Mycobacterial outer membrane is a lipid bilayer and the inner membrane is unusually rich in diacyl phosphatidylinositol dimannosides

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***Mycobacterium* species, including the human pathogen *Mycobacterium tuberculosis*, are unique among Gram-positive bacteria in producing a complex cell wall that contains unusual lipids and functions as a permeability barrier. Lipids in the cell wall were hypothesized to form a bilayer or outer membrane that would prevent the entry of chemotherapeutic agents, but this could not be tested because of the difficulty in extracting only the cell-wall lipids. We used reverse micellar extraction to achieve this goal and carried out a quantitative analysis of both the cell wall and the inner membrane lipids of *Mycobacterium smegmatis*. We found that the outer leaflet of the outer membrane contains a similar number of hydrocarbon chains as the inner leaflet composed of mycolic acids covalently linked to cell-wall arabinogalactan, thus validating the outer membrane model. Furthermore, we found that preliminary extraction with reverse micelles permitted the subsequent complete extraction of inner membrane lipids with chloroform–methanol–water, revealing that one-half of hydrocarbon chains in this membrane are contributed by an unusual lipid, diacyl phosphatidylinositol dimannoside. The inner leaflet of this membrane likely is composed nearly entirely of this lipid. Because it contains four fatty acyl chains within a single molecule, it may produce a bilayer environment of unusually low fluidity and may slow the influx of drugs, contributing to the general drug resistance phenotype of mycobacteria.**

Mycobacterium smegmatis | mycolic acid | phosphoinositides

It is now generally accepted that the organisms belonging to the *Corynebacteria–Mycobacteria–Nocardia* group are covered by a complex cell envelope containing the inner plasma membrane (IM), the peptidoglycan–arabinogalactan complex, and the outer membrane (OM) that is covalently linked to the arabinogalactan (1, 2). However, it has been nearly impossible to isolate the OM without contamination from IM components, and this makes the previous published studies on OM composition (3) and the models of OM (4, 5) less than convincing.

The major limitation here has been the lack of a reliable technique capable of separating the IM and OM of mycobacteria. Although methods such as shaking with glass beads have been used in the past (3), these are random mechanical methods and an absolute selectivity cannot be achieved. Recently we used the technique of reverse micellar solution (RMS) extraction for *Corynebacterium glutamicum* cells and found that complete and selective removal of lipids from the OM was possible (6). Through the quantification of lipids we proved that a member of the *Corynebacterineae* had enough fatty acid chains beyond the peptidoglycan to form a complete OM bilayer barrier around the cell. In the process, we could assign the location of different lipid species to the OM and IM of the corynebacterial envelope. We now extend this approach to *Mycobacterium smegmatis*, which is commonly used as a model species of mycobacteria, a group including *Mycobacterium tuberculosis* that infects one-third of the world population.

Results

Selectivity and Completeness of RMS Extraction Procedure in *M. smegmatis*. We have shown previously that the RMS extraction produces a specific and complete solubilization of free OM lipids of *C. glutamicum* without contamination from IM lipids (6). We found similar specificity when the RMS procedure was applied to *M. smegmatis*; the extract did not contain any major phospholipids (PLs) (Fig. 1*A* and Fig. S1), except some phosphatidylinositol mannosides (PIMs), as will be discussed in the following sections. This absence of PLs seems to be the hallmark of the OM lipids of the *Corynebacteria–Mycobacteria–Nocardia* group (6), and indeed all of the PLs were found in the IM extract, that is, the chloroform–methanol–water (CMW) extract of the RM-treated cells. To confirm that the absence of PLs in the RM extract was not due to repulsion between negatively charged PLs and the detergent, we treated *Escherichia coli* DH5α cells with similar RMS. PLs were seen in the RMS extracts of *E. coli* (Fig. S1). This was as expected because *E. coli* contains PLs in its OM (7). Thus, any possible inability of RMSs to solubilize PLs is ruled out, confirming that the mycobacterial OM is indeed lacking in PLs.

Although it was devoid of PLs, the RMS extract of *M. smegmatis* contained most of the mycolic acid (MA)-containing lipids, the known constituent of OM. As seen in Fig. 1*B*, the alkaline hydrolysis/esterification product of the RMS extract showed the presence of MA methyl esters (MAMEs), whereas that of the IM extract showed only traces of MAMEs. The source of these small amounts of MAMEs in the latter was found to be trehalose monomycolates (TMMs), as will be discussed in the following section. Thus, except TMMs, other MA-containing lipids were confined to the OM, as expected. In addition, Fig. 1*C* shows that the alkali-stable, anthrone-positive glycopeptidolipids (GPLs) were present only in the RMS extracts and absent in the IM extract. (The

Significance

Mycobacteria include human pathogens that still kill millions annually. They are naturally resistant to commonly used antibiotics, presumably because the cell interior is well protected by the presence of the lipid-rich cell wall, which is thought to exist as a membrane, or the outer membrane. However, it was not known whether a sufficient number of lipid molecules existed to form a bilayer within the cell wall. We used a unique method that extracts only cell-wall lipids and showed that there are indeed enough lipids to cover the cell surface entirely as a bilayer. The analysis of inner, cell-membrane lipids also gave an unexpected insight, suggesting that the nature of the inner membrane might also contribute to generalized drug resistance.

