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Identification of the Surface-Exposed Lipids on the Cell Envelopes of *Mycobacterium tuberculosis* and Other Mycobacterial Species

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The surface-exposed lipids of *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium kansasii*, *Mycobacterium gastri*, *Mycobacterium smegmatis*, and *Mycobacterium aurum* were isolated by gentle mechanical treatment of cells with glass beads. Analysis of the exposed lipids demonstrated a selective location of classes of ubiquitous lipids on the surfaces of mycobacteria. While phosphatidylethanolamine and phosphatidylinositol mannosides were exposed in all the species examined, dimycoloyl trehalose ("cord factor") was identified in the surface components of *M. aurum* only. Furthermore, monomycoloyl trehaloses and triacylglycerides were identified in the surface-exposed lipids of *M. avium* and *M. smegmatis* but not in those of the other mycobacterial species examined. The species- and type-species specific lipids were present on the mycobacterial cell surface: phenolic glycolipids, dimycocerosates of phthiocerols, and lipooligosaccharides were identified in the surface-exposed materials of *M. tuberculosis* (Canetti), *M. kansasii*, and *M. gastri*, whereas glycopeptidolipids were identified in the outermost lipid constituents of *M. avium* and *M. smegmatis*. This difference in the surface exposure of lipids of various mycobacterial species may reflect differences in their cell envelope organizations. Brief treatments of *M. tuberculosis* with Tween 80 prior to the use of glass beads led to erosion of regions of the capsule to expose gradually both cord factor and other lipids on the cell surface of the tubercle bacillus, demonstrating that the latter lipids are buried more deeply in the cell envelope and leading to the proposal of a scheme for the location of the capsular lipids of the tubercle bacillus.

Several mycobacterial species, like *Mycobacterium tuberculosis* and *Mycobacterium leprae*, are the causative agents of important human diseases. The former bacterium, which claims 3 million lives per year, is responsible for more deaths annually than any other single bacterial pathogen (8, 38). Knowledge of the architecture of the cell envelopes of mycobacteria is central to our understanding of some unsolved problems of mycobacterial diseases, such as recrudescence of tuberculosis and occurrence of the opportunistic mycobacterioses (43). The cell envelopes of *Mycobacterium* spp. are composed of two structures: a plasma membrane and a cell wall skeleton consisting of two covalently attached macromolecules, peptidoglycan and mycoloyl arabinogalactan (32, 43). These two layers are consistently found among mycobacterial species; however, the outermost layer is considered to have different molecular compositions in the various species (43). The most distinctive structural feature of pathogenic mycobacterial species is the presence of an electron-transparent zone, also called a capsule, which surrounds each bacterium (34) and may be part of the defense mechanism permitting these pathogens to resist killing by phagocytic cells (24).

Previous evidence has shown that inside host cells, *M. leprae* and *Mycobacterium lepraemurium* are surrounded by, respectively, a foamy and a fibrillar capsule (48). These capsules contain characteristic lipids of mycobacterial origin consisting, respectively, of phenolic glycolipids (PGLs [9, 35, 36]) and structurally related dimycocerosates of phthiocerols (DIMs [23, 35]) and glycopeptidolipids (GPLs [25]). Although other mycobacterial species grown on artificial media synthesize lipids, they have not been reported, with the possible exception of a strain of *M. avium*, to produce similar ultrastructurally distinctive capsules inside cells. In the case of *M. avium*, Rulong et al. (54) demonstrated the presence of multilamellar capsule-like structures, probably composed of GPLs, surrounding bacilli growing inside phagosomes of murine macrophages. However, it has been shown that both GPL-positive and GPL-negative strains of *Mycobacterium avium-M. intracellulare* are surrounded by capsules (53). Similarly, some tubercle bacilli, notably the Canetti and Canetti-like strains, synthesize large amounts of PGLs (12, 14, 19), whereas other virulent strains contain only traces, if any at all, of these molecules (11, 12, 51). These observations suggested that PGLs and GPLs are not the major components of the mycobacterial capsule and that *M. leprae* and *M. lepraemurium* are anomalous in producing such very large amounts of lipids (22).

