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The Crucial Role of Trehalose and Structurally Related Oligosaccharides in the Biosynthesis and Transfer of Mycolic Acids in Corynebacterineae*

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Marielle Tropis‡§, Xavier Meniche‡§, Andreas Wolf¶, Henrike Gebhardt¶, Sergey Strelkov¶, Mohamed Chami||, Dietmar Schomburg¶, Reinhard Krämer¶, Susanne Morbach¶, and Mamadou Daffé‡**

From the ‡Department of Molecular Mechanisms of Mycobacterial Infections, Institut de Pharmacologie et Biologie Structurale (UMR 5089 du CNRS et de l'Université Paul Sabatier) 205, route de Narbonne, 31077 Toulouse cedex 04, France, ¶Institut für Biochemie der Universität zu Köln, Zùlpicher Strasse 47, 50674 Köln, Germany, and ||M. E. Müller Institute for Structural Biology, Biozentrum, University of Basel, Klingelbergstrasse 70 CH-4056, Basel, Switzerland

Trehalose (α -D-glucopyranosyl- α' -D-glucopyranoside) is essential for the growth of the human pathogen *Mycobacterium tuberculosis* but not for the viability of the phylogenetically related corynebacteria. To determine the role of trehalose in the physiology of these bacteria, the so-called Corynebacterineae, mutant strains of *Corynebacterium glutamicum* unable to synthesize trehalose due to the knock-out of the genes of the three pathways of trehalose biosynthesis, were biochemically analyzed. We demonstrated that the synthesis of trehalose under standard conditions is a prerequisite for the production of mycolates, major and structurally important constituents of the cell envelope of Corynebacterineae. Consistently, the trehalose-less cells also lack the cell wall fracture plane that typifies mycolate-containing bacteria. Importantly, however, the mutants were able to synthesize mycolates when grown on glucose, maltose, and maltotriose but not on other carbon sources known to be used for the production of internal glucose phosphate such as fructose, acetate, and pyruvate. The mycoloyl residues synthesized by the mutants grown on α -D-glucopyranosyl-containing oligosaccharides were transferred both onto the cell wall and free sugar acceptors. A combination of chemical analytical approaches showed that the newly synthesized glycolipids consisted of 1 mol of mycolate located on carbon 6 of the non reducing glucopyranosyl unit. Additionally, experiments with radioactively labeled trehalose showed that the transfer of mycoloyl residues onto sugars occurs outside the plasma membrane. Finally, and in contradiction to published data, we demonstrated that trehalose 6-phosphate has no impact on mycolate synthesis *in vivo*.

Trehalose (α -D-glucopyranosyl- α' -D-glucopyranoside) is widely distributed in nature; the disaccharide is found in both prokaryotic and eukaryotic cells and can serve as a carbon source, storage carbohydrate, or stress protection compound (for review, see Ref. 1). The most common route of *de novo* trehalose synthesis is by UDP-glucose and glucose 6-phosphate, yielding trehalose 6-phosphate, which is subsequently

dephosphorylated into trehalose (Fig. 1). The reactions are catalyzed in *Escherichia coli* by osmotically regulated trehalose synthesis (Ots)¹ enzymes, OtsA and OtsB. An alternative route for trehalose synthesis in other organisms consists in the conversion of maltose into trehalose, catalyzed by trehalose synthase (TreS), which converts the α (1 \rightarrow 4) glycosidic bond of maltose into an α (1-1) linkage to yield trehalose (Fig. 1). An additional pathway for trehalose synthesis uses oligo/polymaltooligo-dextrins/glycogen as substrate; the terminal α (1 \rightarrow 4) glycosidic bond at the reducing end of the polymer is converted into α (1-1) by maltooligosyltrehalose synthase (TreY), and the resulting terminal trehalosyl unit of the polysaccharide is then released by maltooligosyltrehalose hydrolase (TreZ). Although only one of the three pathways occurs in most bacteria, all three biosynthetic routes were found in mycobacteria and corynebacteria (2–4), suggesting an important role of trehalose in the physiology of microorganisms grouped in the Corynebacterineae suborder. These Gram-positive bacteria are unusual in that, like Gram-negative microorganisms, they contain an outer permeability barrier that may explain both their limited permeability and their rather general insusceptibility to toxic agents (5–7). Although the additional barrier in Gram-negative bacteria is a typical bilayer of phospholipid and lipopolysaccharide, in mycobacteria and corynebacteria it consists of a bilayer composed of a monolayer of mycoloyl residues covalently linked to the cell wall arabinogalactan and a monolayer of a variety of lipids non-covalently attached to the cell wall (6, 8–11). In Corynebacterineae, trehalose derivatives consist exclusively of glycolipids composed mainly of trehalose esterified by long chain (C₃₀–C₉₀) α -alkyl, β -hydroxy fatty acids of various chain lengths and structural features, called mycolic acids. Although in corynebacteria trehalose monocorynomycolate (TMCM) and trehalose dicorynomycolate (TDCM) are certainly involved in the formation of a second permeability barrier functionally similar to the Gram-negative outer membrane (8, 11), trehalose dimycolate is believed to play a key role in the pathogenicity of mycobacteria (12, 13). Importantly, a trehalose analog that inhibits *in vitro* the synthesis of trehalose mycolates and cell-

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§ Both authors contributed equally to the work.

** To whom correspondence should be addressed. Tel.: 33-561-175-569; Fax: 33-561-175-580; E-mail: mamadou.daffe@ipbs.fr.

¹ The abbreviations used are: Ots, osmotically regulated trehalose synthesis; TreS, trehalose synthase; TreY, maltooligosyltrehalose synthase; TreZ, maltooligosyltrehalose hydrolase; CTAB, N-cetyl-N,N,N-trimethylammonium bromide; COSY, correlated spectroscopy; MMCM, maltose monocorynomycolate; MTMCM, maltotriose monocorynomycolate, TMCM, trehalose monocorynomycolate; TDCM, trehalose dicorynomycolate; GC, gas chromatography; MS, mass spectroscopy; MALDI-TOF, matrix-assisted laser-desorption/ionization-time of flight.

bound mycolates has been shown to have a bacteriostatic action on *Mycobacterium aurum* (14). Consistently, the synthesis of trehalose has been recently shown to be essential for the growth of additional mycobacterial species, namely *Mycobacterium smegmatis* (15) and *Mycobacterium tuberculosis* (16). It is not known, however, whether or not the essentiality of trehalose for the mycobacterial growth is directly connected to the metabolism of mycolates. Nevertheless, in the context of the resurgence of tuberculosis and emergence of multidrug-resistant mycobacteria, the enzymes involved in the synthesis of trehalose, which is absent from mammalian cells, and/or the traffic of mycolic acids represent putative targets for the development of new anti-tuberculosis drugs.

Most of the studies devoted to the role of sugars, notably trehalose, in *Corynebacterineae* have been performed in corynebacteria. Pulse labeling experiments with palmitic acid have shown that *Corynebacterium diphtheriae* cell extracts accumulated in the first seconds of incubation a C₃₂- β -keto ester of trehalose, *i.e.* the 6-(2-tetradecyl, 3-keto octadecanoyl)- α -D-trehalose (17). Similar experiments have also demonstrated the stimulating effect of glucose on corynomycolate synthesis by some acellular preparations of corynebacteria (18, 19) and have led to the isolation of glucose and trehalose derivatives in the course of the synthesis of mycolic acids, suggesting a possible role of these compounds in the biosynthesis. Subsequently, trehalose 6-phosphate, but not trehalose, was also shown to stimulate corynomycolate synthesis from palmitate in the presence of ATP into trehalose monomycolate (20). Accordingly, it has been proposed that phosphorylated trehalose is the true activated form implicated in the early step of the synthesis of trehalose mycolates. Functional analysis of the different mycolyltransferase genes has demonstrated that TDCM derives from TCM (21, 22) since the inactivation of these genes results in the accumulation of TCM with the concomitant decrease of TDCM; the inactivation of one of these genes, *csp1* renamed *cmvA* (22), also led to the accumulation of a glucose monomycolate (21). Expectedly, the purified mycolyltransferase from mycobacteria was shown to catalyze the conversion of trehalose monomycolate into trehalose dimycolate (15, 23). In contrast, although the accumulation of TCM correlates in mutants defective in the production of mycolyltransferases with the decrease of cell wall-linked mycolates (21, 22, 24), not all the mutants that elaborate less covalently linked mycolates accumulate trehalose monomycolates (25). Accordingly, further studies are needed to decipher the role of trehalose in *Corynebacterineae*. Interestingly, all the three biosynthetic routes leading to trehalose (Fig. 1) are found in both mycobacteria and corynebacteria (2–4). In contrast to mycobacteria, where trehalose has been shown to be essential for bacterial growth, a targeted gene deletion approach was successful in generating the corynebacterial strains affected in all three pathways of trehalose biosynthesis and was thereby unable to synthesize the disaccharide (3, 4). To gain further insights into the role of trehalose in the physiology of *Corynebacterineae*, we investigated (i) the impact of trehalose deficiency on the lipid metabolism and cell envelope architecture of the triple mutant of *Corynebacterium glutamicum* (4), (ii) the structural requirements of sugars for the production of mycolate-containing glycoconjugates by the trehalose-deficient mutant, and (iii) the possible implication of trehalose 6-phosphate in the synthesis and traffic of mycolates by generating mutants affected in the OtsA/B pathway.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—The various strains affected in one or more pathways of trehalose biosynthesis used in the present study derived from the *C. glutamicum* ATCC13032. In addition to the strains

described by Wolf *et al.* (4), new single and double mutants affected in *otsB* were constructed as indicated below and examined. Culture conditions were identical to those described by Wolf *et al.* (4). To avoid a possible contamination of the culture medium with carbon sources other than sucrose, cells grown on Luria broth were washed 3 times with phosphate-buffered saline, and a portion (1/100) was inoculated on the minimum medium containing 4% sucrose as carbon source. A portion (1/100) of these precultures was re-inoculated in the culture medium containing 4% sucrose as the only carbon source.

