Mycobacterial infections are the leading cause of death from infectious diseases (1). There are approximately 1 billion people presently infected with Mycobacterium tuberculosis. Leprosy, caused by M. leprae, affects 10–12 million people (1). “Atypical mycobacteria,” such as M. avium, M. intracellulare, M. xenopi, M. kansasi, M. chelonae, and M. fortuitum, cause opportunistic infections among AIDS patients. A major problem with the infections caused by mycobacteria is their intrinsic resistance to most general purpose antibiotics (1, 2). This problem is compounded by the emergence of multidrug-resistant strains of M. tuberculosis (3, 4).

The intrinsic drug resistance of mycobacteria has been attributed, at least in part, to the low permeability of the cell wall (2). The influx of small, hydrophilic agents, which are likely to traverse the cell wall through porin channels (5, 6), is extremely slow (7), presumably because the cell wall contains only a small number of low specific activity porin molecules. On the other hand, mycobacterial cell walls are extremely rich in lipids. Lipophilic and amphiphilic agents may therefore be expected to cross the cell wall through its lipid domain, yet most mycobacteria exhibit high levels of intrinsic resistance even to such agents. We are therefore trying to understand how the lipid domain of the mycobacterial cell wall can serve as an effective permeability barrier by studying the physical organization of the cell wall lipids.

The cell wall of mycobacteria contains an arabinogalactan linked to the wall peptidoglycan and esterified with mycolic acids. Mycolic acids and their homologs are long chain β-hydroxy α-branched fatty acids found mainly in the genera Mycobacterium, Nocardia, Rhodococcus, and Corynebacterium (8). Most of the main chain, from C-3 to the methyl-terminal carbon, is the meromycolate branch. Mycobacterial mycolic acids, or simply mycolic acids, are distinct from those of other genera in that: (i) they contain 70–90 total carbon atoms, with the totally saturated α-branch of typically 24 carbons and meromycolate chains of 40–60 carbons; (ii) in the meromycolate branch, there are usually only two positions that may be occupied by double bonds, cyclopropane rings, or other functional groups (9). The more distal of these two positions may contain oxygen-containing functionalities, which allow us to classify mycolic acids into α-mycobactafats (without any oxygen-containing functional groups in the meromycolate branch), ketomycolates, and methoxyoxymycobactafats. In some species, the oxygen-containing group in the meromycolate branch is a carboxylic ester, producing wax ester mycolates. The more proximal of these positions contains a double bond or cyclopropane, and in many species a fraction of these structures is converted from a cis-isomer to a trans-isomer, a reaction that is expected to affect the fluidity of the packed hydrocarbon chains. Mycobacterial cell walls also contain several types of “extractable lipids,” such as trehalose-containing glycolipids, phenolic glycolipids, or glycopeptidolipids (9, 10).

Recent biophysical studies provided some insights into the organization of the cell wall lipids. We (11) applied x-ray diffraction to the purified cell wall of M. chelonae and showed that the bulk of the hydrocarbon chains exist in a tightly packed, parallel, quasicrystalline array and that they are arranged mainly in a direction perpendicular to cell surface. These results provide strong experimental support for a model proposed by Minnikin (8), in which the cell wall is composed of an asymmetric lipid bilayer; the inner leaflet contains mycolic acids covalently linked to arabinogalactan and the outer leaflet...
contains other extractable lipids. More recently, we used DSC and electron spin resonance techniques to study the dynamic properties of lipids in the cell wall of *M. chelonae* (12). DSC showed that much of the lipids underwent major thermal transitions between 30 and 60 °C, suggesting that a significant portion of these lipids existed in a structure of extremely low fluidity in the growing cells. Spin label studies suggested that the mycobacterial cell wall forms an asymmetric bilayer, containing a moderately fluid outer leaflet and a mycolic acid-containing inner leaflet of extremely low fluidity. The low fluidity of the inner leaflet may thus account for the low permeability of the cell wall.

