Importance of mycoloyltransferases on the physiology of Corynebacterium glutamicum

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Mycoloyltransferases (Myts) play an essential role in the biogenesis of the cell envelope of members of the Corynebacterineae, a group of bacteria that includes the mycobacteria and corynebacteria. While the existence of several functional myt genes has been demonstrated in both mycobacteria and corynebacteria (cmyt), the disruption of any of these genes has at best generated cell-wall-defective but always viable strains. To investigate the importance of Myts on the physiology of members of the Corynebacterineae, a double mutant of Corynebacterium glutamicum was constructed by deleting cmytA and cmytB, and the consequences of the deletion on the viability of the mutant, the transfer of corynomycoloyl residues onto its cell-wall arabinogalactan and trehalose derivatives, and on its cell envelope ultrastructure were determined. The double mutant strain failed to grow at 34°C and exhibited a growth defect and formed segmentation-defective cells at 30°C. Biochemical analyses showed that the double mutant elaborated 60% less cell-wall-bound corynomycolates and produced less crystalline surface layer proteins associated with the cell surface than the parent and cmytA-inactivated mutant strains. Freeze-fracture electron microscopy showed that the ΔcmytA ΔcmytB double mutant, unlike the wild-type and cmytA-inactivated single mutant strains, frequently exhibited an additional fracture plane that propagated within the plasma membrane and rarely exposed the S-layer protein. Ultra-thin sectioning of the double mutant cells showed that they were totally devoid of the outermost layer. Complementation of the double mutant with the wild-type cmytA or cmytB gene restored completely or partially this phenotype. The data indicate that Myts are important for the physiology of C. glutamicum and reinforce the concept that these enzymes would represent good targets for the discovery of new drugs against the pathogenic members of the Corynebacterineae.

INTRODUCTION

Mycolic acids, long-chain α-alkyl, β-hydroxy fatty acids, are the hallmark members of the Corynebacterineae, a suprageneric group of actinomycetes which includes the corynebacteria, mycobacteria, nocardia, rhodococci and other related micro-organisms. These fatty acids in mycobacteria, called eumycolic acids, possess a very long

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Abbreviations: Cm, chloramphenicol; Fbp, fibronectin-binding protein; Km, kanamycin; Myt, mycoloyltransferase; Tet, tetracycline; TDCM, trehalose dicorynomycolate; TMCM, trehalose monocorynomycolate.
chain (C\textsubscript{60}–C\textsubscript{90}) and may contain various oxygen functions in addition to the β-hydroxy group, whereas mycolic acids found in other actinomycetes consist of homologous mixtures of saturated and unsaturated acids and contain shorter chains, for example, C\textsubscript{40}–C\textsubscript{50} in nocardomycolic acids and C\textsubscript{25}–C\textsubscript{36} in corynomycolic acids (Collins et al., 1982; Daffe & Draper, 1998; Minnikin, 1982). Mycolic acids, the major lipid constituents of the singular cell envelope of the \textit{Corynebacterineae}, are found covalently linked to the cell-wall arabinogalactan or esterifying trehalose and glycerol. Both types of mycolic-acid-containing components are believed to play a crucial role in the structure and function of this cell envelope, primarily because they are organized with other lipids to form an outer permeability barrier with an extremely low fluidity that confers an exceptionally low permeability upon these bacteria; this may explain the intrinsic resistance of mycobacteria to many antibiotics (Brennan & Nikaido, 1995; Daffe & Draper, 1998, Draper, 1998; Draper, 1998; Minnikin, 1982). From the numerous structural and biochemical analyses performed on both mycobacteria and corynebacteria, it is now considered that this outer permeability barrier consists of an inner monolayer of mycoloyl residues covalently linked to the cell-wall arabinogalactan and includes other lipids which are probably arranged to form a bilayer with the mycoloyl residues (Brennan & Nikaido, 1995; Daffe & Draper, 1998; Draper, 1998; Dubnau et al., 2000; Liu et al., 1995; Minnikin, 1982, Puech et al., 2001; Rastogi, 1991). Although the composition of this additional bilayer is different from that of the Gram-negative outer membrane, its function is probably similar, as confirmed by the presence of porin-like proteins (Kartmann et al., 1999; Lichtinger et al., 1998, 1999, 2001; Mukhopadhyay et al., 1997; Niederweis et al., 1999; Puech et al., 2001; Trias et al., 1992; Trias & Benz, 1994; Rieß et al., 1998; Senaratne et al., 1998). In addition, trehalose mycolates have also been implicated in numerous biological functions related to both the physiology and the virulence of \textit{Mycobacterium tuberculosis} (Daffe & Draper, 1998; Glickman et al., 2000). As a consequence, mycoloyltransferases (Myts), the enzymes that catalyse the transfer of a mycoloyl residue onto trehalose, trehalose monomycolate and/or the cell-wall arabinogalactan, are believed to be important for the physiology of members of the \textit{Corynebacterineae}. For instance, a trehalose analogue that inhibits in vitro up to 60% of the Myt activity of the fibronectin-binding protein (Fbp) C and inhibits, to a certain extent, the synthesis of trehalose mycolates and cell-bound mycolates in \textit{M. tuberculosis}, has been shown to have a bacteriostatic action on \textit{Mycobacterium aurum} (Belisle et al., 1997). Thus, in the context of the resurgence of tuberculosis and the emergence of multidrug-resistant mycobacteria, the traffic of mycolic acids remains an attractive field of research. Accordingly, to gain insight into the functions of Fbps in the whole bacterium, three \textit{fbp}-inactivated single mutants of \textit{M. tuberculosis} (Armitage et al., 2000; Jackson et al., 1999) have been characterized and the active Fbp enzymes were found to be partially redundant (Puech et al., 2002), a phenomenon that hampers this approach. Unfortunately, to date, no double or triple \textit{fbp} mutant has been obtained, preventing further comprehensive functional studies of the Fbp proteins and their influence on the envelope architecture and, consequently, on mycobacterial physiology. As an alternative, we have taken advantage of the reported existence of only one copy of the \textit{csp1} (also called \textit{cop1}) gene (Joliff et al., 1992) in the genome of \textit{Corynebacterium glutamicum}, a member of the \textit{Corynebacterineae}. This gene encodes a protein whose deduced N-terminal region is similar to that of Fbps (Joliff et al., 1992). Indeed, analysis of the \textit{csp1}-disrupted mutant of \textit{C. glutamicum} has established the expected function of the gene product PS1 and has facilitated the identification of the \textit{in vivo} acceptors of the Myt activity of the individual Fbps by expressing the \textit{fbp} genes in the \textit{csp1}-disrupted mutant (Puech et al., 2000). Unexpectedly, however, this mutant has been found to still produce trehalose mycolate and mycoloylated cell-wall arabinogalactan, indicating that, as in mycobacteria, additional \textit{myt} genes exist in \textit{C. glutamicum}. Recently, we and others have identified five new \textit{myt} genes in \textit{C. glutamicum} (Brand et al., 2003; De Sousa-D’Auria et al., 2003) which we renamed \textit{cmytB--F}, for \textit{corynebacterial mycoloyltransferases} (De Sousa-D’Auria et al., 2003). Inactivation of the new-found \textit{cmyt} genes and analyses of the various \textit{cmyt}-disrupted mutants have shown that all strains were viable despite the effects observed on both the trehalose mycolate and cell-wall-bound mycolate contents of most mutant strains, data that established the expected Myt functions of the encoded proteins (De Sousa-D’Auria et al., 2003). Based on the close proximity of \textit{csp1} (renamed \textit{cmytA}) with \textit{cmytB} in \textit{C. glutamicum}, we decided to construct and functionally analyse the \textit{ΔcmytA \DeltacmytB} double mutant in order to address the question of the importance of Myts in the physiology of members of the \textit{Corynebacterineae}.