Cloning of *otsA*, *otsB*, *treY*, and *treS* and Construction of Strains Deleted in Trehalose Biosynthesis Genes—Cloning of the indicated genes and the construction of deletion strains was performed as described previously (4). The *otsB* gene was deleted by the method of allelic replacement based on the selection of a chromosomal deletion by two recombination events. The flanking regions together with the *otsB* gene were amplified by PCR by the use of 5'-CGA TAA ACT TCC CGA TGA TTG CC and 5'-GGA ATT GGA AAC TTC CTT CCA CC as primers. The PCR fragment was cloned into the SmaI site of pUC18. The resulting plasmid was designated pUC18otsB. For the construction of a Δ otsB-deletion allele, an internal fragment of 0.15 bp was excised using the restriction endonucleases NcoI and DraIII, leading to pUC18 Δ otsB. The *otsB* fragment was isolated after digestion with EcoRI and XbaI and ligated with the similarly treated vector pK19mobsacB (26). The resulting plasmid was designated pK19mobsacB Δ otsB. The deletion of *otsB* in the chromosome of 13032 (27) and Cgl Δ treY (4) was carried out as recently described (4).

All enzymes used for recombinant DNA techniques were purchased from NEB (Frankfurt, Germany) or Roche Applied Science. Oligonucleotides were obtained from MWG Biotech (Ebersberg, Germany). Sequence information on the *C. glutamicum* genome was generously provided by the Degussa AG (Hanau, Germany).

Quantification of Cytosolic Trehalose and Trehalose 6-Phosphate—Cytosolic trehalose was quantified by gas chromatography (GC) as described previously (4), whereas the amount of trehalose 6-phosphate was determined by GC-mass spectrometry (GC-MS). For quantifying the latter compound, 40 ml of exponentially growing cells with an optical density of 5 were harvested and washed once in 400 mM KP_i buffer (pH 7.5). Subsequently, cells were sonicated in a methanol bath (2 ml of methanol, 70 °C, 20 min). After centrifugation, the supernatant was collected. To increase the amount of hydrophilic sugar phosphates, the pellet was extracted again with 2 ml of H₂O. The samples were immediately centrifuged after suspension of the pellet. The aqueous supernatant was pooled with the methanol fraction, and 1 ml of CHCl₃ was added. After a phase separation 1 ml of the methanol/water mixture was dried under N₂ at 65 °C, and the putative sugar constituents were converted into methoximes (28) by adding 50 μ l of methoxyamine/pyridine mixture (20 mg of methoxyamine/ml in pyridine). The derivatization was carried out at 30 °C for 90 min. Subsequently, methoximes were trifluoroacetylated by adding 80 μ l of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide, and the samples were incubated at 65 °C for 60 min. GC-MS was performed using a Finnigan Trace-GC/Trace-MS equipped with a J&W Scientific fused-silica column (30 m, 0.25 mm internal diameter, 0.25-mm film thickness) obtained from Agilent (Palo Alto, CA). The samples were applied by split injection (1:25)/PTV (70–280 °C, 14 °C/s). A non-linear temperature gradient from 70 to 76 °C (1 °C/min) and 76 to 325 °C (6 °C/min) was used followed by an isothermic plateau (10 min). Signals that could not be properly distinguished from the base line (signal-to-noise ratio <10) were classified as non-detectable.

Uptake of Glucose, Trehalose, or Betaine—For the uptake experiments cultures of the wild type and Cgl Δ otsA Δ treS Δ treY were grown overnight in CgXII medium with 4% sucrose as the carbon source. Cells were harvested and washed once in KP_i buffer (50 mM KP_i (pH 7.5), 20 mM NaCl). Subsequently, they were suspended in the same buffer and kept on ice. Cells with an optical density of 3 were preheated for 3 min at 30 °C before the uptake measurement was started by the addition of either 500 μ M [¹⁴C]glucose (Hartmann Analytic, Braunschweig, Germany, 100 μ Ci/ml) or 50 μ M [¹⁴C]trehalose (Trenzylme GmbH, Konstanz, Germany, 1 mCi/ml), *i.e.* final concentrations of 0.025 and 0.050 μ Ci/ml of labeled glucose and trehalose, respectively, in the assays. In the case of the betaine uptake measurements osmotic shock was performed by the addition of 600 mM NaCl to activate the betaine uptake carriers of *C. glutamicum*. Cells were preheated for 3 min before the uptake reaction was started by the addition of 250 μ M [¹⁴C]betaine. At different time intervals 100- μ l samples were taken and filtered rapidly through 0.45- μ m glass fiber filters (GF, Schleicher & Schuell GmbH). The filters were washed twice with 2.5 ml of 100 mM LiCl solution, and the radioactivity was determined by liquid scintillation counting.

To determine the location of the label, *i.e.* cell wall fraction or cytosol,

the assay conditions were changed as follows. Cells were prepared and used in the transport assay as described above. After an incubation time of 10 min with 50 μM [^{14}C]trehalose or 250 μM [^{14}C]betaine, 100 μl of cells were filtered on glass fiber filters. They were partly permeabilized by the addition of 1 ml of 50 mM KPi buffer containing 0.1% *N*-cetyl-*N,N,N*-trimethylammonium bromide (CTAB) to release the cytosol without destroying the cell envelope. After 1 min the CTAB solution was filtered, and the remaining cell envelopes were washed twice with 2.5 ml of 100 mM LiCl solution. In a parallel approach the filtered cells were incubated for 1 min in 50 mM KPi buffer instead of the CTAB solution before being washed with LiCl. The radioactivity determined in these control cells represented the total amount of the accumulated substrate present after 10 min of incubation with [^{14}C]trehalose or [^{14}C]betaine. The radioactivity was determined by liquid scintillation counting.

Isolation, Fractionation, and Analysis of Lipids—Lipids were obtained and analyzed as previously described (21). Briefly, lipids were extracted from wet cells for 16 h with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:2, v/v) at room temperature with continuous stirring; the bacterial residues were re-extracted three times with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:1, v/v) and then $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1, v/v); the organic phases were pooled and concentrated. The crude lipid extracts were washed, evaporated to dryness, and partitioned between water and chloroform (1:1, v/v); the organic phase was washed with distilled water, evaporated to dryness to yield the crude lipid extracts from each strain, and comparatively examined by thin layer chromatography (TLC) on silica gel-coated plates (G-60, 0.25-mm thickness, Merck) developed with $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (30:8:1 or 65:25:4, by volume). Detection of all classes of lipids was done by spraying the TLC plates with either rhodamine B or 20% H_2SO_4 in water, the latter followed by heating at 110 $^\circ\text{C}$; glycolipids were revealed by spraying plates with 0.2% anthrone (w/v) in concentrated H_2SO_4 followed by heating at 110 $^\circ\text{C}$. The Dittmer-Lester reagent (29) was used for visualizing phosphorus-containing lipids.

Purification of Glycolipids—The mycolate-containing glycolipids, *i.e.* glucose, maltose, and maltotriose mycolates, were separated from phospholipids by anion exchange chromatography using QMA-silica Sep-Pak Cartridges (Waters). The columns were eluted with a gradient of $\text{CHCl}_3/\text{CH}_3\text{OH}$. Glucose, maltose, and maltotriose mycolates were eluted from the columns, respectively, with 20, 30, and 40% CH_3OH in CHCl_3 (v/v). Phospholipids were retained on the columns and were eluted with 2% NH_4OH in 0.01 M ammonium acetate in $\text{CHCl}_3/\text{CH}_3\text{OH}$ (4:1 v/v).

Quantification of Corynomycolic Acids—The quantification of corynomycolic acids was performed twice in independent experiments as follows. Delipidated cells (300 mg dry weight) and lipid extracts (10 mg) of the various strains were dried under vacuum and saponified (30); the saponified products were acidified with 20% H_2SO_4 . The resulting fatty acids were extracted with diethyl ether, converted to methyl esters with diazomethane, dried under vacuum, and weighed. Portions of fatty acid methyl esters from delipidated cells and extractable lipids (1–2 mg) were treated with trimethylsilyl reagents (31) to derivatize hydroxylated components of the mixtures, *i.e.* corynomycolates, and analyzed by GC. The detector response for the various classes of fatty acid methyl esters was determined using authentic samples of $\text{C}_{16:0}$ and $\text{C}_{32:0}$ corynomycolate methyl esters. Identification of non-hydroxylated fatty acid methyl esters and corynomycolate derivatives was achieved by GC-MS.

GC of fatty acid methyl esters was performed using a Hewlett Packard HP4890A equipped with a fused silica capillary column (25-m length \times 0.22 mm inner diameter) containing WCOT OV-1 (0.3-mm film thickness, spiral). A temperature gradient of 100–300 $^\circ\text{C}$ at 5 $^\circ\text{C min}^{-1}$ followed by a 5-min isotherm plateau at 300 $^\circ\text{C}$ was used. GC-MS analysis was conducted on a Hewlett-Packard 5890 gas chromatograph connected to a Hewlett-Packard 5989A mass spectrometer. Samples were injected in the splitless mode. The column was a 12-m HP-1. A temperature gradient of 100–290 $^\circ\text{C}$ (8 $^\circ\text{C min}^{-1}$) was used.

Mass Spectrometry—Matrix-assisted laser-desorption/ionization-time of flight (MALDI-TOF) mass spectrometry analysis of glycolipids was performed as previously described (32). MALDI-TOF spectra were acquired on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems) equipped with a pulsed nitrogen laser emitting at 337 nm and were analyzed in the Reflectron mode using an extraction delay time set at 100 ns and an accelerating voltage operating in negative ion mode of 20 kV.

Nuclear Magnetic Resonance (NMR) Spectroscopy—Spectra were recorded on a Bruker AMX-500 spectrometer equipped with an Aspect X32 computer and a TXI probe. Sample (1 mg) was dissolved in CDCl_3 (99.96% purity)/ CD_3OD (99.8% purity) (Spin et Techniques, Paris, France), 1:1 (v/v), and analyzed in a 200 \times 5-mm NMR tubes at 25 $^\circ\text{C}$.