In this paper, we extend our previous DSC studies by using several different species of mycobacteria and by using intact cells as well as methyl esters of purified mycolates. Our results showed that cell wall lipids from all species of mycobacteria studied underwent major thermal transition(s) at very high temperatures. There was good correlation between the structure of mycolic acids present and the highest transition temperatures measured in the cell wall of each species. We also demonstrate that the fluidity of mycobacterial cell wall affects its permeability to lipophilic agents. These results strongly suggest that mycolic acids, fatty acids of an unusual structure, are used to produce a bilayer structure of exceptionally low fluidity and low permeability.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—The following strains of *Mycobacterium* species were studied: rapidly growing species *M. smegmatis* mc²-6, *M. smegmatis* mc²-155, *M. vaccae* ATCC15043, *M. aurum* ATCC23030, and *M. chelonae* PS4770; slow growing species *M. tuberculosis* H37Rv, *M. avium* A5, and *M. terrae* ATCC31755. *M. smegmatis* strains were obtained from W. Jacobs via J. Trias. The *M. chelonae* strain has been described (7). *M. tuberculosis* and *M. avium* cells were grown in the P-3 facilities at the Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases, and were used after killing the cells by γ-irradiation with 137Cs. Two species of *Corynebacterium* were also included in the study: *C. pseudotuberculosis* ATCC19110 and *C. bovis* ATCC7715.

Mycobacterial cells were grown in Middlebrook 7H9 broth (Difco) supplemented with 0.2% of glycerol and 10% Middlebrook OADC enrichment (Difco). Cells were harvested at mid-exponential phase. Corynebacterial cells were grown in Brain Heart Infusion Broth (Difco) supplemented with 5% rabbit serum.

**Chemicals**—All chemicals used, except medium ingredients, were of reagent grade. [14C]Norfloxacin (specificity, 14.8 mCi/mmol) was a gift from Merck. [14C]Chenodeoxycholate (specificity, 50 mCi/mmol) was obtained from DuPont NEN.

**Isolation and Purification of the Cell Wall**—The isolation and purification of cell wall was described previously (11). Briefly, mycobacterial cells were broken by sonication. The cell wall fraction was separated from cytoplasmic membrane by centrifugation in a sucrose step gradient. Purified cell walls were treated with trypsin as described (11) before they were subjected to DSC measurement. Corynebacterial cell walls were prepared similarly.

**Preparation of Triton X-114-insoluble Cell Wall Materials**—The preparation of Triton X-114-treated cell wall materials was described (13). Determination of acyl ester groups (14) in Triton-insoluble fraction showed that this treatment extracted about 60% of chloroform-methanol extractable lipids of the cell wall.

**MAME Isolation and Purification**—For *M. tuberculosis*, MAMEs were isolated and purified as described previously (15). Individual classes of mycolic acids (α- keto, and methoxy) were separated by column chromatography on silica gel using 19:1 (v/v) hexane:ethyl acetate as eluant. For *M. avium*, mycolic acids were prepared in one of two ways: (i) for isolation of intact wax ester mycolates, total mycolic acids were extracted using the Mild Tetrabutylammonium Hydroxide methylation method (17). Following esterification, the total mycolates were precipitated with toluene:acetone and purified wax ester mycolates were obtained as follows: 100 mg of total methyl mycolates was dissolved in 5 ml of absolute ethanol, and 50 mg of sodium borohydride was added at room temperature. The reaction was allowed to proceed for 2 h before adding an additional 20 mg of sodium borohydride and stirring at room temperature for an additional 12 h. Water (2 ml) was added slowly, as well as ether (10 ml), and the solution was extracted again with an additional 10 ml of ether. The combined organic layers were washed once with water and dried in vacuo. The remaining mycolates were precipitated, and wax ester mycolates were separated from α-mycolates and reduced ketomycolates by column chromatography as above. These preparations were free of ketomycolates as assessed by H NMR. In addition, thin-layer chromatography was always used to confirm the class assignment of mycolates.

NMR samples were analyzed in deuteriochloroform on a Varian Unity Plus 500 MHz spectrometer. cis/trans-cyclopropane ratios were assessed by integration of the cyclopropane resonances assigned as follows: cis (δ = 0.33 (m, 1H), δ = 0.57 (m, 1H), δ = 0.66 (m, 2H); trans (δ = 0.11 (m, 3H), δ = 0.42 (m, 1H)).

**DSC—DSC** measurements were done on either a Microcal MC-2 differential scanning calorimeter or a Hart model 4110 differential scanning calorimeter. For purified cell wall or Triton X-114-insoluble cell wall materials, data were collected by heating the sample from 10 to 90 °C on the first heating scan. For MAME, the sample was initially heated at 30 °C/h from 10 to 60 °C and cooled at the same rate to 10 °C. This rapid cycle was followed by slower heating and cooling cycles through the same temperature range at 10 °C/h; data were collected at 10-s intervals.

**Assay of Norfloxacin and Chenodeoxycholate Accumulation**—This assay was performed as described (18), with 10 μM substrates, at 23 °C for norfloxacin uptake and 30 °C for chenodeoxycholate uptake.