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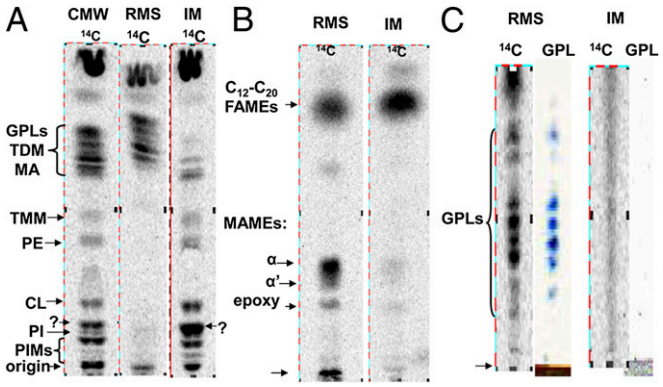

Fig. 1. TLC profiles demonstrate the selective OM-solubilizing ability of RMS in *M. smegmatis* mc^2 155. In all of the figures, CMW and RMS indicate CMW and RMS extracts of intact cells, whereas IM indicates CMW extract of residues of RMS-extracted cells. (*A*) TLC of *M. smegmatis* ^{14}C-labeled lipids developed with CMW (30:8:1), showing that PLs (PE, CL, and PI) are abundant in IM but are totally absent in the RMS extract (OM lipids). (*B*) TLC of the FA esters obtained from ^{14}C-labeled lipids of *M. smegmatis*, developed using petroleum ether:diethylether (85:15), detected by radioactivity. The material from the IM extract shows only traces of MAMEs. (*C*) TLC of alkali-treated lipids of *M. smegmatis*, developed with CM (9:1) and sprayed with anthrone reagent for visualization of GPLs.

IM extract showed traces of anthrone-positive lipids but these were alkali-hydrolyzable and hence not GPLs.) It is important that the IM extract lacked the typical OM lipids such as GPLs and mycolyl lipids (except TMMs), because this shows the completeness of solubilization of all free OM lipids by RMS.

Lipid Composition of the IM of *M. smegmatis*. We note first that reliable composition data on mycobacterial IM does not exist, because CMW extraction of intact cells produces a mixture of IM lipids and extractable OM lipids. Thus, the lipid composition of IM that we present here was previously unknown.

PLs. Fig. 2*A* shows that the IM is composed mostly of PLs, whereas the OM does not contain any major PL groups. The IM lipids were identified using different spray reagents and quantified by phosphorimaging of radioactive TLC profiles, as described in *Experimental Procedures*. The IM contained the typical PLs, cardiolipin (CL), phosphatidylinositol (PI), and phosphatidylethanolamine (PE), constituting ~1.16%, ~0.37%, and ~0.52% of the dry cell mass (dcm), respectively (Fig. 2*A* and Table 1).

However, by far the most abundant lipid group in the IM extract, accounting for up to 42% (by weight) of all of the lipids in the IM extract, had a migration pattern similar to diacyl phosphatidyldimannoside (Ac_2PIM_2) with a retention factor value between that of CL and PI (indicated as "?" in Figs. 1*A* and 2). Although this lipid showed a weak blue coloration with phosphospray, it tested strongly positive with anthrone, indicating significant sugar content (Fig. 2*B*). Although Ac_2PIM_2 was reported in the cell envelope of mycobacteria, it was not known to be the major lipid in its IM (8–11). Even in this study, the CMW extract of intact cells contained a much lower amount of this lipid, almost four times lower (~1.6% of dcm) than that obtained in the CMW extract of RMS-pretreated cells (~5% of dcm), as seen in Figs. 1*A* and 2*A* (compare the intensity of the bands indicated by "?" in the first and the third lanes). This suggests that RMS somehow loosens up the cell envelope so that a much better lipid extraction ensues in the subsequent CMW extraction.

To confirm that this major IM lipid was indeed Ac_2PIM_2, we specifically labeled growing cells with [^{14}C]mannose and found that the lipid of interest was strongly radioactive (Fig. S2*A*). The lipid was isolated by preparative TLC and subjected to electrospray ionization–mass spectrometry (ESI-MS), which identified it as Ac_2PIM_2 (Fig. S2*B*). Also, GC-MS of its constituent fatty acids showed mainly C16:0 (palmitic acid) and C19:0 (most probably 10-methylstearic or tuberculostearic, TS) acids (Fig. S2*D*). These results correspond very well with the predicted structures of Ac_2PIM_2.

