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Fluidity of the lipid domain of cell wall from *Mycobacterium chelonae*

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ABSTRACT **The mycobacterial cell wall contains large amounts of unusual lipids, including mycolic acids that are covalently linked to the underlying arabinogalactan–peptidoglycan complex. Hydrocarbon chains of much of these lipids have been shown to be packed in a direction perpendicular to the plane of the cell surface. In this study we examined the dynamic properties of the organized lipid domains in the cell wall isolated from *Mycobacterium chelonae* grown at 30°C. Differential scanning calorimetry showed that much of the lipids underwent major thermal transitions between 30°C and 65°C, that is at temperatures above the growth temperature, a result suggesting that a significant portion of the lipids existed in a structure of extremely low fluidity in the growing cells. Spin-labeled fatty acid probes were successfully inserted into the more fluid part of the cell wall. Our model of the cell wall suggests that this domain corresponds to the outermost leaflet, a conclusion reinforced by the observation that labeling of intact cells produced electron spin resonance spectra similar to those of the isolated cell wall. Use of stearate labeled at different positions showed that the fluidity within the outer leaflet increased only slightly as the nitroxide group was placed farther away from the surface. These results are consistent with the model of mycobacterial cell wall containing an asymmetric lipid bilayer, with an internal, less fluid mycolic acid leaflet and an external, more fluid leaflet composed of lipids containing shorter chain fatty acids. The presence of the low-fluidity layer will lower the permeability of the cell wall to lipophilic antibiotics and chemotherapeutic agents and may contribute to the well-known intrinsic resistance of mycobacteria to such compounds.**

Mycobacterium tuberculosis causes tuberculosis, a disease that kills 3 million people a year mostly in developing countries. In industrialized countries, the appearance of multiple-drug-resistant strains of *M. tuberculosis* has made it once again a major public health problem (1). Furthermore, *Mycobacterium leprae* causes leprosy in >10 million people worldwide, and several *Mycobacterium* species, including *M. avium* complex, *M. kansasii, M. fortuitum*, and *M. chelonae*, cause opportunistic infections in immunologically compromised patients, such as AIDS patients. The treatment of mycobacterial infection is often difficult because these bacteria are intrinsically resistant to most common antibiotics and chemotherapeutic agents (2). This general resistance is thought to be related to the unusual structure of mycobacterial cell wall, which is located outside the cytoplasmic membrane yet contains large amounts of lipids, many of them with unusual structure. The major fraction of the cell wall is occupied by unusually long-chain fatty acids containing 70–90 carbons, the mycolic acids (3). The peptidoglycan, which contains *N*-glycolylmuramic acid instead of the usual *N*-acetylmuramic acid, is linked to arabinogalactan via a phosphodiester bridge. About 10% of the arabinose residues of arabinogalactan are in turn substituted by mycolic acids, producing the covalently connected structure of the cell wall (4). The cell wall also contains several types of "extractable lipids" that are not covalently linked to this basal skeleton; these include trehalose-containing glycolipids, phenol-phthiocerol glycosides, and glycopeptidolipids (5). The chemistry and immunology of the various components of the mycobacterial cell wall have been studied extensively. However, far less information is available about the physical properties of mycobacterial cell wall. Without such knowledge, it is not possible to understand their function as a permeability barrier and, consequently, to design agents that could overcome this barrier. Recently, we applied x-ray diffraction to study the physical organization of lipids in the cell wall of *M. chelonae* and obtained evidence that lipid hydrocarbon chains in mycobacterial cell wall are arranged in a direction perpendicular to the cell surface and that the cell wall lipids likely form an asymmetric bilayer (6). In this study, we used differential scanning calorimetry (DSC) and electron spin resonance (ESR) techniques to investigate the dynamic properties of mycobacterial cell wall lipids. Our results provide direct evidence that the cell wall of *M. chelonae* forms an asymmetric bilayer, containing a moderately fluid outer leaflet and a mycolate-containing inner leaflet with extremely low fluidity. This construction appears to make the mycobacterial cell wall exceptionally impermeable to lipophilic solutes, including many antibiotics and chemotherapeutic agents.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *M. chelonae* strain PS4770 (7) was grown at 30°C in Middlebrook 7H9 broth (Difco) supplemented with 0.2% glycerol and 10% Middlebrook OADC enrichment (Difco). Cells were harvested in late exponential phase of growth.

