The impact of the absence of glycopeptidolipids on the ultrastructure, cell surface and cell wall properties, and phagocytosis of *Mycobacterium smegmatis*

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Glycopeptidolipids (GPLs) are a class of species- or type-specific mycobacterial lipids and major constituents of the cell envelopes of many non-tuberculous mycobacteria. To determine the function of GPLs in the physiology of these bacteria, a mutant of *Mycobacterium smegmatis* in which the gene encoding a mycobacterial nonribosomal peptide synthetase has been inactivated by transposon mutagenesis was analysed. Labelling experiments indicated that half of the bacterial GPLs were located on the cell surface and represented 85% of the surface-exposed lipids of the parent strain whereas the mutant was defective in the production of the GPLs. Compared to the parent smooth morphotype strain, the GPL-deficient mutant strain exhibited a rough colony morphology, an increase of the cell hydrophobicity and formed huge aggregates. As a consequence, the mutant cells were no longer able to bind ruthenium red, as observed by transmission electron microscopy. The altered surface properties of the mutant cells also affected the phagocytosis of individual bacilli by human monocyte-derived macrophages since mutant cells were internalized more rapidly than cells from the parent strain. Nevertheless, no specific release of surface constituents into the culture broth of the mutant was observed, indicating that the cell surface is composed of substances other than GPLs and that these are essential for maintaining the architecture of the outermost layer of the cell envelope. Importantly, the absence of these major extractable lipids of *M. smegmatis* from the mutant strain has a profound effect on the uptake of the hydrophobic chenodeoxycholate by cells, indicating that GPLs are involved in the cell wall permeability barrier of *M. smegmatis*. Altogether, these data showed that, in addition to being distinctive markers of numerous mycobacterial species, GPLs play a role in the bacterial phenotype, surface properties and cell wall permeability.

Keywords: mycobacteria, cell wall permeability, morphology, ultrastructure, glycolipids

INTRODUCTION

The mycobacterial cell envelope, as revealed by the most recent developments in knowledge of the ultrastructure and chemistry of mycobacteria, is composed of three

structural components: a plasma membrane, a wall and an outer layer (OL) (Daffé & Draper, 1998). While the plasma membrane appears to be a typical bacterial membrane, the complex wall that surrounds this membrane partly resembles a Gram-positive wall, but is unusual in having the thick layer of peptidoglycan covalently linked to a D-arabino-D-galactan, which in turn is esterified by long-chain fatty acids, the so-called
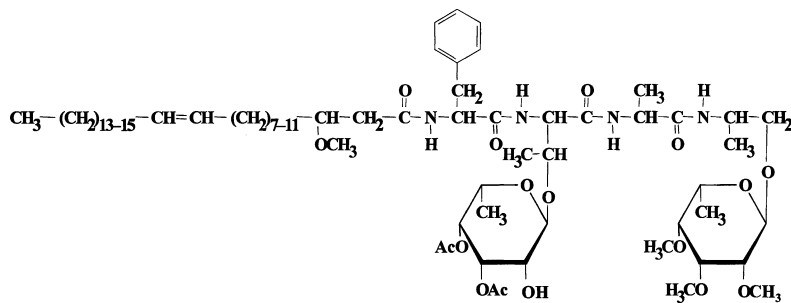
Fig. 1. Structure of the major glycopeptidolipids of *M. smegmatis*. Ac, acetyl.

mycolic acids. Unlike Gram-positive walls, the mycobacterial wall also contains a great variety of other lipids, which are probably arranged to form an asymmetric bilayer with the cell wall mycolate monolayer, but not covalently attached to it (Minnikin, 1982). In addition, pore-forming proteins, typifying Gram-negative bacteria, have been characterized in the walls of several mycobacterial species (Mukhopadhyay *et al.*, 1997; Trias *et al.*, 1992, Trias & Benz, 1994; Senaratne *et al.*, 1998). Consequently, although mycobacteria are phylogenetically classified as Gram-positive bacteria, their wall is more related to that of Gram-negative micro-organisms (Draper, 1998). The outer layer of the cell envelope of mycobacteria consists of a protein–carbohydrate matrix loosely bound to the wall and contains both ubiquitous and species-specific lipids (Daffé & Draper, 1998). Many distinctive features of mycobacteria have been associated with the uncommon composition of their cell envelope. For instance, the well-known acid-fastness of mycobacteria and relatives, i.e. the failure of dilute acids to decolourize bacteria stained with various basic dyes, may be explained by the presence of the asymmetric bilayer, which would represent a permeability barrier to polar molecules (Brennan & Nikaido, 1995). The existence of this barrier could also explain their limited permeability and intrinsic resistance to strong chemicals such as acids, alkalis and hypochlorite (Daffé & Draper, 1998). Likewise, the unusual composition of the mycobacterial cell surface, particularly its remarkable hydrophobicity, may explain the tendency of cells to grow in large aggregates.

The high lipid content of mycobacterial cells, recognized as early as the 1930s, and the extraordinary biological activities associated with some of the purified lipids (for reviews see Daffé & Draper, 1998; Goren & Brennan, 1979), have prompted researchers to devote much effort to the identification of the various types of mycobacterial lipids. Among the characteristic lipids of mycobacteria are the species-specific glycopeptidolipids (GPLs) that typify many non-tuberculous mycobacterial species; these molecules may be subdivided into alkali-stable C-type GPLs and alkali-labile serine-containing GPLs (Daffé & Lemassu, 2000). The C-type GPLs have been found in saprophytic mycobacteria (*Mycobacterium smegmatis*), opportunistic pathogens of man (*Mycobacterium avium–intracellulare*, *Mycobacterium scrofulaceum*, *Mycobacterium peregrinum*, *Mycobacterium chelonae*, *Mycobacterium abscessus*) and of animals (*Mycobacterium lepraemurium*, *Mycobacterium paratuberculosis*, *Mycobacterium porcinum*, *Mycobacterium senegalense*) (Brennan, 1988; Daffé & Lemassu, 2000) whereas the alkali-labile serine-containing GPLs have been found so far only in *Mycobacterium xenopi* (Besra *et al.*, 1993; Rivière & Puzo 1991). C-type GPLs share a common lipopeptidyl core consisting of a mixture of 3-hydroxy and 3-methoxy C_{26-34} fatty acids (Daffé *et al.*, 1983) amidated by a tripeptide D-Phe-D-*allo*-Thr-D-Ala and terminated by L-alaninol (Fig. 1). They differ from one another by the number and the nature of the saccharide units linked to the hydroxyl group of *allo*-Thr and/or alaninol (Brennan, 1988; Daffé & Lemassu, 2000). These glycosyl units are responsible for both the variability and the specificity of the antigenically distinct serovariants within the *M. avium-intracellulare* complex and several other C-type GPL-containing mycobacteria (Brennan, 1988), which implies that these compounds occur on the peripheral bacterial surface, in agreement with their isolation from the outermost compartment of mycobacterial cells (Ortalo-Magné *et al.*, 1996) and their identification as the receptor of mycobacteriophage D4 (Furuchi & Tokunaga, 1972; Goren *et al.*, 1972). Although the role of GPLs in pathogenesis is still unclear (Daffé & Draper, 1998), they accumulate into the phagosome during bacterial intracellular growth, contributing to the formation of a capsule around the bacteria (Draper, 1974; Rulong *et al.*, 1991; Tereletsky & Barrow, 1983). Less is known, however, about the localization of GPLs in deeper compartments of the mycobacterial cell envelope and their possible contribution to the cell wall permeability barrier. Thanks to the availability of *M. smegmatis* mutants with transposon insertions in the genes involved in the synthesis of the C-type GPL core (Billman-Jacobe *et al.*, 1999; Recht *et al.*, 2000), we addressed the question of the effects of the absence of C-type GPLs on the architecture and surface properties of the cell envelope of *M. smegmatis* and on the initial interactions of bacteria with host cells.

