The key role of the mycolic acid content in the functionality of the cell wall permeability barrier in Corynebacterineae

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Recently, it has been shown that trehalose and mycolic acids are essential for the growth of Mycobacterium tuberculosis, the causative agent of tuberculosis, and Mycobacterium smegmatis, and important but not indispensable to the survival of Corynebacterium glutamicum. Therefore, to investigate the function of mycolic acids in both the permeability of the cell wall to small nutrients and antibiotics, and the excretion of amino acids by C. glutamicum, a trehalose-deficient mutant of the L-lysine producer ATCC 21527, designated LPΔtreSΔotsAΔtreY, was constructed. By using different carbon sources in either the presence or the absence of external trehalose, a set of endogenously trehalose-free LPΔtreSΔotsAΔtreY cells that exhibited various mycolate contents was generated. The results showed that the structure of the arabinogalactan of these different cell types of LPΔtreSΔotsAΔtreY was not affected when the mycolic acid layer was either missing or impaired. Nevertheless, cells were more susceptible to antibiotics, and the permeability of their cell walls to glycerol was increased. Interestingly, a concomitant increase in the excretion of both L-lysine and L-glutamate was also observed, indicating that the mycolic acid content of the permeability barrier (and not only the peptidoglycan and/or the arabinogalactan) is implicated in the glutamate excretion process.

INTRODUCTION

Mycolic acids, very-long-chain (C30–C90) α-alkyl, β-hydroxy fatty acids, are major and specific constituents of a distinct group of Gram-positive bacteria, classified in the suborder Corynebacterineae, which includes the causative agents of the human diseases tuberculosis and leprosy, Mycobacterium tuberculosis and Mycobacterium leprae, respectively, and comprises other genera such as Corynebacterium, Rhodococcus and Nocardia. This bacterial group is unusual in that, like Gram-negative bacteria, its members contain an outer permeability barrier that may explain both the limited permeability of their cell walls and the general insusceptibility of these bacteria to toxic agents (Brennan & Nikaido, 1995; Daffé & Draper, 1998). In mycobacteria and corynebacteria, this permeability barrier consists of a bilayer composed of mycolic acids and other lipids. In the innermost monolayer, the mycoloyl residues are covalently linked to the cell wall arabinogalactan. The outermost monolayer is composed of various glycolipids, including trehalose monomycolate and trehalose dimycolate.

The composition and amounts of mycolic acids have been shown to affect the virulence, growth rate, colony morphology and permeability of M. tuberculosis (Dubnau et al., 2000; Glickman et al., 2000; Liu et al., 1996; Yuan et al., 1998). To further investigate the molecular bases of these biological processes, it is important to manipulate the mycolate content of mycobacteria, which includes the construction or isolation of mutants whose cell walls are free from mycolates. No such certified strain is currently available, due to the essentiality of enzymes involved in mycolate biosynthesis for the growth of mycobacteria (Portevin et al., 2004; Vilcheze et al., 2000). Interestingly, the analysis of a wild-type derivative of Corynebacterium glutamicum defective in trehalose biosynthesis has revealed that this strain is also unable to synthesize mycolic acids outside the cell (Tropis et al., 2005). Thus, although important for the growth rate and morphotype of corynebacteria, mycolates are not essential for the physiology of these bacteria (Tzvetkov et al., 2003; Wolf et al., 2003;
Gande et al., 2004; Portevin et al., 2004, 2005; Tropis et al., 2005). In C. glutamicum, the absence of the outer permeability layer slows bacterial growth and changes the surface properties of the trehalose-free wild-type derivative CglΔtreSΔotsAΔtreY, but it is possible to partially or completely restore the wild-type mycolate synthesis by growing the mutant with either glucose or maltose as a carbon source, or by adding trehalose to the sucrose-based medium (Tropis et al., 2005). It was thus interesting, using a ΔtreSΔotsAΔtreY corynebacterial strain, to address the question of how the mycolate content influences, besides the cell-surface properties, the permeability of the C. glutamicum cell wall.

METHODS

Bacterial strains and cultivation conditions. C. glutamicum cells were incubated at 30 °C under aerobic conditions in brain heart infusion (BHI) medium or CGXII medium (Keilhauer et al., 1993). To meet the auxotrophy of C. glutamicum ATCC 25127 (Nakayama et al., 1973), CGXII medium was supplemented with 0.2 g l-leucine l\textsuperscript{-1} and 0.4 g l-homoserine l\textsuperscript{-1}. Four percent sucrose or glucose was routinely used as a carbon source. When indicated, 2% trehalose was added. In general, cells grown in rich medium were washed twice with PBS and used to inoculate CGXII medium. When they reached the exponential or early stationary phase, an aliquot of cells was used to inoculate the main CGXII cultures to initial OD\textsubscript{600} 0.5.