**RESULTS**

**Thermal Transitions of Cell Walls and MAMEs from *M. tuberculosis* and *M. avium*—Melting temperatures of organized lipids serve as rough indicators of the rigidity of the hydrocarbon environment. Thermal transitions of cell wall lipids were therefore examined on various preparations by DSC. The results obtained with *M. tuberculosis* H37Rv and *M. avium* A5 were presented in some detail (Figs. 1 and 2). We first used cell walls purified from *M. tuberculosis* H37Rv without the use of detergents to avoid any possible perturbation of the lipid organization. The cell wall was first treated with trypsin (see “Experimental Procedures”), so that the thermal denaturation of cell wall proteins did not produce peaks in DSC. DSC scanning of such a preparation showed two distinct thermal transitions at 31 and 63 °C (Fig. 1A). In this as well as other analysis of cell walls, scanning was repeated at least four times (two upward scans and two downward scans) to confirm the reversibility of thermal transitions. DSC analysis of intact cells also showed thermal transitions at about the same temperatures (Fig. 1B), suggesting that the transitions seen in the isolated cell wall are not artifacts. Cell wall materials left after Triton TX-114 extraction of much of the loosely associated lipids (see “Experimental Procedures”) showed a melting curve of very similar shape, with a small downward shift of the melting temperatures to 33 and 61 °C (Fig. 1C). This observation suggests that the major transitions are caused by the remaining mycolyl-arabinogalactan complex. Finally, purified MAMEs (see “Experimental Procedures”) also showed a major thermal transition at 41 °C (Fig. 1D), after a few cycles of heating and cooling. Apparently, during these cycles MAMEs become aligned spontaneously to form an organized structure that goes through a cooperative melting process (see Ref. 13). The relatively high transition temperature of MAME is consistent with the notion that the melting of cell wall lipids is unusual at unusually high temperatures (Fig. 1, A-C) and was indeed due to the melting of the mycolate chains. (Methyl esters of conventional fatty acids melt at lower temperatures; methyl myristate...
melts at 19°C and methyl oleate at well below 0°C (19). Although the transition temperature of MAME was considerably lower than in cell walls, this is not surprising because the macromolecular head group, arabinogalactan, is totally absent from MAME. The head group structure affects the melting temperature of phospholipid bilayers (20), and examination of bacterial lipopolysaccharides and Thermus glycolipids suggests that attachment of a larger number of hydrocarbon chains to a single head group raises the melting temperature significantly (21).

Study of *M. avium* A5 led to similar conclusions. Thus, the isolated, trypsinized cell wall showed major thermal transitions at 27 and 66°C (Fig. 2A), intact cells showed the highest transition temperature around 70°C (Fig. 2B), and the Triton-X-114-extracted cell wall showed the highest transition temperature at 64°C (Fig. 2C). The mixture of MAMEs obtained from this strain showed a clear-cut transition at 47°C (Fig. 2D).

**FIG. 1.** Thermal transitions in various fractions from *M. tuberculosis* H37Rv. A, cell walls (dry weight, 4 mg) prepared by sonication and a sucrose step gradient were treated with trypsin before DSC measurement; B, intact cells; C, cell walls prepared by Triton X-114 extraction; D, mixed MAME preparation.

**FIG. 2.** Thermal transitions in various fractions from *M. avium* A5. A, cell walls (dry weight, 4 mg) prepared by sonication and a sucrose step gradient were treated with trypsin before DSC measurement; B, intact cells; C, cell walls prepared by Triton X-114 extraction; D, mixed MAME preparation.

**Mycolic Acid Structure Determines Cell Wall Fluidity**

DSC of Cell Walls Purified from Other Species of Mycobacteria—To understand the structural motif(s) responsible for the observed melting behavior, we extended DSC measurements to cell walls purified from two species of *Corynebacterium* as well as several other species of mycobacteria. The results are shown in Fig. 3 and in Table I. DSC scans of cell walls of *M. terrae*, *M. chelonae*, *M. smegmatis*, *M. aurum*, and *M. vaccae* were similar to those of *M. tuberculosis* H37Rv and *M. avium* A5 in showing two major peaks, whereas cell walls of *M. smegmatis*, *M. aurum*, and *M. vaccae* produced only one major thermal transition each.