Chemicals. Stearic acid spin labels carrying doxyl groups at positions 5, 7, 9, 12, or 16 [5-, 7-, 9-, 12-, or 16-doxylstearic acid spin label (SASL)] were from Molecular Probes.

Isolation and Purification of Cell Wall. The isolation and purification of cell wall have been described (6). Briefly, cells were broken by sonication and French press. The cell wall fraction was separated from cytoplasmic membrane by centrifugation in a sucrose step gradient. The purified cell wall suspensions were stored at −70°C until use.

The phospholipids in the cell wall were determined by assaying for total phosphorus (8).

DSC. Trypsin (2 mg/ml) was added to 4 mg of cell wall in 2 ml of 40 mM phosphate buffer (pH 6.8). The mixture was incubated at 37°C for 12 hr and then centrifuged for 30 min at 15,000 rpm. The cell wall pellet was washed once with distilled water and once with the phosphate buffer and then was resuspended in 2 ml of the phosphate buffer. The samples were

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Abbreviations: DSC, differential scanning calorimetry; 5-, 7-, 9-, 12-, and 16-SASL, 5-, 7-, 9-, 12-, and 16-doxylstearic acid spin label, respectively; ESR, electron spin resonance.

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analyzed with a Microcal MC-2 differential scanning calorimeter.

Spin Labeling of Cell Wall. Fatty acid spin labels (40 nmol) in chloroform were dried at the bottom of a glass tube under a stream of N_2 and kept *in vacuo* at 25°C for at least 6 hr. Purified cell wall (100 μl containing 144 nmol of phosphorus) or intact cells (6×10^9 cells) in 0.1 M sodium borate (pH 9.5) were added to the film, and the tube was agitated in a Vortex mixer vigorously. The suspensions were kept at 25°C for 4 hr. Samples were then washed five times by centrifugation and resuspension to remove excess free spin label. The bound spin labels were about 1% of the total phospholipids of the cell wall. This value corresponds to <0.1% of the total extractable lipids of the cell wall, as phospholipids contain <10% of the fatty acid residues present in the extractable lipids (E.Y.R. and H.N., unpublished).

ESR Spectroscopy. ESR measurements were made on a Varian E-109 spectrometer (Varian) operating at X band, equipped with a variable temperature controller, at the National Biomedical ESR Center (Milwaukee, WI). The magnetic field was stabilized with a Varian field-frequency lock. Temperatures were measured by a thermocouple just outside of the capillary sample tube and were controlled to within 0.1°C. Data acquisition was controlled by a personal computer interfaced to the spectrometer utilizing the VIKING software package (C. C. Felix, National Biomedical ESR Center, Milwaukee, WI).

All spectra were obtained with a scan range of 100 G (1 G = 0.1 mT) and a modulation amplitude of 2.0 G. The spin label concentration was determined by double integration of the ESR spectra followed by comparison with the spectra of standard aqueous TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy, free radical) solution. The order parameter, *S*, was calculated as described (9).

RESULTS

To obtain evidence on the fluidity of lipid interior of the mycobacterial cell wall, heat capacity measurement was carried out with purified cell wall of *M. chelonae*. Since the cell wall contained proteins (6), whose denaturation was expected to absorb heat, the preparation was first treated with trypsin. This treatment removed >99% of the 21-kDa major cell wall protein, based on the scanning of Coomassie blue-stained SDS/polyacrylamide electropherograms (result not shown). We estimate that the protein content of the trypsinized cell wall was <0.2% (wt/wt). The DSC analysis showed major cooperative thermal transitions or melting temperatures near 30°C, 50°C, and 60°C (Fig. 1). These are unusually high transition temperatures for biological lipids.

It was difficult to determine the ΔH of transitions precisely, because of the uncertainty in the baseline. However, the sum of ΔH values with 4 mg of the cell wall preparation (containing roughly 2 mg of lipids), obtained from the area of the peaks, was about 120% of the ΔH observed with the melting of an equivalent amount (2 mg) of dimyristoyl phosphatidylcholine in the DSC scanning carried out on the same day (data not shown). The observation that the ΔH values were of the same order of magnitude suggests that we are not observing the melting of minor, trace components, although precise comparison cannot be made as there are up to 40% differences in the values of ΔH per unit weight among different lipids (10).