METHODS

Bacterial strains and culture. *M. smegmatis* mc²155 and its isogenic GPL-deficient mutant strains TM99, 8-245 and 9-1045 (Billman-Jacobe *et al.*, 1999; Recht *et al.*, 2000) were routinely grown at 37 °C with shaking at 250 r.p.m. (Incubator Shaker, New Brunswick Scientific) in Luria–Bertani (LB broth, Euromedex) medium with the addition of 0·05 % Tween 80.

The strains were also grown on Trypticase-Soy (TS broth, bioMérieux) medium for the determination of surface properties of the cells and on Sauton's medium, without agitation, as surface pellicles when information was needed on extracellular materials. The mutant strains were maintained by kanamycin selection, with kanamycin added to a final concentration of 25 μg ml^{-1}. The generation times of the various strains were determined as described by Raynaud et al. (1998) by weighing cells grown in the LB medium.

The viability of mycobacterial cells was assessed by i) quantifying the level of isocitrate dehydrogenase, an indicator of autolysis, in the culture filtrates (Raynaud et al., 1998) and ii) labelling cells with both propidium iodide and fluorescein diacetate (Cougoule et al., 2002); the percentages of live (fluorescein-positive, green cells) and dead (propidium iodide, red cells) bacteria were determined by counting at least 100 bacteria.

Single-cell suspensions were prepared with late exponential-phase cultures incubated in 100 ml Sauton's medium; the pellicles were harvested by pouring off the medium and were gently shaken for 30 s with 5 g glass beads (4 mm diameter). The declumped cells resulting from this treatment were suspended in 10 ml TS broth plus 0·05% Tween 80 and centrifuged for 10 min 100 g. The OD$_{650}$ of the supernatant, which contains mainly single cells, was adjusted to 1 OD$_{650}$ unit and directly used to inoculate the media (100 μl suspension for 50 ml broth, except where indicated otherwise) for the determination of some cell surface properties. Alternatively cells were washed three times with PBS to give a PBS-washed single cell suspension, or stored at −80 °C in the presence of 20% (w/v) glycerol.

MICs were determined by the agar two-fold dilution method in TS agar (bioMérieux). The drug-containing media were inoculated with 100 μl of a dilution (0·01 OD$_{650}$ unit) of the single cell suspension stored at −80 °C and incubated for 7 days at 37 °C. For all the drugs tested, 99% inhibition of bacterial growth was determined as the MIC of the drug.

Motility. Surface-spreading assays were adapted from Martinez et al. (1999); briefly, Middlebrook 7H9 base medium (Difco) was solidified with 0·4% high grade agarose (Eurogentec). Plates were inoculated into their centre with 10 μl of the 1 OD$_{650}$ unit PBS-washed single cell suspension (see above). Spreading was evaluated after incubation for 5 days at 37 °C in a humidified incubator by measuring the diameter of the halo of growth formed by the mycobacteria.

Cellular aggregation. Mycobacterial strains were cultivated with shaking (250 r.p.m.) in TS broth without Tween 80 for 3 days at 37 °C. The bacterial suspensions were centrifuged for 10 min at 100 g to separate the unicellular mycobacteria from the aggregates, which were pelleted (Cougoule et al., 2002). The single cells were further recovered by centrifugation for 30 min at 3000 g. Cell pellets were dried and weighed, and the cellular aggregation was calculated, i.e. the percentage of aggregate-containing pellets versus total cell weight.

Congo red accumulation. The assay (Cangelosi et al., 1999) was adapted as follows: mycobacteria were cultivated for 3 days at 37 °C with shaking (250 r.p.m.) in TS broth plus 100 μg Congo red ml^{-1} and 0·05% Tween 80. Cells were collected by centrifugation (30 min at 3000 g) and washed extensively with distilled water until the supernatant was colourless. Cells were resuspended in 1 ml acetone, vortexed and gently shaken for 2 h at room temperature; cells were then removed by high-speed microcentrifugation and Congo red in the supernatants was spectrophotometrically measured at 488 nm. The Congo red binding was defined as the A_{488} of the acetone extracts divided by the dry weight (in mg) of the cell pellet.

Hydrophobicity index. Relative hydrophobicities were assessed by the hexadecane partition procedure (Rosenberg et al., 1980). Briefly, 1 OD$_{650}$ unit PBS-washed single cell suspension (see above) of each strain was mixed with 0·3 ml hexadecane (Avocado) by vortexing for 2 min. The hydrophobicity index was defined as the percentage reduction in the OD$_{650}$ of the aqueous phase; this reduction was determined for triplicate samples after allowing 15 min for the hydrocarbon phase to rise completely.

Zeta potential. For determination of the bacterial cell surface charge (Bayer & Sloyer, 1990), zeta-potential (ζ) measurements were performed with 1 OD$_{650}$ unit PBS-washed single cell suspension (see above) in a zetameter Zetasizer 3000 (Malvern Instruments).