Construction of plasmids for the deletion of trehalose biosynthesis genes. In order to delete genes encoding the trehalose biosynthesis enzymes in the genome of C. glutamicum ATCC 25127, the method of allelic replacement was used (Schafer et al., 1994), which is based on the selection of a chromosomal deletion by two recombination events. For this purpose, competent ATCC 25127 cells were transformed with pK19mobsacB derivatives carrying a chromosomal fragment of C. glutamicum, with a deletion in \textit{otsA}, \textit{treS} or \textit{treY}, as described by Wolf et al. (2003). Plasmid integration in the genome was verified by selecting \textit{kanamycin-resistant} and sucrose-sensitive colonies. To promote re- excursion of the plasmid DNA, positive clones grown overnight in Luria–Bertani (LB) broth (10 g tryptone l\textsuperscript{-1}, 10 g NaCl l\textsuperscript{-1}, 5 g yeast extract l\textsuperscript{-1}) containing 2% glucose were plated on LB agar supplemented with 10% sucrose at different dilutions, usually between 10\textsuperscript{-2} and 10\textsuperscript{-5}. Kanamycin-sensitive and sucrose-resistant colonies obtained from the subsequent selection were tested by PCR to verify that the gene of interest carried the desired deletion in the genome. To generate the multiple gene deletion strain LP\textit{AttreS\textit{AttreY}}, the single deletion strain LP\textit{AttreS} or the double deletion strain LP\textit{AttreS\textit{AttreA}} was used instead of ATCC 25127 for allelic replacement.

Quantification of internal trehalose and excreted amino acids. Analysis of the internal trehalose content was performed by GC, as described by Wolf et al. (2003). HPLC analysis was used for the quantification of excreted amino acids, as described by Wolf et al. (2003).

Extraction of non-covalently bound lipids and analysis of trehalose-linked corynomycolic acids. Lipids were extracted from wet cells for 16 h with chloroform/methanol (1:2, v/v) at room temperature; the cells were re-extracted with chloroform/methanol (1:1, v/v) for 16 h and chloroform/methanol (2:1, v/v) for 16 h. The three organic phases were pooled and concentrated by means of rotary evaporation. The crude lipid extracts were partitioned between the aqueous and organic phases arising from chloroform/water mixture (1:1, v/v). The lower organic phases were collected and evaporated to dryness to yield the crude lipid extracts from each strain. Subsequently, 10 µg of lipids from each sample was comparatively examined using TLC on silica gel-coated plates (Durasil-25, 0.25 mm thick; Macherey–Nagel) developed with chloroform/methanol/water (30:8:1 or 65:25:4, by vol.). Glycolipids were detected by spraying plates with 0.2% anthrone in concentrated H\textsubscript{2}SO\textsubscript{4} followed by heating. The mycolate content of extractable lipids was determined in three independent experiments as follows: lipid extracts (100 µg) of the various strains were dried under vacuum and saponified (Daffe et al., 1983), and the saponified products were acetylated with 20% H\textsubscript{2}SO\textsubscript{4}. The resulting fatty acids were extracted with diethyl ether, washed with water, converted to methyl esters with diazomethane, dried under vacuum, and weighed. The fatty acid methyl esters were separated from contaminants on a silica gel column irrigated with different concentrations of diethyl ether in petroleum ether (0, 5, 10, 20 and 100%, v/v). Fractions were analysed by TLC, developed with dichloromethane. Lipids were detected by spraying plates with rhodamine B, and fractions containing mycolates were pooled and weighed.

Arabinogalactan-bound mycolic acids. The arabinogalactan-bound mycolic acids present in the delipidated cells, i.e., the material after the chloroform/methanol extraction (described above), were saponified with 40% KOH in 2-methoxyethanol (1:7, v/v) at 110 °C for 3 h (Daffe et al., 1983), and the solution was neutralized using 20% H\textsubscript{2}SO\textsubscript{4}. The released mycolic acids were extracted with diethyl ether and methylated with diazomethane. The resulting fatty acid methyl esters were examined using TLC on silica gel-coated plates, developed as above. When significant amounts of fatty acids other than mycolic acids were present in the saponification products, mycolates were isolated by chromatography on a silica gel column, as described above, and weighed.

Isolation, purification and analysis of the cell wall arabinogalactan. Cell walls were prepared as previously described (Daffe et al., 1990, 1993). Briefly, wet cells were resuspended in PBS (50 mM, pH 7.2) at a concentration of 5 g per 20 ml, and broken in a cell disrupter (2.7 kbar). After three cell-disrupter cycles, intact cells were removed by centrifugation (3000 g), and the supernatant containing the cell walls was centrifuged (27 000 g, 1 h). The resulting cell wall pellet was treated with aqueous 2% SDS at 95 °C for 1 h and sedimented again at 27 000 g. Cell walls were washed twice with 80% (v/v) acetone in water, and subsequently in water, to remove SDS, and lyophilized. The glycolyl composition of purified cell walls was determined by hydrolysing an aliquot with 2 M trifluoroacetic acid (CF\textsubscript{3}COOH) for 2 h at 110 °C, followed by trimethylsilylation (Sweeley et al., 1963) and GC analysis of the resulting products.