To examine the local fluidity within the cell wall structure, spin-labeled fatty acid probes were inserted, through spontaneous partition, into the isolated *M. chelonae* cell wall. Typical ESR spectrum of 5-SASL-labeled cell wall is shown in Fig. 2 *Top*. Only traces of unbound spin label were present in this as well as other experiments. A single homogeneous low field peak was observed from the probe inserted into the cell wall, over the temperature range examined. The hyperfine splitting

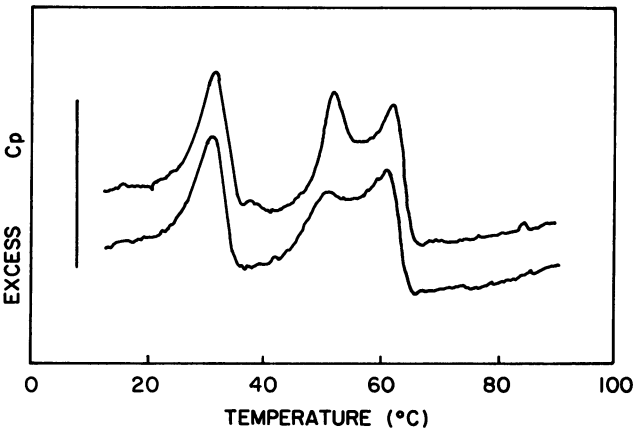

FIG. 1. Thermal transitions of *M. chelonae* cell wall as revealed by DSC. Trypsinized cell wall (4 mg of dry weight) was heated at a rate of 45°C/hr in a Microcal MC-2 differential scanning calorimeter. The lower curve shows the rescanning of the same sample after cooling to 10°C; no obvious hysteresis was observed except at the 50°C peak. The vertical bar corresponds to 1 $J \cdot g^{-1} \cdot degree^{-1}$. Cp, heat capacity at constant pressure.

parameter, $2T_{\|}$, is related to the angular motion of the spin label and therefore reports the local fluidity of the lipid environment (11, 12). In general, high values of $2T_{\|}$ reflect low fluidity. The $2T_{\|}$ values of 5-SASL in cell wall at different temperatures (Fig. 3) suggested that the spin label probe was located in a relatively organized, yet somewhat fluid, environment. For a rough comparison, the $2T_{\|}$ values of 5-SASL in

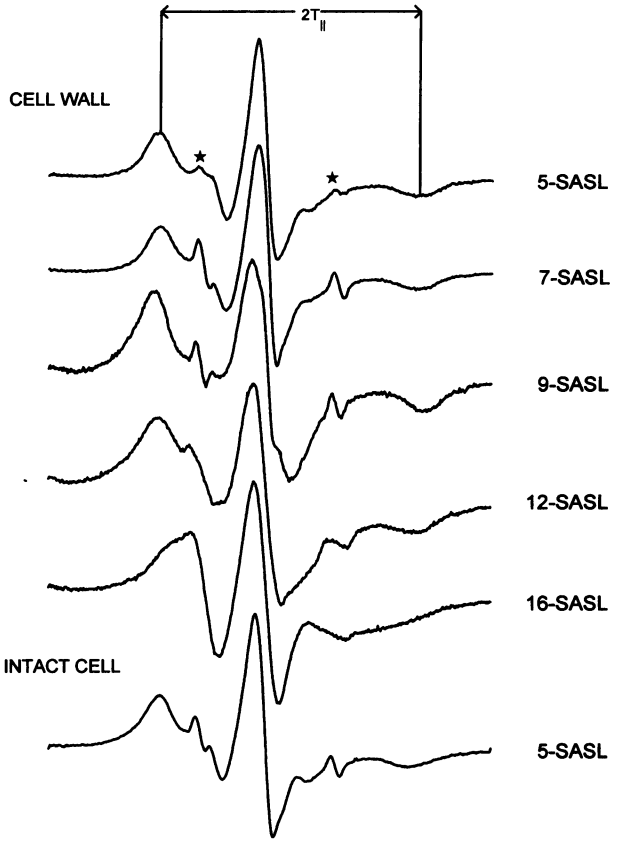

FIG. 2. ESR spectra of *M. chelonae* cell wall labeled with spin-labeled stearate probes at 25°C. Hyperfine splitting ($2T_{\|}$) as well as signals from unbound probes [indicated with stars (★)] are shown in the 5-SASL-labeled spectrum. For comparison, the spectrum of intact *M. chelonae* cells labeled with 5-SASL is shown at the bottom.

