Identification of a Stress-Induced Factor of *Corynebacterineae* That Is Involved in the Regulation of the Outer Membrane Lipid Composition

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*Corynebacterineae* are gram-positive bacteria that possess a true outer membrane composed of mycolic acids and other lipids. Little is known concerning the modulation of mycolic acid composition and content in response to changes in the bacterial environment, especially temperature variations. To address this question, we investigated the function of the Rv3802c gene, a gene conserved in *Corynebacterineae* and located within a gene cluster involved in mycolic acid biosynthesis. We showed that the Rv3802 ortholog is essential in *Mycobacterium smegmatis*, while its *Corynebacterium glutamicum* ortholog, NCgl2775, is not. We provided evidence that the NCgl2775 gene is transcriptionally induced under heat stress conditions, and while the corresponding protein has no detectable activity under normal growth conditions, the increase in its expression triggers an increase in mycolic acid biosynthesis concomitant with a decrease in phospholipid content. We demonstrated that these lipid modifications are part of a larger outer membrane remodeling that occurs in response to exposure to a moderately elevated temperature (42°C). In addition to showing an increase in the ratio of saturated corynomycolates to unsaturated corynomycolates, our results strongly suggested that the balance between mycolic acids and phospholipids is modified inside the outer membrane following a heat challenge. Furthermore, we showed that these lipid modifications help the bacteria to protect against heat damage. The NCgl2775 protein and its orthologs thus appear to be a protein family that plays a role in the regulation of the outer membrane lipid composition of *Corynebacterineae* under stress conditions. We therefore propose to name this protein family the envelope lipids regulation factor (ElrF) family.

Mycolic acids (MA), high-molecular-weight 2-alkyl and 3-hydroxy fatty acids (FA), are the major lipid constituents of the singular cell envelope of *Corynebacterineae* (9, 29). They occur either as esters of trehalose or as esters of the terminal penta-arabinofuranosyl units of the cell wall arabinogalactan and form with other lipids an additional bilayer functionally similar to the gram-negative outer membrane (29, 37, 50). In addition to their essential structural function, MA have also been implicated in functions related to the virulence of *Mycobacterium tuberculosis* (9, 14, 38). The structure and biosynthesis of MA have been the subject of intense efforts of research for many decades (13, 46), but still little is known regarding the modulation of their composition and content at a molecular level in response to changes in the bacterial environment, especially temperature variations.

In response to temperature shifts, bacteria are able to modify their membrane composition to maintain the appropriate fluidity of membrane lipids (11, 17, 42, 45). The most common mechanism used by microorganisms to modulate their plasmic membrane fluidity is a change in the proportion of long-chain and saturated FA of the glycerophospholipids (GPL) (24–26). Changes in the FA composition of lipid A, the lipid moiety of the lipopolysaccharide that constitutes the outer leaflet of the outer membrane of gram-negative bacteria, in response to suboptimal temperature exposure have also been reported (7, 39). Similar regulations are expected for *Corynebacterineae*, since these gram-positive bacteria possess a true outer membrane that constitutes a barrier between these bacteria and the external medium. Indeed, changing the growth temperature has been shown to affect the MA compositions of different species of mycobacteria (1, 19, 47). In addition, correlations between MA structures and the melting points of the cell wall strongly suggest that alteration of MA composition by temperature regulates the fluidity of the outer membrane (22, 23). However, almost nothing is known about the proteins involved in the thermal regulation of the *Corynebacterineae* cell wall composition. Changes in the expression level of enzymes in-
volved in MA metabolism (KasA and the so-called Ag85 complex) in response to an elevated growth temperature (55°C) in Mycobacterium thermoresistibile, concomitant with significant changes in the MA content and composition, have been described previously (19). At a global level, transcriptional analyses performed with mycobacteria or corynobacteria revealed a complex heat shock response that varies with both temperature and time exposure (2, 30, 33, 44). Recent analysis showed that a large number of genes in Corynobacterium glutamicum are differentially expressed under moderate (40°C) and severe (50°C) heat shock conditions, and this includes genes involved in cell wall biogenesis and lipid metabolism (3).

Among the important genes involved in MA biosynthesis are the three genes required for the FA condensation step (pks13, accD4, and fadD32) (35, 36), and those encoding mycolyltransferases, the enzymes that catalyze the transfer of a mycoloyl residue onto trehalose, trehalose mononucleotide, and/or the cell wall arabinogalactan (5, 12). Among these genes and highly conserved in Coryrnobaetinae is the Rv3802c gene (locus tag in M. tuberculosis; the ortholog in C. glutamicum is the NCgl2775 gene), the protein of which has been recently shown to possess lipase and thioesterase activities in vitro (32, 49).

In this report, we showed that the Rv3802c protein is essential for mycobacterial physiology, in contrast to the case for its cornybacterial ortholog, NCgl2775. Accordingly, the latter protein is dispensable for normal growth at temperature up to 40°C but is involved in the management of thermal stress at higher temperatures. We characterized the effects of the deletion of the NCgl2775 gene on the growth and viability of heat-shocked cells and further investigated the effects of this deletion or overexpression of the protein on the cell wall lipid content and composition. We showed that the NCgl2775 protein is involved in the regulation of the ratio between MA and phospholipids in response to an increase in temperature exposure of the cells.

MATERIALS AND METHODS

Strains and culture conditions. C. glutamicum RES167 (15) was cultured on brain heart infusion (BHI) medium (Difco). M. smegmatis mc155 was grown on Middlebrook 7H9 medium (Difco) supplemented with 0.05% Tween 80 to prevent aggregation. Escherichia coli DH5a was used for the construction of plasmids and grown on Luria Bertani (LB) medium (Difco). Ampicillin, kanamycin (Km), hygromycin (Hyg), chloramphenicol (Cm), and sucrose (Suc) were added when required at final concentrations of 100 µg/ml ampicillin, 40 µg/ml (for M. smegmatis) or 25 µg/ml Km (for C. glutamicum and E. coli), 50 µg/ml Hyg, 15 µg/ml or 30 µg/ml Cm (for C. glutamicum or E. coli, respectively), and 5% Suc (wt/vol).