Cell walls were O-methylated by a slight modification of the Hakomori procedure (York et al., 1986). Cell walls (100 mg) were suspended in 3 ml DMSO (Pierce), 300 µM 5.2 M dimethylsulfoxide carbamion was added, and the mixture was stirred for 5 h. An equimolecular amount of methyl iodide (CH\textsubscript{3}I) (120 µl; Aldrich) was slowly added and the mixture was stirred for 2 h. Addition of the base and CH\textsubscript{3}I was repeated twice, and a large excess (2 ml) of CH\textsubscript{3}I was added at the end of the third cycle. After 2 h of stirring, the reaction mixture was partially evaporated with nitrogen, an equal volume of water was added, the mixture was dialysed and the retentate was lyophilized. Aliquot fractions of per-O-methylated cell walls were hydrolysed with 2 M CF\textsubscript{3}COOH for 2 h at 110 °C, reduced with sodium tetrahydroborate, and acetylated. The resulting per-O-methylated alditol acetates were analysed by GC/MS, as previously described (Daffe et al., 1990, 1993). GC/MS of the partially O-methylated, partially O-acetylated alditols was performed on a Hewlett-Packard 5890 gas chromatograph connected to a Hewlett Packard 5989 mass selective detector. The temperature of the injector was 260 °C and that of the transfer line was
300 °C. The column was a 12 m HP-1 (Hewlett-Packard). The oven was programmed to hold at 60 °C for 3 min, followed by a rise of 20 °C min⁻¹ to 100 °C, and a rise of 8 °C min⁻¹ to 290 °C, and an 8 min hold. The mass spectrometer was set to scan from 50 to 500 Da.

For NMR analysis, purified cell walls were treated with 2 M NaOH (16 h, 80 °C) to cleave the phosphodiester link between arabinogalactan and peptidoglycan (Daffe et al., 1990, 1993). The supernatant, which contained the soluble arabinogalactan, was obtained by centrifugation (27 000 g, 30 min), neutralized with acetic acid, and dialysed to remove salts. A precipitate formed during the treatment was removed by centrifugation. The supernatant was treated with ethanol (80% in water), kept at 4 °C overnight to precipitate the polysaccharide that was recovered by centrifugation, and lyophilized. For ¹H- and ¹³C-NMR analysis, arabinogalactans were solubilized in ²H₂O (20 mg per 0.5 ml) and analysed at 600 and 150.9 MHz, respectively, on a Bruker Avance 600 MHz NMR spectrometer equipped with a TCI cryobore. ¹H decoupled ¹³C-spectra were obtained with a DEPT 135 pulse sequence in a D2O acquisition mode.

**Antibiotic susceptibility tests.** For quantitative determination of susceptibility to erythromycin, Etest strips containing the antibiotic were used (AB Biodisk). For this purpose, strains were pre-cultured overnight in CGXII containing glucose or sucrose as carbon source, and 2% trehalose when indicated. On the next day, 25 ml of the overnight culture (OD 600 1) and cultivated again for 24 h at 30 °C. Then, the Etest strips were dispensed, washed twice in 50 mM potassium phosphate (pH 7.5) and 20 mM NaCl containing cells at OD 600 3. The transport rates were expressed as nmoles 20 mM NaCl and 10 mM glucose, before their use for uptake measurement. The cell suspension was diluted to OD600 1 and spread onto the agar plates containing the same carbon source and trehalose when indicated. Then, the Etest strips were applied. The plates were incubated at 30 °C for 2–3 days, after which, the MIC was determined.

**Determination of glycerol transport rates.** Prior to uptake measurements, cells of ATCC 21527 or LPAtreSαotsAAtreY were grown twice in minimal medium supplemented with sucrose to ensure that LPAtreSαotsAAtreY did not contain any mycolates in the cell wall. After 16 h of growth, cells of the second culture were harvested, washed once in 50 mM potassium phosphate buffer containing 20 mM NaCl and 10 mM glucose, before their use for uptake measurements. [¹⁴C]Glycerol (3.7 MBq ml⁻¹, 5.3 GBq mmol⁻¹) was purchased from PerkinElmer. [¹⁴C]Glycerol 20–100 μM (37 kBq ml⁻¹) was added to 1 ml 50 mM potassium phosphate buffer (pH 7.5) and 20 mM NaCl containing cells at OD600 3. Aliquots of cells (0.1 ml) were taken after 6, 12, 18, 24, 30 and 60 min, and were separated from the assay buffer by filtration. Subsequently, incorporated radioactivity was determined by liquid scintillation counting. The transport rates were expressed as nmoles glycerol accumulated mm⁻¹ (mg cell dry weight)⁻¹.

**Assay for glycerol kinase activity.** Strain ATCC 21527 and its LPAtreSαotsAAtreY mutant were grown to exponential phase, harvested, washed twice in 50 mM potassium phosphate (pH 7.5) and suspended in 2 ml 50 mM Tris/HCl (pH 7.5), 10 mM MgCl₂, and disrupted by glass beads. The cell debris was removed by centrifugation in a microcentrifuge (12 000 r.p.m.) at 4 °C. The enzyme assays were carried out in 100 mM sodium phosphate (pH 6) according to the method of Kwakman & Postma (1994). The protein content in the assay was varied between 2 and 5 mg ml⁻¹.