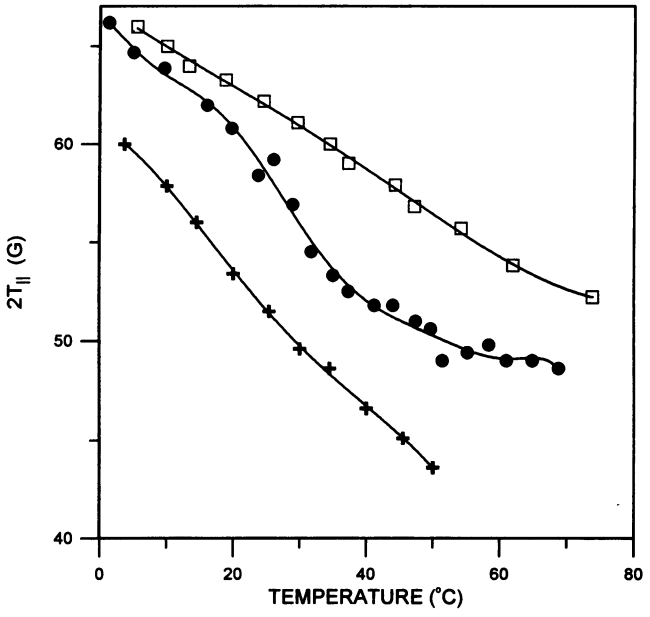

FIG. 3. Temperature dependence of hyperfine splitting parameter ($2T_{\parallel}$). Hyperfine splitting values measured with 5-SASL-labeled *M. chelonae* cell wall are shown (●). For comparison, we also indicate hyperfine splitting values that were taken from the literature (13), obtained with bilayers of *Salmonella typhimurium* phospholipids (+) and lipopolysaccharides (□), both labeled with 5-SASL.

reconstituted bilayers of phospholipids and lipopolysaccharides, each isolated from *S. typhimurium* (13), are also plotted in Fig. 3. The rotational mobility of 5-SASL in the cell wall of *M. chelonae* was lower than in the bilayer of membrane phospholipids but higher than that in isolated lipopolysaccharides. These results suggest that a certain degree of fluidity exists at least in a certain area(s) of mycobacterial cell wall.

We believe that this more fluid domain corresponds to the external leaflet of the cell wall, as defined in our model (see *Discussion*). When spin-labeled fatty acids are added to structures containing domains of different degrees of fluidity—for example, the outer membrane of Gram-negative bacteria (14)—they become inserted nearly exclusively into the most fluid domain, as can be seen from the observation that the fluidity reported by such probes (15, 16) is fairly close to that of the fluid phospholipid domain (13, 16) and very far away from that of the much less fluid lipopolysaccharide domain (13, 16). We have shown, furthermore, that 5-SASL added to intact *M. chelonae* cells produced ESR spectra of a similar $2T_{\parallel}$ value as that added to the isolated cell wall (Fig. 2 *Bottom*), suggesting strongly that the label becomes inserted into the exposed, external surface of the cell wall.

Each of the four other fatty acid spin labels, 7-SASL, 9-SASL, 12-SASL, and 16-SASL, was added to the isolated cell wall to define the fluidity in more detail. In these probes, the nitroxide-carrying doxyl group is located farther away from the carboxyl end, which presumably remains at the external surface of the cell wall owing to its negative charge. ESR spectra of these probes are also shown in Fig. 2. Again, a single symmetric low field peak was always observed except with 16-SASL. (For this reason, the 16-SASL spectrum was not included in subsequent analysis.) To compare the fluidity at different depths from the cell wall surface, the values of order parameter, S, were determined for each probe at 25°C (Fig. 4). For a sample that is oriented in a completely uniform manner, $S = 1$, and for a sample oriented in a totally random manner, $S = 0$. When S was plotted as a function of the position of the nitroxide spin label along the stearic acid chain, with the usual phospholipid bilayer a steeply increasing gradient of flexibility