Other PIMs such as $AcPIM_2$, $AcPIM_4$/Ac_2PIM_4, and $AcPIM_6$/Ac_2PIM_6, accounting for up to 3% of the dcm, were seen in the IM extract. When the IM extract was subjected to SDS/PAGE with 15% tricine gel, a sugar-containing band was seen with basic fuchsin and also with silver staining at around 4–5 kDa (Fig. S3 *A* and *B*). This band contained mannose, because it contained radioactivity when prepared from [^{14}C]mannose-labeled cells (Fig. S3*C*). The band was almost absent in the water wash following IM extract, and we suspect that it may be mannolipid biosynthetic intermediates between $AcPIM_6$/Ac_2PIM_6 and lipomannans (LMs). We found that PIMs with up to four mannose residues were confined to the IM, whereas PIM_6 and higher were also detected in traces in the RMS extract (Fig. 2*A* and Fig. S3). (Ac)PIM_6 and higher were also released when intact cells were treated with aqueous lysozyme solutions, suggesting that they may be cell wall-associated (Fig. S3*D*).

Other Lipids. The IM contained a lipid with a TLC migration pattern similar to that of TMM. This lipid band appeared bluish-violet with anthrone, consistent with this identification. When this band was isolated by preparative TLC and subjected to alkaline hydrolysis, it was indeed found to contain MAs (Fig. S4). TMM has been believed to be involved in transporting MAs from their site of synthesis in the cytosol to the outer cell envelope, where they may be esterified to the cell wall or remain as trehalose dimyoclate (TDM) (12, 13). In contrast to these studies, other workers (14) indicated that in *C. glutamicum* TMM is synthesized in the outer cell wall and hence cannot be present in the IM.

In addition to PLs and TMM, the IM showed traces of anthrone-positive lipids with a migration pattern similar to that of GPLs and TDM (Fig. 3*A*). However, these were not GPLs because these glycolipids were alkali-hydrolyzable (Fig. 1*C*) or TDM, as revealed

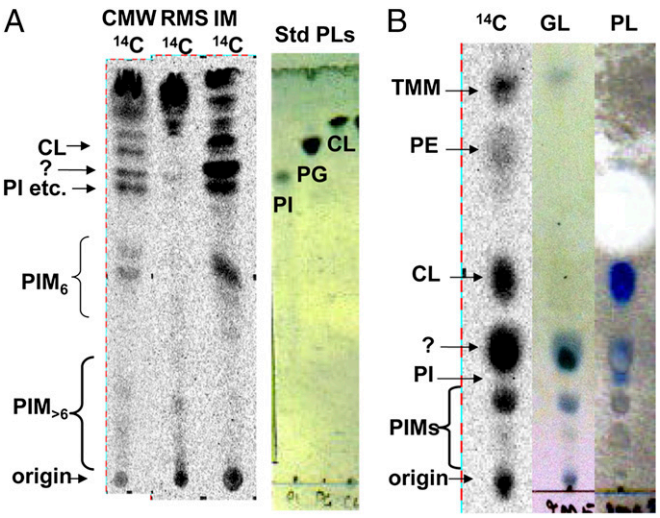

Fig. 2. TLC profiles of polar lipids from *M. smegmatis*. (*A*) TLC profiles of ^{14}C-labeled lipids in different extracts, developed with chloroform–methanol–13 M ammonia–1 M ammonium acetate–water (180:140:9:9:23, vol/vol). Question mark indicates the suspected diacyl phosphatidylinositol dimannosides (Ac_2PIM_2). The band labeled "PI etc." seems to contain not only PI but also $AcPIM_2$, PIM_2, and PIM_4. For comparison, standard phospholipids (std PLs) were developed in the same solvent and visualized with phosphospray. (*B*) TLC profiles of IM lipids developed using chloroform–methanol–water (65:25:4, vol/vol) sprayed with anthrone for glycolipids (GLs) or phsophospray for PLs.

Table 1. Quantitative distribution of fatty acyl chains in the cell envelope

Lipid species	Lipid mass, % dcm	Average MW, Da	Acyl chain per lipid molecule	Millimoles of acyl chain per 100 g of dry cells
IM lipids				
$AcPIM_6$*	1.5	2,060	3 FA	1.1 FA
Ac_2PIM_6*		2,298	4 FA	1.3 FA
$AcPIM_2$†	1.5	1,412	3 FA	3.2 FA
PI‡	0.37	853	2 FA	0.9 FA
Ac_2PIM_2†	5.00	1,653	4 FA	12.1 FA
CL‡	1.16	1,419	4 FA	3.3 FA
PE‡	0.52	735	2 FA	1.4 FA
TMM§	0.35	1,530	1 MA	0.22 MA
Apolar lipids	1.1	?**		
Total extractable lipids in IM	11.5			23.3 FA and 0.22 MA
OM lipids‖				
TDM§	0.23	2,717	2 MA	0.17 MA
GPLs¶	5.56	1,269	1 FA	4.4 FA
DAG‡	0.45	629	2 FA	1.40 FA
Free MAs and FAs§	2.06	1,188	1 MA	1.7 MA
TAG‡	2.54	833	3 FA	9.10 FA
Apolar lipids	0.28	?**		
**Total extractable lipids in OM	11.12			14.9 FA and 1.9 MA
Cell-wall-bound MAs§	4.6	1,188	1 FA	3.9 MA