**RESULTS**

**Construction and phenotypical analysis of a trehalose-deficient L-lysine producer**

It has recently been shown that *C. glutamicum*, like *M. tuberculosis*, possesses three different pathways for the biosynthesis of trehalose, namely the OtsAB, TreS and TreYZ pathways (De Smet et al., 2000; Tzvetkov et al., 2003; Wolf et al., 2003). These pathways use activated glucose-6-phosphate and UDP-glucose, maltose and maltodextrins, respectively, as substrates for trehalose biosynthesis. In order to construct a trehalose-deficient mutant of the L-lysine producer *C. glutamicum* ATCC 21527, the genes otsA, treY and treS were deleted stepwise from the genome of ATCC 21527 by allelic replacement. The resulting strain was designated LPAtreSαotsAAtreY. The analysis of the cytosolic trehalose content revealed that the parental strain ATCC 21527 had a concentration of 47 μmol (g cell dry mass)⁻¹, whereas in LPAtreSαotsAAtreY, no trehalose was detectable at all, which agrees with previously published data on the CgltreSαotsAAtreY mutant strain derived from the type strain ATCC 13032 of *C. glutamicum* (Wolf et al., 2003).

We have recently shown that the trehalose-deficient CgltreSαotsAAtreY is also unable to accumulate mycolates when grown on sucrose-based minimal medium, proving that endogenously synthesized trehalose is an essential precursor for the production of detectable mycolic acids in *C. glutamicum* (Tropis et al., 2005). By the addition of glucose or maltose, or by supplementing the sucrose-based medium with trehalose, it is possible to completely or partially restore mycolate production, and manipulate to some extent the composition of the extractable mycolic acids (Tropis et al., 2005). In order to modulate the composition of the cell wall of the trehalose-deficient L-lysine producer, LPAtreSαotsAAtreY and its parental strain ATCC 21527 were grown in either sucrose- or glucose-based minimal medium in the presence or absence of trehalose. Lipid analysis of both strains revealed that with sucrose as a substrate no mycolic acids were detectable in the mutant strain, whereas in the presence of trehalose, mycolates were produced and found linked to trehalose in extractable lipids and attached to arabinogalactan (Fig. 1A, Table 1). Trehalose monooctanoylmycolates (TMCs; Puech et al., 2000) represent the major mycolate-containing glycoconjugates in the extractable lipids of both the parental and the trehalose-free mutant grown in medium supplemented with the disaccharide. This observation is in agreement with the data obtained from the wild-type strain of *C. glutamicum* and its derivative CgltreSαotsAAtreY (Tropis et al., 2005), in which trehalose dicocyanomylcates (TDCMs; Puech et al., 2000) represent minor glycoconjugates under these cultivation conditions. The use of glucose as a carbon source leads to the synthesis of significant amounts of glucose monooctanoylmycolates (GMCNs; Puech et al., 2000) in the mutant, which represents, however, a minor constituent in both the parental and mutant strain grown in glucose-based media supplemented with trehalose (Fig. 1A, Table 1). Compared to the L-lysine producer ATCC 21527 grown on minimal medium supplemented with glucose, the LPAtreSαotsAAtreY mutant produced only half the quantity of both cell wall-linked and trehalose-containing mycolates (Table 1). It was
thus possible to modulate the mycolate content of the LP mutant by growing cells in defined minimal medium with different carbon sources.

In order to investigate the influence of the mycolic acid composition on the physiology of ATCC 21527 and LP\textsuperscript{ΔtreSDotsADtreY} cells, we first determined the growth behaviour of the mutant (Fig. 1B). For this purpose, cells were grown in minimal medium containing either sucrose or glucose as a carbon source. In a second step, trehalose, which cannot be taken up by \textit{C. glutamicum} (Tropis \textit{et al.}, 2005), was added to the different cultures in a concentration of 2\%, in order to complement the defect in mycolic acid synthesis. Compared to the parental strain ATCC 21527, the growth of LP\textsuperscript{ΔtreSDotsADtreY} on either sucrose- or glucose-based minimal medium was severely impaired. Not only was the growth rate lowered, but also the final yield of cell mass was reduced. This effect was more pronounced when sucrose was used as a carbon source. If sucrose-based medium was supplemented with 2\% trehalose, the growth defect was partly rectified, whereas growth was more or less identical to that of the parental strain ATCC 21527 when cells were grown in glucose-based medium supplemented with trehalose (Fig. 1B).

**Influence of corynomycolate production on the excretion of amino acids**

The ATCC 21527 and LP\textsuperscript{ΔtreSDotsADtreY} strains were able to excrete L-lysine into the medium. Under our growth
Table 1. Quantification of corynomycolates of ATCC 21527 and LPΔtreSΔotsAΔtreY