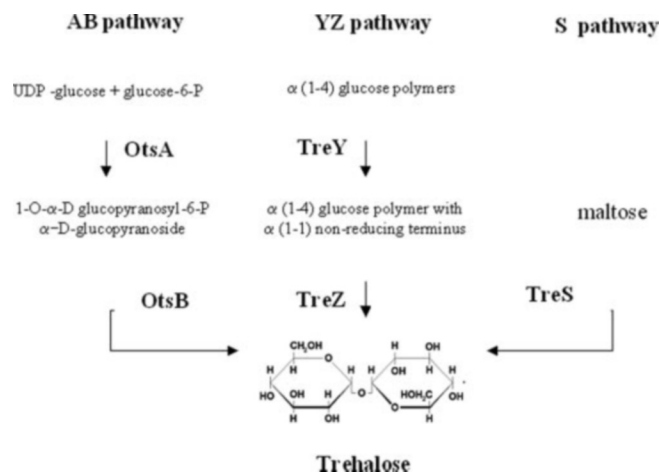


FIG. 1. The three trehalose biosynthesis pathways identified in mycobacteria and corynebacteria.

Chemical shifts are expressed in ppm downfield from the signal for chloroform (δ_{H} /tetramethylsilane 7.27). The one-dimensional proton (^1H) spectrum was measured using a 45 $^\circ$ tipping angle for the pulse and 3 s as a recycle delay between each of the 1000 acquisitions. The spectral width of 7500 Hz was collected in 16,000 complex points that were multiplied by a sine bell (ssb = 2) before processing to 32,000 real points in the frequency domain. After Fourier transformation, the spectra were base line-corrected with a fourth order polynomial function.

All two-dimensional NMR data sets were recorded without sample spinning, and data were acquired in the phase-sensitive mode using the time proportional phase increment method (33) unless otherwise indicated. The spectral width was 4006 Hz in each dimension, and the relaxation delay was 3 s. 450 spectra of 4096 data points with 32 scans per t_1 increment were recorded. For processing, a sine bell shift (11b = 2) was applied in both dimensions, and the data matrix was expanded to 4000 \times 1000 data points. A two-dimensional correlated spectroscopy (COSY) spectrum was obtained with standard Bruker pulse sequence. A two-dimensional total correlation spectroscopy spectrum was recorded using a MLEV-17 mixing sequence of 160 ms (34).

Freeze Etch Electron Microscopy—For freeze-fracture experiments bacterial suspensions were centrifuged at 13,000 $\times g$. A drop of the pellet was placed between a thin copper holder and a thin copper plate before quenching in liquid propane, as described by Aggerbeck and Gulik-Krywicki (35). The frozen samples were fractured in a Balzers 301 freeze-fracture unit at -125 $^\circ\text{C}$ *in vacuo* of about 1.3×10^{-5} pascal by removing the upper plate with a liquid nitrogen-cooled knife.

The fractured sample was etched at -100 $^\circ\text{C}$ for 5 min and then replicated with 1–1.5 nm of deposits of platinum-carbon and backed with about 20 nm of carbon. The replicas were cleaned overnight with chromic acid, washed with distilled water, and observed with a Philips 410 electron microscope operating at 80 kV.

RESULTS

In contrast to most bacterial genera, corynebacteria and mycobacteria possess three different pathways leading to the synthesis of trehalose (Fig. 1). The genes for three different trehalose synthesis pathways have been identified in *M. tuberculosis* (2), and in the *C. glutamicum* genome three comparable pathways are found as well (3, 4). According to the respective genes, they are named OtsAB, TreYZ, and TreS pathways. To define the individual roles of these putative trehalose synthesis pathways, a number of strains deleted in different trehalose biosynthesis genes was constructed (4). In the case of pathways comprising sequential catalytic steps, the gene coding for the first enzyme of the sequence was deleted, thereby avoiding accumulation of potentially toxic intermediates. The trehalose content of the various mutant cells grown on sucrose was determined (Table I). The abolition of one of the three pathways did not much affect the trehalose content of the mutant cells. The wild type and strain *Cgl* Δ *treS* had similar internal trehalose concentrations, whereas *Cgl* Δ *otsA* and Δ *treY* possessed $\frac{3}{4}$ and less than half of the trehalose content of the wild

TABLE I

Contents in cytoplasmic trehalose and mycolates esterifying extractable lipids and covalently linked to the cell walls of various strains of *C. glutamicum* (Cgl) defective in individual trehalose synthesis pathways and grown in minimal medium

The concentration of trehalose is expressed relative to the cell dry mass (cdm).

Strain (carbon source)	Cytoplasmic trehalose $\mu\text{mol/g of cdm}$	% Corynomycolates	
		Extractable lipids ^a	Wall-linked mycolates ^b
Wild type (sucrose)	41	15	1.7
CglΔotsA (sucrose)	32	14	2.5
CglΔtreY (sucrose)	17	20	1.4
CglΔtreS (sucrose)	46	24	2.2
CglΔotsAΔtreY (sucrose)	0	0	0
CglΔotsAΔtreYΔtreS (sucrose)	0	0	0
Wild type (maltose)	269	47	1.7
CglΔotsAΔtreY (maltose)	74	20	1.9
CglΔotsAΔtreYΔtreS (maltose)	0	41	1.4

^a The % corynomycolates in extractable lipids was determined by GC analysis of fatty acid methyl esters that composed the lipids extracted from bacterial cells with organic solvents.

^b The resulting delipidated cells were saponified, and their corynomycolate content was determined by weighing.

type strain. These data indicated that the contribution of the TreYZ pathway is more important for the synthesis of trehalose than that of OtsAB, that of TreS being zero. Consistently, no trehalose at all could be isolated from CglΔotsAΔtreY and CglΔotsAΔtreYΔtreS cells grown on sucrose. Obviously, TreS does not contribute at all to the internal trehalose pool, and trehalose synthesis under these growth conditions is solely OtsAB- and TreYZ-mediated. It has to be noted that this redundancy in the trehalose biosynthesis pathways in *C. glutamicum* (3, 4) and *M. smegmatis* (16) is in sharp contrast with the situation of *M. tuberculosis*, where the former pathway was found to be essential for trehalose biosynthesis (16).

Consequences of Trehalose Deficiency on the Bacterial Ultrastructure—In addition to an increase of osmo-sensitivity (4), the CglΔotsAΔtreY and CglΔotsAΔtreYΔtreS cells grown on sucrose exhibited a strong aggregation of cells in liquid culture (data not shown). Centrifugation of the mutant strains resulted in the observation of a translucent layer in top of the bacterial pellet. The upper layer was devoid of any protein but contained high molecular weight cell wall constituents composed of arabinose, mannose, and glucose. These compounds probably correspond to substances that form the outermost cell envelope compartment of corynebacteria, namely glucan and arabinomannan (8, 11). Analysis of the supernatant of CglΔotsAΔtreSΔtreY cultures revealed that the protein concentration was roughly 7-fold higher than in the wild type supernatant (307.3 ± 9.5 versus 41.5 ± 1.7 $\mu\text{g/ml}$, respectively).

To characterize the changes in the cell walls of strains CglΔotsAΔtreY and CglΔotsAΔtreSΔtreY provoked by the absence of trehalose, cells of the two strains were subjected to freeze-fracture electron microscopy (Fig. 2), a technique that has been successfully applied to corynebacterial cells (8, 11, 36). Because the planes are theoretically produced by hydrophobic structures at low temperatures (8), two fracture planes are expected to occur in Corynebacterineae, *i.e.* in the ubiquitous plasma membrane and in the cell wall mycolate layer. In practice, however, only one fracture plane is generally observed in freeze-fracture preparations of Corynebacterineae, which occurs within the cell wall near the cell surface (8, 11, 36). This plane is postulated to propagate between the mycolate mono-

layer formed by the mycoloyl residues, which esterify the arabinose termini, and the lipid layer of the outer barrier of these organisms (8, 11). In mycolate-less corynebacterial cells the fracture plane is seen within the plasma membrane, exposing the protein-rich cytosolic content (11). Cells of both trehalose-less double and triple mutants lacked the typical cell wall fracture plane found in the wild type but exhibited the fracture plane located deeply below the cell surface correlating with the plasma membrane. A similar observation has been made with the naturally occurring mycolate-free *Corynebacterium amycolatum* (11). Consequently, the CglΔotsAΔtreY and CglΔotsAΔtreYΔtreS strains grown on sucrose were examined for the production of mycolates (Table I, Fig. 3A and data not shown for the CglΔotsAΔtreY). Interestingly, the two trehalose-less strains lacked both trehalose-containing glycolipids, *i.e.* TMCM and TDCM, and mycolates covalently bound to the cell wall arabinogalactan. These data clearly support the current view that the cell wall fracture plane in corynebacteria corresponds to the region of weakness where mycolates are arranged with other lipids to form a bilayer-like structure (8, 11).

Influence of Carbon Sources on the Production of Mycolates—To study the functionality of the TreS pathway, maltose has been recently used as the carbon source for growing different mutants (3, 4). It has been shown that the wild type cells produced 6–7-fold more trehalose when grown on maltose than cultivated on sucrose. Consistently, in the presence of maltose a significant trehalose pool was built up by TreS activity in CglΔotsAΔtreY cells (compare also Table I), whereas no trehalose was found under the same conditions in strain CglΔotsAΔtreSΔtreY. Besides the accumulation of trehalose, a beneficial effect on the growth rate was detected in CglΔotsAΔtreY when grown on maltose. This observation has led Wolf *et al.* (4) to pose the question of whether the growth enhancement noted on maltose was caused by the occurrence of mycolates in the cell wall of CglΔotsAΔtreY. Thus, in this work the influence of maltose on the mycolate content was investigated. Interestingly, although more internal trehalose was found in the strain CglΔotsAΔtreY grown on maltose compared with wild type cells grown on sucrose (Table I), no trehalose mycolates, *i.e.* TMCM and TDCM, were present in the double mutant strain, which, however, synthesized new glycolipids (Fig. 3B and data not shown for the double mutant). To characterize the new glycolipids the purified compounds were analyzed by MALDI-TOF. Importantly, the major pseudomolecular ion $[M+Na]^+$ peaks of the MALDI-TOF mass spectrum of the purified glycoconjugates (Fig. 4B) were observed at 843.3 and 869.3 m/z and corresponded to the mass values of a series of a hexose esterified by one molecule of C_{32:0} or C_{34:1} mycolate. Sugar compositional analysis of the glycoconjugates, determined by alkaline hydrolysis followed by trimethylsilylation and GC analysis, showed that they contained maltose as the only sugar constituent, suggesting that the external maltose was used for mycolate synthesis and thereby questioning the so-far assumed concept that trehalose is necessary for the production of mycolates. This conclusion was further supported by the production of maltose mycolates by the CglΔotsAΔtreSΔtreY strain that is completely devoid of trehalose (Table I). Consequently, the synthesis of mycolates by the mutant strains grown on various carbon sources that included different sugars was investigated (Table II).