Construction of plasmids and strains. (i) Construction of an M. smegmatis MSMEG_6394 conditional mutant. The M. smegmatis conditional mutant was constructed using a strategy previously described (35). Briefly, a DNA fragment, overlapping the Rv3802 gene ortholog from M. smegmatis, was amplified by PCR from the M. smegmatis total DNA by using primers 3802A (5'-CACGACGGCGTACCGGGAAG-3') and 3802B (5'-GGACTAGTGTCAGCTCCTCTACGTGCGCG-3'), mapping 1,528 bp upstream of the start codon and 1,534 bp downstream of the stop codon, respectively. PCR was performed using M. smegmatis genomic DNA. The 2-kb fragment was purified and digested with EcoRI and inserted between the SmaI and EcoRI restriction sites of pBluescript to give pBS3802. A Hyg resistance cassette was inserted into the SphI restriction site of pBluescript and inserted between the SmaI and EcoRI restriction sites of pBluescript to give pBS3802. A Hyg resistance cassette was inserted into the SphI restriction site of pBluescript to yield pBS3802hyg. In this construction, the hyg gene is in the same orientation as the MSMEG_6394 gene. A 3.5-kb EcoRf fragment carrying the disrupted MSMEG_6394::hyg allele was then inserted into the SmaI site of pJQ200 to yield pDP386. Plasmid pDP386 was transferred into M. smegmatis by electroporation and transformants were selected on Hyg-containing plates. Transformants in which pDP386 had been integrated by a single crossover between the wild-type and mutated copies of MSMEG_6394 were characterized by PCR, using primers 3802C (5'-TCCGACACCATCAATGAACACA3') and 3802D (5'-GTGGGATGGGTCAGGACAA-3'), H1 (5'-AGACCC AGCGGTTCGCTGG-3'), and H2 (5'-TCAAGCACTTCCGGACGGTTCG-3'). One clone, giving the pattern corresponding to the insertion of the plasmid by single homologous recombination event was retained for further analysis and named PDP386. To produce the complementation plasmid, the MSMEG_6394 gene was amplified by PCR from M. smegmatis total DNA by using primers 3802E (5'-CGGGATCCGGAAGAAGCGTCGAGGTAACG-3') and 3802F (5'-GGACAGGTGTCATCGCTCTCTACGTGCGCG-3'). The 1-kb fragment was purified, digested with BamHI and SpeI, and inserted between the BamHI and SpeI restriction sites of pDP26, a derivative of the thermosensitive mycobacterial plasmid pCG63 (16) containing the mycobacterial expression cassette from pMP12 (21). The resulting plasmid, named pDP87, contained the MSMEG_6394 gene under the control of the pBlaF5 promoter (21). Plasmid pDP87 was transferred by electroporation into PMP87, and transformants were selected on plates containing Km and Hyg. The second crossover event at the chromosomal MSMEG_6394 locus was selected by plating a liquid culture of strain PMP87-pDP87 grown at 32°C on plates containing Km, Hyg, and Suc (5%), which were then incubated at 32°C. Colonies were screened by PCR using primers 3802C, 3802D, H1, and H2. One strain (PMP88::pDP87), in which the wild-type chromosomal copy of the MSMEG_6394 gene was replaced by the mutated MSMEG_6394::hyg allele, was retained for further analysis.

(ii) Construction of NCgl2775 deletion strain. Deletion of the NCgl2775 gene was done using a strategy described previously (35). In brief, two DNA fragments overlapping the NCgl2775 gene ortholog at 5' and 3' extremities were amplified at 5' and 3' extremities, respectively. The MSMEG_6394 ORF was amplified using primers 2775-AM (5'-ACAAGGGCGTTACGTCAAAGG-3') and 2775-AP (5'-TTATCCACGAGTACCTCGGAGAGAG-3') (592 bp) and 2775-AM (5'-TCGATGAGCCGCGAGGATG-3') and 2775-AP (5'-GGACTAGTGTCAGCTCCTCTACGTGCGCG-3') (737 bp), respectively. These fragments were purified and flanked inserting a Km resistance cassette into plasmid pMC95 (MoBiTec, Göttingen, Germany). The resulting plasmid was transferred into E. coli by electroporation (40) and transformants were selected on Km-containing plates. Transformants in which allelic replacement had occurred were selected by PCR analysis using combinations of primers localized upstream and downstream of the NCgl2775 gene and in the aphIII sequences. After sequencing of the PCR products, one strain (2775S) was selected for further studies.

(iii) Construction of expression vectors. Expression vectors encoding NCgl2775 (pCGL492-2775) and MSMEG_6394 (pCGL482-6394) proteins were constructed using pCGL482 (34) as the cloning vector. We chose to clone these open reading frames (ORFs) under the control of the mytA promoter (named PmytA hereafter). The coding sequence of the NCgl2775 gene and a DNA fragment containing the mytA promoter (355 bp upstream of the mytA start codon) were amplified by PCR from C. glutamicum ATCC 13032 chromosomal DNA by using primers 2775-AM (5'-CCAAGGCGATCCGAGGATCAGG-3') and 2775-AP (5'-GACGGTCGTGACGGATCGATAG-3'). The resulting plasmid MSMEG_6394 ORF was amplified using primers 2802-AM (5'-AAACCTACGGAAAGAAAGG-3') and 2802-AP (5'-CGGGATCCGATCCTATTAGCAAGG-3') and cloned into pCGL482 to give an in-frame fusion of the NCgl2775 operon to C. glutamicum mytA promoter and a C. glutamicum mytA locus. We used pCGL482 as cloning vector because this vector allows the expression of a single homologous fragment of a gene, which is flanked by two short ORFs, one upstream and one downstream of the inserted ORF, and can be used for simultaneous ligation of a fragment containing the PmytA promoter and a fragment containing one ORF and the appropriately digested pCGL482. Transformants were selected on Km-containing plates.