Isolation, fractionation and analysis of the extracellular and surface-exposed components. Surface-exposed material (SEM) and extracellular compounds were extracted and analysed as previously described (Lemassu et al., 1996; Ortalo-Magné et al. 1996). Briefly, mycobacterial cells were harvested by centrifugation (30 min at 3000 g) and the culture broths were sterilized by filtration through 0·20 μm pore-size sterile filters (Nalgene). Cells were shaken for 1 min with 10 g glass beads (4 mm diameter) per 2 g (wet weight) cells (Ortalo-Magné et al., 1995), resuspended in distilled water (50 ml per flask) and then the cells were removed by filtration. A portion of the crude filtrate, which contains the SEMs, and of the culture broths, which contains the extracellular materials, were concentrated separately under vacuum, extensively dialysed against distilled water and analysed by colorimetric assays for their carbohydrate content (Dische, 1962) and protein content by the Lowry method. Chloroform and methanol were added to the remaining portions to obtain partition mixtures composed of chloroform/methanol/water (3:4:3, by vol.); the organic phases were concentrated, washed with water, evaporated to dryness to yield crude lipid extracts, and weighed. The lipid extracts were dissolved in chloroform and analysed by TLC on silica gel Durasil 25-precoated plates (0·25 mm thickness; Macherey-Nagel). The lipids were resolved by TLC run in the following solvent mixtures: petroleum ether/diethyl ether (9:1, v/v) for analysing triacyl glycerols, chloroform/methanol (9:1, v/v) for GPLs and trehalose dimycolates and chloroform/methanol/water (60:30:8 by vol.) for trehalose monomycolates and phospholipids. Sugar-containing compounds (GPLs, trehalose dimycolates, trehalose monomycolates and phosphatidylinositol mannosides) were visualized by spraying the plates with 0·2% anthrone in concentrated sulfuric acid, followed by heating at 110 °C. The Dittmer–Lester reagent (Dittmer & Lester, 1964) was used to detect phosphorus-containing substances. The ninhydrin reagent was used to reveal the presence of free amino groups and a spray with 10% molybdophosphoric acid in ethanol solution, followed by heating at 110 °C, was used to detect all of the lipid spots, including triacyl glycerols.

Labelling of lipids. The various classes of extractable and cell surface lipids were quantified by labelling; 1·2 MBq sodium [^{14}C]acetate (Amersham) was added to 100 ml 2-day-old cultures containing mid-exponential-phase bacteria. After 16 h incorporation, the reaction was stopped by centrifugation and the SEMs were isolated by extraction with glass beads (Ortalo-Magné et al., 1995). Lipids from these latter materials and those from bead-treated cells were extracted with chloroform/methanol (1:2, v/v). Both types of lipid extracts

were analysed by TLC using the solvent mixtures described above; the radioactivity was located and counted on plates using an automatic TLC linear analyser (Berthold LB 2832). Then the lipid spots were visualized by spraying with the appropriate reagents, with heating when necessary.

Permeability assays. The permeability of the strains of *M. smegmatis* to chenodeoxycholate was assessed as previously described (Bardou *et al.*, 1998; Jackson *et al.*, 1999). Exponentially grown cells (2-day-old cultures) were first labelled for 16 h with [5,6-^{3}H]uracil (20 μM, 1·85 GBq mmol^{-1}; DuPont NEN) to quantify the biomass present in aliquots used in the accumulation assays. Then, cells were collected by centrifugation and washed with 10 mM HEPES, pH 7·2. Aliquots of labelled cells were counted, dried and weighed to correlate ^{3}H labelling with cell dry weight. Accumulation assays were performed under continuous agitation. [^{14}C]chenodeoxycholate (20 μM, 1·8 GBq mmol^{-1}; DuPont NEN) was added to a 1 ml mixture of HEPES containing about 40 mg ^{3}H-labelled cells. Aliquots (0·1 ml) were removed at different time intervals and added to the top of an Eppendorf centrifuge tube containing 0·25 ml silicon oil/paraffin oil (1:0·2, v/v). Cells were separated from the accumulation medium by centrifugation (13 000 *g*, 1 min). Centrifuged tubes were frozen on dry ice and the pellets were dropped into counting flasks by cutting the cone top. Then the scintillation solution (Aqualuma) was added and the vials were sonicated for 30 min in a water bath to disperse cells.

Transmission electron microscopy. The method for preparation of samples for transmission electron microscopy was based on the procedures of Paul & Beveridge (1992). *M. smegmatis* mc^{2}155 and the GPL-deficient mutant TM99 were grown for 72 h in both the LB broth and Sauton's medium, then subcultured into fresh broths by making a 1/100 dilution. The fresh broth was incubated overnight and cells were harvested by centrifugation (8000 *g*, 10 min), and washed twice in PBS. The resulting pellets were fixed in 2·5% (w/v) glutaraldehyde, 0·05% (w/v) ruthenium red in cacodylate buffer for 2 h in the dark at room temperature. Cells were washed three times in cacodylate buffer (0·1 M, pH 6·8), postfixed for 2 h in the dark in 1% (w/v) osmium tetroxide, 0·05% (w/v) ruthenium red and then washed twice each in cacodylate buffer and in water. Cells were dehydrated through a graded ethanol series of 10, 20, 30, 40, 50, 60, 70, 80 and 95% for 5 min each then washed twice for 15 min each in 100% ethanol, then twice for 15 min each in propylene oxide. Cells were suspended in 1:1 propylene oxide/Spurr resin for 2 h in an open tube. After infiltration overnight, the tubes were opened for 2 h; then samples were transferred to 100% Spurr resin and left overnight. Resin was replenished the next morning and samples were left to cure at 60 °C overnight. Blocks were thin-sectioned on a Reichert-Jung microtome and mounted on copper grids. Sections were poststained with uranyl acetate and Reynold's lead citrate. Microscopy was performed on a JEOL 120 EX electron microscope.

Phagocytosis. Macrophages were obtained by isolating monocytes from human peripheral blood as previously described by Astarie-Dequeker *et al.* (1999); these monocyte-derived macrophages (MDMs) were cultured at 37 °C in 5% CO$_2$ for 6 to 7 days on sterile glass coverslips in 24-well tissue culture plates (5 × 10^5 cells per well) containing RPMI medium supplemented with 10% inactivated foetal calf serum and antibiotic; the culture medium was renewed on the third day. Before use, MDMs were washed twice with fresh serum-free RPMI and equilibrated for 20 min at 37 °C in 5% CO$_2$. Single cells of *M. smegmatis* were prepared and fluorescently labelled

with FITC as previously described (N'Diaye *et al.*, 1998). MDMs were incubated with *M. smegmatis* in serum-free RPMI, for the time indicated, and then washed twice with fresh medium to remove unbound particles. Phagocytosis of FITC-stained bacteria was determined as previously described (Peyron *et al.*, 2000). Briefly, MDMs were fixed with 3·7% paraformaldehyde in PBS containing 15 mM sucrose, pH 7·4, for 20 min at room temperature. After neutralization with 50 mM NH$_4$Cl, extracellular mycobacteria were labelled with rabbit polyclonal antibodies directed against mycobacteria (1/50), revealed by TRITC-conjugated secondary antibodies. MDMs containing at least one FITC-stained bacterium (∼100 cells) were counted in duplicate samples.