The corynomycolates that composed the lipids extracted from bacterial cells with organic solvents (extractable lipids) and those that remained attached to delipidated cells (wall-linked corynomycolates) were determined after saponification of these materials, followed by methylation of the resulting fatty acids, purification of the corynomycolates by chromatography on silica gel columns, and weighing, as described in Methods. The percentages were determined relative to the masses of extractable lipids and delipidated cells. The values are the means of at least three independent experiments. WT, ATCC 21527; ΔSAY, LPΔtreSΔotsAΔtreY.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Wall-linked corynomycolates</th>
<th>Corynomycolates in extractable lipids</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>ASAY</td>
</tr>
<tr>
<td>4% Sucrose</td>
<td>1.0±0.1 %</td>
<td>0.0</td>
</tr>
<tr>
<td>4% Glucose</td>
<td>0.7±0.1 %</td>
<td>0.4±0.1 %</td>
</tr>
<tr>
<td>2% Sucrose + 2% trehalose</td>
<td>0.9±0.1 %</td>
<td>0.8±0.1 %</td>
</tr>
<tr>
<td>2% Glucose + 2% trehalose</td>
<td>0.5±0.1 %</td>
<td>0.7±0.1 %</td>
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conditions, a final yield of up to 43±2 mM was reached after growth on sucrose. Since ATCC 21527 and LPΔtreSΔotsAΔtreY showed large differences in the growth rate, and consequently in the mass of cells that were able to excrete L-lysine, we compared the amount of L-lysine produced per gram of cell dry mass. This revealed that when glucose was used as carbon source, almost no difference was observed between the trehalose-free LPΔtreSΔotsAΔtreY and the parental strain, in terms of productivity of lysine (Fig. 2). In sharp contrast, when sucrose was used as a carbon source, LPΔtreSΔotsAΔtreY cells excreted threefold more lysine [9 mmol (g cell dry mass)⁻¹] than did the parental strain [3 mmol (g cell dry mass)⁻¹]. Adding 2% trehalose to the medium resulted in decreased excretion of lysine by mutant cells, to the level of that of the parental strain (Fig. 2).

In standard growth conditions, C. glutamicum does not excrete L-glutamate into the medium; L-glutamate efflux can be induced by applying certain treatments, e.g. the addition of penicillin, Tween 60 or ethambutol (Eggeling & Sahm, 2003; Kimura, 2005; Radmacher et al., 2005). However, it is well known that the treatment of sensitive bacteria with ethambutol, by inhibiting arabinosyl transferases (Belanger et al., 1996), also decreases their mycolate content (Takayama et al., 1979). Since we showed that, depending on the carbon source, the trehalose deficiency of LPΔtreSΔotsAΔtreY led to a severe change in the mycolic acid content of the corynebacterial cell wall, we analysed the L-glutamate content of the medium of LPΔtreSΔotsAΔtreY and the parental strain ATCC 21527 grown on various carbon sources. Independent of the chosen carbon source, the ATCC 21527 strain did not excrete L-glutamate. In contrast, the loss of mycolate biosynthesis in the cell wall led to a significant accumulation of L-glutamate in the medium (Fig. 3). Interestingly, the production of mycolates in a glucose-based medium was not sufficient to abolish the excretion of glutamate, whereas it was possible to reduce drastically the L-glutamate excretion by adding 2% trehalose to sucrose- or glucose-containing medium. It was
thus concluded that the mycolate content, as opposed to the production of these lipids, is important to switch off the stimulus for L-glutamate excretion.

Structural features of the arabinogalactans of the L-lysine producer and mutant strains

Taken together, the results of the excretion of amino acids were consistent with the fact that the changes in mycolic acid composition provoked changes in excretion of both L-lysine and L-glutamate in strain LP\textsubscript{treS}otsA\textsubscript{treY}. Thus, it seemed reasonable to postulate that the content and composition of the mycolic acid layer directly influence the permeability of the cell wall. Nevertheless, since it has been reported that inhibition of arabinogalactan synthesis by treatment with ethambutol also results in an increased excretion of glutamate (Radmacher et al., 2005), it remained possible that the increased excretion of amino acids by the mutant strain was a consequence of changes in the structure of the cell wall arabinogalactan, which may have followed the deficiency in mycolate. In addition, such a comparative analysis should have given important information on the sequence of events during the building of the mycolylated cell wall. Accordingly, cell walls from both the parental and corynomycolate-deficient strains were isolated and purified (Daffe\textsuperscript{e} et al., 1990, 1993). Per-O-methylation of the cell walls, followed by acid hydrolysis, reduction and O-acetylation, resulted in the production of partially O-methylated, partially O-acetylated alditols that were then analysed by GC/MS to determine the glycosyl linkage composition of the purified cell walls. No obvious qualitative or quantitative difference was seen between the compositions of the purified cell walls from the two origins; they were composed of t-Araf, 2-Araf, 5-Araf, 2,5-Araf, 3,5-Ara\textsubscript{d}, t-Gal\textsubscript{d}, 5-Gal\textsubscript{d}, 6-Gal\textsubscript{d}, 5,6-Gal\textsubscript{d} and t-Rha\textsubscript{p}. This composition was identical to that found in the type strain of C. glutamicum (Alderwick et al., 2005), and similar to those of other corynebacteria (Puech et al., 2001) and related genera (Daffe\textsuperscript{e} et al., 1993).