(iv) Construction of the PmytA::lacZ reporter strain. To create a transcriptional fusion of the mytA-NCgl2775-NCgl2776 operon to lacZ, we used pCGL529, a derivative plasmid of pMP2 that contains the lacZ operon and can be used for integration at the icel locus for single-copy reporter fusion, as previously described (43). A fragment containing the PmytA promoter (271 bp) was amplified from C. glutamicum chromosomal DNA by using primers MytA-F (5'-TCTTGTGCGATGATCAGCGG-3') and MytA-R (5'-ATGCGGATGCAGTTTACGTTCG-3') and cloned into pCGL529 to give an in-frame fusion of the PmytA and lacZ. The resulting plasmid (pCGL529pop) was transferred into C. glutamicum ATCC 13032 by electroporation, leading to its integration into the chromosome at either the icel locus or the mytA-NCgl2775-NCgl2776 locus. Two independent insertions were analyzed by PCR to determine the exact localization of the inserted plasmid. One recombinant strain that has pCGL529pop inserted by homologous recombination at the icel locus was used for further study.

RNA isolation, RT-PCR, and transcriptional activity measurement. Total RNA was extracted from an exponentially growing C. glutamicum culture at 30°C
as described previously (27). Reverse transcription (RT) was performed using Transcriptor reverse transcriptase (Roche) according to the manufacturer's recommendations and DNase- and RNase-free (Roche). Total RNA was transcribed into cDNA by using primer 2775CLrev (5'-GGTGGCCCAATCCACA ACCC-3'). The RT step was carried out at 50°C for 1 h 30 min, followed by a period of 10 min at 85°C to inactivate the reverse transcriptase. The cDNA was used for PCR amplification with primers 2775CLrev and 2775CL (5'-TACGCA ACTCTTGGCTTTGGCG-3') or 277CLrev (5'-CCCGGATCTGTTGCAACG G-3') and PS1Δ (5'-GCTGATGCACGACCCTT-3'). As a control reaction, the same experiment was performed without reverse transcriptase.

β-Galactosidase assay. β-Galactosidase activity was measured in cytosolic extracts as described previously (43). The protein concentration of the extracts was determined by the Bradford method (Bio-Rad protein assay). Specific activity was expressed as A$_{420}$ (420 nm) $\times$ min $^{-1}$ (µg protein)$^{-1}$.

Stress conditions. Stress experiments were performed with mid-exponential-phase cultures (optical density at 600 nm [OD$_{600}$] = 5). Cells were collected by centrifugation (4,000 rpm for 10 min at 4°C), and aliquots were resuspended under different conditions. For heat shock, the cultures were resuspended in the growth media set at 42°C. For acid and alkaline stresses, the cultures were resuspended in the growth media at pH 5 and pH 9. For oxidative stress, hydrogen peroxide (10 mM) was added to the media. For hyperosmotic stress, cells were added to media containing 1 M NaCl. Finally, for nutritional stress, cells were resuspended in a 50 mM phosphate buffer, pH 8.

In all experiments, transcriptional activity (i.e., β-galactosidase activity) was measured after 2.5 h of incubation under the stress condition at 30°C. Heat shock (42°C or 50°C) and cold shock (melting ice, 0°C) experiments were performed as described above, and transcriptional activity (i.e., β-galactosidase activity) was measured as a function of time.

Heat stress resistance assay and survival. For mild-temperature stress, bacteria that had been cultured at mid-exponential phase at 30°C were placed under 42°C or 45°C conditions without any treatment for about 18 h (without shaking) and then replaced in culture conditions at 30°C or 42°C or 45°C conditions without any treatment for about 18 h (without shaking) and then replaced in culture conditions at 30°C or 30, 45, or 60 min. Surviving cells were enumerated on BHI agar plates, and the surviving fraction was expressed as the percentage of the viable cell count before heat challenge.

Extraction and quantification of lipids. Lipids were extracted from wet cells for 16 h with CHCl$_3$-CH$_3$OH (1:2, vol/vol) at room temperature, as described previously (37); the cells were reextracted with CHCl$_3$-CH$_3$OH (1:1, vol/vol) and CHCl$_3$-CH$_3$OH (2:1, vol/vol) 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 the organic phases arising from a mixture of CHCl$_3$-H$_2$O (1:1, vol/vol). The lower organic phases were collected and evaporated to dryness to yield the crude lipid extracts from each strain. Subsequently, the resulting lipids were resolved by thin-layer chromatography (TLC) on silica gel-coated plates of 0.25-mm thickness (Durasil-25; Macherey-Nagel) developed with CHCl$_3$-CH$_3$OH-H$_2$O (30:8.1 or 65:25.4, vol/vol). Glycerolipids were detected by spraying plates with 0.2% anthrone in concentrated H$_2$SO$_4$, followed by heating.

The corynomycolate contents of extractable lipids and delipidated cells were determined in at least three independent experiments as follows. Lipid extracts (100 mg) and delipidated cells of the various strains were dried under a vacuum, weighed, and saponified (10); the saponified products were then acidified with H$_2$SO$_4$; Sigma releasing 5-thio-2-nitrobenzoate ([DNTB]$_2$) as substrates. The assay mixture contained 50 mM phosphate buffer (pH 8), 0.46 mM DTNB, and palmitoyl-CoA (Sigma) at various concentrations (8 µM to 0.25 mM).