We recently isolated the outermost capsular components of *M. tuberculosis* and demonstrated that they consist principally of polysaccharides and proteins (50); small amounts of lipids (1 to 6% of the dried bacterial mass) were also detected in the surface-exposed material (50), whereas only traces of lipids were found in the material shed into the culture medium (41). Because the interaction of the outermost constituents of the bacterial cell envelope with the phagocyte determines in part the immunological response of the host, the surface-exposed

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TABLE 1. Lipid composition and amounts of the materials extracted from the cell envelope of *M. tuberculosis* (Canetti)

Treatment	Lipids present[a]											Amt of recovered material[b]
	PGLs	LOSs	DIMs I	DIMs II	PE	PIMs	DATs	PPTs	DMTs	MMTs	TAGs	
Glass beads	+	+	+	+	+	+	+	−	−	−	−	2.4
Tween, 1 h	+	+	+	+	+	+	+	+	−	−	−	4.7
Tween, 4 h	+	+	+	+	+	+	+	+	Tr	−	−	5.8
Glass beads after Tween 1 h	+	+	+	+	+	+	+	+	+	−	−	1.4
Glass beads after Tween 4 h	+	+	+	+	+	+	+	+	+	−	−	0.8
Tween, 16 h	+	+	+	+	+	+	+	+	+	+	+	8.0

[a] DIMs, dimycocerosates of phthiocerols (I) or phthiodiolone (II). +, detected; −, not detected.
[b] Expressed as the mean value (in milligrams) of material per 100 mg of bacteria.

lipids may play a role in the pathogenesis of mycobacterial diseases. It is worthy of note that both PGLs and GPLs have been shown to be located on the cell surface (3, 9, 21, 29) and to exhibit various immunomodulatory properties (10, 26, 44, 52, 56). In the present study, we adopted a novel approach to study the outermost capsular components, based on the release of superficial components by gentle mechanical abrasion, to define the chemical nature of the surface-exposed lipids of *M. tuberculosis* and other pathogenic and nonpathogenic mycobacterial species. An attempt to distinguish between lipids on the actual surface and others buried more deeply in the capsule of *M. tuberculosis* was also made.

MATERIALS AND METHODS

M. tuberculosis H37Rv (ATCC 27294) and Canetti (CIPT 1400100-059), *M. avium* (ATCC 15769), *Mycobacterium kansasii* (ATCC 12478), *Mycobacterium gastri* (W471), and *Mycobacterium smegmatis* (ATCC 607) were grown on Sauton's medium (100 ml per flask) as surface pellicles at 37°C. *M. aurum* (strain A+) and *M. smegmatis* were grown on 7H9 Middlebrook broth enriched with 0.5% (wt/vol) Casitone and 1% (wt/vol) glucose at 37°C and harvested in the mid-log phase by centrifugation.