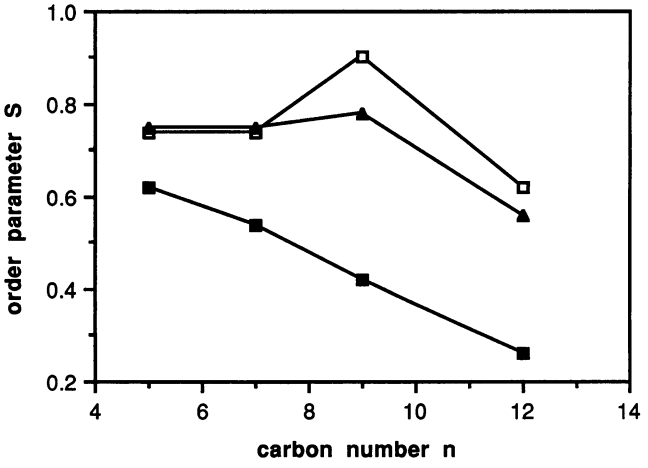

FIG. 4. Dependence of order parameter (S) on the position of the label. Order parameters, at 25°C, calculated for isolated cell wall (□) or intact cell (▲) labeled with various stearate spin label probes are shown. The order parameters of the same set of spin labels in egg phosphatidylcholine bilayers are also shown for comparison (■); these data are from Knowles *et al.* (17).

was seen as one approached the center of the bilayer (Fig. 4). In the mycobacterial cell wall, a similar downward trend was evident, suggesting that the outermost lipids constitute a leaflet. However, the gradient was much shallower than in the phospholipid bilayer, and the order parameter with 9-SASL was actually slightly higher than that of 5-SASL in the isolated cell wall.

DISCUSSION

The model of mycobacterial cell wall proposed by Minnikin (3) predicted that mycolic acid chains are packed side by side in a direction perpendicular to the plane of the cell surface. It seems likely that this mycolic acid-containing inner leaflet is covered by an outer leaflet composed of extractable lipids, the whole structure producing an asymmetric lipid bilayer (ref. 18; see Fig. 5). This model received an experimental support from our recent x-ray diffraction study of purified *M. chelonae* cell wall, which showed that a significant portion of the hydrocarbon chains in the cell wall was tightly packed in a direction perpendicular to cell surface (6).

Mycolic acid residues, comprising the inner leaflet of the bilayer, contain 70–90 carbons and yet only two linkages that can be unsaturated (3). The inner leaflet is thus expected to have very low fluidity, since lipids containing longer hydrocarbon chains and fewer double bonds tend to become packed tightly against each other. In contrast, the extractable lipids of *M. chelonae* contain the usual mixture of saponifiable fatty acids mainly composed of 16:0, 16:1, and 18:1 (E.Y.R. and H.N., unpublished), in addition to glycopeptidolipids, reported to contain C_{28} to C_{34} unsaponifiable fatty acids that often contain double bonds (3). Thus, the outer leaflet is likely to be much higher in fluidity, and a steep gradient of fluidity is likely to exist across the thickness of the mycobacterial cell wall.

Consideration of the chemical structure of mycolic acids suggests that there may also be a more localized gradient of fluidity within the thickness of the inner leaflet. The mycolic acid contains a shorter, totally saturated, α-branch of typically 24 carbon atoms and a longer meromycolate branch that contains 50–60 carbon atoms (except α′-mycolic acid that occurs as a minor species) (3). The meromycolate branch contains only two positions that could be occupied by cis double bonds or *cis*-cyclopropane groups that produce kinks in the chain and thus increase the local fluidity (3). Furthermore, in highly drug-resistant, fast-growing species such as *M. che-*