**METHODS**

**Plasmids, strains and media.** The various plasmids used in the present study are listed in Table 1. Strains were grown in Brain–Heart Infusion (BHI; 3.7% Difco Brain–Heart Infusion) with shaking (250 r.p.m.). The parent strain of \textit{C. glutamicum} (CGL2005) and the \textit{cmytA} (\textit{csp1})-inactivated (CGL2022) and \textit{cmytB}-disrupted (CGL2026) strains were cultivated at 34°C, whereas the \textit{ΔcmytA \ΔcmytB} double mutant strain (CGL2029) and its complemented derivative strains were cultivated at 30°C. Transformation of \textit{C. glutamicum} by electroporation was performed as described by Bonamy et al. (1990). Antibiotics were added to a final concentration of 25 μg ml\textsuperscript{-1} for kanamycin (Km); 15 μg ml\textsuperscript{-1} (\textit{C. glutamicum}) or 30 μg ml\textsuperscript{-1} (\textit{Escherichia coli}) for chloramphenicol (Cm); and 1 μg ml\textsuperscript{-1} (\textit{C. glutamicum}) or 10 μg ml\textsuperscript{-1} (\textit{E. coli}) for tetracycline (Tet). \textit{E. coli} strains DH5α and TOP10 (Invitrogen) were grown on Luria–Bertani medium.

**DNA manipulations.** Recombinant DNA techniques were performed as described by Sambrook et al. (1989). Plasmid DNA was isolated from \textit{C. glutamicum} or \textit{E. coli} using Promega or Qiagen kits for DNA purification. \textit{C. glutamicum} chromosomal DNA was extracted as described by Ausubel et al. (1987). Restriction endonucleases and DNA-modifying enzymes were obtained from Promega and used according to the manufacturer’s instructions.
Table 1. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Characteristics</th>
<th>Source or reference</th>
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<tr>
<td>pCR2.1-TOPO</td>
<td>E. coli cloning vector fl and pUC origins; lacZa; KmR, AmpR</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pUN121</td>
<td>E. coli cloning vector in which the tet gene is under the control of the repressor protein encoded by cl of the λ phage; AmpR</td>
<td>Nilsson et al. (1983)</td>
</tr>
<tr>
<td>pTomyB</td>
<td>pCR2.1-TOPO carrying the cmytB gene; KmR, AmpR</td>
<td>This work</td>
</tr>
<tr>
<td>pTAcmytA-cmytB</td>
<td>pCR2.1-TOPO carrying 424 bp of cmytA and 827 bp of cmytB; KmR, AmpR</td>
<td>This work</td>
</tr>
<tr>
<td>pUNΔcmytA-myB</td>
<td>pUN121 containing the 1-25 kb BamHI–NsiI fragment of pTAcmytA-cmytB; AmpR, TetR</td>
<td>This work</td>
</tr>
<tr>
<td>p::pcons</td>
<td>pCR2.1-TOPO carrying 753 bp of the pcons gene; KmR, AmpR</td>
<td>This work</td>
</tr>
<tr>
<td>pCGL243</td>
<td>Shuttle vector E. coli–C. glutamicum, derivative of pBL1; KmR</td>
<td>Reyes et al. (1991)</td>
</tr>
<tr>
<td>pCGL482</td>
<td>Shuttle vector E. coli–C. glutamicum, derivative of pCGL243; CmR</td>
<td>Peyret et al. (1993)</td>
</tr>
<tr>
<td>pCGL284</td>
<td>Derivative of pCGL482 containing the cmytA gene of C. glutamicum contained in 753 bp of pUN121.</td>
<td>Peyret et al. (1993)</td>
</tr>
<tr>
<td>pCGL2221</td>
<td>pUNΔcmytA-cmytB containing KmR from pCGL243 as a SacI fragment; AmpR, TetR, KmR</td>
<td>This work</td>
</tr>
<tr>
<td>pCGL2302</td>
<td>Derivative of pCGL482 containing the cmytB gene of C. glutamicum (1964 bp); CmR</td>
<td>De Sousa-D’Auria et al. (2003)</td>
</tr>
<tr>
<td>pCGL2303</td>
<td>Derivative of pCGL482 containing the cmytA gene of C. glutamicum (2977 bp); CmR</td>
<td>De Sousa-D’Auria et al. (2003)</td>
</tr>
<tr>
<td>pCGL2317</td>
<td>Derivative of pCGL482 containing the pcons gene of C. glutamicum (1870 bp); CmR</td>
<td>This work</td>
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Oligonucleotide primers were synthesized by Genosys. PCR amplifications were performed in a GenAmp PCR 2400 thermocycler (Perkin Elmer) using Taq (Promega) or Pfu (Promega) DNA polymerases and, for fragments of more than 3 kb long, JumpStart REDAccuTaq DNA polymerase (Sigma). PCR fragments were systematically purified (Qiaquick PCR purification kit; Qiagen). All DNA sequencing was carried out by Q-biogene and Genome Express.