Mycobacterial cells were harvested by pouring off the medium and gently shaken for 1 min with 10 g of glass beads (4-mm diameter) per 2 g (wet weight) of cells (50). In a parallel experiment, the original culture medium of actively growing *M. tuberculosis* H37Rv was replaced by fresh Sauton's medium containing 1% (wt/vol) Tween 80, and the cells were shaken at 37°C for 1, 4, or 16 h. The cells were then harvested by centrifugation and treated with glass beads as described above. The declumped cells resulting from the treatment of bacteria with glass beads were suspended in distilled water (50 ml per flask) and immediately filtered through a 0.2-μm-pore-size sterile filter (Nalgene) to yield sterile surface-exposed materials. The filtrates derived from the treatment of bacteria with Tween 80 and/or glass beads were concentrated separately under vacuum to 1/10 of the original volume. Chloroform and methanol were added to the aqueous solutions to obtain a partition mixture composed of chloroform-methanol-water (3:4:3, vol/vol/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 a few milliliters of chloroform and analyzed by thin-layer chromatography (TLC) on Silica Gel 60-precoated plates (0.25-mm thickness; E. Merck, Darmstadt, Germany). The plates were developed with the following: petroleum ether-diethyl ether (9:1 and 7:3 [vol/vol], respectively) for analyzing DIMs (15) and triacyl glycerols (TAGs); chloroform-methanol (99.5:0.5 [vol/vol]) for polyphthienoyl trehaloses (PPTs [13]); chloroform-methanol (9:1 [vol/vol]) for PGLs, GPLs, and 6,6′-dimycoloyl trehaloses (DMTs); and chloroform-methanol-water (30:8:1 [vol/vol/vol]) for 6-monomycoloyl trehaloses (MMTs), 2,3-diacyl trehaloses (DATs), and lipooligosaccharides (LOSs) (18, 42). Phospholipids were analyzed by developing the TLC plates with chloroform-methanol-water (65:25:4 [vol/vol/vol]). When the crude materials extracted with Tween 80 were analyzed, it was difficult to observe well-resolved spots, probably because of the presence of huge amounts of the detergent. Thus, the crude lipid extracts derived from the treatment of cells with Tween 80 were precipitated twice with 6 volumes of cold ethanol, and the precipitates were applied to Florisil columns (60/100 mesh), which were eluted successively with 100 ml of chloroform, with 100 ml of increasing concentrations of methanol in chloroform (5, 10, 20, 30, and 50% [vol/vol]), and finally with 100 ml of a mixture of chloroform, methanol, and water (65:25:4 [vol/vol/vol]). The crude lipid extracts derived from the mechanical treatment of cells with glass beads were also fractionated on Florisil, as described above. The eluates were concentrated and analyzed by TLC. Sugar-containing compounds were visualized by spraying plates with 0.2% anthrone in concentrated sulfuric acid, followed by heating at 110°C. The Dittmer-Lester reagent (20) was used for revealing phosphorus-containing substances. A ninhydrin reagent was used to reveal the presence of free amino groups.

Cells were extracted with chloroform-methanol (2:1 [vol/vol]), and the lipid extracts were evaporated to dryness. Delipidated cells, lipid extracts, and the various fractions eluted from the Florisil column were saponified; the fatty acids were methylated and analyzed by gas chromatography as described previously (16). Mycolic methyl esters were analyzed by TLC with petroleum ether-diethyl ether (9:1 [vol/vol], four runs) and dichoromethane as developing solvents (16). For sugar analyses, a sample (1 mg) of the chromatographic fractions or purified lipids was routinely hydrolyzed with 2 M aqueous trifluoroacetic acid (Sigma, St. Louis, Mo.) solution at 110°C for 1 h. The hydrolysates were then partitioned between chloroform and water. The aqueous phases were dried under nitrogen, subjected to trimethylsilylation, and analyzed by gas chromatography with a Girdel G-30 apparatus equipped with a fused silica column (25-m length by 0.22-mm internal diameter) coated with OV-1 (0.3-mm film thickness). A temperature gradient of 100 to 280°C (2°C min^{-1}) was used.

The bacteria were examined by scanning electron microscopy as previously described (50).

RESULTS AND DISCUSSION

The tubercle bacillus and most other mycobacterial species are known to form large clumps, especially when growing in stationary liquid cultures (32). This phenomenon is illustrated in Fig. 1A. Gentle shaking of pellicles of *M. tuberculosis* with 4-mm-diameter glass beads declumps cells by extracting the amorphous covering material but does not affect their integrity (50). In the present study, this method of isolation of the outermost surface constituents was combined with shaking of exponentially grown tubercle bacilli with Tween 80 for 1 or 4 h or overnight. The latter treatment also extracted the surface-exposed material and prevented cell clumping (Fig. 1B). When treatment with Tween 80 was followed by the gentle mechanical shaking of the treated cells with glass beads, we expected the exposure of compounds which were buried more deeply in the capsules of the intact bacilli.