Thioesterase activity was assayed spectrophotometrically by following the release of coenzyme A (CoA) from acyl-CoA reacting with DTNB [5,5′-dithiobis(2-nitrobenzoic acid); Sigma] releasing 5-thio-2-nitrobenzoate ([DNTB]$_2$ = 13,700 M$^{-1}$ cm$^{-1}$ at 410 nm). The assay mixture contained 50 mM phosphate buffer (pH 8), 0.46 mM DTNB, and palmitoyl-CoA (Sigma) at various concentrations (8 µM to 250 µM). Assays were done in 96-well plates (path length, 0.5 cm) in a 200 µl reaction volume. For kinetic parameter determination, the reaction was started by addition of 100 µl of the assay mixture containing the substrate at the appropriate concentration to 100 µl of 50 mM phosphate buffer (pH 8) containing 0.1% Triton X-100 and 0.30, 0.45, or 0.75 µg of the protein mixture from f250 or f250A. The contents were incubated at 37°C, and the reaction was followed spectrophotometrically at 410 nm for 30 min. The protein concentration of the extracts was determined by the Bradford method (Bio-Rad protein assay). Kinetic constants (K$_m$ and V$_{max}$) were obtained by nonlinear curve fitting using the software Origin7 (OriginLab).

RESULTS

Genetic organization of the Rv3802c locus in Corynebacteriaceae. As shown in Fig. 1, the Rv3802c gene is localized just upstream of the three genes (pks13, acc4d, and fadD32) whose products are required for the final condensation step of mycolate biosynthesis (35, 36). A search for homologous proteins showed that the Rv3802 protein is present in all the members of the Corynebacteriaceae suborder but not in other bacteria. Its corresponding C. glutamicum ortholog gene is annotated as Ngl2775 (Fig. 1). Amino acid sequences of Rv3802 protein orthologs are very well conserved, with a predicted cutinase motif (PFAM accession number PF01083) (4) and a predicted...
signal sequence followed by a proline-rich region. In both *C. glutamicum* and *M. smegmatis*, but not in *M. tuberculosis*, the Rv3802c gene ortholog is preceded by a small ORF (the NCgl2776 gene in *C. glutamicum*) that would encode a protein of unknown function. Because of the small intergenic distances between these two genes in the different genomes, it is very likely that, when present, the gene pair forms an operon. In all *Corynebacterineae*, two or more genes encoding mycoloyltransferases are found upstream of these ORFs. In *C. glutamicum*, like in other corynebacteria (except in *C. ulcerans*), the very small distances between mytA and the NCgl2776 gene and between NCgl2776 and NCgl2775 genes (five and six nucleotides, respectively) strongly suggest that these three genes form a polycistronic transcriptional unit. To test this hypothesis, RT-PCR was performed on total RNA by using primer pairs designed to span the putative operon, giving overlapping amplification products. RT amplification products were observed with each primer pair used, showing that, as expected, these genes are transcriptionally linked (data not shown).

**Essentiality of the Rv3802c gene orthologs.** In their screening by transposition insertion experiments for genes that impaired growth in *M. tuberculosis* and *M. bovis*, Sassetti et al. annotated the Rv3802c gene as a probable essential gene (40). However, as presumption of essentiality did not always work with mycobacteria, we tentatively deleted the Rv3802c ortholog (the MSMEG_6394 gene) in *M. smegmatis*. First, a merodiploid strain was generated by single crossover between the wild-type chromosomal MSMEG_6394 gene and a mutated allele carried by a nonreplicative plasmid containing the counterselectable marker sacB (PMM87) (Fig. 2). Attempts to select the allelic exchange mutant by plating a culture of PMM87 on solid LB medium containing Suc and Hyg failed, suggesting that null mutation of the MSMEG_6394 gene might be lethal for *M. smegmatis* on the growth condition tested. To validate the assumption, a functional copy of this gene was then provided in trans on a thermosensitive mycobacterial plasmid. Selection of Sac<sup>−</sup> Hyg<sup>−</sup> clones at 32°C allowed the isolation of a conditional mutant, named PMM88, in which the MSMEG_6394 chromosomal allele was disrupted by the Hyg resistance cassette and a functional copy of this gene was expressed from the thermosensitive plasmid (Fig. 2). Streaking this recombinant strain on Hyg-containing plates at 32°C or 42°C revealed that it grew as a single-crossover mutant at 32°C but was unable to form colonies at the high temperature (Fig. 2). These data demonstrated that, as expected, the MSMEG_
6394 gene is essential for the survival of M. smegmatis under these conditions.

In contrast to what was observed for M. smegmatis, a C. glutamicum deletion mutant (Δ2775) in which the entire NCgl2775 gene was replaced by a Km resistance cassette (aphIII) via a double crossover was easily obtained. The allelic replacement of the wild-type copy of the gene with the mutated one was verified by PCR using appropriate combinations of primers (data not shown).

Investigation of the putative role of the Rv3802 ortholog in MA biosynthesis. In view of the genetic context of Rv3802c, it was tempting to postulate that the gene product is involved in MA metabolism. This hypothesis is supported by the recent data showing that in vitro the esterase activity of the Rv3802 protein can be inhibited by tetrahydrodipalmitin, an antituberculoc compound with an unknown mechanism but that causes a decrease in the production of MA (32). However, there is no experimental evidence for a specific function of this protein in MA biosynthesis in vivo. Accordingly, on account of the non-essentiality of the NCgl2775 gene, we investigated the possible involvement of the gene product in MA metabolism. In comparison to the wild-type strain, the Δ2775 mutant exhibited no significant phenotypic changes and had similar growth patterns at 30 and 34°C. A detailed comparative lipid analysis of Δ2775 and the wild-type strain was then performed using bacterial cultures grown at 30°C. Lipids extracted with organic solvents were analyzed by TLC and quantified by being weighed. Concomitantly, cell wall-linked corynomycolates were isolated from delipidated cells, purified by chromatography, and weighed. The extractable lipids from the two strains exhibited qualitatively similar profiles; they consisted mainly of trehalose monocorynomycolate (TMCM), trehalose dicorynomycolate (TDCM), and phospholipids (data not shown). Likewise, no significant differences were observed between the Δ2775 and wild-type strains in terms of amounts of corynomycolates esterifying trehalose, i.e., TMCM and TDCM, or of corynomycolates covalently bound to arabinogalactan.