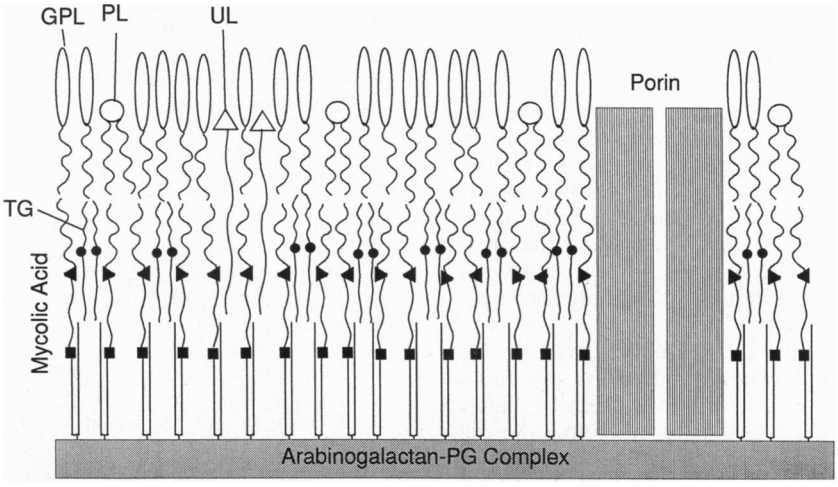

FIG. 5. Model of *M. chelonae* cell wall. This model is based on that proposed earlier by Minnikin (3). It is modified, however, in several ways. (*i*) We emphasize the presence of a fluidity gradient across the cell wall and, indeed, even within the mycolate-containing inner leaflet. This is based on the knowledge that the proximal double bond in the mycolic acid of *M. chelonae* (■) is at least 50% trans, on the basis of gas chromatography/mass spectroscopy data of Kaneda *et al.* (19). (*ii*) We assume that much of the difference in length between the two "arms" of mycolate is made up by the intercalation of triglycerides (TG). This is because the number of C_{14} to C_{19} saponifiable fatty acids present in the *M. chelonae* cell wall is about twice the number of mycolic acid residues, and about 80% of such "short-chain" fatty acids are found in TG (E.Y.R. and H.N., unpublished). (*iii*) We assume that much of the external surface of the cell wall is covered by glycopeptidolipids (GPL), since the number of GPL molecules present appears to be close to that of mycolate residues (E.Y.R. and H.N., unpublished). Some glycerophospholipids (PL) also appear to be present in the external leaflet (18). In addition, there are lipids of unknown structure (UL) containing fatty acids of intermediate chain length (between C_{20} and C_{40}) (E.Y.R. and H.N., unpublished). The position of the more distal double bond in the mycolic acid residue, which is always cis, is indicated by ▲. PG, peptidoglycan.

lonae, M. fortuitum, and *M. smegmatis*, most of the proximal double bonds that are typically 16–20 carbons away from the carboxyl end (■ in Fig. 5) are converted into trans double bonds (3, 19), that are incapable of producing kinks (20). These considerations suggest that the deepest section of the inner leaflet has the lowest fluidity in the cell wall. In contrast, the more distal position that is usually 30 or more carbons away from the carboxyl end (▲ in Fig. 5) is always occupied by cis double bonds or cyclopropane groups or by oxygen-containing structures that are likely to prevent the tight lateral packing of hydrocarbon chains (3, 18, 19). Thus, the more distal portion of the mycolate residue may produce a layer of intermediate fluidity.

The results of DSC study were fully compatible with the presence of one or more domains of extraordinarily low fluidity in the mycobacterial cell wall. (*i*) The observation of large cooperative thermal transitions was consistent with the presence of organized lipid domains. (*ii*) The transition temperatures observed were exceptionally high for lipids of biological membranes. Thus, the cytoplasmic membrane lipids of *Escherichia coli* grown at 37°C start to melt at −10°C and the thermal transition is essentially complete at 30°C (21), but the transition temperatures observed with mycobacterial cell wall were much higher than this, suggesting that, at growth temperature, significant portions of the lipids are in a "frozen" or gel-like state.

To our knowledge the melting behavior of mycobacterial cell wall has not been examined earlier. However, Durand *et al.* (22) studied the monolayers of a mycolate-containing lipid, trehalose dimycolate. They found that even the compounds containing corynemycolates from corynebacteria, which are much smaller (C_{32}) in comparison with the mycolates from mycobacteria (C_{70} to C_{90}) and therefore are expected to melt at much lower temperature, showed a major thermal transition at 41°C. These results are thus consistent with those of the present study.