*The combined yield of Ac_2PIM_6 and $AcPIM_6$ determined in this work was 1.5% dcm, and for calculations it was assumed that each of them were present at 0.75% dcm. MWs of $AcPIM_2$ and Ac_2PIM_6/$AcPIM_6$ are based on ref. 9.
†The MW of the major species of Ac_2PIM_2 determined in this work (Fig. 3) was used for calculations.
‡MWs of lipids were calculated assuming that C16:0 and TS are the constituting fatty acids.
§MWs of lipids were calculated assuming an average mycolic acid weight of 1,188 (34).
¶MW of GPLs is an average of those for the major *M. smegmatis* mc^2 155 GPLs reported in refs. 5 and 21.
‖Extraction of whole cells produced LAM (0.05% dcm) and LM (0.2% dcm), but they were not included in the calculation because they are expected to contribute little to the surface area of the bilayer.
**Since apolar lipids were not identified, their MW is unknown.

by 2D TLC (Fig. S5). Large amounts of triacylglycerols (TAGs) corresponding to >10.3% of the dcm were seen in the IM extracts (Fig. 3*B*). However, most of these TAGs probably originated from within the cytoplasm and not from the IM, because cytoplasmic inclusion bodies composed of TAGs are common in *M. smegmatis* (15). Similar to Ac_2PIM_2, TAGs were also released in larger amounts when CMW extraction was done with RMS-treated cells (~10.3% dcm) than with intact cells (~7.6% dcm). Finally, the IM contained highly apolar lipids at ~1.1% of dcm (Fig. 3*B*). Alkaline hydrolysis of these apolar lipids did not result in liberation of MAs, ruling out their being mycolyl glycerols (16, 17). These apolar lipids remain unidentified at present.

Lipid Composition of the OM of *M. smegmatis*. Almost half of the lipids found in the RMS extract, thus the noncovalently held lipids of the OM, were GPLs corresponding to ~5.6% of the dcm (Figs. 1*A* and 3*A* and Table 1). GPLs were separated from other glycolipids, namely sulfolipids (SLs) and TDMs, by 2D TLC and by their property of being alkali-stable (Fig. 1*C* and Figs. S5 and S6). TDM accounted for up to ~0.23% of dcm. SLs were detected by radioactivity when cells were grown in the presence of $Na_2{}^{35}SO_4$ (Fig. S6). However, their amount was very small, because they were below the detection limit when cells were grown in the presence of [U-^{14}C]glucose (Fig. S6).

Diacyl gycerols (DAGs) (~0.45% of dcm), TAGs (~2.5% of dcm), and free MA (FMA) (~2% of dcm) (with some free fatty acids) were the other major components the *M. smegmatis* RMS extract (Fig. 3*B* and Figs. S7 and S8). In addition, a few unidentified, extremely nonpolar lipids (~0.3% dcm) were detected in the RMS extract.

In our previous work with *C. glutamicum,* alkaline hydrolysis of delipidated cells, that is, cells after CMW extraction, released corynomycolic acids and large amounts of C12–C20 fatty acids (6). The source of these C12–C20 fatty acids was peptidoglycan-embedded CLs, which apparently make up a significant part of the inner leaflet of OM, and were released when CMW extraction was done after disrupting the cell wall. In *M. smegmatis*, hydrolysis of delipidated cells released mostly α, α′, and epoxy MA (~5% dcm), and only traces of low-molecular-weight fatty acids (Fig. 3*C*). No additional CLs or other lipids were released when CMW extraction was done after subjecting the delipidated cells to lysozyme or ultrasonication treatment (data not shown). This suggests that covalently bound MA form completely the inner leaflet of the OM in *M. semgmatis*.