The materials extracted from the tubercle bacillus with glass beads and/or Tween 80 represented 1 to 8% of the bacterial dry mass, depending on the method used for extraction (Table 1). Treatment of the cells for 4 h with the detergent extracted more material than did 1 h of the same treatment. Similarly, treatment of cells for 16 h with Tween 80 led to a higher yield than did treatment for 4 h. These data suggested a gradual removal of the constituents from the cell surface. The different recovered materials were partitioned between chloroform, methanol, and water, and the organic phases were compared by TLC. As shown in Table 1, marked differences between the lipid compositions of the materials derived from the different treatments were observed.

Identification of the surface-exposed lipids of *M. tuberculosis*. The outermost surface-exposed lipids, i.e., the organic phase, represented 1 to 6% of the material removed by the mechan-

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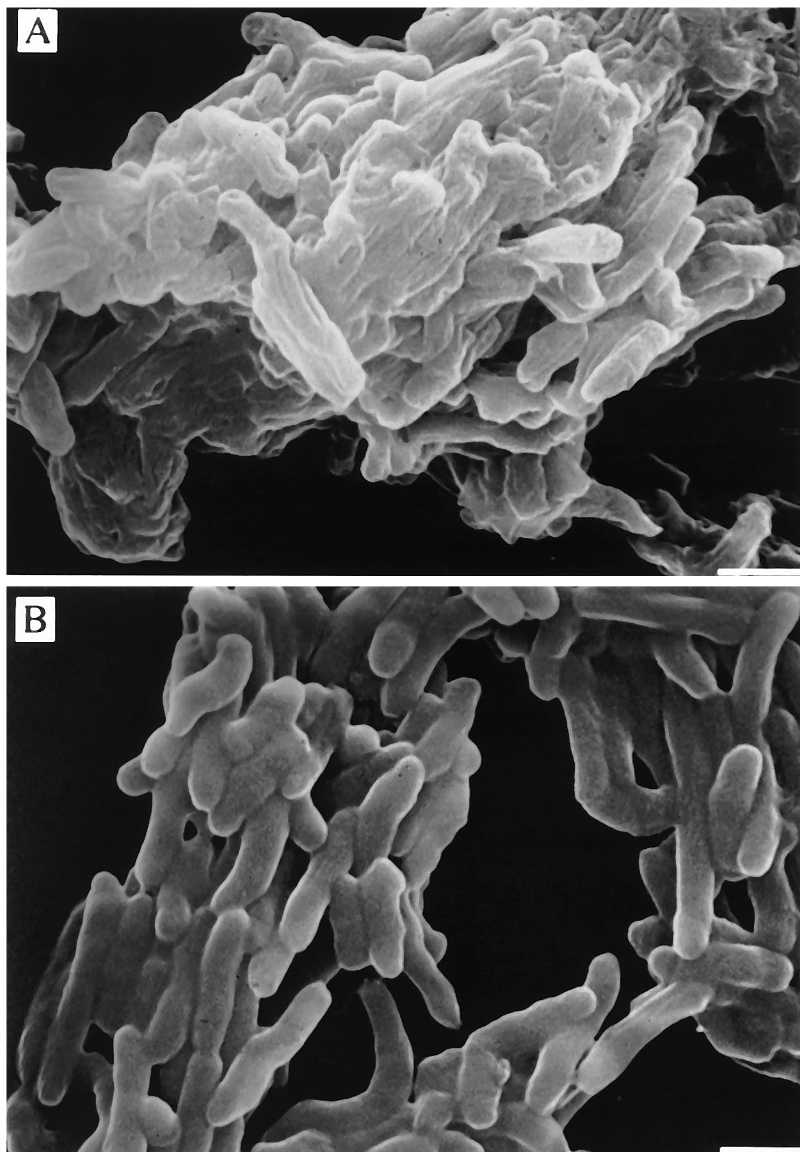