Nevertheless, it was possible to further explore the function of the protein by overexpressing Rv3802 orthologs and comparing the lipid profiles of the wild type, Δ2775, Δ2775(NCgl2775), and Δ2775 in which the MSMEG_6394 gene was overexpressed [Δ2775(MS6394)]. While no significant difference between the amounts of covalently linked corynomycolates of the different strains could be detected (data not shown), quantitative determination of the extractable lipids of the various corynebacterial cells indicated that the two overproducing strains [Δ2775(Δ2775) and Δ2775(ΔMS6394)] exhibited the same behavior and accumulated more trehalose corynomycolates (about a 30% increase) and elaborated fewer phospholipids (about a 15% decrease in the amount of FA relative to the levels for the parent strains [wild type and Δ2775]) (Fig. 3). As a result, the ratios of MA to FA, which were very similar for the wild-type and the Δ2775 strains, were almost two times higher for the overproducing strains than for the wild-type and the Δ2775 strains (Fig. 3). Thus, the overexpression of Rv3802 orthologs, the NCgl2775 and MSMEG_6394 genes, in C. glutamicum clearly impacts on the lipid composition of the resulting overexpressing strains.

Induction of the NCgl2775-NCgl2776-mytA operon by stress conditions. Taken together, the results described above strongly suggest that under normal growth conditions, the NCgl2775 gene has no detectable contribution to MA biosynthesis in C. glutamicum. They also point to a possible role of the protein under defined conditions, e.g., overexpression. To determine whether the NCgl2775 protein might function only in specific environmental conditions, we investigated the transcriptional activity of the NCgl2775-NCgl2776-mytA operon promoter (P_{op}) under different stress conditions. For that purpose, a transcriptional fusion of a DNA fragment corresponding to the 355 bp upstream of the start codon of mytA and the promoterless lacZ gene (P_{op}-lacZ) was constructed and inserted into the bacterial chromosome of the wild-type strain at the icd locus. To measure P_{op} activity, cells harboring the P_{op}-lacZ construct were grown at 30°C in the exponential phase, harvested by centrifugation, and subjected to various stress conditions. No change in β-galactosidase expression was seen after 2.5-h exposure to cold, hypo-osmotic, or hyperosmotic shock, acidic or alkaline pH, or hydrogen peroxide and carbon starvation relative to that under unexposed control conditions (data not shown). The only condition tested that led to a significant change in β-galactosidase expression was exposure to an elevated temperature (42°C). We thus performed a kinetic experiment of P_{op}-lacZ expression following a temperature shift from 30°C to 42°C. We observed that induction was relatively slow and increased at least until 3 h at a level approximately threefold higher than that of the control (Fig. 4).

Effects of NCgl2775 gene deletion on growth and heat resistance. The heat response of NCgl2775 gene expression led us to investigate the behavior of the Δ2775 mutant strain under heat stress conditions. We first examined its growth kinetics over the 30-to-42°C temperature range. For this purpose, fresh medium was inoculated with cells grown overnight at 30°C. Growth of the mutant was indistinguishable from that of the wild type at temperatures up to 40°C, which is the upper limit of the normal growth temperature for C. glutamicum (data not shown). Growth deficiency became apparent when the temperature was raised to 41°C. Under this stress condition, the mutant strain displayed a slight growth defect that was detectable...
3 h after the beginning of culture (data not shown). This growth defect was much more visible at 42°C, as the \( \Delta 2775 \) strain stopped growing after about one generation (Fig. 5A). Surprisingly, complementation of the \( \Delta 2775 \) mutant strain \([\Delta 2775(\text{NCgl2775}) \text{ strain}]\) resulted in growth that was slightly slower than those of the mutant and wild-type strains at temperatures above 37°C (data not shown). The reason for this growth defect is unclear but could be due to the high level of NCgl2775 protein produced by the strain (see below), which could be toxic under elevated growth temperature conditions. As expected from its behavior at elevated temperatures, the complemented strain did not restore the temperature effect (Fig. 5A).

We next examined the ability of the cells to survive and grow after a heat challenge at mild (\( \leq 45°C \)) and high temperatures (\( \geq 50°C \)). Mild temperatures are defined as conditions in which a significant fraction of the \( C. \text{ glutamicum} \) population can survive at least 24 h, whereas high temperatures are conditions under which all the bacteria are killed in 1 h or less. Cells from exponentially growing cultures at 30°C were diluted in fresh BHI medium, incubated 16 h at 42°C and then cultivated again at 30°C. A significant increase in the delay of the recovery period before growth resumption was systematically observed for the \( \Delta 2775 \) relative to the parental strain (Fig. 5B). This difference in growth retardation was not due to a difference in the number of viable cells, since bacterial viability was not affected by the exposure at 42°C (data not shown). However, when the same experiment was performed at 45°C instead of 42°C, a loss in bacterial viability of 50- to 100-fold was consistently observed for the \( \Delta 2775 \) strain relative to the level for the wild-type strain (Fig. 5C). Again, these effects could not be explained by a difference in the number of viable cells.

![FIG. 4. Expression of P\(_{\text{op}}\) under heat stress conditions. The reporter strain contains the promoter region of the NCgl2775-NCg2776-mytA operon (P\(_{\text{op}}\)) fused to the lacZ gene. The strain was grown at 30°C at mid-exponential phase and divided into two samples (time zero). One was incubated at 30°C (white bars) and the other at 42°C (gray bars). P\(_{\text{op}}\) expression was quantified for each sample at 1, 2, and 3 h by determining the \( \beta \)-galactosidase activity as described in Materials and Methods. Values are means of three determinations \( \pm \) standard deviations. prot, protein.](http://jb.asm.org/)

![FIG. 5. Effects of mild temperature on growth and heat resistance.](http://jb.asm.org/)