Location of LMs and Lipoarabinomannans in the Cell Envelope of *M. smegmatis*. As described above, PI and its mannosylated forms containing up to six mannose residues were confined to the IM, although higher PIMs were seen in the RMS extract. Location of the hyper-mannosylated forms of PIMs, namely LMs and lipoarabinomannans (LAMs), has always been a subject of debate (18–20). Their location in OM was assumed on the basis of their being absent from an IM or spheroplast preparation (18, 20). The poor solubility of these lipopolysaccharides in either aqueous or conventional organic solvents has also been an additional obstacle in their extraction. Disruptive conditions, such as phenol and ethanol at high temperatures, have usually been used (see, e.g., refs. 19 and 20), presumably because they are the only solvents that can effectively solubilize LMs and LAMs. RMSs extract components solely from the OM, but they have limited solvent activity for polysaccharides. Nevertheless we checked our RMS extracts and the aqueous cell washes following RMS extraction for the presence of LMs and LAMs.

Whereas the organic RMS extract did not contain any LAMs, the aqueous wash following RMS extraction did show a purple-colored band corresponding to LAMs between 25 and 35 kDa, when the gel was stained with basic fuchsin (Fig. 4). Probing with CS-35 antibody specific to LAM confirmed the presence of LAMs in the OM of *M. smegmatis* (Fig. 4). No LMs were seen in

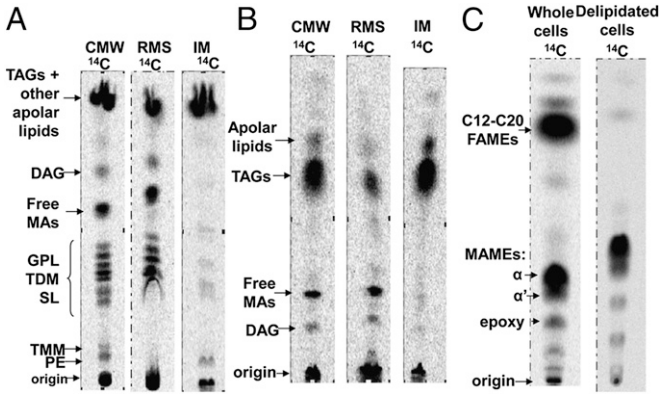

Fig. 3. TLC of medium- and low-polarity lipids from *M. smegmatis*. (*A*) Development with CMW (100:14:0.8), showing that these lipids are confined to OM (RMS extract), except TAG. (*B*) Development with hexane–diethyl ether–acetic acid (70:30:1). (*C*) FA and mycolic acid methyl esters (FAMEs and MAMEs) obtained from whole cells or delipidated (CMW-extracted) cells, developed with petroleum ether: diethylether (85:15).

any of the extracts. They may have precipitated out during these extraction steps owing to solubility limitations.

Complete recovery and quantification of LMs/LAMs were attempted by phenol–water extraction of delipidated cells. LMs and LAMs were analyzed by SDS/PAGE using 15% tricine gel (Fig. 4). Using a known amount of standard *E. coli* LPS and using ImageJ software, we determined that *M. smegmatis* contained approximately ∼0.05% dcm of LMs and ∼0.2% dcm of LAMs, an amount much higher than that found in the aqueous wash after RMS extraction, where poor solubility was probably the limiting factor.

Fatty Acid Content of the OM and IM. The results obtained show us the location and amount of various lipids in the OM and IM (Table 1); this analysis was repeated at least three times to confirm reproducibility. To calculate the contribution of these lipids to the surface area of the bilayer structure, we took into account the average molecular weight (MW) of each species and converted the mass of each into molar amounts of fatty acid (FA) chains in IM and OM (Table 1). For example, GPLs (MW 1,157–1,185 (21)), which account for ∼5% of the dcm in the OM, contain only one FA chain per molecule. However, the major lipid of the IM, Ac_2PIM_2 (MW of the major species is 1,653 as determined in this work) also accounts for 5% of the dcm but contains four acyl chains per molecule. Therefore, whereas GPLs contribute approximately 4.3 mmol of FAs per 100 g of dry cells, Ac_2PIM_2 contributes approximately 12.1 mmol of FAs per 100 g of dry cells. When the FA or MA contribution of individual lipid species was considered, we found that the OM contained noncovalently held FAs and MAs, presumably in the outer leaflet, in sufficient quantity (about 15 mmol FA and 1.9 mmol MA, per 100 g dcm) to cover over the entire area of IM, containing about 24 mmol FA total, and thus 12 mmol FA for each leaflet. The covalently linked MA was found at about 4 mmol per 100 g dry cells. This amount is clearly insufficient to cover the area of OM if MA folds to produce two hydrocarbon chains of unequal length (8 mmol of hydrocarbon chains compared with 12 mmol in the IM or 15 mmol of FA in the outer leaflet of OM). However, because MA apparently folds in a "W" form containing four hydrocarbon chains (22, 23) this amount would correspond to 16 mmol of hydrocarbon chains, close to the number of hydrocarbon chains in the outer leaflet.

FA composition was examined for the lipids in the CMW extract of intact cells, RMS, and IM extracts after alkaline hydrolysis, as described in *Experimental Procedures*. The composition, determined by GC-MS, is shown only for CMW extract in Fig. S9. TS acid and palmitic acid each constitute around 30% of the total, as expected.