RESULTS

Phenotypic characterization of a GPL-deficient mutant

The TM99 mutant strain is derived from *M. smegmatis* mc^{2}155 by transposon insertion mutagenesis (Billman-Jacobe *et al.*, 1999). Genetic analysis revealed that this phenotype was the result of the insertion of a transposon in the *mps* gene encoding a mycobacterial nonribosomal peptide synthetase, while lipid compositional analysis indicated that the strain was defective in the production of GPLs (Billman-Jacobe *et al.*, 1999). Strain TM99 displayed a rough colony morphology, compared to the smooth aspect of the mc^{2}155 parent strain (Fig. 2); these data were consistent with previous observations that showed a correlation between the absence of C-type GPL and the rough morphotype (Barrow & Brennan, 1982; Belisle *et al.*, 1991; Recht *et al.*, 2000).

When the motility of the strains on agar plates were compared (Table 1), the TM99 strain was found to display a spreading halo lower than that of the parent mc^{2}155 strain. Knowing that the diameter of the spreading halo is highly susceptible to the experimental conditions, depending particularly on the moisture at the surface of the medium, the above result was better expressed in terms of relative motility; accordingly, the relative motility of the mutant strain was consistently three- to fivefold smaller than that of the parent strain. The mutant strain exhibited also identical macroscopic aspects in broth cultures, both in shaken and static (in pellicles) growth conditions: under agitation and even in the presence of Tween 80, the mutant grew as huge aggregates, which became perceptible to the naked eye within 3 days, whereas mc^{2}155 rarely displayed aggregates, visible only by microscopy and composed of 3 to 5 cells. In addition, while the parent strain formed thin (1 mm height) pellicles that could hardly persist for more than 4 days on Sauton's medium, cells from the mutant strain were heavily clumped and gave thick and robust (3–5 mm height) pellicles that persisted at the surface of the medium for more than 10 days. These phenotypic changes were neither attributable to the growth rates of the strains, since they exhibited a similar generation time (3·5–4 h in LB medium), nor to cell lysis. These data indicated that the absence of GPL resulted in marked effects on the surface properties of the mutant cells.
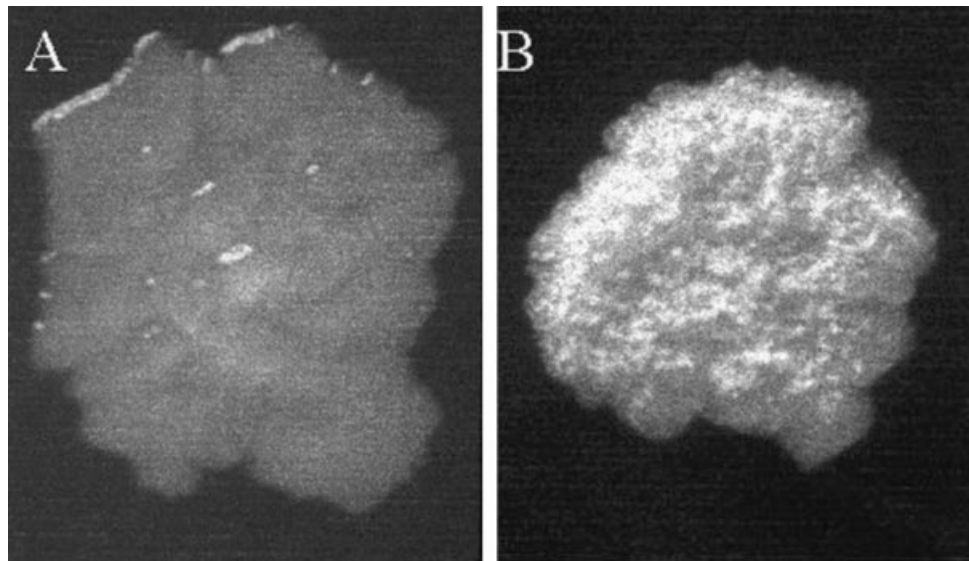
Fig. 2. Colony morphology of *M. smegmatis* mc²155 (A) and its isogenic *mps*-inactivated mutant TM99 (B) on Luria agar.

Table 1. Phenotypic and surface properties of the wild-type (mc²155) and *mps*-inactivated (TM99) strains of *M. smegmatis*

Results are expressed as mean ± SD of at least three independent experiments.

Surface properties	mc²155	TM99
Motility*	29·1 ± 18·7	8·7 ± 1·9#
Cellular aggregation†	20·1 ± 3·1	97·9 ± 0·4#
Congo red binding‡	211 ± 81	439 ± 97¶
Hydrophobicity index§	34·9 ± 19·7	51·7 ± 18·5#
Zeta potential‖	−27·6 ± 1·2	−28·1 ± 3·6

* Diameter (in mm) of the halo formed.

† Percentage of aggregated cells.

‡ $10^{-4} \times A_{488}$.

§ Arbitrary units, expressed as the percentage of cells recovered in the organic phase.

‖ Expressed in mV.

¶ $P < 0.05$ — when compared with control calculated with paired (motility and hydrophobicity) or unpaired Student's t test.
\# $P < 0.01$