The highest melting temperatures are listed in Table I. In contrast to corynebacterial cell wall, the cell walls of all mycobacterial species melted at dramatically high temperatures, between 60 and 70°C, suggesting an interior of extremely low fluidity at the growth temperature. However, the highest transition temperatures were not identical among different species. Among the fast growers, the melting temperatures of cell walls from *M. vaccae* and *M. aurum* were about 2–4°C lower than those of the cell walls from *M. chelonae* and *M. smegmatis* grown at the same temperature. Compared with the fast growers, cell walls of the three slow growers showed significantly higher melting temperatures, with the exception of *M. tuberculosis* H37Rv.

Melting Temperatures and Structures of Isolated Mycolate Subclasses—To investigate the relationship between the structure of mycolate present and thermal transition temperature, we purified each of the individual mycolic acid subclasses from *M. tuberculosis* H37Rv (*α*, keto, and methoxy) and *M. avium* A5 (*α*, keto, and wax ester) and determined the ratio of trans- to cis-cyclopropane at the proximal position (the position closer to the carboxylate group) by 1H NMR (see “Experimental Procedures”). The mycolates of *M. tuberculosis* were easily separable by column chromatography. The separation of *M. avium* mycolates was more difficult due to the tendency of the mycolate moiety of wax ester mycolates to become cleaved during the initial saponification procedure. Isolation of wax ester mycolates was achieved by gentler saponification followed by the reduction of ketomycolates into hydroxymycolates (see “Experimental Procedures”). Each MAME subclass usually exhibited one major thermal transition peak. However, the ketomycolates from *M. tuberculosis*, containing 17% trans-cyclopropane, were an exception and always showed two well separated tran-
sitions at 40.5 and 49.5 °C. Since the other mycolate subclases containing no trans-cyclopropane all melt around 40 °C, we interpret this result to mean that the cis-cyclopropane-containing species and trans-cyclopropane-containing species become laterally segregated, and melt independently at 40.5 and 49.5 °C, respectively. When the observed transition temperatures of various MAMEs were plotted against the ratio of trans-to cis-cyclopropane structure at the proximal position, an excellent correlation was observed, regardless of which moiety was present at the distal position (Fig. 4).

trans/cis Ratio and Whole Cell Thermal Transitions of M. avium Grown at Different Temperatures—To extend this analysis to whole organisms, we grew M. avium A5 at four temperatures between 30 °C and 45 °C. Analysis of the whole cell phase transition temperatures revealed that M. avium responded to increasing growth temperature by increasing the transition temperature over a 4.5 °C range (Fig. 5). We purified total mycolates from M. avium grown at each of these temperatures by hydrolysis with 5% tetrabutylammonium hydroxide (to preserve wax ester mycolates intact) and analyzed each of these mixed mycolate preparations by 1H NMR. The ratio of trans/cis-mycolates varied from 0.53 for organisms grown at 30 °C to 0.75 for organisms grown at 40 or 45 °C (Fig. 5), showing a good correlation with the transition temperature. We also examined these mycolate preparations by pyrolytic gas chromatography-mass spectrometry to determine whether any changes occurred in the length of the α-branch. Using the conditions described earlier (23), we confirmed that M. avium from each growth temperature released only tetracosanoic acid, suggesting that the length of the α-branch does not change in response to growth temperature, consistent with the results reported in M. smegmatis (24). Analysis by reverse-phase HPLC of p-bromophenacyl esters of these mycolates revealed only small variations in total chain length, at most a shift in two carbons in each of the major mycolate species between organisms grown at 30 °C and 45 °C (data not shown).

DSC of Cell Walls Purified from M. smegmatis Grown at Different Temperatures—We also compared the mc2-6 strain of M. smegmatis grown at different temperatures. In the cell walls from organisms grown at 18, 30, 37 or 45 °C, the highest thermal transitions occurred at 49.8, 60.3, 63.5, and 65.6 °C, respectively. Earlier studies indeed showed that the composition of mycolates showed a significant change in M. smegmatis depending on growth temperature (24, 25). An increase in the growth temperature from 20 to 45 °C increased the chain length somewhat, so that the major α-mycolate species at these two temperatures were C72–76 and C79, respectively. Furthermore, in the series containing 78 or 79 carbon atoms, the species containing a trans double bond (i.e. the species with an odd number of carbon atoms) increased from 19% at 20 °C to 59% at 45 °C. These alterations would be expected to raise the melting temperature in cells grown at 45 °C. We have also confirmed, by HPLC analysis of mycolate p-bromophenacyl esters, that the increase in growth temperature produced a small increase in the average chain length of mycolic acids (data not shown). It thus appears that mycobacteria alter the fluidity of their cell wall matrix by altering the structure of the mycolic acids produced.