Discussion

Qualitative Composition of OM and IM. In this study, we used the RMS extraction method that was shown previously to extract "free" lipids only from the OM (6) in *C. glutamicum.* Similar to our finding in *C. glutamicum*, conventional glycerophospholipids such as PE or CL, typical components of IM, were totally absent in the RMS extract (Figs. 1 and 2 and Table 1), showing that we have obtained lipids from OM only, not at all contaminated by the IM lipids. This can be contrasted with the composition of OM lipids obtained by crude mechanical shearing where PE was reported to be present (3). Even in an OM preparation purified by gradient centrifugation (24), glycerophospholids were found to be present (2). In *C. glutamicum* (6) the OM contained a large amount of tightly bound, nonextractable CL. In contrast, *M. smegmatis* OM contained bound MAs (Table 1) in the inner leaflet, as expected from the Minnikin model (1), instead of CLs. This obligatory presence of MAs in the OM is consistent with the observation that mycobacteria cannot (but *C. glutamicum* can) survive without trehalose, which is needed for the export of MA residues to the OM (13, 14).

TAGs were found in the OM (Fig. 3 and Table 1). Although TAGs are abundant in the cytosol, the use of the RMS extraction procedure ensured that these compounds are also components of the OM, as has been surmised by the use of less stringent extraction protocols (3). Lipids such as SLs, LAMs, and LMs are known antigens involved in the initiation of the host immune response. These were present in very small amounts in the cell envelope. SLs were not even detected when cells were labeled with [U-^{14}C]glucose. LAMs and LMs were not more than 0.25% of the total radioactivity. GPLs, present in large amounts, are presumably important structural component of the cell envelope. These major lipids likely provide the effective permeability barrier exhibited by this group of organisms.

As for the IM lipids, aside from the conventional glycerophospholipids we note the presence of PI and PIMs (Table 1). We note that in addition to the early intermediates Ac_1/Ac_2PIM_2, the extensively mannosylated forms Ac_1/Ac_2PIM_6 were also found predominantly in the IM, confirming the pathway proposed by Besra and coworkers (25). It is also important that TMM was exclusively found in the IM, but TDM only in the OM (Table 1).

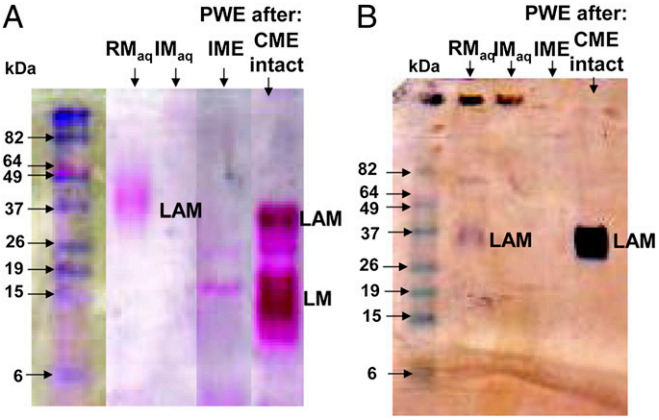

Fig. 4. SDS/PAGE profile of lipoglycans in various aqueous and phenol water extracts of *M. smegmatis*. RM_{aq} and IM_{aq} denote aqueous washes following RMS extraction and following CMW extraction of RMS-treated cells, respectively. "PWE after IME" and "PWE after CME intact" indicate the phenol–water extracts of residues obtained after IM extraction and after CMW extraction of intact cells, respectively. The leftmost lane shows MW standards, the size indicated in kilodaltons. (*A*) Staining with basic fuschin. (*B*) Visualization with the CS-35 antibody probe specific for LAMs.

Quantitative Composition of IM and OM. Table 1 shows that 100 g of dcm of *M. smegmatis* contained 23.3 mmol of FA chains in the IM. Thus, one leaflet of the IM is expected to contain about 12 mmol FA chains. We would expect to see about the same (or slightly higher) amount of hydrocarbon chains in the OM. Indeed, in the inner leaflet there were 3.9 mmol of bound MA, which would produce 15.6 mmol of hydrocarbon chains, according to the model in which one mycobacterial MA folds to generate four hydrocarbon chains (22, 23). This is clearly sufficient to cover the cell surface. The outer leaflet of OM should then be composed of the extractable OM lipids (14.9 mmol FA chains plus 0.7 mmol hydrocarbon chains from TDM), an amount that fits nearly perfectly with the hydrocarbon chain content of the inner leaflet. The OM in addition contains 1.7 mmol of FMA, which would produce 7.6 mmol hydrocarbon chains. If all of this were in the outer leaflet, its surface area would be too large. However, *M. tuberculosis* and *M. smegmatis* are known to produce biofilms composed of FMA when grown without detergents (26), and the FMA material we found likely comes from extracellular material. Not surprisingly, GPL was an abundant component of the outer leaflet, but it is noteworthy that we found substantial amounts of TAG. Compounds such as phthiocerol dimycocerosate and lipooligosaccharides, reported to be present in the *M. smegmatis* cell envelope (3), apparently represent minor components and thus do not affect the balance among major constituents. A model of the cell envelope based on this quantitative analysis is shown in Fig. 5.