- **A** Stationary-phase cells from the wild-type (WT; open squares), \( \Delta 2775 \) (filled squares), and \( \Delta 2775(\text{NCgl2775}) \) (filled triangles) strains grown at 30°C were diluted directly into fresh medium and transferred to 42°C. Growth was monitored by OD\(_{600}\) measurements.
- **B** Cells from the wild type (WT; open squares) and \( \Delta 2775 \) (filled squares) grown to mid-exponential phase at 30°C were diluted into fresh BHI medium (OD\(_{600}\) = 1), shifted to 42°C for 16 h without shaking, and then transferred again to 30°C under culture conditions. Growth recovery at 30°C was monitored by OD\(_{600}\) measurements.
- **C** Cells from the wild type (WT) and \( \Delta 2775 \) grown to mid-exponential phase at 30°C were diluted directly into fresh BHI medium at an OD\(_{600}\) of 1 (corresponding to \( 10^8 \) bacteria/ml), shifted to 45°C for 16 h without shaking, and spotted (10 \( \mu \)l) onto BHI agar plates at different bacterial dilutions (0 to \( 10^{-5} \)). The absence of growth during the incubation period at 45°C was checked before spotting. Plates were incubated at 30°C, and growth was visualized 48 h after spotting. All the data are representative of the results for three independent experiments.
complemented by the overexpression of the NCgl2775 protein in the mutant strain. In contrast to what was observed at 45°C, no significant difference in the survival rate of bacteria could be detected between the two strains after exposure at 50°C (data not shown). Altogether, these results indicated that the NCgl2775 protein is involved in both bacterial resistance and viability following a heat insult but only when temperature conditions are not too severe. This conclusion is consistent with the very slow kinetic of the transcriptional activity we observed for the NCgl2775 promoter (P\text{op}) (Fig. 4) that must be compared to the time course of survival at mild temperatures (several hours or days), while killing of bacteria takes minutes at high temperatures.

**Effect of the NCgl2775 gene deletion on the lipid envelope composition at high temperature.** Temperature is known to significantly affect the content and composition of microbial lipids in response to variations in membrane fluidity. In this context, considering the effects of a long exposure at temperatures higher than 40°C on the Δ2775 mutant, we investigated a possible role of NCgl2775 in lipid metabolism in response to thermal stress. The *C. glutamicum* lipodome comprises principally corynomycolates esterifying trehalose (TMCM and TDCM) and arabinogalactan and three GPL classes, namely phosphatidylglycerol, phosphatidylinositol, and cardiolipin (31, 37). We thus analyzed the effect of a growth at 42°C on FA and MA compositions. As palmitoyl (C\text{16:0}) and oleoyl (C\text{18:1}) constitute 95% of the fatty acyl moieties of *C. glutamicum* ATCC 13032 GPL (31), determination of the C\text{16:0}/C\text{18:1} ratio (rFA) is a good index of the balance between saturated and unsaturated FA and of the amount of GPL. Similar ratios were observed for the wild-type and mutant strains cultivated either at 30°C (rFAs of 0.85 ± 0.12 for the wild type and 0.79 ± 0.15 for Δ2775) or at 42°C (rFAs of 0.67 ± 0.19 for the wild type and 0.85 ± 0.21 for Δ2775).

*C. glutamicum* ATCC 13032 (wild type) synthesizes different classes of corynomycolates, mainly C\text{32:0}, C\text{34:0}, and C\text{34:1} and, to a lesser extent, C\text{36:2}, C\text{36:1}, and C\text{36:0}. At 30°C, the ratios of saturated corynomycolates to unsaturated corynomycolates (rMA) are 1.04 ± 0.1 for the wild type and 1.02 ± 0.33 for Δ2775. Interestingly, and in sharp contrast with what was observed for GPL, culturing *C. glutamicum* at 42°C had a drastic effect on the balance between saturated and unsaturated mycolates for the wild-type strain (rMA = 3.88 ± 0.27). A similar effect was observed for the mutant strain (rMA = 3.95 ± 1.25). It is unlikely that these quantitative modifications are accompanied by structural changes (e.g., length of mycolates), as no shift of the amounts of C\text{32:0}, C\text{34:0}, and C\text{34:1} could be observed while increasing the growth temperature either for the wild type or the Δ2775 strain (data not shown).

Modification of MA composition was not the only change induced by the temperature; we also observed an important variation in the MA/FA ratio. This ratio, which represents the amounts of TDCM and TMCM relative to GPL, increased about seven times for the wild-type strain (7.4 ± 2.6) when the temperature was increased from 30°C to 42°C. In contrast, the same stress conditions caused only a threefold increase in the MA/FA ratio for the Δ2775 strain (2.8 ± 1.05). Taken together, these results indicated that an important reorganization of the outer membrane lipids occurred when *C. glutamicum* was exposed to high temperatures and that the NCgl2775 protein was involved in this process. Based on the data obtained by overexpressing the NCgl2775 or MSMEG_6394 gene at 30°C (see above), which has an effect on lipid composition quite comparable to that of an elevated growth temperature, these results are in agreement with a mechanism in which an increase in temperature induces an overexpression of the NCgl2775 protein, resulting in turn in a modification of the balance between trehalose corynomycolates and GPL.

**Partial purification of the NCgl2775 protein from *C. glutamicum*.** Though our results clearly demonstrated that the NCgl2775 protein is involved in the cell wall lipid modifications related to heat stress management, we decided to perform an in vitro analysis of the enzymatic activity(ies) of the NCgl2775 protein in order to gain insights into its possible mechanism of action. According to the esterase/cutinase characteristics of the Rv3802 protein family, and because cutinases display hydrolytic activity toward a broad variety of esters, which include soluble synthetic esters, we tested pNP esters of FA as substrates (8). Preliminary experiments performed on whole-cell extracts (after lysis by microbead treatment) and culture supernatants of wild-type and Δ2775 strains showed that most of the hydrolysis activity of pNPD occurred in the culture supernatant (Table 1). Only a small difference between the hydrolysis activities of the wild-type and Δ2775 strains was detected, in both fractions, indicating that *C. glutamicum* possesses another esterase(s), besides NCgl2775, mainly secreted. The same experiment performed on Δ2775 (NCgl2775) showed that the overexpression of NCgl2775 in *C. glutamicum* resulted in a >100-fold increase in the hydrolysis activity of pNPD, essentially in the cell extract (Table 1). The purification procedure and hydrolysis activity observed with pNPD, used as the substrate, at each purification step are summarized in Table 2. As can be seen, the pNPD activity was clearly associated with the French press membrane vesicle fraction. Treatment of these vesicles with 5 M urea did not extract the protein in contrast to 1% Triton X-100, which fully solubilized pNPD activity. The Triton X-100 extract was then subjected to DEAE Sephacel chromatography, and the pNPD activity was found to elute with a 250 mM NaCl concentration. Thereafter, this fraction containing the NCgl2775 gene was named f250+. As a control, the same purification procedure was performed using the Δ2775 strain as starting material (Table 2). Comparison of the DEAE fraction f250+ with the same fraction from the control (f250Δ) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a broad but intense band at around 40 kDa, corresponding to the NCgl2775 protein, which was subsequently used for enzymatic measurements. It should be

