoglycan precursors, also the

arabinogalactan precursor is built on a decaprenol carrier. GlcNAc and rhamnose serve as linker, whereon the galactose chain is built and flipped to the periplasm by a so far unknown mechanism (→6) (54, 55). Arabinose is provided as decaprenylmonophosphoryl-D-arabinose (DPA), another polyprenol-dependent precursor, involving the enzyme UbiA (→7) (56). DPA is most likely reoriented to the periplasm by a transporter encoded by cg0234 (rv3789 in *M. tuberculosis*) (57) (→8) and serves as arabinose donor both for arabinogalactan (→9) and lipoarabinomannan (LAM) (→10) before being recycled (→11). Some chains of arabinogalactan are capped by rhamnose (→12) (58). The arabinogalactan-precursor is then presumably transferred onto peptidoglycan by the LcpA protein characterized in this study (59, 60) (→13) and the decaprenol-phosphate becomes recycled (→14). The mycolic acids are also synthesized within the cell and most of them are transported across the membrane as trehalose monomycolates or trehalose dimycolates (61, 62) (→15). Subsequently the mycolic acids are transferred onto arabinogalactan by corynomycolic acid transferases (encoded by *cop1* & *cmt1-5*) (→16) (63).