FIG. 1. Scanning electron microscopy of strain H37Rv of *M. tuberculosis* before (A) and after (B) overnight treatment with Tween 80. Bars = 1 μm.

ical treatment of bacilli with glass beads. It has to be noted that extraction of the material derived from the mechanical treatment of cells with petroleum ether-diethyl ether (1:1 [vol/vol]) also yielded small amounts of lipids, quantitatively comparable to those obtained by using water to recover the material extracted with glass beads (50). This observation suggested that the amount of recovered lipids reflected the quantity of lipids in the mixture of surface-exposed components. The remaining 95 to 99% was composed of proteins and polysaccharides (50). The surface-exposed lipids consisted of the type-specific PGLs (14) and LOSs (18) of the Canetti strain, the species-specific DIMs (15, 46) and DATs (2, 6, 42) of *M. tuberculosis*, and the ubiquitous phosphatidylinositol mannosides (PIMs) and phosphatidylethanolamine (PE). In contrast, the ubiquitous DMTs, MMTs, and TAGs (32, 45), as well as the type-specific PPTs (13), were not detected in the material removed with glass beads (Table 1). The failure to detect DMTs in this material was surprising in view of the old published data. Historically, DMTs were isolated from virulent cord-forming tubercle bacilli and were called cord factor because it was believed that they were responsible for the formation of cords (7, 32). Indeed, all the ubiquitous lipids, including DMTs, were detected in the lipids extracted from the glass bead-treated cells. The failure to detect these lipids among the surface-exposed components was not due to their hydrophobicity, as less polar compounds, such as DIMs, were easily detectable in the surface-exposed material extracted with glass beads.

Identification of buried capsular lipids of *M. tuberculosis*. When *M. tuberculosis* was treated for 1 h with Tween 80, the recovered material contained PPTs which were not detectable in the outermost material extracted with glass beads (Table 1). While treatment of bacilli with the detergent for 4 h led to the detection of traces of DMTs, treatment for 1 h with the same agent, followed by the subsequent treatment of cells with glass beads, was needed for the detection of significant amounts of DMTs in the cell surface-exposed material of the tubercle bacillus. This observation further substantiated the fact that the previous failure to detect this family of glycolipids was not

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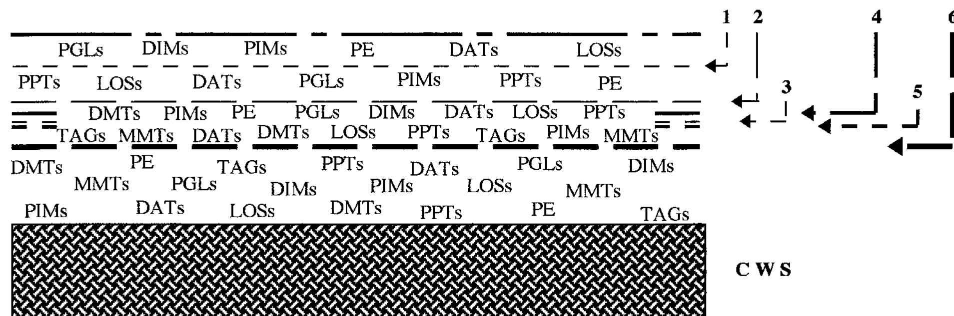

FIG. 2. Proposed scheme for selective location of classes of lipids in the polysaccharide-protein matrix of the capsule of the tubercle bacillus, based on data shown in Table 1, and illustration of the action of treatment of bacilli with glass beads and/or Tween 80. Materials were extracted with glass beads (1), Tween 80 for 1 h (2), glass beads after treatment with Tween 80 for 1 h (3), Tween 80 for 4 h (4), glass beads after treatment with Tween 80 for 4 h (5), and Tween 80 overnight (6). While the different extracted materials are drawn to a scale of relative amounts, the extent of the innermost layers is arbitrary. CWS, cell wall skeleton (peptidoglycan-arabinogalactan-mycolate).