Among the sugars tested, in addition to sucrose and maltose, the wild type ATCC13032 and the CglΔotsAΔtreSΔtreY strains were able to use glucose, fructose, and maltotriose as carbon sources. In contrast, both strains were unable to grow on arabinose, galactose, cellobiose, lactose, maltitol, melibiose, and trehalose and grew only poorly on mannose. This observation

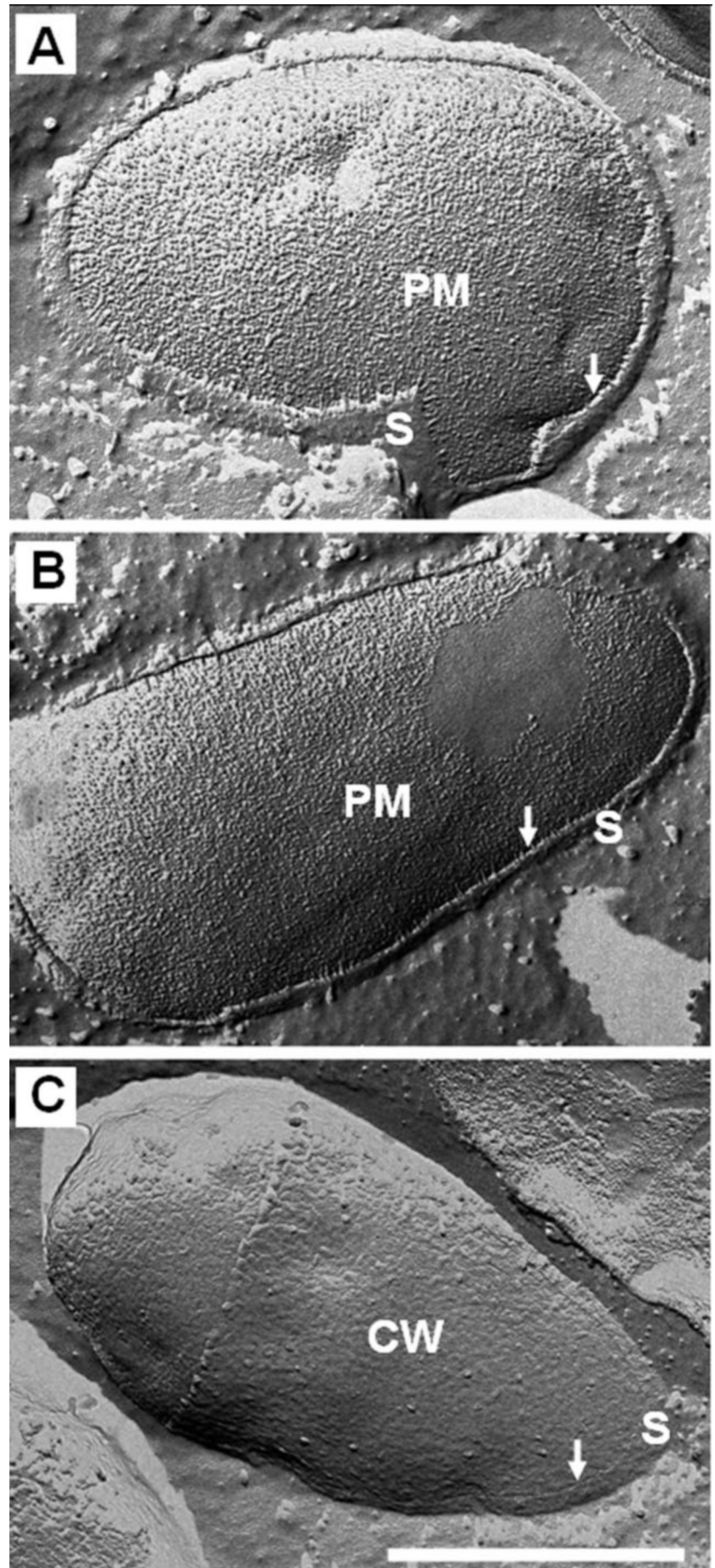


FIG. 2. Freeze-fractured and deep-etched preparations of trehalose-less *CglΔotsAΔtreY* (A), *CglΔotsAΔtreYΔtreS* (B), and the wild type ATCC 13032 (C) strains of *C. glutamicum* grown on minimal medium-containing agar plates. The fracture plane is seen in the cell wall of the wild type strain (CW), close to the bacterial surface (S), whereas the fracture occurs in the plasma membrane (PM) of the mutants. The fracture lines are indicated by arrows. The scale bar represents 0.5 μm.

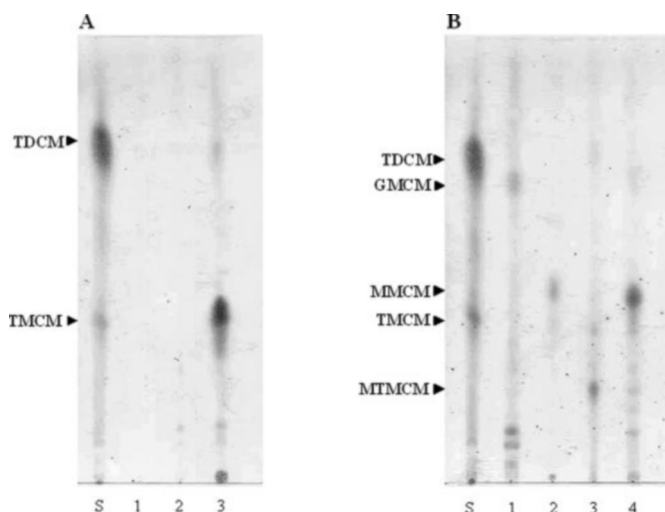


FIG. 3. TLC analysis of lipids extracted with organic solvents from the wild type ATCC 13032 strain and its isogenic trehalose-less *CglΔotsAΔtreYΔtreS* mutant of *C. glutamicum* grown on various carbon sources. A, lipids of the mutant grown on acetate (lane 1), sucrose (lane 2), and sucrose and trehalose (lane 3). B, TLC of the lipids extracted with organic solvents from the mutant grown on glucose (lane 1), maltose (lane 2), maltotriose (lane 3), and glucose, maltose, and maltotriose (lane 4). Glycolipids were visualized by spraying the plates with 0.2% anthrone (*w/v*) in concentrated H_2SO_4 followed by heating. Arrowheads indicate the mobilities of mycolate-containing glycoconjugates characterized in the present study. TLC was run in $CHCl_3/CH_3OH/H_2O$ (30:8:1, by volume). S, sample of TDCM and TMCM. GMCM, glucose monocorynomycolate.

pointed to the importance of both the conformations of the sugar residues and the configuration of the linkage in the oligosaccharides tested as carbon source (Table II). In monosaccharides the *gluco* configuration seemed to be required for their internalization and/or use as carbon sources, whereas a terminal α -glucosyl sugar unit linked to a position other than the anomeric one, *i.e.* trehalose, seemed necessary in di- and trisaccharides. Analysis of the mycolic acid content of strains grown on various carbon sources revealed that whenever the wild type cells can grow they also contain mycolic acids in their cell walls. In contrast, mycolic acids were found in the cell walls of the trehalose-deficient strain *CglΔotsAΔtreYΔtreS* only when glucose, maltose, or maltotriose was used as a carbon source, although growth was possible on a broader variety of carbon sources (Table II).

When grown on glucose both the *CglΔotsAΔtreY* and *CglΔotsAΔtreYΔtreS* cells produced a glycolipid whose chromatographic mobility (Fig. 3B and data not shown for the double mutant) was similar to that of glucose monocorynomycolate (21). The *m/z* values of the major pseudomolecular ion $[M+Na]^+$ peaks observed in the MALDI-TOF mass spectrum (Fig. 4A) were consistent with this hypothesis. The major peak was seen at 681.4 *m/z* and corresponded to a hexose esterified by one molecule of $C_{32:0}$ corynomycolic acid. Furthermore, alkaline hydrolysis of the glycolipid followed by trimethylsilylation and GC analysis led to the identification of corynomycolates and glucose. Finally, the NMR spectrum of the compound was superimposable with that of glucose 6-corynomycolate (21), establishing the structure of the glycolipid. Both strains also elaborated glycolipids when grown on maltotriose (Fig. 3B). Again, only one molecule of corynomycolate was present per trisaccharide as determined by MALDI-TOF mass spectrometry (Fig. 4C). The *m/z* values of the major pseudomolecular ion peaks, observed at 1005.6 and 1031.6 *m/z*, corresponded to an oligosaccharide esterified by one molecule of $C_{32:0}$ or $C_{34:1}$ corynomycolic acid. The preferred glucose-containing oligosaccharide substrate used by the *CglΔotsAΔtreYΔtreS* strain was

likely an oligosaccharide since both the wild type and trehalose-less mutants elaborated three times more corynomycolate-containing glycolipids when grown on maltose or maltotriose than glucose. When grown on a mixture of glucose, maltose, and maltotriose, added in similar amounts to 4% sucrose, maltose monocorynomycolates were the predominant glycolipids produced by the triple mutant strain (Fig. 3B). These data are consistent with the fact that the natural substrate of mycolates in fractions extractable with organic solvents in both corynebacteria and mycobacteria is also a disaccharide, *i.e.* trehalose (6, 8).