It is difficult to give a definitive interpretation for the presence of more than one thermal transition (Fig. 1). In view of their large size, each of these transitions is likely to be the result of a cooperative gel-to-liquid crystalline transition. Such multiple transitions have been seen only when lipids were segregated either into separate lateral domains (23) or perhaps into separate leaflets (24). Since lateral phase separation is unlikely to occur in the inner leaflet of mycobacterial cell wall in which the mycolic acid residues are covalently fixed onto the arabinogalactan–peptidoglycan backbone, existence of more-or-less separate domains within the thickness of the bilayer, along the lines described above, seems to explain the results better. Although it is not possible to identify precise domain or layer that melts at each transition temperature, the considerations outlined suggest that the highest transition temperature may correspond to that of the deepest layer of the inner, or the mycolate, leaflet. The observation also suggests the presence of a domain(s) of much higher fluidity, a prediction borne out by the ESR studies.

The spin label experiments always produced a single, homogeneous signal from the bound spin label (except with 16-SASL). This suggests that the probe sampled only one, relatively homogeneous, environment. Spin-labeled fatty acids with their bulky doxyl groups are likely to become inserted into the region of highest fluidity. In our model (Fig. 5), this corresponds to the outer leaflet of the cell wall, composed of short-chain, often unsaturated, extractable lipids. In contrast, the opposite side would represent the least fluid part of the structure. We have shown, indeed, that addition of spin-labeled fatty acids to intact cells produced ESR spectra similar to that obtained with the isolated cell wall. As shown in *Results*, this outermost layer had considerable fluidity, although it was somewhat lower than that of the typical phospholipid bilayer. The marked temperature dependence of the mobility of spin label fatty acids was also consistent with the idea that the local environment of these probes is an organized lipid structure.

The fluidity (or flexibility) increased gradually as the nitroxide probe was positioned farther away from the surface (Fig. 4). This is as expected for a bilayer structure, as the lateral interaction between the polar head groups immobilizes the nearby methylene groups of the hydrocarbon chain, whereas there are more chances of C—C bonds taking non-*gauche* conformation as one travels farther toward the methyl end of the chain. Interestingly, however, the gradient of the fluidity

was much shallower than that commonly seen in phospholipid bilayer membranes. This could be due to one or more of the following possible causes: (*i*) the intercalated structure of the cell wall (Fig. 5), (*ii*) the unusual structure of the main outer leaflet lipid, glycopeptidolipid (3, 18), and (*iii*) the influence exerted by the much less fluid inner leaflet composed of mycolic acid residues. There is, indeed, a precedent for the last interpretation. Thus, 5-SASL inserted into the presumably more fluid phospholipid leaflet of the bacterial outer membrane reports fluidity values (15, 16) slightly lower than that inserted into phospholipid bilayer (13, 16), possibly because of the immobilizing effect of the less fluid lipopolysaccharide leaflet.

In this work, we studied the fluidity of the hydrocarbon domain in the cell wall of *M. chelonae.* Because the genus *Mycobacterium* comprises a rather tight cluster of highly related organisms (25), it can be safely assumed that the cell walls of other mycobacterial species, including those of *M. tuberculosis* and *M. leprae*, would behave in a manner at least qualitatively similar to that of *M. chelonae.* DSC and spin label studies here suggest that the cell wall contains domains of strikingly different degrees of fluidity. Taken together, these results are consistent with the presence of an asymmetric bilayer structure, with the mycolic acid-containing, less fluid, inner leaflet and the more fluid outer leaflet composed of extractable lipids. Because spin-labeled probes tend to become inserted only into the most fluid regions of the structure, it was not possible to measure directly the fluidity of the most rigid region of the cell wall. Nevertheless, the previous x-ray diffraction data (6) and the very high thermal transition temperature of 60°C, detected by DSC, suggest that the fluidity of this region is, indeed, very low. The lipopolysaccharide outer leaflet of the Gram-negative outer membrane, with its low fluidity interior (14), is known to slow down the influx of lipophilic compounds by a factor of 50–100 in comparison with the lipid bilayer of plasma membrane (26). Since the fluidity of the innermost layer of the mycobacterial cell wall is expected to be much lower than that in the lipopolysaccharide leaflet, this observation may explain, at least in part, the extraordinary resistance of many mycobacterial species to lipophilic antibiotics and chemotherapeutic agents (2). We have shown, indeed, that cultivation of *M. smegmatis* at 42°C produced cell wall lipids that melted at a higher temperature than those of the same organism grown at 25°C and, at the same time, decreased the influx of a moderately lipophilic antibacterial agent, norfloxacin, into the cell (unpublished).

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