due to the use of glass beads. The structurally related MMTs, as well as the ubiquitous TAGs, were detected only after treating cells for 16 h with the detergent (Table 1). These results demonstrated that not all the different classes of lipids were exposed on the surfaces of intact tubercle bacilli. While PGLs, DIMs, LOSs, DATs, and phospholipids were present in the outermost compartment of the capsule, DMTs, TAGs, and MMTs were located in an inner region of the capsule, TAGs and MMTs being buried more deeply in the capsule than DMTs. The lipid extracts from the various Tween-treated cells still contained all the classes of lipids, demonstrating that the type-specific as well as the species-specific lipids were not located only in the outermost compartment of the capsule. These data, schematized in Fig. 2, supported a concept of gradual extraction of the capsular material with glass beads and Tween 80. This concept was further substantiated by the lipid compositions of the different extracted materials, in that all the lipids found in the outermost capsular material were also detected in the Tween extracts (Table 1). It has to be noted that in the proposed scheme (Fig. 2), classes of lipids are selectively located in a polysaccharide-protein matrix.

Identification of the surface-exposed lipids of other mycobacteria. The failure to detect all the classes of lipids in the outermost capsular material of *M. tuberculosis* led us to investigate the distribution of lipids in the cell envelopes of other mycobacterial species. This was done by comparing the surface lipid extracts derived from the mechanical shaking of cells with glass beads (Table 2). The materials extracted with glass beads from the cell surfaces of the different species represented roughly 2 to 3% of the dried bacterial mass and contained

TABLE 2. Compositions of lipids extracted from the cell surfaces of various mycobacterial species by gentle shaking of cells with glass beads

Species	Lipids extracted[a]									
	PGLs	LOSs	DIMs I	DIMs II	GPLs	DMTs	MMTs	TAGs	PE	PIMs
M. kansasii	+	+	*	+	*	−	−	−	+	+
M. gastri	+	+	*	+	*	−	−	−	+	+
M. avium	*	*	*	*	+	−	+	+	+	+
M. smegmatis	*	*	*	*	+	−	+	+	+	+
M. aurum	*	*	*	*	*	+	+	+	+	+

[a] DIMs, dimycocerosates of phthiocerols (I) or phthiodiolone (II). +, detected; −, not detected; *, absent from the whole lipid extract of the strain.

small amounts of lipids, as in the case of *M. tuberculosis*. They represented 1 to 5% of the materials removed by the treatment with glass beads, the remaining constituents also being composed of polysaccharides and proteins (40). Among the ubiquitous mycobacterial lipids (32, 45), PE and PIMs were the lipids found in the outermost compartments of all the species. The presence of phospholipids as surface-exposed lipids is notable and merits consideration. The phospholipid compositions of mycobacterial cell fragments were studied earlier by several authors (1, 31, 49). In those studies, however, all the individual phospholipids were present in substantial proportions in all the fractions examined, and the technical problems involved in obtaining representative fractions were recognized (45). Thus, the impression that phospholipids were certainly membrane components which probably contaminated cell wall fractions prevailed. Therefore, our results clearly demonstrated that, in addition to being found in the plasma membrane, phospholipids are also located in the outermost region of the mycobacterial envelope.

M. kansasii and *M. gastri* showed the same profile as *M. tuberculosis*: PGLs (27, 57), DIMs (15, 46), and LOSs (30, 37) were surface exposed in these species, but DMTs, MMTs, and TAGs were not. The surface exposure of these species-specific lipids was in agreement with the immunocytochemical data showing the surface location of PGLs and LOSs on *M. kansasii* (4, 29) and with the identification of PGLs and DIMs in the petroleum ether extract of this species (39). In connection with that agreement, it is interesting that DIMs, PGLs, and TAGs were detected in a petroleum ether extract of *M. kansasii* (39). It is likely that the organic solvent extracted not only the surface-exposed material but also other cell constituents of bacilli.