Location of the Mycoloyl Residue in the Newly Identified Glycolipids—The major purified glycoconjugates produced by the mutant strains when grown on maltose and maltotriose consisted of maltose and maltotriose monocorynomycolates as determined by compositional analyses and MALDI-TOF mass spectrometry (see above). To determine the location of the corynomycoloyl substituent on the carbohydrate moieties, the two purified glycolipids were analyzed by 1H NMR (Fig. 5). This technique is known to easily identify the acylated positions of glycolipids whose resonances are downshifted when compared with those found in non-acylated saccharides. The resonances of protons bearing acylated hydroxyl groups on carbons 1–4 appear in the region of anomeric resonances (4.8–5.6 ppm), whereas those of C6s are usually found to be >4.0 ppm (37–39).

The 1H NMR spectra of both the maltose monocorynomycolate (MMCM) and maltotriose monocorynomycolate (MTMCM) were very similar (Fig. 5). The most deshielded proton resonances in both spectra were observed at 5.35 ppm and were assigned to those of the ethylenic protons in unsaturated corynomycoloyl residues that substituted maltose. The two signals centered at 5.09 ppm (Fig. 5A) observed in spectrum of MMCM were assigned to the anomeric proton (H-1) resonances of the terminal non-reducing glucosyl residue (residue A, Table III) whose resonance is sensitive to the anomeric configuration of the reducing glucosyl unit (residue B) of MMCM. The resonances of the α -anomer of this latter residue was observed at 5.16 ppm (0.6 H), whereas those of the β -anomer were seen at 4.52 ppm (0.4 H). Likewise, the H-1 resonance of the terminal non-reducing glucosyl residue A in MTMCM was observed at 5.08 ppm (1H), whereas those of the residue B and the α -anomer of reducing glucosyl unit C were seen at 5.15 ppm (1.6 H). The resonance of the β -anomer of reducing glucosyl unit C was observed at 4.52 ppm (0.4 H). Because no other deshielded resonances were observed in the region of anomeric and other deshielded proton resonances of both glycolipids, the corynomycoloyl residue was not located on C-1 through C-4 of the glucosyl residues in both MMCM and MTMCM (37–39). Consequently, the fatty acyl was expected to be located on one C-6 of both glycolipids. To determine the exact location of the corynomycoloyl residue, the purified MMCM and MTMCM were analyzed by two-dimensional COSY spectroscopy. Analysis of the latter spectra allowed the assignment of the resonances of the H-1 through H-5 of all glucosyl residues (Table III) but not those of the H-6s, due to the overlapping of cross-peaks (data not shown). These uncertainties were removed by performing two-dimensional total correlation spectroscopy experiments (data not shown). Analysis of the latter spectra confirmed the previous assignments and extended the assignment to all the proton resonances with the exception of those of some H-6s (Table III). From the chemical shift values of the various sugar proton resonances it clearly appeared that the corynomycoloyl residue was located on the C-6 of the non-reducing glucosyl unit of both MMCM and MTMCM, whose H-6 resonances were deshielded at 4.57 and 4.55 ppm, respectively,

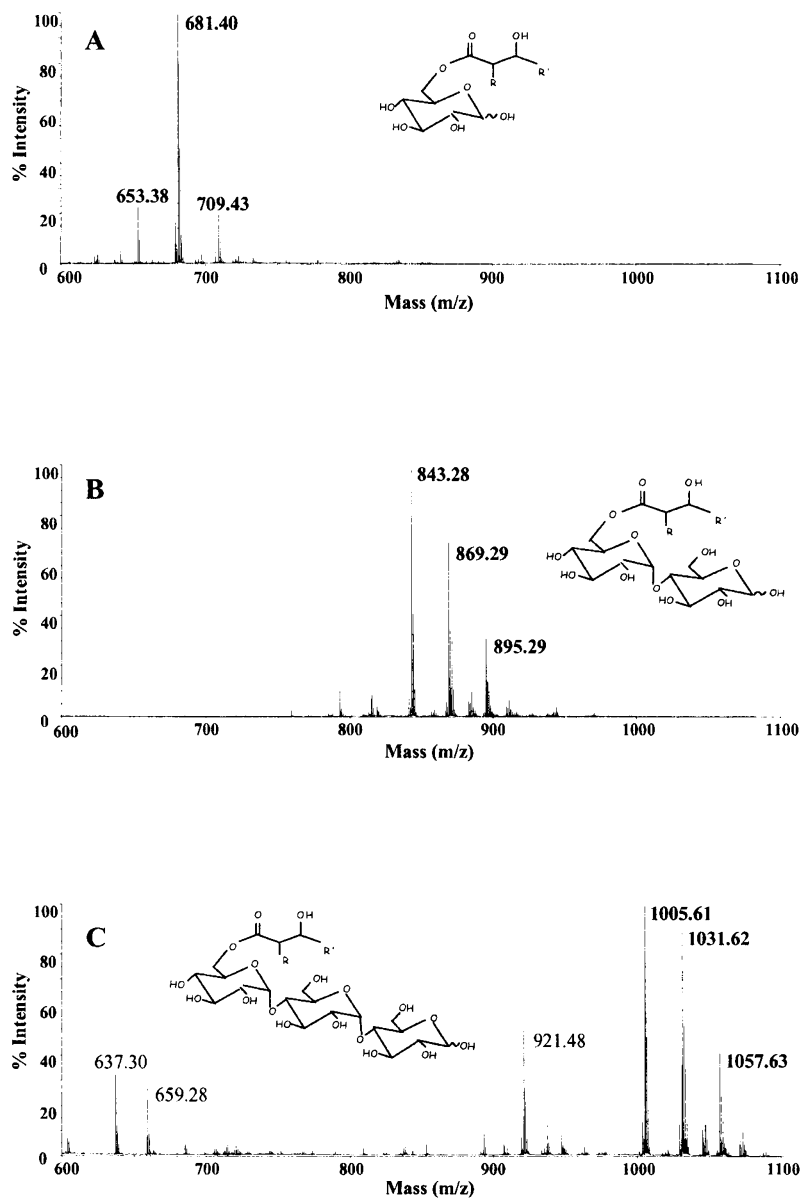


FIG. 4. MALDI-TOF mass spectra of purified glucose monocorynomycolate (A), maltose monocorynomycolate (B), and maltotriose monocorynomycolate (C) from the trehalose-less *CglΔotsAΔtreYΔtreS* mutant of *C. glutamicum* grown on glucose, maltose, and maltotriose, respectively. The structures of the characterized glycoconjugates are shown in the corresponding panels. *R* and *R'* represent C_{14-16} and C_{15-17} acyl residues, respectively, which may contain a double bond.

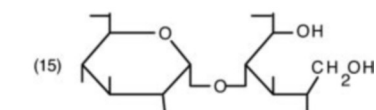
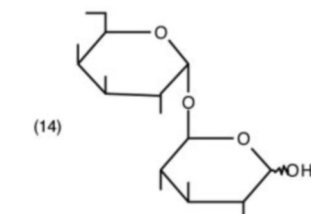
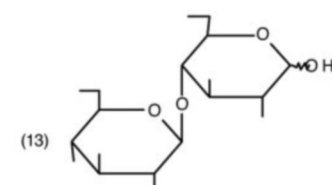
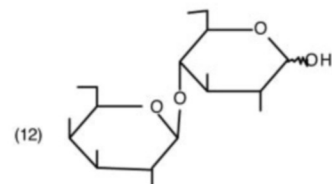
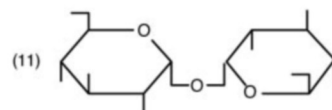
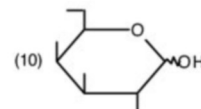
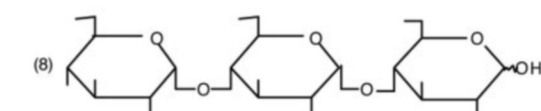
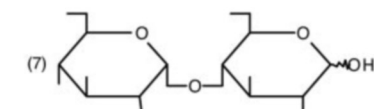
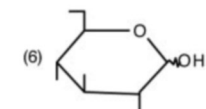
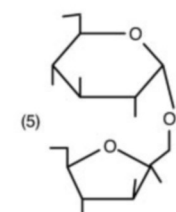
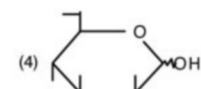
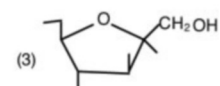
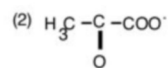
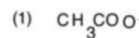
instead of <4.00 ppm (Table III, Fig. 5). It followed then that the MMCM and MTMCM corresponded, respectively, to 6-corynomycoloyl- α -D-glucopyranosyl (1 \rightarrow 4)- α -D-glucose and 6-corynomycoloyl- α -D-glucopyranosyl-(1 \rightarrow 4)- α -D-glucopyranosyl-(1 \rightarrow 4)- α -D-glucose.

Definition of the Structural Requirements for Sugars to Induce the Synthesis of Mycolates—To address the question of the sugars used as mycolate acceptors we decoupled the inability to use a defined sugar as a carbon source and its putative role as mycolate acceptor by growing the wild type and the triple mutant strains on sucrose as the carbon source. Various sugars unable to be used by the bacteria were then added to the cultures, and the mycolate contents of the strains grown under these conditions were determined (Table II). Interestingly, the *CglΔotsAΔtreYΔtreS* strain grown on sucrose produced mycolate-containing glycolipids, *i.e.* TCMC, only when trehalose was added to the medium. The amount of the glycolipid in the mutant grown under these conditions was roughly 3-fold higher than that of the wild type grown on sucrose. Importantly, corynomycolic acids were also found to esterify the cell wall polysaccharide of the mutant grown on sucrose with external trehalose. The amount of cell wall-linked corynomycolates in the mutant was half that of the wild type grown on

sucrose. Consistent with our observation, Tzvetkov *et al.* (3) has also recently shown that the addition of external trehalose to minimal medium resulted in the production of TCMC by a trehalose-deficient mutant of *C. glutamicum*. Furthermore, we noticed that sugars that were not used by the bacteria as a carbon source, including D-arabinose, which is found esterified with mycolic acids in the cell wall arabinogalactans of *Corynebacterineae*, were not used by the mutant as an acceptor of mycolic acid (Table II). These data together with the finding that glucose, maltose, or maltotriose are esterified with mycolates clearly demonstrated that a terminal α -glucosyl sugar unit is required for the synthesis and transfer of mycolates onto the sugar residue. Importantly, the triple mutant grown either on acetate, pyruvate, fructose, or sucrose, known to be good carbon sources and to be metabolized into glucose phosphates, neither synthesized glucose mycolates nor corynomycolic acids bound to the cell wall arabinogalactan, thus indicating that the synthesis of internal glucose phosphate was not sufficient for the production and/or transfer of mycolates in the cell wall. Because the carbon sources were present in excess, it was unlikely that the lack of synthesis of mycolates was due to the rapid utilization of internal glucose for the production of energy and essential compounds. These data led to the suggestion that