Construction of a ΔcmytA ΔcmytB double mutant of C. glutamicum. To generate a deletion in the cmytA–cmytB DNA region, the suicide vector pCGL2221 was constructed. Plasmid pTomyB, corresponding to pCR2.1-TOPO in which a PCR product of the cmytB gene had been cloned, was first digested with SacI and XhoI to give a linearized vector which contains only the 5’ end of the cmytB gene [827 bp containing the first 68 bp of the open reading frame (ORF)]. A 3’-end fragment of the cmytA gene (424 bp containing the last 65 bp of the ORF) was amplified by PCR. As an artificial XhoI site was added to the downstream primer and a natural SacI site localized at the beginning of this fragment, the PCR product was digested with both enzymes and cloned in between the sites of the digested pTomyB to give pTAcmytA-cmytB. Therefore, the 1-25 kb fragment containing the downstream region of cmytA and the upstream region of cmytB was excised from pTAcmytA-cmytB with BamHI/NsiI and cloned into the repressor gene of the λ phage (cl) of vector pUN121 (Nilsson et al., 1983) digested with NsiI/BclI to give plasmid pUNAcmytA-cmytB, which contains a unique SacI site between the cmytA and cmytB fragments. Disruption of cl allowed the constitutive expression of the tet gene. Finally, pCGL2221 was obtained by the insertion of a Km-resistance cassette, excised from pCGL243 (Reyes et al., 1991) by SacI, into plasmid pUNAcmytA-cmytB digested with SacI (Fig. 1). C. glutamicum CGL2005 was transformed with pCGL2221 and recombinants were selected on BHI plates containing 25 μg Km ml⁻¹. Presence of a double crossing-over was tested by the sensitivity of the Km⁺ transformants to Tet (1 μg ml⁻¹).

Disruption of the pcons gene. To disrupt the pcons gene, an internal fragment (753 bp) was amplified from C. glutamicum CGL2005 chromosomal DNA and cloned into pCR2.1-TOPO. The resulting plasmid (p::pcons) was used to electrotransform C. glutamicum CGL2005. Transformants generated by integration of the

Fig. 1. Disruption of the cmytA–cmytB region of C. glutamicum. (a) A schematic representation of the cmytA- and cmytB-containing region of the chromosome of C. glutamicum CGL2005. The bars under the chromosome represent the two DNA fragments which were cloned into the pUN121 vector and used as sites for the homologous recombination events. (b) Linearized pCGL2221 used for transformation of C. glutamicum CGL2005. (c) Genomic organization of the ΔcmytA ΔcmytB double mutant after the allelic exchange. The four primers used for the verification of the correct deletion are shown.
The plasmid was selected on BHI plates containing 25 μg Km ml⁻¹ and called CGL2031. The integration of the plasmid in the expected site was evaluated by three PCR amplifications using two primers localized on the p:cons gene and flanking the insertion sites and the M13 forward and reverse primers localized in the pCR2.1-TOPO vector.

**Construction of a plasmid carrying the pcons gene.** A 2.5 kb DNA fragment containing the pcons gene was amplified from C. glutamicum CGL2005 genomic DNA and subcloned into pCR2.1-TOPO. A 1.87 kb fragment (421 bp upstream of the start codon of the pcons gene and 85 bp downstream of the stop codon) was re-isolated using Acc65I and XhoI. The resulting insert was purified and ligated to pCGL482 digested with XhoI and KpnI. The plasmid was introduced into the C. glutamicum ΔacytA ΔacytB double mutant strain CGL2029 by electroproporation and the transformants were selected on BHI plates containing Cm (15 μg ml⁻¹).

**Preparation of proteins for SDS-PAGE and Western blot analysis.** Corynebacterium cells (equivalent of 2 ml bacterial suspension at OD₅₅₀ 1) were centrifuged at 16,000 g for 10 min. The supernatant, which contained only cell-wall-associated and not cytoplasmic proteins (Peyret et al., 1993), was recovered. Proteins were separated by SDS-PAGE (Laemmli, 1970). After electrophoresis, gels were either stained with Coomassie brilliant blue R-250 or blotted onto nitrocellulose membranes (Towbin et al., 1979). In the latter case, proteins were probed with rabbit polyclonal antibodies, anti-P2 (Peyret et al., 1993). Bands were detected using alkaline-phosphatase-conjugated antibodies and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate p-toluidine (Promega) as substrates.

**Isolation and analysis of extracellular materials.** Bacteria were grown for 24 h in BHI and the culture filtrates were recovered by centrifugation. Cells were dried and weighed, and CHCl₃ and CH₃OH were added to the filtrates, as well as a negative control consisting of BHI, to give 2-8 ml final one-phase mixtures of H₂O/CHCl₃/CH₃OH (0:8:1:2, by vol.) (Bligh & Dyer, 1959), and lipids were extracted for 1 h at room temperature; two-phase partition mixtures were obtained by adding 1 ml CHCl₃/H₂O (1:1, v/v) to the one-phase solutions and lipids were recovered in the lower organic phases, dried and analysed by TLC as described below. The aqueous phases were concentrated under vacuum to 1/10 of the original volumes and cold ethanol (6 vol.) was added; after centrifugation of the ethanol precipitates, the pellets were dialysed extensively against distilled H₂O and extracellular materials were obtained and analysed for carbohydrate and protein content as described previously (Lemassu & Daffe, 1994).

**Isolation, fractionation and analysis of whole-cell lipids.** Lipids were obtained and analysed as described previously (Puech et al., 2000). Briefly, lipids were extracted from 5 to 10 g of wet cells for 16 h with CHCl₃/CH₃OH (1:1, v/v) at room temperature with continuous stirring; the bacterial residues were re-extracted three times with CHCl₃/CH₃OH (2:1, v/v) and the organic phases were pooled and concentrated. The crude lipid extracts were partitioned between the aqueous and organic phases arising from a mixture of CHCl₃/CH₃OH/H₂O (8:4:2, by vol.); the lower organic phases were collected, evaporated to dryness to yield the crude lipid extracts from each strain and comparatively examined by TLC on silica-gel-coated plates (60, 0.25 mm thickness; Merck) developed with CHCl₃/CH₃OH (9:1, v/v) or CHCl₃/CH₃OH/H₂O (30:8:1 or 65:25:4, by vol.). Detection of all classes of lipids was achieved by spraying the TLC plates with either Rhodamine B (0.01% w/v) or 20% HSO₄ in H₂O, the latter followed by heating at 110 °C; glycolipids were revealed by spraying plates with 0.2% (w/v) anthrone in concentrated H₂SO₄, followed by heating at 110 °C. The Ditter–Lester reagent (Ditter & Lester, 1964) was used for visualizing phosphorus-containing lipids.