It should be pointed out that this bilayer model of the outer membrane, with the folded conformation of mycolic acid residues (22, 23), explains why the outer membrane visualized by cryo-electron microscopy (27, 28) has a thickness only marginally larger than that of the IM and the puzzle that the *M. smegmatis* porin MspA has a hydrophobic exterior that would not traverse the exceptionally thick hydrocarbon region of OM (29), expected from the extended chain model of mycolic acid residues.

The quantitative composition of the IM needs a comment. Remarkably, more than one-half of the FA chains came from Ac_1/Ac_2PIM_2; as detailed in *Results*, this unexpectedly large yield is the result of the preextraction with RMS. Thus, the application of the RMS extraction procedure presumably weakened the association of these lipids with other components of the envelope and revealed the true composition of the IM. Interestingly, enzymes involved in the synthesis of polar PIMs are reported to reside in a special subdomain of IM, with a strong interaction with the rest of the envelope (11). According to the proposed biosynthetic pathway (25), these lipids should be present mostly in the inner leaflet (of the IM), which is thus expected to be composed almost entirely of these compounds (Fig. 5). (However, we should emphasize that our analysis was carried out only with the late-exponential-phase cells, and for cells from different growth phases the results might be different.) The outer leaflet of the IM is then expected to contain other lipids, including Ac_1/Ac_2PIM_6 and TMM, in addition to the conventional glycerophospholipids such as CL, PE, and PI.

Ac_1/Ac_2PIM_2, the predominant component of the inner leaflet of IM, contains three or four FA chains. At least one membrane lipid containing three fatty acyl chains, Gal-Glc-(*N*-acyl)GlcN-Glc-diglyceride, from an extreme thermophile *Thermus* (30), is known to raise the thermal transition temperature of the membrane to close to 80 °C, although the FAs are not of exceptional lengths (31). The physical behavior of acyl PIMs does not seem to have been investigated, but most intriguingly polar lipids from *Mycobacterium bovis* bacillus Calmette–Guérin, containing roughly 34% (by weight) Ac_1/Ac_2PIM_2, were found to form liposomes only at temperatures above 55 °C (32). The authors ascribe this high thermal transition point to the presence of C19:0 TS acid, but this may not be the whole explanation. The physical properties of acyl PIMs should be a worthwhile subject of study, because if they produce a less fluid IM, they could contribute to the generally high intrinsic resistance levels of *M. smegmatis* to antimicrobial agents (see, e.g., ref. 33) by decreasing the rate of their transmembrane diffusion.

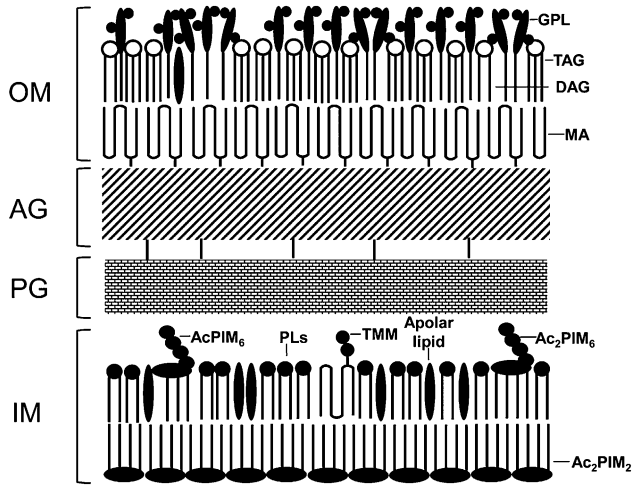

Fig. 5. A probable model of mycobacterial cell envelope. Based on the lipid composition data obtained in this study, a model of the OM and IM is proposed. The ratios between various components reflect the quantitative composition data, but very roughly. In the OM, TDM is not shown because it is present in an amount corresponding to less than 1 in the scheme. Free MA is not shown because it is likely to exist largely as an extracellular material. The thickness of peptidoglycan (PG) and arabinogalactan (AG) layers is arbitrary.

Experimental Procedures

Bacterial Growth and Radiolabeling. *M. smegmatis* mc^2155 was grown in Middlebrook 7H9 broth (Difco), supplemented with 10% (vol/vol) albumin–dextrose–catalase enrichment, at 37 °C with aeration and shaking. The final medium contained 0.2% glucose but no glycerol or surfactant.