TABLE II
Growth and mycolic acid (M.A.) content of the wild type (WT) of *C. glutamicum* ATCC13032 and its isogenic *CglΔotsAΔtreYΔtreS* mutant (AYS) grown on various carbon sources

Carbon Sources	growth	
	WT	AYS
1 Acetate	+	+
2 Pyruvate	+	+
3 Fructose	+	+
4 Mannose	+/-	+/-
5 Sucrose	+	+
6 Glucose	+	+
7 Maltose	+	+
8 Maltotriose	+	+
9 Trehalose	-	-
10 Arabinose	-	-
11 Galactose	-	-
12 Lactose	-	-
13 Cellobiose	-	-
14 Melibiose	-	-
15 Maltitol	-	-
Trehalose + Sucrose	+	+
Arabinose + Sucrose	+	-
Galactose + Sucrose	+	-
Lactose + Sucrose	+	-
Cellobiose + Sucrose	+	-
Melibiose + Sucrose	+	-
Maltitol + Sucrose	+	-
Mannose + Sucrose	+	-



the synthesis and transfer of mycolates onto appropriated acceptors takes place only when glucose, maltose, or trehalose is available outside the cells.

Determination of the Site of Production of Trehalose Mycolates—To determine the bacterial cell compartment in which trehalose mycolates are synthesized, we performed uptake ex-

periments with radiolabeled trehalose. If trehalose can be shown not to be taken up by *C. glutamicum*, which is strongly suggested by the fact that this sugar cannot be used as a carbon source, this would prove that the transfer of mycoloyl residues onto the sugar acceptors occurs outside the cell. Consequently, the wild type and *CglΔotsAΔtreYΔtreS* strains were grown in

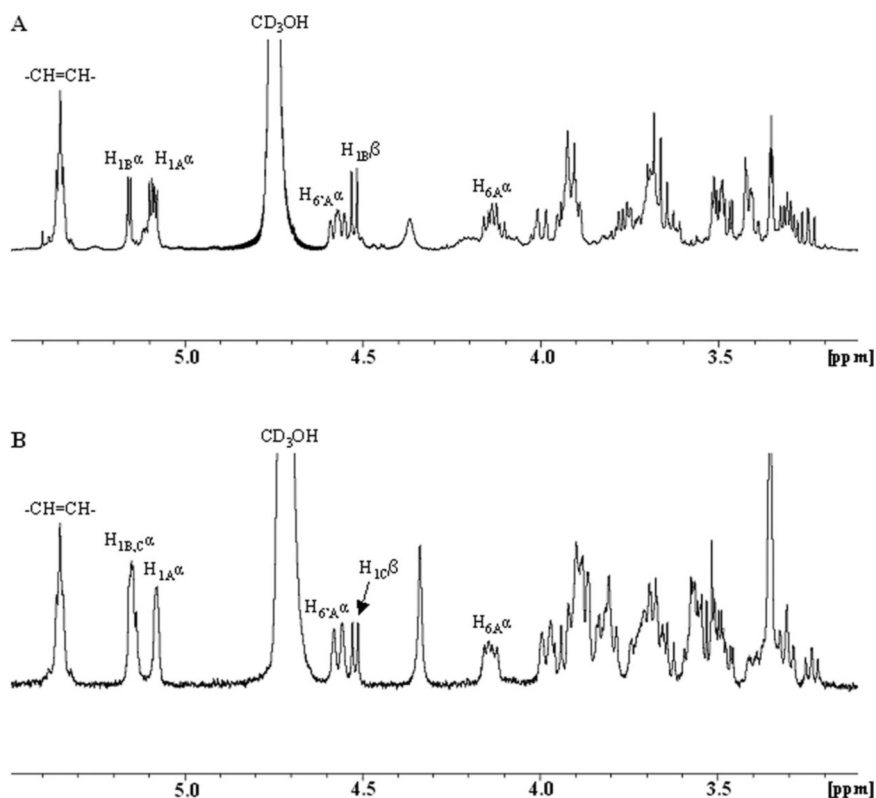


FIG. 5. Partial ^1H NMR spectra of the maltose monocorynomycolate (A) and maltotriose monocorynomycolate (B) purified from the trehaloseless *CglΔotsAΔtreYΔtreS* mutant of *C. glutamicum* grown on maltose and maltotriose, respectively. Spectra were obtained in CHCl_3 . The anomeric proton resonances are indicated.

TABLE III

Chemical shifts (in ppm) and coupling constant values (in Hz) deduced from the analysis of the ^1H NMR and two-dimensional COSY and two-dimensional total correlation spectroscopy spectra of the new mycolate-containing glycolipids found in the *CglΔotsAΔtreYΔtreS* mutant of *C. glutamicum* grown on maltose and maltotriose

Sugar residue	Maltose monocorynomycolate			Maltotriose monocorynomycolate		
	Proton	Resonance (δ)	Coupling constant (J)	Proton	Resonance (δ)	Coupling constant (J)
A	H-1	5.09	$J_{1,2} = 3.7$	H-1	5.08	$J_{1,2} = 3.1$
	H-2	3.51	$J_{2,3} = 6.7$	H-2	3.49	$J_{2,3} = 7.1$
	H-3	3.67	$J_{3,4} = 9.3$	H-3	3.66	$J_{3,4} = /$
	H-4	3.28	$J_{4,5} = /$	H-4	3.31	$J_{4,5} = 8.9$
	H-5	3.91	$J_{5,6} = /$	H-5	3.90	$J_{5,6} = 11.4$
	H-6	4.12		H-6	4.13	
	H-6'	4.57		H-6'	4.55	
B α -Anomer	H-1	5.16	$J_{1,2} = 3.6$	H-1	5.15	$J_{1,2} = /$
	H-2	3.47	$J_{2,3} = 9.6$	H-2	3.55	$J_{2,3} = /$
	H-3	3.92	$J_{3,4} = /$	H-3	3.88	$J_{3,4} = /$
	H-4	3.36	$J_{4,5} = /$	H-4	3.80	$J_{4,5} = /$
	H-5	3.75	$J_{5,6} = /$	H-5	3.37	$J_{5,6} = /$
	H-6,6'	/		H-6,6'	/	
β -Anomer	H-1	4.52	$J_{1,2} = 7.8$			
	H-2	3.24	$J_{2,3} = 7.8$			
	H-3	3.62	$J_{3,4} = 9.2$			
	H-4	3.39	$J_{4,5} = /$			
	H-5	3.73	$J_{5,6} = /$			
	H-6,6'	3.99				
C α -Anomer				H-1	5.15	$J_{1,2} = 3.5$
				H-2	3.45	$J_{2,3} = 9.8$
				H-3	3.95	$J_{3,4} = 9.7$
				H-4	3.56	$J_{4,5} = /$
				H-5	3.71	$J_{5,6} = /$
				H-6,6'	3.97	
β -Anomer				H-1	4.52	$J_{1,2} = 7.4$
				H-2	3.22	$J_{2,3} = 8.5$
				H-3	3.63	$J_{3,4} = /$
				H-4	3.56	$J_{4,5} = /$
				H-5	3.40	$J_{5,6} = /$
				H-6,6'	/	

TABLE IV
Accumulation of radiolabeled trehalose and betaine in *C. glutamicum* cells

In each measurement 1.1 mg of cell dry mass were incubated for 10 min either with [¹⁴C]trehalose or with [¹⁴C]betaine. The concentration of trehalose is expressed relative to the cell dry mass (cdm).

Strain	Trehalose accumulation in whole cells	Trehalose accumulation in cell walls	Betaine accumulation in whole cells	Betaine accumulation in cell walls
	<i>nmol/mg of cdm</i>	<i>nmol/mg of cdm</i>	<i>nmol/mg of cdm</i>	<i>nmol/mg of cdm</i>
ATCC 13032	1.6 ± 0.5	1.5 ± 0.3	85.7 ± 10	0.5 ± 0.05 ^a
CglΔotsAΔtreYΔtreS	0.1 ± 0.4 ^a	0.2 ± 0.1 ^a	60.2 ± 6.6	0.3 ± 0.06 ^a

^a In these measurements the detected radioactivity was below 70 counts/min, which is the level of background radioactivity.

minimal medium with sucrose. Under these conditions the cell wall of CglΔotsAΔtreYΔtreS was proven to be devoid of mycolates (Table I). To test the suitability of these conditions for sugar uptake, [¹⁴C]glucose was used as a control. Under these conditions both strains showed an identical uptake rate of 9 ± 0.7 nmol/min/mg (of cell dry mass) for glucose. In contrast, the uptake of [¹⁴C]trehalose was found to be different for the wild type and mutant strains. Whereas wild type cells accumulated [¹⁴C]trehalose with a very small but significant rate of 0.13 ± 0.07 nmol/min/mg (of cell dry mass), no uptake of trehalose at all was detected in the CglΔotsAΔtreYΔtreS mutant cells. We have shown that the cell architecture of CglΔotsAΔtreYΔtreS is completely different from that of the wild type cells, missing the mycolic acids and, thus, the second fracture plane of the cell wall (Fig. 2). Thus, the label found in wild type cells may originate from trehalose mycolates known to be localized primarily in the cell wall (8). To test this hypothesis we separated the cytoplasm from the cell envelope of both strains by partial permeabilization of the cells with the detergent CTAB. A concentration of 0.1% CTAB was sufficient to disrupt the plasma membrane and to release the cytosol without destroying the cell envelope. In fact all radioactive-labeled trehalose detected in wild type cells was found in the cell envelope, whereas no labeling was determined to be associated with that of CglΔotsAΔtreYΔtreS (see Table IV). As a control for the permeabilization and washing conditions, we used [¹⁴C]betaine, which is known to be accumulated in the cytosol of *C. glutamicum* after a hyperosmotic shock but is not metabolized (40). After induction of betaine, up to 40% of the added betaine was taken up within 10 min by the cells. As shown in Table IV, no betaine label was detected in the cell envelope fractions of cells treated with CTAB, indicating that the conditions used for the permeabilization of cells were suitable. Taken together, the results of the uptake experiments demonstrated that trehalose cannot be taken up by *C. glutamicum* but is used to synthesize TCM (Fig. 3A), due to the availability of mycolates and the activity of the mycolyltransferases located in the cell wall of the wild type strain of *C. glutamicum* (8).