The various classes of extractable lipids were also analysed by labelling. Briefly, 5 μCi (185 kBq) of [1-14C]acetate sodium salt (2-11 μCi mmol⁻¹; Amersham) were added to 100 ml exponential-phase-grown bacteria for 1, 2 and 5 h. The reaction was stopped by centrifugation (8000 g for 10 min) and the cell pellets were extracted with CHCl₃/CH₃OH (9:1, v/v) for 16 h. The organic solutions were separated from the delipidated cells by filtration and dried; the crude lipid extracts were resuspended in CHCl₃ and analysed by TLC using CHCl₃/CH₃OH/H₂O (30:8:1, by vol.) as the developing solvent. Radioactivity was located and counted on plates using an automatic TLC linear analyser (Berthold LB 2832). Then, the lipids were visualized by spraying with anthrone followed by charring.

Quantification of corynomycolic acids was performed as follows. Delipidated cells (1-5 g dry weight) and lipids extracts (100 mg) of the various strains were dried under vacuum prior to weighing and saponified (Daffe et al., 1983). The saponified products were acidified with 20% H₂SO₄ and the resulting fatty acids were extracted with diethyl ether, converted to methyl esters with diazomethane and dried under vacuum, dissolved in petroleum ether and applied to a Florisil (60–100 mesh; Merck) column equilibrated in petroleum ether. The column was irrigated step-wise with increasing concentrations of diethyl ether in petroleum ether. Fractions were monitored by TLC on silica-gel-coated plates using dichloromethane, and fractions containing the same lipid compounds [non-hydroxylated fatty acid methyl esters (FAMEs) or corynomycolates] were pooled and weighed. Three sequential determinations from separate preparations of delipidated cells were performed (Puech et al., 2000). Mycolates were also isolated from both acetate-labelled extractable lipids (50–100 mg) and delipidated cells (300–500 mg), and purified as described above; the specific radioactivity of the mycolates purified from extractable lipids and delipidated cells was determined by counting (in d.p.m.) using a Packard Tricarb 1900 TR equipped with a 3H/Ca programme.

FAMEs from corynebacteria, delipidated cells and extractable lipids (1–2 mg) were treated with trimethylsilyl reagents (Sweeney et al., 1963) to derivatize hydroxylated components of the mixtures, i.e. corynomycolates, and analysed by GC. The detector response for the various classes of FAMEs was determined using authentic samples of C₁₆:₀ and C₁₂:₀ corynomycolate methyl esters. Identification of non-hydroxylated FAMEs and corynomycolate derivatives was achieved by GC-MS.

**MS and GC.** GC was performed using a Hewlett Packard HP4890A apparatus equipped with a fused silica capillary column (25 m length × 0.22 mm i.d.) containing WCOT OV-1 (0.3 mm film thickness; Spiral). A temperature gradient of 100–290 °C (5 °C min⁻¹), followed by a 10 min isothermal plateau at 290 °C, was used. GC-MS analysis was conducted on a Hewlett Packard 5890 Gas Chromatograph connected to a Hewlett Packard 5998A Mass Spectrometer. Samples were injected in the splitless mode. The column was a 12 m HP-1. A temperature gradient of 100–290 °C (8 °C min⁻¹) was used.

**Transmission electron microscopy.** For ultra-thin sectioning of cells, bacterial pellets were fixed for 1 h at 4 °C with 2.5% (w/v) glutaraldehyde and 0.05 M lysine in a 0-1 M cacodylate buffer.
(pH 7–4) with or without 0.075% (w/v) ruthenium red as appropriate. Cells were washed five times in the same buffer, i.e. with or without 0.075% (w/v) ruthenium red, and post-fixed for 1 h at room temperature in 1% (w/v) osmium tetroxide with or without 0.075% (w/v) aqueous uranyl acetate for 1 h at room temperature and then washed five times in distilled H₂O. Bacteria were suspended in 1% (w/v) aqueous uranyl acetate for 1 h at room temperature and then washed five times in distilled H₂O. Suspended cells were embedded in 2% molten agar type IX (Sigma) before dehydration through a graded ethanol series. Dehydrated cells were embedded in Spurr medium with intermediate 1,2-epoxypropane infiltration. Blocks were cut conventionally, stained and examined using a Philips CM12 microscope operating under standard conditions.

For freeze-fracture experiments, bacterial suspensions were centrifuged at 13000 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 & Gulik-Krzywicket (1986). The frozen samples were fractured in a Balzers 301 freeze-fracture unit at −125°C in vacuo (about 1 × 10⁻⁸ Pa) and then replicated with 1–1.5 nm of deposits of platinum–carbon, and backed with about 20 nm of carbon. The samples were cleaned overnight with chromic acid, washed with distilled H₂O and then observed using a Philips CM12 electron microscope.

**RESULTS**

We have previously reported the consequences of the disruption of the cmytA (formerly csp1) gene on the cell envelope of *C. glutamicum* (Puech et al., 2000). Despite the 50% decrease of the cell-wall-linked mycolate content and the accumulation of trehalose monomycolate (TDCM) with a concomitant decrease of trehalose dicorynomycolate (TDCM), the csp1-inactivated mutant exhibited a growth rate similar to that of its parent strain (Puech et al., 2000). More recently, we and others have demonstrated the existence of additional cmyt genes in *C. glutamicum* (Brand et al., 2003; De Sousa-D’Auria et al., 2003). Inactivation of cmytB in *C. glutamicum* (De Sousa-D’Auria et al., 2003) has also resulted in a phenotype very similar to that observed with the cmytA-disrupted mutant strain (Puech et al., 2000). These data prompted us to construct a ΔcmytA ΔcmytB double mutant in order to evaluate the importance of these two cmyt genes on the physiology of *C. glutamicum*.