To decipher the structural features of the arabinogalactans from the parental and mutant LP strains, the polysaccharides were released from the cell walls by means of alkaline hydrolysis (Daffe\textsuperscript{e} et al., 1990, 1993) and were comparatively analysed. As expected from the glycosyl composition, the \textsuperscript{1}H-NMR spectra of the arabinogalactans from the two strains were superimposable (data not shown) and similar to those of mycobacteria and rhodococci, but different from those of nocardiae (Daffe\textsuperscript{e} et al., 1990, 1993). Anomeric proton signals of the arabinogalactans were seen at $\delta$ 5.0–5.3, consistent with the presence of $\alpha$- and/or $\beta$-furanosyl residues and/or $\alpha$-pyranosyl units (Daffe\textsuperscript{e} et al., 1993). Resonances of the other sugar protons were grouped at $\delta$ 4.3–3.6. Since \textsuperscript{13}C-NMR has proved to be an excellent investigative tool, both as an aid to structural determination and in fingerprinting for taxonomic purposes (Daffe\textsuperscript{e} et al., 1990, 1993), the arabinogalactans from the two isogenic strains were analysed comparatively by this technique (Fig. 4).

The \textsuperscript{13}C-NMR spectra of the arabinogalactans from the two LP strains were superimposable (Fig. 4) and had many similarities with those from other Corynebacterineae (Daffe\textsuperscript{e} et al., 1993). Based on the previous assignments deduced from the spectra of M. tuberculosis (Daffe\textsuperscript{e} et al., 1990) and other Corynebacterineae (Daffe\textsuperscript{e} et al., 1993), resonances attributable to the C-1s of t-$\beta$-Ara\textsubscript{t} and 2-linked-$\alpha$-Ara\textsubscript{t} of the non-reducing end of the arabinogalactan molecules were seen at 101–102 p.p.m. and 106–107 p.p.m., respectively. The resonances of the C-2s of 2-linked-$\alpha$-Ara\textsubscript{t} were identified at $\delta$ 87–89, while that of the C-5 of t-$\beta$-Ara\textsubscript{t} was seen at 64 p.p.m. (Daffe\textsuperscript{e} et al., 1990, 1993). Importantly, the signals of C-1s of 2-linked-$\alpha$-Ara\textsubscript{t} of C. glutamicum ($\delta$ 106–107) were more complex than those of all arabinogalactans examined...
so far; an observation that is consistent with the complexity of the corresponding C-2s (δ 87–90). Furthermore, two groups of resonances of C-2s of 2-linked-α-Araf were observed. The major asymmetrical pair signals were seen at 87.8 and 88.1 p.p.m., whereas the minor series were observed at 89.0 and 89.3 p.p.m. This correlates with the occurrence of an asymmetrical pair of signals attributable to C-1s of t-β-Araf (δ 101–102). This suggests the occurrence of specific combinations of t-β-Araf and 2-linked-α-Araf residues, different from those of the non-reducing tri- and penta-arabinosyls found in the arabinogalactans of other Corynebacterinae (Daffe et al., 1990, 1993). Further detailed structural studies of the polysaccharides of corynebacterial origin are clearly needed. The furanoid nature of the remaining Ara and Gal residues was also obvious from the chemical shift values of the C-1 resonances (δ 108–110). The resonances of primary alcohol carbons of unsubstituted C-5s of α-Araf and C-6s of β-Galf were observed at δ 61–62, whereas those of 5- and 3,5-Araf and 6- and 5,6-Gal f were seen at δ 67.8. These assignments were based on the glycosyl linkage composition and 13C-NMR spectroscopy in the DEPT mode (Fig. 4), in agreement with previously published data (Daffe et al., 1990, 1993). The signal at 103.3 p.p.m., absent from the NMR spectra of the various arabinogalactans analysed so far, was attributed to the resonance of t-Rha p, a glycosyl residue that has so far been found to be specific to the arabinogalactan of C. glutamicum. It was thus concluded that the gross structure of the arabinogalactan of the LP strain is not affected by mutations that result in a total lack of production of trehalose and mycolates. It follows that the enhanced excretion of amino acids by the LP mutant strain was attributable to the absence of the mycoloyl layer, the restoration of which fully abolished the observed excess excretion.

Influence of mycolate content on the susceptibility of C. glutamicum to antibiotics

The integrity of the mycolic acid layer has been shown to be important for the resistance of M. tuberculosis to antibiotics (Brennan & Nikaido, 1995; Daffe & Draper, 1998). Thus, we investigated whether the absence of mycolic acids or variations in the mycolic acid content of the C. glutamicum cell wall resulted in a higher susceptibility to antibiotics. For this purpose, cells grown on liquid minimal medium (with either sucrose or glucose as a carbon source) were spread on agar plates containing the same growth medium, and the MIC of the hydrophobic compound erythromycin was determined (Fig. 5). Analysis of the susceptibility of the parental lysine producer and the mutant strains to the antibiotic clearly showed that the mycolate deficiency of LPΔtreSΔotsAΔtreY resulted in a higher permeability of the cell wall, independent of the carbon source. Similar results were also obtained with the hydrophilic ethambutol (data not shown), proving that the mycolates act as a permeability barrier, at least for antibiotics.