The observation that trehalose mycolates are synthesized outside the cytoplasm raised the question of the existence of a specific pathway whose enzymes would be located in the cell wall and dedicated to the synthesis of trehalose destined to be mycoloylated. Examination of the mycolate content of the CglΔotsA and CglΔtreY single mutants grown on sucrose, *i.e.* in this case the TreS pathway does not participate in trehalose synthesis, showed that both strains synthesize trehalose mycolates in amounts comparable with those of the wild type strain (Table I). The deletion of both *otsA* and *treY* was necessary to generate a mycolate-less strain that was also devoid of trehalose (Table I). These data demonstrated that neither the AB pathway nor the YZ pathway, the two main pathways of trehalose synthesis in Corynebacterineae (2, 3, 4), is specially dedicated to the production of trehalose mycolates.

Role of Trehalose 6-Phosphate in the Synthesis of Mycolates—According to the current model of mycolate synthesis in coryne-

bacteria (20), trehalose phosphate is a precursor molecule for mycolate synthesis. To test the validity of this assumption, we first checked whether OtsA is the only trehalose-6-phosphate synthase of *C. glutamicum* (Fig. 1). Accordingly, the cytoplasmic trehalose 6-phosphate content was determined in a variety of strains deleted or not in the *otsA*-gene and related to the mycolate content of the respective strains. As documented in Table V, in all *otsA*-lacking strains trehalose 6-phosphate was under the detection limit, indicating that OtsA is in fact the only trehalose 6-phosphate-generating enzyme of *C. glutamicum*. Consequently, if trehalose 6-phosphate is necessary for mycolate synthesis, all *otsA*-deleted strains should be devoid of mycolates. Surprisingly, the results presented in Table I are clearly in disagreement with this hypothesis. Mycolates were only absent in strains devoid of trehalose, *i.e.* CglΔotsAΔtreY and CglΔotsAΔtreSΔtreY. In addition, strains CglΔotsB and CglΔotsBΔtreY, which accumulated trehalose 6-phosphate in cytoplasmic concentrations of at least 2 orders of magnitude higher than the wild type due to the lack of the trehalose-6-phosphate phosphatase OtsB (Table V), exhibited no significant difference in their mycolate content compared with CglΔotsA. Obviously, trehalose 6-phosphate plays no particular role in the synthesis of mycolic acids in *C. glutamicum in vivo*. The synthesis of mycolates apparently depends on the availability of trehalose and related sugars (Tables I and II).

DISCUSSION

The present study was undertaken to determine why trehalose is essential for the physiology of mycobacterial species such as *M. smegmatis* (15) and *M. tuberculosis* (16). Because the disaccharide can serve as a carbon source, storage carbohydrate, or stress protection compound (1) in both prokaryotic and eukaryotic cells, several possibilities may explain its crucial role in mycobacteria. In the case of *M. smegmatis* a dual role has been suggested for trehalose, *i.e.* as both a thermoprotectant and a precursor of critical cell wall metabolites (15). The latter suggested role, which consists of a link between the synthesis of trehalose and the production of trehalose mycolates, is particularly attractive knowing that these glycolipids are abundant substances in Corynebacterineae (6, 8) and both structurally and functionally important cell wall constituents of mycobacteria (6, 10, 12, 13). To determine the role of trehalose in this group of bacteria we used corynebacteria that we previously showed to represent a convenient model for the study of specific but essential functions of mycobacteria (24, 41, 42). Accordingly, we biochemically analyzed strains deleted in various trehalose synthesis pathways. Among these the *C. glutamicum* CglΔotsAΔtreSΔtreY was constitutively unable to synthesize trehalose. Under routine growth conditions on minimal medium with sucrose as the carbon source this strain is devoid of cytoplasmic trehalose (4). Suggestive of the crucial role of trehalose and the cell wall properties, the mutant grown on minimal medium exhibited distinct changes in the cell surface properties such as cell clumping and adhesion to glass surfaces such as shake flasks. Furthermore, strains

TABLE V
Cytoplasmic trehalose 6-phosphate and mycolate contents of the wild type and various isogenic strains of *C. glutamicum* (Cgl) grown in minimal medium

The concentration of trehalose 6-phosphate is expressed relative to the cell dry mass (cdm).

Strain	Cytoplasmic trehalose 6-phosphate ($\mu\text{mol/g}$ of cdm)	% Mycolates ^a	
		Extractable lipids	Wall-linked mycolates
Wild type ATCC13032	0.08 \pm 0.03	15	1.9
Cgl Δ otsA	Non-detectable	14	2.5
Cgl Δ otsB	23.2 \pm 5.7	25	2.3
Cgl Δ otsA Δ treY	Non-detectable	0	0
Cgl Δ otsB Δ treY	146.7 \pm 15.9	30	2.3
Cgl Δ otsA Δ treY Δ treS	Non-detectable	0	0

^a The % of corynomycolates were determined as described in Table I.

Cgl Δ otsA Δ treY and Cgl Δ otsA Δ treS Δ treY were found to be devoid of the cell wall fracture plane, as does *C. amycolatum*, a naturally occurring mycolate-free corynebacterium (11).

Examination of the mycolate contents of the Cgl Δ otsA Δ treY and Cgl Δ otsA Δ treS Δ treY mutant strains grown on sucrose demonstrated that they were devoid of trehalose mycolates. The "rescue experiment" was also provided and confirmed the observation that if trehalose is added to the medium together with sucrose, they were able to synthesize trehalose monocorynomycolates. Trehalose seemed, thus, necessary for mycolic acid synthesis in *C. glutamicum* but, importantly, was shown not to be a prerequisite for mycolate synthesis since mycolates were produced by the constitutively trehalose-deficient triple mutant grown on either glucose, maltose, or maltotriose. In this case mycolic acids esterified both the sugar used as carbon source and the cell wall arabinogalactan of Cgl Δ otsA Δ treS Δ treY. Thus, in principle mycolate synthesis is generally possible if an α -glucosyl-containing sugar is present in the medium. These observations were the first hints that the biosynthesis of mycolates does not depend on activated trehalose provided from the cytoplasm. An additional important observation was the fact that the conditional trehalose producing Cgl Δ otsA Δ treY strain grown on maltose, despite the presence of cytoplasmic trehalose, produced maltose mycolates but was devoid of trehalose mycolates. These data again suggested that the synthesis of mycoloylated glycolipids occurs in cell compartments other than the cytoplasm. This hypothesis was supported by the synthesis of trehalose monomycolate by the trehalose-deficient strain Cgl Δ otsA Δ treS Δ treY upon the addition of external trehalose to the minimum medium. Consistently, although glucose phosphate is produced inside cells grown on either sucrose, fructose, pyruvate, or acetate used as carbon source, no mycolate was found in the trehalose-less triple mutant cells, whereas glucose mycolates were identified in the same cells grown on glucose. The final proof for this hypothesis was given by the fact that [¹⁴C]trehalose cannot be taken up by *C. glutamicum* cells, an observation that implies that the detected trehalose monomycolates must indeed be synthesized outside the cell. Altogether, these experiments demonstrated that the transfer of the mycoloyl residue onto trehalose, glucose, maltose, or maltotriose occurs outside the cells, as expected from the production of trehalose corynomycolates in cell-free systems consisting of cell walls (18, 19). They also reinforce the concept that the essentiality of trehalose for mycobacterial growth is due to its use for building the cell wall glycolipids since the trehalose-less mutant of *M. smegmatis*, when grown under optimal conditions, does require exogenous trehalose but not intracellular trehalose (15). It has to be noted that the demonstration of the synthesis of mycoloylated trehalose outside the cells contradicts a recent model in which the production of trehalose monomycolate is proposed to occur in the cytosol (43).

The trehalose-less strains were not only devoid of trehalose mycolates but also of mycolates covalently attached to the cell wall arabinogalactan (8). This observation is in agreement with the postulated role of trehalose monomycolates as the precursor of both cell wall mycolates and trehalose dimycolates. However, when glucose, maltose, or maltotriose were used as the carbon source, the triple mutant cells produce both cell wall-linked and sugar mycolates, indicating that these glycolipids are also capable of transferring mycoloyl residues onto the cell wall arabinogalactan. This result is consistent with the accumulation of glucose monomycolate in a strain of *C. glutamicum* partly affected in the transfer of mycoloyl residues onto its cell wall (21) and, in agreement with the hypothesis, that this glycolipid represents a putative intermediate in the transfer of mycolates onto the cell wall (17, 19). Thus, the synthesis of trehalose monomycolates is not essential for the production of mycoloylated cell walls. Besides, structural analysis of the newly found mycolate-containing glycolipids showed that only one mycoloyl residue was found to esterify either glucose or glucose-containing oligosaccharides, whereas no sugar dimycolate was detected in the trehalose-less mutant grown under these conditions. This observation is consistent with the previous data of Shimakata and Minatogawa (20), which have demonstrated an inhibitory effect of the production of trehalose dimycolate in the presence of an excess of trehalose.