**Construction of a ΔcmytA ΔcmytB double mutant strain of *C. glutamicum***

Analysis of gene locations on the *Corynebacterium diphtheriae* chromosome showed that cmytA is in close proximity to cmytB, this gene organization is similar to that of the fbpA and fbpC1 (also called fbpD) genes in *M. tuberculosis*. Sequencing of the region containing cmytA and cmytB in *C. glutamicum* CGL2005 has shown that cmytB is separated from cmytA by one ORF transcribed in a direction opposite to that of the two cmyt genes (Fig. 1). This unknown ORF encodes a hypothetical protein, Pcons, which has no homology with the six cMyts identified so far (Brand et al., 2003; De Sousa-D’Auria et al., 2003). A similar gene organization was also found in *C. glutamicum* ATCC 13032T, whose genome sequence has been published during the course of this study. We took advantage of the fact that cmytA and cmytB are in close proximity on the chromosome to delete the corresponding DNA region (Fig. 1a). As pcons was only present in *C. glutamicum* but absent from both *C. diphtheriae* and *M. tuberculosis* while the organization of the surrounding genes is highly conserved in all these bacteria, it seems unlikely that Pcons has a function related to the Myt activity of CMytA and CMytB. Accordingly, the region including cmytA, the unknown ORF and cmytB was deleted by allelic exchange and replaced by a Km cassette. The integrative vector pCGL2221 (Table 1, Fig. 1b) was introduced into strain CGL2005 and KmR transformants were selected. An allelic exchange resulting from a homologous recombination with double crossing-over would insert the aphIII gene but not the tet gene, leading to a KmR TetS phenotype for the deletion mutants. One TetS clone (CGL2029) was obtained from seven KmR transformants. Its genomic DNA was analysed by PCR using two sets of primers (km1/MA and km2/MB, respectively; Fig. 1c), each constituting of an aphIII primer and a primer in the genome outside the region used for making the disruption vector pCGL2221. Two PCR products, which were both of the expected sizes, were sequenced and the results showed that the cmytA–cmytB region was deleted, as expected, and replaced by the aphIII cassette (data not shown). When PCR amplifications were done on genomic DNA from CGL2029, using primer pairs in the cmytA or cmytB deleted part of each gene, no PCR product was detected, confirming the absence of any duplication event.

**Phenotypic analysis of the ΔcmytA ΔcmytB double mutant strain of *C. glutamicum***

The ΔcmytA ΔcmytB mutant showed virtually no growth at 34°C on solid and in liquid media and exhibited a growth defect at 30°C (Fig. 2). Complementation of the double mutant cells with either the wild-type cmytA or the wild-type cmytB gene alleviated the growth defect of the double mutant strain; the cmytA-complemented double mutant strain CGL2029(pCGL2303) exhibited a growth rate slightly slower than that of the wild-type strain, but the two strains produced similar amounts of bacterial mass at the stationary growth phase at 30 and 34°C. In contrast, the expression of cmytB in the double mutant strain CGL2029(pCGL2302) led to a growth rate that was invariably slower than those of the wild-type strain and the double mutant complemented with cmytA at both 30 and 34°C; consistent with this observation, the biomass produced by CGL2029(pCGL2302) at the stationary growth phase was lower than those of the former strains, though always higher than that of the ΔcmytA ΔcmytB mutant CGL2029. Complementation of the double mutant with pcons had no effect on the phenotype of the CGL2029(pCGL2317) transformant, as expected from its absence from both *C. diphtheriae* and *M. tuberculosis*. Furthermore, inactivation of the pcons gene (strain CGL2301) did not lead to any reduction of the growth rate of the mutant (data not shown). These data clearly indicated that the phenotype observed for the double
mutant was really due to the deletion of the cmytA and cmytB genes only.

Light microscopy revealed that cells from the double mutant strain (Fig. 3b) were longer and wider than those of the wild-type cells (Fig. 3a). Since the sizes of the cmytA-inactivated or cmytB-disrupted single mutant cells were similar to those of the wild-type strain (data not shown), the simultaneous mutations of the two genes, as opposed to the single mutations, have an effect on cell division. The abnormal morphological aspect of the double mutant cells was fully reversed by complementation with the wild-type cmytA gene (Fig. 3d). Expression of cmytB in the double mutant also restored the segmentation defect phenotype but segmentation-defective cells may be observed in the culture of the transformants (Fig. 3c). Again, complementation of the double mutant with pcons did not reverse the segmentation defect of the mutant and pcons disruption did not affect cell morphology (data not shown).

Extracellular and cell-wall-protein profiles of the ΔcmytA ΔcmytB double mutant strain of C. glutamicum

Comparative analyses of the extracellular and cell-wall proteins from the wild-type strain and the single and double mutants by SDS-PAGE demonstrated the presence of numerous bands in the materials from the ΔcmytA ΔcmytB mutant strain CGL2029 (Fig. 4a). The protein profile of the ΔcmytA ΔcmytB mutant also revealed that the strain elaborated less PS2 in the cell wall than did the parent strain CGL2005 (Fig. 4a). PS2 is the major corynebacterial secreted protein that forms the crystalline surface layer in several corynebacterial species and is anchored in the cell wall, presumably in the external mycolic acid bilayer, via a C-terminal hydrophobic sequence of 21 residues (Chami et al., 1993). This phenomenon was clearly visible when cspB, the gene that encodes PS2, was overexpressed in the parent strain, the cmytA-inactivated mutant CGL2022 or the ΔcmytA ΔcmytB mutants. While the overexpressed PS2 was found to be almost exclusively associated with the cell-wall materials of the parent strain and the cmytA-inactivated mutant, more than half of the protein was found in the culture filtrate of the ΔcmytA ΔcmytB double mutant strain CGL2029 (Fig. 4b). Western blot analysis of the cell walls and extracellular materials of the double mutant strain, probed with anti-PS2 antibodies, showed specific bands of molecular mass lower than PS2 (Fig. 4c), indicating a partial degradation of the protein. Besides this finding, this analysis also confirmed the absence of cMytA in the double mutant (Fig. 4c). These results suggested a profound alteration of the cell-wall architecture of the double mutant. Complementation of the double mutant strain with the wild-type cmytA or cmytB gene restored the protein profiles, except for the S-layer PS2 protein; while PS2 is present almost exclusively in the cell walls of the parent strain, this protein was found in the culture filtrates and cell-wall fractions from the double mutant and its two complemented strains in roughly equal amounts (Fig. 4a, c). In addition, the extracellular material of the double mutant complemented with cmytA contained large amounts of the overexpressed cMytA protein (Fig. 4a).