Glycerol transport rates in relation to production of mycolic acids

Glycerol is a membrane-permeable substance that can enter the cells of Corynebacterinae by diffusion across their thick cell walls that contain the mycolic acid layer as a second membrane-like structure, and their plasma membranes. It has been shown that the diffusion of glycerol can be used as a marker of the permeability of the mycobacterial cell wall (Bardou et al., 1998; Jackson et al., 1999). Thus, in order to characterize the function of the mycolic acid layer in C. glutamicum, the velocity of glycerol transport was determined in the ATCC 21527 and LPΔtreSΔotsAΔtreY strains grown on sucrose-containing minimal medium. Under
these conditions, the LPAtreSΔotsAΔtreY strain was, in contrast to the wild-type cells, completely free of cell wall-located mycolic acids (see above), and thus free of the outer permeability membrane-like structure in the cell wall (Puech et al., 2000; Portevin et al., 2004; Tropis et al., 2005).

At a concentration range of 30–100 μM, glycerol transport by the corynebacterial cells by diffusion depended linearly on the glycerol concentration. In the LPAtreSΔotsAΔtreY strain, the transport rate was approximately three times higher than that in its parental ATCC 21527 strain (Fig. 6), indicating that the loss of mycolic acids indeed facilitates the diffusion of glycerol into the cell, in agreement with previous data (Puech et al., 2000). In order to exclude the possibility that the mutant strain has a higher glycerol kinase activity that results in a faster conversion of glycerol to glycerol phosphate, and thus in a higher concentration gradient of non-phosphorylated glycerol across the membrane, we determined the glycerol kinase activity of cells used for the transport assay. Both strains exhibited comparable glycerol kinase activities of $0.25 ± 0.01 \mu$mol min$^{-1}$ (g cell dry mass)$^{-1}$, which were 100-fold higher than the glycerol transport rates. Thus, it was concluded that the observed differences in the rate of transport of glycerol by the strains were attributable to their different mycolate contents.

**DISCUSSION**

We have recently established the importance of trehalose biosynthesis for the production of detectable amounts of mycolic acids in *C. glutamicum*, by showing that a derivative of the type strain ATCC 13032, which is not able to synthesize trehalose (Wolf et al., 2003), is devoid of cell wall mycolic acids when sucrose is used as carbon source (Tropis et al., 2005). Interestingly, this defect is partly rectified when sucrose is exchanged by glucose or a carbon source possessing a terminal α-(1→6)-glucopyranosyl unit (Tropis et al., 2005). Under these conditions, the mycolates have been shown to be synthesized and to esterify both the sugar used as carbon source, in the form of monomycolate, and the cell wall-linked arabinogalactan (Tropis et al., 2005). Using this knowledge, we manipulated the cell wall composition of LPAtreSΔotsAΔtreY, a trehalose-deficient derivative of the L-lysine-producing *C. glutamicum* strain ATCC 21527, by adding different carbon sources for growth. The mutant cells varied in their cell wall composition, from being completely devoid of any detectable mycolates, to having a mycolate composition quantitatively similar to that of the parental strain. Accordingly, it was possible to investigate the influence of the presence and amount of mycolates in *C. glutamicum* on the permeability of the cell wall to both uptake and excretion of different substances.

To ensure that mycolates, as opposed to the whole cell wall core, which includes arabinogalactan and peptidoglycan, are the sole parameter concerned in these studies, we showed that the absence of mycolates had no influence on the production and structural features of the cell wall arabinogalactan. In mycobacteria, in which the cell wall polysaccharide represents the major mycolate-containing compound (Brennan & Nikaido, 1995; Daffé & Draper, 1998), mycolic acids are found esterifying the four primary alcohol groups of half (two-thirds in *M. tuberculosis*) of the non-reducing terminal penta-arabinosyl motifs of the polysaccharide (McNeil et al., 1991). There are at least two possible explanations for these structural characteristics: either the whole arabinogalactan is synthesized and transferred to the cell wall compartment prior to the selective and specific mycoloylation of some motifs, or it is the mycoloylated penta-arabinosyl motifs that are added to
the arabinogalactan during its synthesis. Based on our results and on the similarities in the cell wall composition and architecture between mycobacteria and corynebacteria, the first hypothesis seems more likely. This hypothesis is further strengthened by the fact that we have recently shown that the biosynthesis of TCMC, the likely precursor molecule for the formation of cell wall-linked mycolates, takes place within the cell wall and not in the cytoplasm (Tropis et al., 2005). Therefore, by modulating the amount and composition of the mycolates in the trehalose-free mutant by varying the carbon source, the effects of the membrane-like mycolic acid layer on permeability could be evaluated.

The effects exerted by the composition of the mycolate layer on the excretion of amino acids could be important for biotechnological purposes, if they can be used as a tool to increase the productivity of C. glutamicum strains used for amino acid synthesis. Indeed, the investigation of the L-lysine production of strain LPΔtreSΔotsAΔtreY revealed that, after fermentation in sucrose medium, a threefold higher productivity [g L-lysine (g cell dry mass)⁻¹] was detected in comparison to that in strain ATCC 21527. This indicates that the mycolate layer may in fact contribute to the efficient excretion of a charged amino acid like L-lysine. The effect can be explained by the following assumption: due to the hydrophobic character of the outer membrane-like mycolic acid layer in the parental strain ATCC 21527, L-lysine has to pass through the cell wall via the cation-selective porin PorA, which has recently been described in C. glutamicum (Lichtinger et al., 2001; Costa-Riu et al., 2003). If this permeability barrier is missing in the trehalose-free mutant, L-lysine may be excreted faster. As the L-lysine excretion was decreased to the level of the parental strain in cells grown on glucose, this implies that half of the mycolate content is sufficient to restore the permeability barrier for this amino acid. In addition, these results show that mycolate deficiency may in fact serve as a tool to increase product yields, although the observed severe growth impairment would have to be overcome.