Cell surface properties of the *mps*-inactivated strain TM99

To tentatively quantify the aggregation level of the mycobacterial cells, we used the method of fractionation of mycobacterial cultures into large and small aggregates, and single cells (Cougoule *et al.*, 2002) and determined the percentages of cells (wet weight) that pelleted at 100 *g*, i.e. larger clumps. Cells from strain TM99 were found to aggregate fivefold more than did the parent strain (Table 1). This phenomenon could be due to the change of the cell surface hydrophobicity, which is known to play a major role in the cellular adhesion of *Corynebacterineae* (Bendinger *et al.*, 1993; Borrego *et al.*, 2000; see also Goren & Brennan, 1979), and useful information may be obtained with use of Congo red, a planar and hydrophobic diazo-dye that binds lipids, lipoproteins and a broad range of other macromolecules (Cangelosi *et al.*, 1999). Consistent with an increase of the cell surface hydrophobicity, cells from strain TM99 bound twice as much dye as the mc²155 strain (Table 1). As the Congo red binding assay was performed on non-fractionated cultures, the possibility existed that the observed difference above was, at least in part, due to a trapping of the dye in large cellular clumps of TM99 rather than a real cellular accumulation per se. To address this question, mycobacterial cultures grown for 3 days in the presence of Congo red were washed, and aliquots were sonicated for various time periods, to disrupt aggregates of variable sizes into single cells. The resulting cells were washed again until colourless supernatants were obtained; the dye was then extracted from single cells and the binding determined. Whatever the sonication times used (data not shown) similar amounts of dye per mg wet weight were consistently associated with cells; in addition, this phenomenon was independent of the proportion of aggregates in the suspensions. It was thus concluded that the observed Congo red accumulation likely reflected the surface properties of individual cells. To further ascertain the difference in hydrophobicity between the parent and mutant strains, the hexadecane partition procedure was used (Rosenberg *et al.*, 1980). This rapid quantitative assay is based on the degree of interaction of cells with liquid hydrocarbon following a brief period of mixing. Again, cells from strain TM99 exhibited a hydrophobicity index significantly greater than that of the parent strain (Table 1); in a typical experiment,
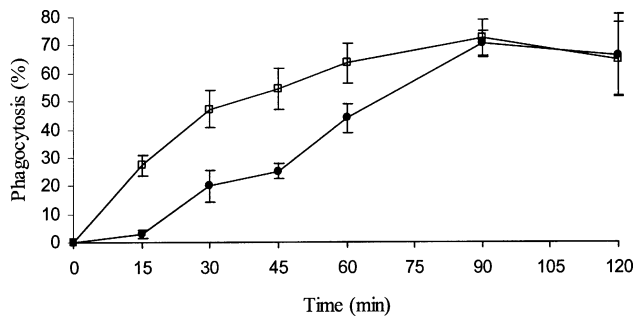
Fig. 3. Phagocytosis of the mc²155 (●) and the *mps*-inactivated TM99 (□) strains of *M. smegmatis* by human MDMs. MDMs were exposed for various times to FITC-labelled mycobacteria (cell:bacteria ratio, 1:50), then washed and fixed. At least 100 cells per slide were counted under fluorescence microscopy. The percentage of phagocytic cells having ingested at least one bacterium is expressed as the mean±SD of at least three independent experiments performed in duplicate.

52·5% of cells from strain TM99 cells were extracted with hexadecane whereas only 27·9% of those from strain mc²155 were recovered in the organic phase.

Most prokaryotic cells present a negative surface charge, a parameter that may contribute to the adhesive properties of cells (Van Loosdrecht *et al.*, 1987; Bendinger *et al.*, 1993). Although C-type GPLs of *M. smegmatis* are neutral lipids (Daffé *et al.*, 1983), the absence of these molecules may induce a change in the distribution of surface-exposed lipids of the mutant, resulting in a relative abundance of phospholipids already present in the outermost compartment of the envelope (Ortalo-Magné *et al.*, 1996) whose phosphate groups may influence the surface net charge of cells. Because electrophoresis allows the examination of large populations of microorganisms and has been demonstrated to be the most appropriate method among those experimentally examined for measuring the net charge of bacteria cells (Bayer & Sloyer, 1990), strain TM99 and its parent strain were compared by this technique. No significant difference was found between the negative zeta-potential displayed by the two strains (Table 1).

Internalization of the *mps*-disrupted mutant

M. smegmatis is internalized by MDMs through receptors that include the mannose receptor (Astarie-Dequeker *et al.*, 1999). To investigate the consequence of the phenotypic changes induced by the absence of C-type GPLs from the surface of strain TM99 on the nonopsonic phagocytosis of the bacterial cells, the kinetics of internalization of individualized cells from the wild-type and mutant strains were compared (Fig. 3). A high rate of phagocytosis of both strains of *M. smegmatis* (70%) was observed when bacterial cells were incubated with MDMs for 90 min. Interestingly, while only 5% of MDMs internalized the wild-type strain in the early 15 min, mutant cells were phagocytosed by 40% of the MDMs during the same period; a significant difference in the uptake rate of individual

bacterial cells from the two strains was still observed up to 1 h after the addition of bacteria. After this time period, however, the percentages of MDMs having internalized both types of mycobacterial cells were very similar. This observation indicated that the absence of C-type GPLs from the bacterial cell surface induced a very rapid internalization of *M. smegmatis* cells by MDMs.

Composition of the extracellular material of the *mps*-inactivated mutant

In most published chemical models of the mycobacterial cell envelope, type- and species-specific lipids such as GPLs are proposed to form a monolayer of lipid that covers the entire mycobacterial surface (Brennan & Nikaido, 1995; Liu *et al.*, 1995; Minnikin, 1982). One possible consequence of the absence of GPLs from the mutant cells may be the release of surface-exposed components of the mutant into the culture medium, assuming that GPLs play a crucial structural role in the architecture of the outermost layer of the parent strain; accordingly, the absence of C-type GPLs would cause a disorganization of the layer and the release of some of its components. To test this hypothesis, the composition of the extracellular material of the mutant was compared to that of the parent strain. The amounts of extracellular compounds in LB culture broth could not be determined, due to the high content of peptones and carbohydrate in this medium. Nevertheless, reasoning that the LB medium of strain TM99 should contain some lipids if the bacterial outermost constituents were released from the bacterial surface, the LB culture fluids from the mutant and parent strains were extracted with organic solvents and the organic phases were analysed. No significant amount of lipids was detected in these extracts. To compare the amounts of materials released into the culture fluids, the two strains were grown in synthetic Sauton's medium. Similar amounts of carbohydrate and protein were found in the culture fluids of the two strains which exhibited the same sugar composition (data not shown) that was similar to that previously found for *M. smegmatis* (Lemassu *et al.*, 1996). Thus, the absence of GPLs from the mutant strain does not induce the release of surface components into the culture fluid.

Composition of the SEM of the *mps*-disrupted mutant

Even if GPLs are not important for the architecture of the outermost layer, they are assumed to be abundant and known to be surface-located. If so, the absence of GPLs from the surface of mutant cells should induce the exposure of the substances (such as trehalose dimycolates) that are known to be located in deeper compartments of the cell envelope of the parent strain (Ortalo-Magné *et al.*, 1996). To address this question, the nature and relative amounts of the SEM of the mutant were determined and compared to those of the parent strain (Table 2). Gentle shaking of the bacterial cells with glass beads, a technique that declumps aggregates by extract-

Table 2. Lipid composition of the wild-type (mc²155) and *mps*-inactivated (TM99) strains of *M. smegmatis*

The lipid composition is shown as relative percentages of radioactivity incorporated into lipids.