Lipid analysis of the ΔcmytA ΔcmytB double mutant strain of C. glutamicum

Corynomycolates are found both covalently linked to the cell-wall arabinogalactan and esterifying trehalose as TMCM and TDCM (Puech et al., 2001); the latter glycoconjugates are non-covalently associated to the cell wall and can be extracted from bacterial cells with organic solvents. Quantitative determination of the lipid contents of the various corynebacterial cells indicated that the double mutant produced slightly less lipid than the parent strain and the cmytA-inactivated and cmytB-disrupted single mutant strains (Table 2). TLC analysis and labelling of the extractable lipids of the double mutant showed that this strain accumulated more TMCM and elaborated less TDCM than the wild-type strain CGL2005 and the cmytA-inactivated and cmytB-disrupted single mutant strains (Fig. 5, Table 2). When the amounts of covalently linked corynomycolates were measured, the double mutant strain was found to contain less corynomycolates bound to the cell-wall arabinogalactan than the parental strain CGL2005 and the cmytA-inactivated and cmytB-disrupted single mutant strains, CGL2022 and CGL2026, respectively.

Fig. 2. Growth curves for the wild-type strain of C. glutamicum, its derivative ΔcmytA ΔcmytB double mutant and complemented strains. Cultures were grown in BHI medium as described in Methods at (a) 30 °C or (b) 34 °C. ●, Strain CGL2005; ▲, strain CGL2029; □, strain CGL2029(pCGL2302); ■, strain CGL2029(pCGL2303).

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The residual production of both trehalose mycolates and mycoloylated cell-wall arabinogalactan by the double mutant is consistent with the demonstrated occurrence of additional and functionally active cMyt(s) protein(s) in *C. glutamicum* CGL2005 (De Sousa-D’Auria et al., 2003; Brand et al., 2003). Complementation of the double mutant strain by either the wild-type *cmytA* or *cmytB* gene, but not with *pcons*, resulted in the

![Fig. 3](https://www.microbiologyresearch.org/article/Fig3.jpg)

**Fig. 3.** Light microscopy of cells from *C. glutamicum* CGL2005 and ΔcmytA ΔcmytB mutant strains. Light microscopy analysis was done using an Axioplan (Zeiss) microscope with phase-contrast on exponentially growing cells. (a) Wild-type strain CGL2005; (b) ΔcmytA ΔcmytB mutant strain CGL2029; (c) ΔcmytA ΔcmytB mutant complemented with the *cmytB* gene, strain CGL2029(pCGL2302); (d) ΔcmytA ΔcmytB mutant complemented with the *cmytA* gene, strain CGL2029(pCGL2303).

![Fig. 4](https://www.microbiologyresearch.org/article/Fig4.jpg)

**Fig. 4.** SDS-PAGE and Western blot analysis of cell-wall and extracellular proteins from the ΔcmytA ΔcmytB double mutant and from strains overexpressing *cspB*. Procedures were performed as described in Methods. S, supernatant containing the secreted proteins; CW, cell-wall fraction. (a) SDS-PAGE of proteins from the wild-type strain (2005), the ΔcmytA ΔcmytB mutant (2029) and the corresponding *cmytA*- and *cmytB*-complemented strains [2029(2303) and 2029(2302), respectively]. (b) SDS-PAGE of proteins from the wild-type [2005(824)], the *cmytA*-inactivated [2022(824)] and the ΔcmytA ΔcmytB mutant strain [2029(824)] containing overexpressed *cspB* gene. (a, b) M, molecular mass markers (in kDa). (c) Western blotting of all the samples analysed in (a) and the ΔcmytA ΔcmytB double mutant with *cspB* [CGL2029(pCGL24)] analysed in (b), using as a probe anti-PS2 antibodies, which also recognize the cMytA protein.
production of more extractable lipids and fully restored the balance of trehalose mycolates, but it only partially restored the amounts of wall-linked mycolates (Table 2).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant phenotype</th>
<th>Lipid extract*</th>
<th>TDCM/TMCM†</th>
<th>Wall-linked mycolates‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGL2005</td>
<td>Wild-type</td>
<td>7.0±0.1</td>
<td>0.71</td>
<td>100</td>
</tr>
<tr>
<td>CGL2022</td>
<td>cmytA inactivated</td>
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<td>0.15</td>
<td>50±1</td>
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<tr>
<td>CGL2026</td>
<td>cmytB disrupted</td>
<td>6.9±0.3</td>
<td>0.30</td>
<td>53±4</td>
</tr>
<tr>
<td>CGL2029</td>
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<td>0.05</td>
<td>39±2</td>
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<tr>
<td>CGL2031</td>
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<td>7.1±0.5</td>
<td>0.66</td>
<td>90±6</td>
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<tr>
<td>CGL2029(pCGL2317)</td>
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<td>0.05</td>
<td>41±2</td>
</tr>
<tr>
<td>CGL2029(pCGL2303)</td>
<td>ΔcmytA ΔcmytB, complemented with cmytA</td>
<td>7.8±0.2</td>
<td>0.73</td>
<td>57±4</td>
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<tr>
<td>CGL2029(pCGL2302)</td>
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<td>7.9±0.2</td>
<td>0.74</td>
<td>59±6</td>
</tr>
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</table>

*Percentages of the dry mass of extractable lipids relative to that of cells. The values represent the means of at least three independent determinations.
†Cells were labelled with [14C]acetate for 5 h and then extracted with organic solvents; the extractable lipids were analysed by radio-TLC and the radioactivity incorporated into TDCM and TMCM was determined separately. The molar ratio of TDCM was obtained by dividing the radioactivity in TDCM by a factor of 2, based on the occurrence of two molecules of mycolates in these molecules while only one mycoloyl residue is found in TMCM.
‡Cells were extracted with organic solvents; delipidated cells were saponified and their mycolate content was determined both by weighing and by GC (see Methods). The values correspond to the percentage of mycolates relative to that of the parental strain CGL2005 (100%).