The LPΔtreSΔotsAΔtreY strain, but not the parent strain ATCC 21527, was able to excrete L-glutamate spontaneously into the medium. This effect in LPΔtreSΔotsAΔtreY was almost completely reversed if trehalose was added to the medium. Although the general mechanism of L-glutamate efflux is rather poorly understood, a common feature of several of these manipulations is to affect the integrity of the cell wall of C. glutamicum (Eggeling & Sahm, 2003; Daffe, 2005; Radmacher et al., 2005). While it could not be determined in previous experiments whether the trigger for L-glutamate excretion originated from a change in the plasma membrane and/or the outer membrane-like mycolic acid layer, our data demonstrate that the lack of mycolate is sufficient to induce glutamate excretion. For this process, however, the amount of mycolates seems to be more critical than for the excretion of lysine. The growth of the LP mutant on glucose, which led to the production of half the quantity of the mycolates produced by the parental strain, and abolished L-lysine excretion, still resulted in the excretion of glutamate. Nevertheless, further work is required to reveal whether a change in the structure of the cell wall is both sufficient and a direct reason for the induction of glutamate excretion, or whether the disordering of the cell wall is somehow detected by the cell, which in turn leads to a particular cellular response, ultimately resulting in glutamate excretion. In this context, it should be noted that a decrease in the activity and expression level of 2-oxoglutarate dehydrogenase, which is the key enzyme for the carbon flux within the TCA cycle, has been observed after the induction of glutamate excretion (Kawahara et al., 1997; Kataoka et al., 2006). The resulting shift in the ratio between the carbon flux via 2-oxoglutarate dehydrogenase and that via glutamate dehydrogenase may also be a factor which contributes to glutamate excretion.

In order to define the impact of the mycolic acid layer on the permeability of the cell wall in the Corynebacteriaeae in more detail, we studied changes in uptake activities in the C. glutamicum strain LPΔtreSΔotsAΔtreY. These investigations are only possible to a limited extent in mycobacteria, due to the impossibility of generating viable mycolate-free mycobacterial strains. We used glycerol, a membrane-permeable substance that is known to diffuse across the thick cell wall of Corynebacteriaeae, as a marker for the permeability of the cell wall, as established previously for both mycobacteria (Bardou et al., 1998; Jackson et al., 1999) and corynebacteria (Puech et al., 2000). We determined the velocity of glycerol diffusion by the ATCC 21527 and LPΔtreSΔotsAΔtreY strains of C. glutamicum grown on sucrose-containing minimal medium, conditions in which the LPΔtreSΔotsAΔtreY strain was completely free of cell wall-linked and trehalose-containing mycolic acids. We showed that diffusion of glycerol in LPΔtreSΔotsAΔtreY was approximately three times faster than that in its parental strain ATCC 21527, indicating that the loss of mycolic acids indeed facilitates the transport of glycerol into the cells. This was not attributable to a higher glycerol kinase activity in the mutant, which would have resulted in a faster conversion of glycerol to glycerol phosphate, and thus to a higher driving force for inward diffusion of non-phosphorylated glycerol across the membrane, since the parental and mutant strains exhibited comparable glycerol kinase activities that were 100-fold higher than the glycerol transport rates.

In mycobacteria, the mycolate layer is of unusual thickness and low fluidity. These properties are thought to result in high resistance to most of the common antibiotics (Brennan & Nikaido, 1995). Although the mycolic acid layer in C. glutamicum is thinner than that in mycobacteria, due to a shorter chain length of the mycolic acids, susceptibility to antibiotics can also be used as a measure of permeability in C. glutamicum. By using erythromycin, we intended to ensure that the permeability of the cell wall, and not that of the plasma membrane, determined the inhibitory effect. This macrolide antibiotic was selected as an example of a
large hydrophobic molecule that has to pass through the cell wall of the parent strain by diffusion across the hydrophobic lipid bilayer, and not through the hydrophilic channels of porins, as recently shown for penicillin G (Costa-Riu et al., 2003). Analysis of the susceptibility of the parental and mutant lysine-producer strains to erythromycin showed that the mycolate deficiency of LPatEsAotsAAtreY resulted in a higher permeability of the cell wall, proving that the mycolates act as a permeability barrier for this antibiotic.

In conclusion, we showed that modulation of the content of the mycolic acid layer of \textit{C. glutamicum} can be used to analyse the function of the outer membrane-like structure. Cells with a reduction in mycolate content showed a higher permeability for substances taken up into the cells or excreted into the culture medium. Based on the observation that the structure of the arabinogalactan layer was not affected when the mycolic layer was either missing or impaired, the detected permeability changes can be solely attributed to the mycolic acid layer of the cell wall.

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