Lipid	mc²155		TM99	
	Bead-treated bacterial lipids	Surface-exposed lipids	Bead-treated bacterial lipids	Surface-exposed lipids
Triacylglycerols	13·9	Trace	16·1	Trace
Glycopeptidolipids	18·3	84·6	0·0	0·0
Trehalose dimycolates	8·8	0·0	10·4	0·0
Trehalose monomycolates	11·2	Trace	13·1	Trace
Phospholipids	47·8	13·3	60·8	95·0

ing the amorphous material covering cells as observed by scanning electron microscopy (Ortalo-Magné *et al.*, 1995, 1996), was used. The amount of SEM extracted with glass beads from the surface of the mutant was similar to that obtained from the parent strain (15 to 16% of the cell dry weight). The carbohydrate and protein contents of the SEMs from the two strains were comparable. The percentages of carbohydrate in the SEMs of the parent and the mutant strains were estimated to 13·2% and 13·8% of the dry weight, respectively; the sugar compositions of the SEMs from both origins were similar and consisted of arabinose, mannose, xylose and glucose (Lemassu *et al.*, 1996). Protein represented 59·3% and 75·4% of the dry weight of the SEMs of the parent and mutant strains, respectively. Surface lipids represent a very minor fraction (0·02–0·1% bacterial dry weight) of mycobacteria grown on synthetic Sauton's medium (Lemassu *et al.*, 1996; Ortalo-Magné *et al.*, 1996). Consequently, bacterial cells of the two strains grown to exponential phase cells were labelled with sodium [¹⁴C]acetate before extracting SEMs to easily quantify surface-exposed lipids. In contrast, larger amounts of lipids were extracted from the SEMs of both strains grown on LB medium. They represented 3·4% and 2·3% bacterial dry weight of the parent and mutant strains, respectively. C-type GPLs represented 85% of the radioactivity found in the surface-exposed lipids from the wild-type strain grown on both media and were not detected in lipids from strain TM99 (Table 2), as expected. Lipids extracted from the parent and mutant strains after bead treatment represented 10·5 and 11·2% of the cell dry weight, respectively. The lipid composition of the materials originated from both strains was similar, except for the absence of C-type GPLs, and consisted of triacyl glycerols, trehalose monomycolates, trehalose dimyco-lates and phospholipids. Importantly, it has to be noted that trehalose dimycolates, which were not detected in the surface-exposed lipids from the parent strain, were also absent from the SEM of strain TM99. This data indicated that the absence of GPLs from the mutant strain does not induce the exposure of lipids that are buried in deeper compartments of the cell envelope.

GPLs represented the major lipid constituents of the

parent strain of *M. smegmatis* grown on LB both (Table 2); they remained a prominent class of lipids of bacterial cells grown in the Sauton's medium, although more triacyl glycerols were produced by bacteria grown on this glycerol-rich medium (data not shown). Based on the relative percentages of labelled lipids and the amounts of lipids extracted from SEMs it was estimated that 50% of GPLs were localized in the outermost layer of the parent strain of *M. smegmatis* grown in LB medium. The remaining GPLs were present in deeper compartments of the cell envelope of the parent strain and extracted after bead treatment of the cells with organic solvents.

Ultrastructural features of the *mps*-inactivated strain TM99

Cells from *M. smegmatis* strain mc²155 and its isogenic TM99 mutant were grown in both LB and Sauton's media and examined by electron microscopy (Fig. 4). Ultrathin sections of cells were stained with ruthenium red, a stain that strongly reacts with the surface of mycobacteria (Rastogi *et al.*, 1984). Examination of thin sections revealed a cell envelope structure of *M. smeg-matis* strain mc²155 (Fig. 4A) composed of (i) a plasma membrane, (ii) a thick internal electron-dense layer, (iii) an electron-transparent layer and (iv) a thick electron-dense outer layer (OL); the space observed between the plasma membrane and the thick electron-dense layer corresponds to the hypothetical periplasmic space (Daffé & Draper, 1998). This ultrastructural appearance is similar to that previously found in other mycobacterial species (Daffé & Draper, 1998; Draper, 1982; Paul & Beveridge, 1992; Rastogi *et al.*, 1986). In sharp contrast, the thick electron-dense OL stained with ruthenium red and observed on the thin sections of the parent *M. smegmatis* strain mc²155 (Fig. 4A) was unlabelled in those of the cells from the isogenic *mps*-inactivated strain TM99 (Fig. 4B). However, the thickness of the electron-transparent layer of the mutant roughly corresponded to the sum of the thickness of the electron-transparent layer and that of the OL of the parent strain. These data suggested that the absence of staining of the OL of the mutant strain was not due to the loss of this layer but, rather, may be attributed to absence of GPLs
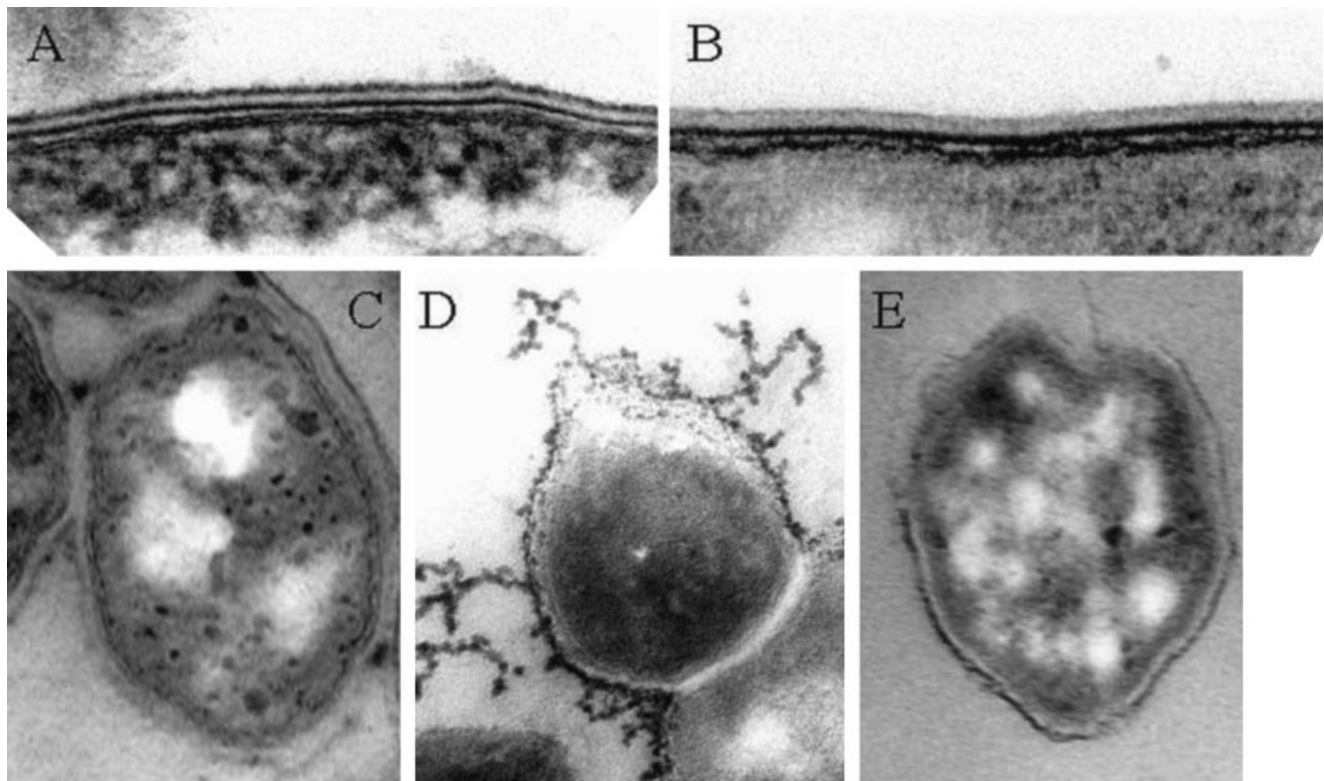
Fig. 4. Transmission electron micrographs of *M. smegmatis* strain mc²155 (A, D and E) and its isogenic *mps*-inactivated strain TM99 (B and C). Strains were grown either in LB (A, B and D) or Sauton's medium (C and E).