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<th>Strain</th>
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<td>WT</td>
<td>0.28</td>
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<tr>
<td>Δ2775</td>
<td>0.19</td>
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<td>Δ2775 (NCgl2775)</td>
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* Stationary-phase cultures were centrifuged, the pellet was resuspended in 50 mM phosphate buffer, and the cells were broken using microbeads. pNPD (0.25 mM) activity was measured using the broken cell extract and the culture supernatant as protein sources. WT, wild type.

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**TABLE 1. Hydrolysis activity of pNPD in the wild type, Δ2775, and the overexpressing Δ2775 (NCgl2775) strain**
noted that the apparent molecular mass of the NCgl2775 protein is greater than the calculated mass of the deduced amino acid sequence (32,615 Da). Our purification procedure showed that the NCgl2775 protein is associated with a membrane fraction and could be solubilized only by added detergent, a result consistent with the NCgl2775 protein being anchored in the membrane. The hydrophobic profile of the NCgl2775 protein predicts only one N-terminal transmembrane segment (residue 5 to 27) that is also predicted as a possible signal sequence with a more probable signal peptidase cleavage site located between residues 24 and 25 or 27 and 28 (SignalP3.0 and LipoP1.0). No lipid attachment site in NCgl2775 could be predicted (LipoP1.0 and DOLOP).

**In vitro activity of NCgl2775.** During the course of this work, two studies involving independent functional analyses of the recombinant His-tagged Rv3802 protein have been published (32, 49). Taken together, these reports showed that Rv3802 possesses in vitro esterase, thioesterase, and phospholipase activities. On the basis of the huge difference observed between the \(K_m\) values of an esterase substrate (\(p\)NBP; \(K_m = 23.5\) mM) and a thioester substrate (palmitoyl-CoA; \(K_m = 50\) \(\mu\)M), it was proposed that the Rv3802 protein could be a thioesterase in vivo (32). Considering the difference in acyl chain length of the substrates used in that study (\(C_4\) versus \(C_{16}\)), we chose to compare the NCgl2775 hydrolisis activities of different \(p\)NP esters of FA (\(p\)NPB \(C_6\), \(p\)NPD \(C_{10}\), and \(p\)NPP \(C_{16}\)) with that of palmitoyl-CoA (\(C_{16}\)). For this purpose, time-dependent hydrolysis of substrates was measured using \(250 + 1\) and \(250\Delta\) as enzyme sources. As \(250 + 1\) was active toward all the substrates tested and \(250\Delta\) was inactive in all assays, we deduced that the measured kinetic values reflected the NCgl2775 activity alone. Kinetic parameters are given in Table 3 (those for \(p\)NBP, which exhibited a non-Michaelian behavior under our conditions, are not shown). Identical \(V_{\text{max}}\) values were obtained for \(p\)NPP and palmitoyl-CoA, while a very slight difference between \(K_m\) values (a factor of 3) was observed. As the two substrates share the same acyl chain (palmitoyl) and thus are especially appropriate for discrimination between a thioesterase and an esterase function, we concluded that the small difference observed between the kinetic parameters of these substrates is not sufficient to favor a thioesterase activity rather than an esterase one. Besides, similar values of \(K_m\) and \(V_{\text{max}}\) were also obtained for \(p\)NPP and \(p\)NPD, indicating that, in the \(C_{10}\)-to-\(C_{16}\) range, the acyl chain length has a very small influence, if any, on the enzyme affinity and turnover. Finally, the temperature (20, 30, or 42°C) had no influence on NCgl2775 activity with \(p\)NPD as the substrate (data not shown).

**DISCUSSION**

In this study, we examined the in vivo function of the *C. glutamicum* ortholog of the Rv3802 protein, the NCgl2775 protein, and found that it is involved in the reorganization of outer membrane lipid composition following heat stress. When subjected to temperatures greater than their physiological growth temperature, bacteria have to cope with membrane fluidization that can alter essential physiological functions (11, 17, 42, 45). For bacteria possessing two membranes, the outer one is the first component that suffers damage. This is illustrated by the important disorganization and drastic increase in permeability that occur in the outer membrane of *E. coli* after a severe heat shock (18, 41, 48). Although it is obvious that changes must occur to prevent or repair membrane alterations due to heat treatment, the nature and mechanisms of these modifications are poorly documented in the case of the bacterial outer membrane. In the present study, we showed that important changes are induced in the outer membrane of *C. glutamicum* in response to mild heat exposure (42°C). First, the ratio of saturated MA to unsaturated MA increases by a factor 4. A change in the saturated-to-unsaturated FA balance is a common mechanism used by various organisms to adjust the fluidity of their plasma membranes in response to temperature variations. *C. glutamicum* uses this mechanism to decrease the viscosity of its outer membrane but not that of its inner one, as no change in the GPL acyl chain composition could be detected after several hour of exposure to heat. This result is in agreement with the results of Özcan et al., who showed that the \(C_{16}\)\(\alpha\)/\(C_{18}\)\(\alpha\) ratio remained unchanged when *C. glutamicum* bacteria were grown at 30°C, 37°C, and 40°C (31). Second, the ratio of extractable MA (TDCM and TMCC) to FA also increases drastically. Noncovalently linked corynomycolates have been postulated to participate with GPL in the structure of both leaflets of the corynobic bacterial outer membrane (37, 50). Our results strongly suggest that following a heat challenge, the balance between these two classes of lipids is modified inside the outer membrane. The physiological effect of this membrane remodeling is not clear. Indeed, while trehalose corynomycolates, which are \(C_{12}\)-to-\(C_{36}\) corynomycolates that contain two \(C_{16}\)-to-\(C_{18}\) chains, are comparable in size to GPL, they largely differ in their structure and by their head group. In consequence, changes in the balance between MA and GPL could modify the packing of the lipids and the surface charge...
of the membrane and thus influence not only the fluidity but also the permeability of the outer membrane. We showed in the present study that the NCgl2775 protein participates in the outer membrane restructuring caused by heat exposure. Indeed, when the NCgl2775 protein was inactivated, we observed a loss of regulation of the MA and GPL contents induced by heat exposure. However, this loss of regulation is partial, suggesting that the NCgl2775 protein is not the only actor involved in this regulation. Importantly, NCgl2775 overexpression provokes both an increase in the MA contents and a decrease in the FA contents independently of any thermal challenge, confirming that an increase in NCgl2775 concentration alone is able to induce lipid content modifications.