Ultrastructural appearance of cmyt-deleted mutants of C. glutamicum

While both the cmytA-disrupted and the cmytB-inactivated single mutant of C. glutamicum, as well as the wild-type strain CGL2005 (data not shown), exhibited a typical fracture plane within their cell walls (Fig. 6a, b), freeze-fracturing of the double mutant cells revealed a heterogeneous pattern of fracture, with multilayered structures (Fig. 6c, e). In addition, a significant number of bacteria

![Fig. 5. TLC of trehalose lipids from the ΔcmytA ΔcmytB double mutant and complemented strains of C. glutamicum. Glycolipids from exponential-phase-grown bacteria were prepared as described in Methods. Lanes: 1, CGL2005 (wild-type); 2, CGL2022 (cmytA inactivated); 3, CGL2029 (ΔcmytA ΔcmytB); 4, CGL2029(pCGL2303), cmytA overexpressed; 5, CGL2029(pCGL2302), cmytB overexpressed.](image)

![Fig. 6. Freeze-fracture electron micrographs of corynebacterial strains. (a) C. glutamicum CGL2026 (cmytB disrupted); (b) C. glutamicum CGL2022 (cmytA inactivated); (c–f) C. glutamicum CGL2029 (ΔcmytA ΔcmytB). Black arrows in (a), (b), (d) and (f) indicate ordered arrays, whereas the white arrows in (c) and (e) show the multiple fracture planes observed in the cell wall (CW) of the double mutant which also may present a fracture plane in its cytoplasmic membrane (CM). Bar, 500 nm.](image)
exhibited a fracture plane in the plasma membrane (Fig. 6d, f). Similarly, and in sharp contrast to the single cmyt-inactivated strains and their parent strain, which all exhibited S-layer ordered arrays at their surface (see black arrows, Fig. 6a, b), only few ordered regions were visible on the surface of the double mutant cells (see black arrows, Fig. 6d, f). These data indicated that the mutations had a profound impact on the integrity of the outermost part of cell envelope of the double mutant strain.

Examination by electron microscopy of ultra-thin sections from conventionally fixed corynebacterial cells from the parent strain (Fig. 7a) revealed a cell envelope structure composed of four different layers: a plasma membrane, typified by its two leaflets, tightly associated to a thick electron-dense layer, which in turn is surrounded by a thin electron-transparent layer and an outer layer. The thin section of the double mutant cells revealed that their envelope was virtually devoid of the electron-transparent layer and did not bind ruthenium red, the dye that reveals the outer layer (Fig. 7b). Complementation of the double mutant by either cmytA or cmytB resulted in an ultrastructural appearance of the complemented strains identical to that of the parent strain (data not shown).

To determine whether the absence of the outermost layer around the double mutant cells was due to the lack of synthesis of its constituents or, more likely, to their release into the extracellular environment as a consequence of the disorganization of the cell envelope of the mutant, the culture fluids from the various corynebacterial strains were analysed. As expected from the later hypothesis, the material from the double mutant contained significantly more lipid, protein and carbohydrate than that of its parent strain (Table 3). The extracellular lipids probably originated from the cell envelope since they contained all the different classes of lipids extractable from the corynebacterial cells, i.e. trehalose corynomycolates and phospholipids (Puech et al., 2001). Similarly, the extracellular materials of the double mutant strain contained the various types of polysaccharides found associated to the cell envelope of the parent strain, i.e. glucan, arabinomannan and mannan (Puech et al., 2001).

Accordingly, sugar compositional analysis of the extracellular material of the double mutant strain revealed the presence of glucose, mannose and arabinose. Neither galactose, a component of the cell-wall arabinogalactan, nor free fatty acids were detected in the extracellular material of the double mutant, indicating that this material did not contain significant amounts of bacterial fragments or cell lysis products. Complementation of the double mutant strain with the wild-type cmytA gene fully restored the wild-type phenotype in terms of the amounts of extracellular compounds produced, whereas overexpression of the cmytB gene only partially restored this phenotype (Table 3).

**DISCUSSION**

The present study was undertaken in order to determine the importance of mycolic acids in the physiology of members...
of the Corynebacterineae. This was done by evaluating the contribution of Myt-encoding genes to the physiology of C. glutamicum. Four such genes exist in M. tuberculosis (Cole et al., 1998), namely fbpA, fbpB, fbpC and fbpD, and six cmyt genes have been identified in C. glutamicum (Brand et al., 2003; De Sousa-D’Auria et al., 2003; Joliff et al., 1992). Disruption of each of the fbp genes in mycobacteria and the cmyt genes in corynebacteria has resulted in viable mutant strains which still produce significant amounts of mycoloylated cell walls and trehalose mycolates (Armitage et al., 2000; De Sousa-D’Auria et al., 2003; Jackson et al., 1999; Puech et al., 2002). These results suggest a functional redundancy for Fbp and cMyt proteins and led us to postulate that the multiplicity of the fbp and cmyt genes is a consequence of the importance of the physiological functions these proteins may play in members of the Corynebacterineae. Because of this redundancy, a significant advantage in the comprehension of the importance of Myts in cell-wall structure and bacterial physiology could only be obtained by the simultaneous knockout of several Myt-encoding genes. To date, no fbp double mutant has been described, presumably because of the non-viability of the double mutant.