from the surface of the mutant cells which would bind ruthenium red. This conclusion was further supported by the observation of the thickness of this layer in adjacent cells (Fig. 4C). That the staining of OL in the parent strain by ruthenium red is attributable to GPLs is supported by the correlation between the ultrastructural appearance of stained cells and the amounts of surface-exposed GPLs. When large amounts of GPLs are present on the cell surface of the parent strain (growth in the LB medium, see above), the whole surface of the bacilli is stained by the dye that also interacts with GPLs budding from the cell surface and with an appearance of filamentous and rope-like structures (Fig. 4D). When cells exposed less amounts of GPLs on their surface (growth in Sauton's medium, see above) they are stained less by ruthenium red (Fig. 4E). These data establish that the dye binds to the OL through interactions with surface-exposed GPLs.

Cell wall permeability of the mutant

Because GPLs are major lipids of *M. smegmatis* (Table 2) and at least 50 % of GLPs are not surface-exposed but located in deeper compartments of the cell envelope, it was interesting to address the question of the possible implication of C-type GPLs in the cell wall permeability barrier. To determine whether this barrier was altered in the *mps*-inactivated mutant of *M. smegmatis*, the uptake of the hydrophobic chenodeoxycholate by cells of the
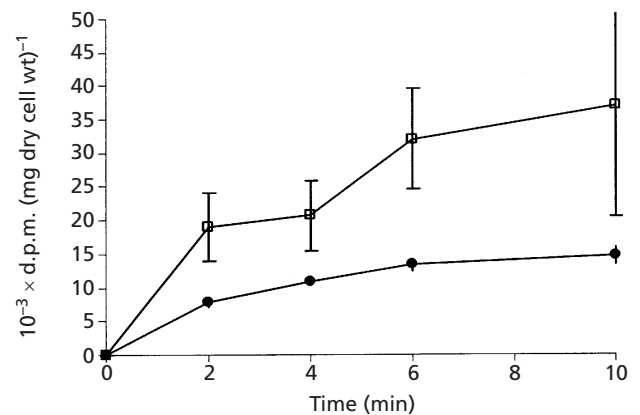

Fig. 5. [^{14}C]-Chenodeoxycholate uptake by the mc²155 (●) and the *mps*-inactivated TM99 (□) strains of *M. smegmatis*. Exponential-phase cells were first incubated overnight with [5,6-^{3}H]uracil to quantify the biomass present in aliquots used in the accumulation assays. Accumulation assays of [^{14}C]chenodeoxycholate were performed in 1 ml HEPES buffer under continuous agitation; aliquots were taken at different time intervals and counting (in d.p.m.) was performed. The values are the mean±SD of at least three independent experiments.

wild-type strain mc²155 and the TM99 mutant were compared (Fig. 5). This probe is a negatively charged hydrophobic molecule that has been previously used to

evaluate the fluidity of the mycobacterial cell wall lipids (Liu *et al.*, 1996; Yuan *et al.*, 1997). The mutant showed a significantly higher initial rate of uptake and accumulation of chenodeoxycholate than did the parent strain. This result indicated that C-type GPLs contribute to the permeability barrier of the cell wall of *M. smegmatis*.

The permeability of the cell wall will determine whether certain antibiotics can enter bacteria and achieve their inhibitory action; we therefore investigated the susceptibility of strain TM99 and its parent to various molecules. The MICs of the molecules examined were unaffected by the mutation; these included the relatively hydrophobic rifampicin (256 μg ml^{-1}), chloramphenicol (32 μg ml^{-1}) and novobiocin (32 μg ml^{-1}), and the hydrophilic hygromycin B (64 μg ml^{-1}), isoniazid (4 μg ml^{-1}) and streptomycin (2 μg ml^{-1}). These data indicated that the diffusion of these antibiotics through the cell wall barrier was not the limiting step for their activity. A similar observation has been made for the *fbpC*-inactivated mutant of *Mycobacterium tuberculosis*, which exhibited a change in its cell wall permeability to chenodeoxycholate due to a 40% reduction of covalently linked mycolates (Jackson *et al.*, 1999).