Shimakata and Minatogawa (20) have also proposed a model for mycolate synthesis in *Corynebacterium matruchotii* and have suggested that trehalose 6-phosphate would act as an intermediate acceptor of already synthesized free mycolate (20). The resulting product, trehalose 6-monomycolate, then would serve as a carrier to transfer mycolate onto its final acceptors, *i.e.* the cell wall arabinogalactan and trehalose dimycolates, with the liberation of free trehalose in the overall reaction balance. To test this hypothesis we analyzed the mycolate content of strains of *C. glutamicum* devoid of the sugar phosphate. Not only the Cgl Δ otsA strain devoid of trehalose 6-phosphate synthesizes mycolates, but we found no significant difference in its mycolate content compared with strains Cgl Δ otsB and Cgl Δ otsB Δ treY, which accumulated the sugar phosphate in cytoplasmic concentrations of at least 2 orders of magnitude higher than the wild type due to the lack of the trehalose-6-phosphate phosphatase OtsB. Furthermore, strain Cgl Δ otsA Δ treS Δ treY, which is deficient in the production of trehalose 6-phosphate and, consequently, expected to be devoid of trehalose corynomycolates if trehalose 6-phosphate is the acceptor of pre-synthesized mycolate, was capable of synthesizing trehalose mycolates when trehalose was added to the external medium. Assuming that the biosynthetic pathways leading to the production of corynomycolates are common to all members of the genus, these data showed that, in contradiction to literature data (20), trehalose 6-phosphate is not required *in vivo* for mycolate synthesis in *C. glutamicum*.

As far as the mode of action of sugars in the biosynthesis of mycolates is concerned, the stimulating effect of glucose in some acellular preparations (18, 19) and the isolation of glucose and trehalose derivatives in the course of the synthesis of mycolic acids have led some authors to imagine possible mechanisms for the action of these compounds in the mycolate biosynthesis. Indeed, glucose is required for the biosynthetic activity of the cell-free system of *C. matruchotii* (19), where the resulting corynomycolate was shown to occur as esters of glucose and trehalose. Glucose and trehalose palmitate were also synthesized in these cell-free systems. In *C. diphtheriae* the condensation product after a pulse-labeling experiment with palmitate has been shown to be a 6-(2-tetradecyl 3-keto octadecanoyl)- α -D-trehalose. Because this lipid is the condensation

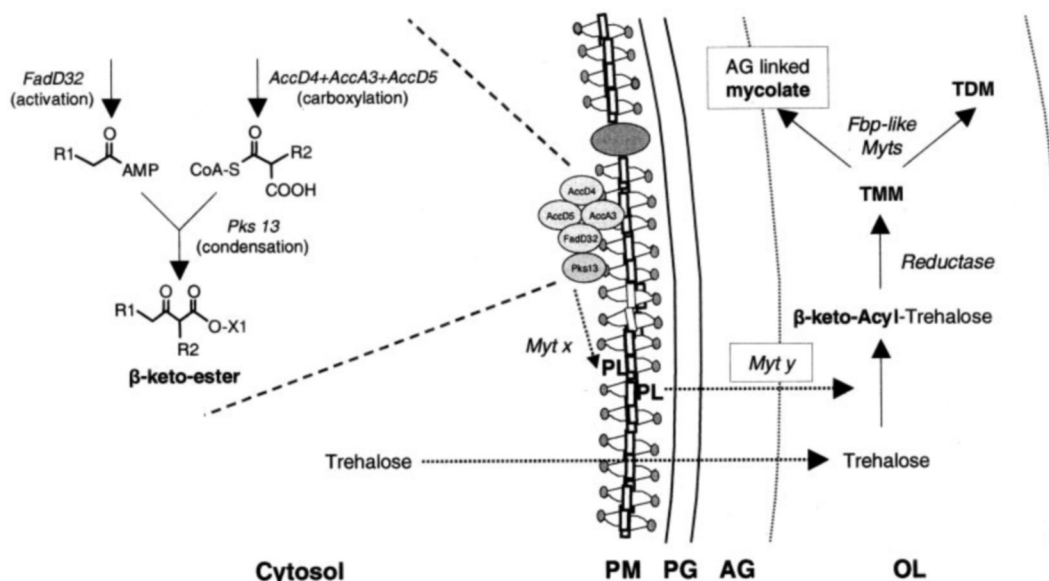


FIG. 6. A working model of the final steps of mycolate biosynthesis and transfer in *Corynebacterineae*. Because cell-free systems able to produce mycolates have been shown to be composed of cell envelopes, the final steps of biosynthesis of mycolates probably take place in the cell envelopes of *Corynebacterineae* *i.e.* the plasma membrane (PM), the cell wall peptidoglycan (PG)-arabinogalactan (AG)-mycolate complex, and the outer layer (OL). The two fatty acid substrates activated by the acyl-AMP ligase FadD32 and the AccD4-, AccD5-, and AccA3-containing acyl-CoA carboxylase are condensed by Pks13. The condensation product, a 2-alkyl 3-keto fatty acyl molecule, would then be transferred by a putative mycoloyltransferase (*Myt*) onto a phosphorylated lipid (PL) to form an oxo-mycoloylated phosphorylated lipid. The trehalose molecule synthesized in the cytosol is transported to the cell wall compartment, where its position 6 is esterified by the 2-alkyl 3-keto fatty acyl molecule originated from the oxo-mycoloylated PL. The trehalose oxo-mycolate is then reduced to form trehalose monomycolate (TMM), the precursor of both trehalose dimycolates (TDM) and cell wall AG-linked mycolates. The latter transfer of mycolates involves the fibronectin-binding proteins (Fbp) in mycobacteria and their homologous Fbp-like proteins in corynebacteria. R1 and R2 correspond to alkyl chains whose sizes vary according to the *Corynebacterineae* species. X1 corresponds to the carrier molecule on which the newly synthesized oxo-mycolate is transferred.

product detected in the first second of incubation time, it has been proposed that trehalose could be implicated at an early step of the synthesis, *e.g.* in locating at a favorable distance for the condensation reaction the two putative activated palmitoyl and tetradecylmalonyl residues that would esterify the 6 and 6' positions of a trehalose diester (44). An alternative role of trehalose in the catabolism of corynomycolic acid has also been proposed based on the fact that the latter lipid is easily decomposed into palmitate. Trehalose would serve as a matrix that locates the functional groups fixed on the 6 position at the right interacting distance and, thus, permits the degradation of the C₃₂- β -keto ester into dipalmitate esters (retro-Claisen process). However, based on the lack of production of the postulated C₃₂- β -keto ester, the putative oxo precursor of mycolates, by trehalose-less mutants of *C. glutamicum*, it is likely that trehalose- and glucose-containing oligosaccharides are necessary for the synthesis rather than degradation of mycolates. Furthermore, with the recent identification of the condensase, Pks13 (41), the acyl-AMP ligase FadD32, and the AccD4-, AccD5-, and AccA3-containing acyl-CoA carboxylase (42), the concept of trehalose as the carrier molecule of fatty acid substrates to be condensed should be revised. The present working model of the putative roles of the disaccharide and the other identified intermediates in the biosynthesis of mycolates by *Corynebacterineae* is represented in Fig. 6. Because various cell-free systems able to produce mycolates are composed of cell envelopes (17–20, 45–48), *i.e.* the plasma membrane, the cell wall peptidoglycan-arabinogalactan-mycolate complex, and the outer layer (6), we propose that the biosynthesis of mycolates takes place in the cell envelope of *Corynebacterineae*. Accordingly, although Pks13 possesses no putative transmembrane domain, the enzyme as well as FadD32 and the acyl-CoA carboxylase, which activates the fatty acid substrates to be condensed by Pks13, have to interact with the plasma membrane. The condensation product, a 2-alkyl 3-keto fatty acyl molecule,

would then be transferred by a putative mycoloyltransferase to be identified onto a phosphorylated lipid to form an oxo-mycoloylated phosphorylated lipid whose presumed location is the plasma membrane. Consistent with our finding that trehalose corynomycolates are synthesized outside the plasma membrane, we suggest that trehalose synthesized in the cytosol would be transported to the cell wall compartment, where its position 6 would be esterified by the 2-alkyl 3-keto fatty acyl molecule, which originated from the oxo-mycoloylated phosphorylated lipid. The oxo-mycolate would then be reduced to form trehalose monomycolate, the putative precursor of both trehalose dimycolates and cell wall arabinogalactan-linked mycolates. The latter transfer has been convincingly shown to involve the fibronectin-binding proteins, formerly known as the antigen 85 complex, and their homologous proteins in mycobacteria and corynebacteria (14, 21–25). The proposed model (Fig. 6) is consistent with the identification of a 3-oxo-2-tetradecyloctadecanoate-containing phospholipid in *C. diphtheriae* (49) and a 6-*O*-mycoloyl-mannosylphosphoprenol in mycobacteria (50) and of a 6-(2-tetradecyl 3-keto octadecanoyl)- α -D-trehalose among the first products synthesized by a cytosol-free system of *C. diphtheriae* (17). The demonstration in the present study that trehalose monocorynomycolate is produced in the outermost compartment of corynebacterial cells when trehalose is supplied is also in agreement with the measurement of extracellular trehalose in the wild type strain of *C. glutamicum* (3, 51). The fact that an isogenic strain Cgl Δ otsA Δ treB Δ treZ that synthesizes intracellular trehalose does not export the disaccharide when grown on maltose (3) may be due to the production of maltose mycolates from the excess of the carbon source as demonstrated herein in the corresponding mutant strain Cgl Δ otsA Δ treY grown in similar conditions. A direct proof of export of trehalose under physiological conditions is, however, still lacking, and the transport system that would export the disaccharide remains to be identified. Further stud-

ies are needed to both challenge the proposed model and functionally characterize the unknown enzymes. For instance, it is not known whether or not additional intermediate acceptors of the mycoloyl precursors exist and what type of reduction system is used outside of the plasma membrane to transform the oxo-mycolates into mycolates.

In conclusion, although trehalose has some significance as a stress protectant under certain conditions, e.g. osmotic up-shift under nitrogen limitation (4), our data suggest that mycolate synthesis and its impact on cell wall composition and biogenesis represents the main reason for the essentiality of trehalose in mycobacteria (15, 16). In this context and knowing that trehalose is absent from mammalian cells, it is tempting to propose the targeting of trehalose synthesis for drug development against pathogenic mycobacterial species. Despite the redundancy of trehalose synthesis in corynebacteria (3, 4) and *M. smegmatis* (15) that would hamper this approach, the inhibition of both *M. aurum* and *M. smegmatis* by trehalose analogs is documented (14, 52). The approach is even more attractive in the case of *M. tuberculosis*, the species responsible for the greatest morbidity and mortality, where the OtsAB was shown to be the dominant pathway for trehalose biosynthesis and its loss cannot be compensated by either of the two alternative pathways (16).

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