For uniform radiolabeling of cellular components, [U-^{14}C]glucose (9.25–13.3 GB·mmol^{-1}) was added to the culture (0.25 μCi·mL^{-1}), when OD_{660} reached 0.5. Cells were harvested at an OD_{660} of ~3 and were washed 10 times with distilled water.

For specific labeling of mannose-containing lipoglycans a method described earlier (11) was used. Unlabeled cells from ~100 mL culture in their mid-exponential phase were incubated in the medium described above, at a concentration of 0.2 g wet cells·mL^{-1}, for 5 min at 37 °C. Cells were then labeled with 4 μCi·mL^{-1} [^{14}C]mannose (55 mCi·mmol^{-1}) for 15 min, immediately cooled, harvested, and washed several times with distilled water. Specific labeling of sufolipids with $Na_2{}^{35}SO_4$ was carried out as described (35).

Extraction of Lipids and Lipoglycans. The strategies used to extract lipids completely and selectively from the OM and IM of *M. semgamtis* are based on those developed in our previous work with *C. glutamicum* (6). For complete recovery of all noncovalently held lipids, an aliquot of cells corresponding to 10 mg dry weight was extracted with 3 mL and then reextracted four more times with 2 mL each of CMW (2:1:0.1). For recovery of all noncovalent lipids selectively from the OM, the same amount of cells was extracted five times with 1 mL of RMS [10 mM sulfosuccinic acid 1,4-bis (2-ethylhexyl) ester sodium salt (AOT) in heptane]. In both cases, the first extraction was overnight and the remaining four were for 15–30 min each. For the quantification of IM lipids, RMS-extracted cells were thoroughly washed with water and then extracted with CMW.

We made certain that all extractions were carried out in monophasic solutions. For extracting the cell wall-associated lipids, cells were treated with aqueous lysozyme solutions as described previously (6).

Extraction of LMs and LAMs. Delipidated cells obtained after CMW extraction of intact or RMS-treated cells (starting dcm ~100 mg) were broken by ultrasonication and then extracted with 50% ethanol as described (36). The extract was dried and the material was solubilized in a single-phase 50% (vol/vol) phenol at 75 °C, and LAM and LM were partitioned into the aqueous phase separated by the cooling of the mixture (36).

Analysis of Lipids. Lipids in various extracts were resolved by TLC using aluminum-backed silica gel plates (silica gel 60 F254; Merck). TLC profiles of

radiolabeled lipids were determined by a phosphorimager (Typhoon 9400; Molecular Dynamics). Alternatively, lipids were visualized by spraying with various agents (6).

For quantification, the total radioactivity of cells labeled using [U-^{14}C]glucose was determined by scintillation counting and correlated with their dcm. From the relative intensity of various lipid subgroups resolved in a TLC profile and the total radioactivity applied to that lane, we could thus calculate the mass of various lipid species in different layers of the cell envelope in a unit cell mass.

Analysis of FAs. Lipids in extracted fractions were converted into FA methylesters by phase-transfer catalysis (36). Covalently linked FAs (including MAs) were treated similarly except that intact cells or CMW-extracted residues of the cells were used as the starting material.

Quantitative data on the relative content of C16–C20 FAs in an extract was obtained by GC-MS of FA methyl esters a DB-XLB capillary column (30 m × 0.25 mm, 0.25-μm film thickness). An 8 °C/min gradient of temperature from 140 to 280 °C was used, and the detection used a quadrupole mass spectrometer. The GC system, however, did not allow the elution of mycolate esters, which were thus quantitated by TLC separation of methyl esters of MA and common FAs [developed with petroleum ether/ether (85/15, vol/vol)] and by phosphorimager.

Analysis of Lipoglycan. LAM and LM in the various extracts obtained after treatment of cells with CMW, RMS, or ethanol refluxing followed by phenol–water treatment were analyzed using 15% tricine SDS-polyacrylamide gels. The lipoglycans were visualized using periodic acid/Schiff reagent (Basic Fuschin kit; Sigma) or by using the Pro-Q emerald glycoprotein stain (Invitrogen). Alternatively, blotting to nitrocellulose membrane was performed at 50 V for 1 h. Immunodetection was performed with monoclonal antibody CS-35 and Con A conjugated to peroxidase (Sigma). The membranes probed with Con A–peroxidase were subsequently developed with the 4-chloro-1-naphthol 3,3-diaminobenzidine tetrahydrochloride substrate kit (Pierce).

ESI-MS. Lipids were isolated by preparative TLC using Analtech silica gel G plates. The isolated lipids were then analyzed in negative-ion mode by either Waters Q-Tof Premier mass spectrometer or by Thermo Finnigan LTQ-FT mass spectrometer (for high-resolution data), both equipped with a nanospray ion source.

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