DISCUSSION

The present study addressed the question of the function of C-type GPLs, one of the major species- or type-specific lipid constituents of the cell envelopes of many non-tuberculous mycobacteria (Brennan, 1988; Daffé & Lemassu, 2000), in the physiology of these bacteria. For this purpose, a mutant derived from *M. smegmatis* mc²155 by transposon insertion mutagenesis, strain TM99, and previously shown to be defective in the production of C-type GPLs (Billman-Jacobe *et al.*, 1999) was analysed. Genetic analysis has revealed that the mutation in TM99 was the result of the insertion of the transposon in the *mps* gene encoding a mycobacterial nonribosomal peptide synthase. The same phenotype has been found for two insertional mutants of *M. smegmatis* mc²155, namely strains 9-1045 and 8-245 (Billman-Jacobe *et al.*, 1999; Recht *et al.*, 2000). While the transposon was localized in the *mps* gene in strain 9-1045, in strain 8-245 the mutation occurred in the *tmtpC* ORF encoding a putative transport membrane protein (Recht *et al.*, 2000). The *tmtpC*-disrupted mutant has been shown by labelling experiments to be unable to synthesize GPLs, suggesting that the encoded protein may be involved, not only in the transport of the glycolipids, but also in the production of GPLs (Recht *et al.*, 2000). Compared to the wild-type smooth morphotype strain, the C-type-GPL-deficient mutant strains TM99, 9-1045 and 8-245 exhibited a rough colony morphology (this work, Recht *et al.*, 2000). This observation was in agreement with previous studies on GPL-deficient *M. avium–intracellulare* (Barrow & Brennan, 1982; Belisle *et al.*, 1991). The absence of C-type GPLs also affects the surface properties of strain TM99. This mutant strain, as well as strains 9-1045 and 8-245 (this work, Recht *et al.*, 2000), was found to display a spreading halo smaller

than that of the parent mc²155 strain, but similar to those of the other C-type GPL-negative mutants. In addition, the cell hydrophobicity of the mutants were significantly higher than that of their parent strain. As a consequence, the three mutant strains also exhibited identical macroscopic aspects, both in shaken and static cultures. A similar observation has been made for strains of *Gordona* devoid of a specific glycolipid; colonies of these latter strains exhibited a rough morphology and were composed of highly aggregated cells possessing a more hydrophobic cell surface than the smooth morphotypes (Moormann *et al.*, 1997).

In *M. smegmatis*, the phenotypic changes were associated with the absence of binding of ruthenium red to the surface of the mutant cells, as observed by transmission electron microscopy. This data implies that the surface-exposed C-type GPLs of *M. smegmatis* are the substances that react with ruthenium red to give the electron-dense appearance of the outermost cell envelope layer. The absence of these compounds, however, does not cause the disorganization of the outer layer since no specific release of surface constituents into the culture broth of the mutant was observed. Consistent with this observation, compounds such as trehalose dimycolates that are located in deeper compartments of the cell envelope of the parent strain (Ortalo-Magné *et al.*, 1996) were not surface-exposed in the mutant. These data reinforce the credibility of the recent model (Daffé & Draper, 1998) in which the surface of mycobacteria is composed of polysaccharides, proteins and lipids, as opposed to models in which the surface consists of a lipid layer (Brennan & Nikaido, 1995; Liu *et al.*, 1995; Minnikin, 1982). Nevertheless, at the interface between the bacterial and host cells, surface-exposed GPLs may play a role in the internalization of C-type GPL-containing non-tuberculous mycobacteria by macrophages which are believed to use glycoconjugates as ligands of many of their receptors (Ehlers & Daffé, 1998). Accordingly, phagocytosis of the wild-type strain and its isogenic *mps*-inactivated strain of *M. smegmatis* by human MDMs was investigated. As expected from the existence of numerous receptors capable of internalizing mycobacteria, both strains were found in phagosomes but the kinetics of phagocytosis of the mutant was much more rapid than that of the parent strain. This result suggests that either GPLs may mask some as yet undefined ligands that interact with highly efficient macrophage receptor(s) or, alternatively, the bacterial cell surface hydrophobicity may play an important role in the rate of internalization of bacteria. Further studies are warranted to determine the precise role of GPLs in the internalization of mycobacteria.

GPLs are the major extractable lipids of *M. smegmatis* and other non-tuberculous mycobacterial species containing these molecules and a large portion of these substances are localized in the deeper compartments of the wild-type *M. smegmatis* envelope, presumably in the mycolic acid-containing asymmetric bilayer. It was therefore important to investigate the consequence of the absence of GPLs on cell wall architecture, especially

its implication in the outer permeability barrier. In all currently proposed models (Brennan & Nikaido, 1995; Daffé & Draper, 1998; Liu *et al.*, 1995; Minnikin, 1982; Rastogi, 1991) the outer permeability barrier of mycobacteria consists of a monolayer of mycoloyl residues covalently linked to the cell wall arabino-galactan and includes other lipids which are probably arranged to form a bilayer with the mycoloyl residues. Although no sign of a second lipid bilayer has ever been reported in thin sections of mycobacterial cells (Draper, 1998), freeze-fractured samples of mycobacteria (Barks-dale & Kim, 1977; Benedetti *et al.*, 1984; Takeo *et al.*, 1984) showed that these organisms had two such planes of weakness in their envelopes; in addition to the expected plasma membrane fracture, a second fracture plane, close to the cell surface of mycobacteria, was observed. The cell wall-linked mycolates certainly participate in this barrier since the disruption of a gene that encodes a mycoloyltransferase, namely antigen-85C, causes a decrease in the amount of cell wall-bound mycolates and affects the permeability of the envelope of the mutant (Jackson *et al.*, 1999). Evidence has also been presented that the chemical structure of mycolic acids plays a role in determining the fluidity and permeability of the mycobacterial cell wall (George *et al.*, 1995; Liu *et al.*, 1996; Dubnau *et al.*, 2000). To date, however, the implication of non-covalently bound lipids in the wall bilayer has been demonstrated for only phthiocerol dimycocerosates of *M. tuberculosis* (Camacho *et al.*, 2001). The present work demonstrated that the absence of C-type GPLs from the cell envelope of *M. smegmatis* has a profound effect on the uptake of chenodeoxy-cholate, a hydrophobic molecule that diffuses through lipid domains of the mycobacterial cell wall (Liu *et al.*, 1996; Yuan *et al.*, 1997). Based on the localization of cell wall fracture plane in mycobacteria (Barksdale & Kim, 1977; Benedetti *et al.*, 1984; Takeo *et al.*, 1984), far from the cell surface, it is likely that the GPLs located in inner compartments of the cell envelope, but not surface-exposed GPLs, contribute to the permeability barrier.

In conclusion, in addition to be distinctive markers of numerous mycobacterial species that may help in the diagnosis of non-tuberculous diseases and for taxonomic purposes, GPLs are also involved in the wall permeability barrier that certainly plays a role in bacterial physiology.

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