The species-specific GPLs were also detected in the materials extracted with glass beads from *M. avium* and *M. smegmatis* (Table 2). The surface location of GPLs was in accordance with the isolation and characterization of GPLs from the capsule of *M. avium-M. intracellulare* (3, 21), with the identification of this class of mycobacterial glycolipid as the receptor of mycobacteriophage D4 (28, 33), and with GPLs being the Schaefer typing antigens (32). The immunochemical data obtained for *M. avium-M. intracellulare* also supported the location of GPLs in the outermost region of the cell envelope. The ubiquitous MMTs and TAGs were also surface exposed in these two species, but DMTs were not. It is worth noting that the detection of TAGs in the surface-exposed components of three mycobacteria, namely, *M. avium*, *M. aurum*, and *M. smeg-*

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matis (Table 2), and in the inner region of the capsule of *M. tuberculosis* (Table 1) was surprising in that these lipids have been regarded as storage compounds found in the bacterial cytoplasm (32).

M. aurum was the only species in which DMTs were exposed on the cell surface (Table 2). As the strain of *M. aurum* used was grown on a modified Middlebrook medium (because it did not grow on glycerol-containing media), we also analyzed the surface-exposed lipids of *M. smegmatis* grown on the same medium. No difference was observed between the lipid composition of the material removed from the cells of *M. smegmatis* grown on Sauton's medium and that of the material removed from cells grown on Middlebrook medium by the mechanical treatment with glass beads (data not shown).

In conclusion, the present study demonstrates that mycobacteria are not identical in terms of surface exposure of the various classes of lipids, probably reflecting differences in their cell envelope organizations. This finding is interesting in that no such differences between species in the arrangement of surface-exposed lipids are mentioned in the general models which have been proposed. The present study did not investigate the arrangement of the peptidoglycan-arabinogalactan-mycolate and associated lipids. Thus, the mono- or bilayer models currently proposed for this mycobacterial cell envelope region still remain possible, providing opportunities for further studies. Nevertheless, the data presented in Fig. 2 show that lipids are present in the outermost region of the mycobacterial cell envelope in a protein-polysaccharide matrix, requiring models to be modified accordingly.

On the basis of the surface-exposed lipid compositions described here, the six mycobacterial species investigated may be arranged into three groups. The first group is composed of *M. tuberculosis*, *M. kansasii*, and *M. gastri*; on outermost cell surfaces of these species, species-specific lipids (PGLs, LOSs, and DIMs) are exposed but ubiquitous lipids (DMTs, MMTs, and TAGs) are not. The second group consists of *M. avium* and *M. smegmatis*; their species-specific lipids (GPLs) and some of the ubiquitous lipids (MMTs and TAGs) are exposed on the cell surface. The third group is composed of *M. aurum*; all the classes of lipids so far described for this species are exposed at the outermost region of the cell.

Finally, the location of PGLs and GPLs on the cell surface deserves consideration, as the outermost constituents of the bacilli, in the course of infection, are in contact with the host cells and may affect their response. It must be recalled that both classes of specific mycobacterial glycolipids may function as virulence factors in the pathogenesis of mycobacterial diseases. PGLs have been shown to inhibit the lymphoproliferative response (26, 44), to suppress monocyte oxidative responses (56), and to scavenge oxygen radicals (47). Similarly, GPLs, which are mainly the most simple version of these molecules, have been shown to inhibit both the nonspecific mitogen-induced proliferation of mononuclear cells (10) and mitochondrial oxidative phosphorylation (55). Keeping in mind that bacterial pathogenicity and virulence are multifactorial, it is not surprising that some nonpathogenic mycobacterial species, like *M. gastri*, synthesize PGLs (15, 57), whereas both virulent smooth transparent and avirulent smooth opaque strains of *M. avium-M. intracellulare* elaborate GPLs (5), as does the nonpathogenic *M. smegmatis* (17), while some virulent strains are devoid of these glycolipids (15, 18, 53).

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