It is clear from our results that the NCgl2775 protein is membrane anchored. Various computer programs predict only one putative membrane-spanning helix overlapping a potential signal sequence but do not detect any lipid posttranslational-modification motif. It is thus likely that the N-terminal hydrophobic segment is not processed and serves as an anchor for the NCgl2775 protein in the plasma membrane, leaving a very short N-terminal extension in the cytosol and the rest of the protein in the periplasmic space. However, further experimental validation is required to confirm these topology predictions. Taken together, our data lead us to propose the following scheme. (i) NCgl2775 activity is localized in or near the plasma membrane and depends on protein concentration. This concentration is increased by heat stress conditions, resulting in the activation of the NCgl2775 protein. (ii) When active, the NCgl2775 protein allows an increase in MA synthesis, concomitant with a decrease in GPL. The precise activity of the NCgl2775 protein is unknown, but the enzyme possesses comparable esterase and thioesterase activities in vitro. (iii) These changes, together with a modification of the saturated/unsaturated balance of the MA, lead to a dramatic outer membrane remodeling that protects the outer membrane and, consequently, the bacteria from heat damage. Although it is clear from our data that the NCgl2775 protein enhanced MA biosynthesis, the mechanism by which the protein acts remains elusive. One hypothesis is a direct control of the MA flux by the NCgl2775 protein via an interaction with an enzyme of the MA biosynthetic machinery. This would require an effective interaction only when NCgl2775 is activated (heat stress conditions) and would result in an acceleration of the release of the MA from the enzyme, increasing the global rate of MA synthesis, which in turn would influence GPL synthesis. Such an interaction has been suggested by Parker et al., who proposed that the Rv3802 protein could function as an additional thioesterase for Pks13 (32), the enzyme that catalyzes the condensation of two FA to give MA (32, 35). It is noteworthy that a Pks13/NCgl2775 interaction is not consistent with the predicted topological model of NCgl2775, since the active domain of the protein is predicted to be in the periplasm, while Pks13 is believed to be located on the cytosolic side. Mature MA, obtained from the reduction of the β-ketoester product of Pks13 by CmrA (20), are believed to be translocated across the plasma membrane by an unknown mechanism before being attached to trehalose and transferred onto arabinogalactan. It is thus conceivable from this scheme that the NCgl2775 protein could control the translocation step or interact with a cell wall mycoloyltransferase. Alternatively, and based on the demonstrated phospholipase activity of Rv3802 (32), it is conceivable that when activated, the NCgl2775 protein would hydrolyze a GPL in the plasma membrane, which in turn could serve as a signal for outer membrane restructuring. Though our results do not allow discrimination between the aforementioned hypotheses, it is obvious that NCgl2775 is part of a large regulation process that involves other proteins. Two mycoloyltransferase-encoding genes are found upregulated during a heat stress: mytA, which is cotranscribed with the NCgl2775 gene, and mytB (our unpublished results). An increase in MytA and MytB activities is quite consistent with an increase in MA synthesis, since these two proteins contribute to the transfer of MA onto their final acceptors. The search for other partners is under way.

NCgl2775 is part of an ortholog family exclusively found in all Corynebacterineae. Conservation of locus synten and of protein sequences strongly suggests that the function of all this protein family is also well conserved and specific to this sub-order. Indeed, we showed in the present study that overexpression of the MSMEG_6394 protein in C. glutamicum is able to modify the MA/GPL ratio in the same way as its NCgl2775 ortholog does. However, the stress conditions that trigger lipid modifications induced by this protein family could be different from one bacterium to another. Indeed, Miltner et al. found the Mycobacterium avium Rv3802c ortholog to be probably involved in bacterial invasion of the intestinal epithelium and showed that its expression is increased under both high-osmolarity and low-oxygen conditions, conditions that mimic the intestinal environment (28). We thus proposed that this protein family plays a role in the regulation of outer membrane lipid composition by influencing the balance between MA and other lipids under stress conditions. We therefore propose to name this protein on the envelope lipid-regulating factor (ElrF).

The balance between MA and other lipids could be more or less important for bacterial physiology among the different genera of Corynebacterineae. It is well established that mycobacteria are very sensitive to changes in their outer membrane composition (e.g., mycolates) than corynebacteria. This may explain why NCgl2775 is needed only under stress conditions while the M. smegmatis ortholog is essential under physiological conditions. Further investigations to provide insight into this regulation process are in progress. Finally, the crucial, though yet unknown, function of Rv3802 in mycobacteria suggests the use of the protein as a putative drug target in the search for new antituberculous drugs, which are urgently needed.

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