The chromosomal organization of cmytB and cmytA, in close proximity on the genome of C. glutamicum CGL2005, allowed us to construct a ΔcmytA ΔcmytB double mutant. This mutant exhibited virtually no growth at 34°C and showed a growth defect at 30°C. At the permissive temperature, the double mutant was defective in division and formed bigger and longer cells than the wild-type strain and the cmytA-inactivated and cmytB-disrupted single mutant strains. The double mutant was severely affected in its cell-wall composition, as demonstrated by the decrease of its covalently bound corynomycolate content and the defect in anchoring the cell-wall protein PS2. The fact that the double mutant still produced both mycoloylated cell walls and trehalose was certainly due to the recent demonstration of the existence in C. glutamicum of other functional cmyt genes (Brand et al., 2003; De Sousa-D’Auria et al., 2003). The cell-wall-defective phenotype of the ΔcmytA ΔcmytB double mutant was further supported by the observations from electron microscopy. Freeze-fracturing showed that, in contrast to the parental and the cmytA mutant strains that all exhibited almost exclusively a fracture plane within their cell walls, the ΔcmytA ΔcmytB double mutant cells contained multiple fracture planes in their cell walls. This phenotype may be related to the profound defect in covalently linked mycolates and the ability of trehalose corynomycolates to spontaneously form bilayers (Puech et al., 2001). This may, in turn, explain the absence of the outermost layer surrounding cells in thin sections. The above assumption is supported by the recent demonstration of the importance of trehalose corynomycolates for the physiology of corynebacterial cells (Tzvetkov et al., 2003; Wolf et al., 2003). Inactivation of trehalose production has resulted in both impaired growth (Tzvetkov et al., 2003; Wolf et al., 2003) and altered cell-wall-lipid composition of trehalose-deficient mutant strains (Tzvetkov et al., 2003). In fact, the growth problems of the mutants may be connected to their inability to synthesize TMC and TDCM, two important constituents of the outer cell-wall lipid layer (Puech et al., 2001). In addition, it has been shown that trehalose, through trehalose 6-phosphate, plays a key role in the entire process of corynomycolic acid synthesis (Shimakata & Minatogawa, 2000). Since trehalose 6-phosphate is considered to serve as an acceptor for newly synthesized corynomycolic acid, the trehalose-deficient mutants may be impaired in the production of both trehalose corynomycolates and cell-wall-linked mycolates. This hypothesis is currently under investigation.

Because deletion of cmytA and cmytB resulted in the loss of pcons, a gene of unknown function, it was important to investigate the role of this gene in the phenotype of the mutant. Inactivation of the pcons gene had no effect on the growth rate and cell morphology of the resulting single mutant, compared to the wild-type strain. Likewise, lipid analysis of the mutant strain showed no difference between it and the wild-type strain. Furthermore, complementation of the ΔcmytA ΔcmytB double mutant with pcons had no impact on the phenotype of the mutant. It followed then that Pcons plays no detectable role in the observed phenotype of the double mutant. In contrast, all the phenotypic changes of the double mutant, except the wall-linked mycolate content, were fully reversed by complementation with cmytA, but were often only partially reversed with cmytB. These data demonstrate that cmytA and cmytB are not fully redundant, consistent with the structural differences between the encoded proteins. While the cmytB gene encodes a protein that is highly similar to Fbps, the length of the cMytA protein is twice that of the other Myts and only the N-terminal region of the protein is similar to that of Fbps (Joliff et al., 1992). This observation can not explain, however, the lack of complementation of the cell-wall mycolylation of the ΔcmytA ΔcmytB double mutant since both the wild-type cmytA and the wild-type cmytB gene are able to fully compensate the mycolylation defect of the cmyt-disrupted single mutants (De Sousa-D’Auria et al., 2003). As an alternative, differences in the cell localization and/or specificities of the two proteins may well explain the need for both cMytA and cMytB for the full restoration of the cell-wall mycolylation. Nevertheless, and most importantly, the lack of complementation of the cell-wall mycolylation of the ΔcmytA ΔcmytB double mutant raised the question of the importance of the amount of cell-wall-bound mycolate content for the bacterial physiology. It has to be recalled that Fbp- and cMyt-defective single mutants exhibit a normal growth phenotype while possessing up to 50% less wall-linked mycolates than their parent strain (Jackson et al., 1999; Puech et al., 2000; De Sousa-D’Auria et al., 2003; Table 2). When the covalently bound mycolate content was lowered by 60% (Table 2), the ΔcmytA ΔcmytB double mutant was severely affected in its growth features. Interestingly, a growth inhibition of M. aurum has been reported when the cell-wall-linked mycolates were lowered.
by 60% (Belisle et al., 1997). When the covalently bound mycolate content of the double mutant was raised above 50% by complementation with either cmytA or cmytB the complemented strains recovered their normal growth and morphological features. These data suggest that these bacteria can tolerate up to a 50% defect in cell-wall-linked mycolates but no more.

In this connection an enlarged cell volume phenotype has recently been reported for a single cmytA-deleted mutant derived from C. glutamicum ATCC 13032T (Brand et al., 2003), a phenotype that was not observed for the cmytA-inactivated strain CGL2022 derived from C. glutamicum CGL2005 (Joliff et al., 1992; De Sousa-D’Auria et al., 2003). One major difference between the two studies resides in the fact that C. glutamicum ATCC 13032T was grown on a minimal medium whereas C. glutamicum CGL2005 and derivatives used in our study were unable to grow in a minimal medium. To determine the origin of the phenotypic difference between the two cmytA-inactivated mutants of C. glutamicum, we constructed a cmytA-disrupted mutant of ATCC 13032T and showed that the mutant exhibited the enlarged cell volume phenotype when grown on a minimal medium but not on the rich BHI medium (our unpublished results). Although further studies are warranted to clarify the relationship between the growth conditions and mycolate contents of the strains, the phenotypic difference observed between the two cmytA-inactivated single mutants indicates that variations in the expression of Myts occurs according to the culture media used. We thus speculate, based on our data on the ΔcmytA ΔcmytB double mutant, that the enlarged cell volume phenotype of the mutant cultivated on a minimal medium was caused by a cell-wall-linked mycolate content lower than the 50% found for strain CGL2022 grown on a rich medium (Puech et al., 2000). Besides, it appears unlikely that the C-terminal domain of cMtyA, which distinguishes this protein from all other cMyts and Fbps, plays a role in the cell shape formation as suggested by Brand et al. (2003) since the enlarged cell volume phenotype of the ΔcmytA ΔcmytB mutant was fully reversed to the wild-type phenotype by complementation with the cmytB gene alone.

In conclusion, our data on the ΔcmytA ΔcmytB double mutant of C. glutamicum indicate that, although several partially redundant Myts are present, cmytA and cmytB play an important role in corynebacterial physiology. These data, in the context of the resurgence of mycobacterioses and multidrug-resistant bacilli, reinforce the concept that Fbps represent potential good drug targets. This concept, originally formulated by Belisle et al. (1997) who showed that an in vitro inhibitor of Fbp activity possesses a bacteriostatic action on M. aurum, has been recently validated by using antisense technology (Harth et al., 2002).

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