Accumulation of Lipophilic Agents by M. smegmatis Cells Grown at Different Temperatures—To investigate the correlation between the fluidity of mycobacterial cell wall and its permeability to antimicrobial agents, we examined the accumulation of norfloxacin and chenodeoxycholate by intact cells of M. smegmatis (strain mc2-6) grown at 30 and 45 °C. Typical accumulation curves are shown in Fig. 6. M. smegmatis grown at 30 °C showed significantly higher initial rates of accumulation for both probes than the same organism grown at 45 °C, a result suggesting that at least portions of these agents penetrate the cell wall through the lipid domains, which are less fluid in M. smegmatis cells grown at 45 °C. Similarly, higher initial rates of accumulation for chenodeoxycholate were observed in M. avium A5 grown at lower temperatures (data not shown).

DISCUSSION

Previous studies have suggested that the M. chelonae cell wall contains a lipid bilayer in which the mycolic acid chains are packed side by side in a direction perpendicular to the cell surface and that this mycolic acid-containing inner leaflet is presumably covered by an outer leaflet composed of extractable, shorter chain lipids (11). The direct consequence of this arrangement is the existence of a fluidity gradient across the bilayer with the minimum fluidity occurring in the innermost part. The very low fluidity of this domain was suggested by the sharp 4.2-Å x-ray diffraction ring arising from the quasi-crystalline packing of the hydrocarbon chains (11) and was confirmed by a high melting temperature, around 60 °C, of the nonprotein domain observed by DSC (12). It seemed likely that this melting temperature reflected the thermal transition of mycolic acid-containing layer, because mycolic acids are a major component of the cell wall. To test this assumption, we extended the DSC studies to several other species.

The low fluidity of cell wall lipids was not limited to the previously studied M. chelonae and was indeed found in all of the mycobacterial species studied (Table I). These data further suggest that the major components melting at the highest transition temperatures are indeed mycolic acid residues and that the transition temperature is determined by at least two structural features of mycolic acid. The first feature is the overall length of the hydrocarbon chains. Thus, corynebacterial...
cell walls, containing 32–38 carbon corynemycolates (26), melted at 36–39 °C, in striking contrast to mycobacterial cell walls, which contained 74–80 carbon mycolates and melted in a much higher temperature range, above 59°C. The second walls, which contained 74–80 carbon mycolates and melted in 36–39°C, in striking contrast to mycobacterial cell walls, containing 32–38 carbon corynemycolates (26), which have a cyclopropane group in the proximal position, all the other species listed contain double bonds in this position. Values were estimated from published data (27).

All strains were grown at 37 °C.

Total carbon number (average, see Refs. 26–27).

Except for M. tuberculosis H37Rv and M. avium, which have a cyclopropane group in the proximal position, the other species listed contain double bonds in this position. Values were estimated from published data (27).

M. tuberculosis contained, however, ketomycolates, of which 17% had trans cyclopropane (see "Results").

M. avium contained, however, large amounts of keto- and wax-ester mycolates, of which 60 and 64%, respectively, had trans cyclopropane (see "Results").

### Table I

| Species | Mycolate size<sup>b</sup> | % trans at proximal position in α-mycolate<sup>c</sup> | Highest transition temperature<sup>c</sup> |
|---------|--------------------------|---------------------------------|------------------------------------------|
| C. bovis | 32                       |                                 | 35.9                                     |
| C. pseudotuberculosis | 38                  | <10                             | 39.4                                     |
| M. vaccae | 74                   |                                 | 59.3                                     |
| M. aurum | 76                       | <20                             | 60.3                                     |
| M. chelonae | 77                   |                                 | 62.0                                     |
| M. tuberculosis H37Rv | 80                 | 0<sup>d</sup>                   | 62.8                                     |
| M. smegmatis mc<sup>2</sup>-6 | 77            | 68                              | 65.5                                     |
| M. smegmatis mc<sup>2</sup>-155 | 77        | 68                              | 64.0                                     |
| M. avium | 80                       | 9<sup>e</sup>                   | 65.3                                     |
| M. terrae | 79                      |                                 | 66.9 (major)                             |

<sup>a</sup> All strains were grown at 37 °C.

<sup>b</sup> Total carbon number (average, see Refs. 26–27).

<sup>c</sup> Mycolate structure and thermal transition temperature.

<sup>d</sup> M. tuberculosis contained, however, ketomycolates, of which 17% had trans cyclopropane (see "Results").

<sup>e</sup> M. avium contained, however, large amounts of keto- and wax-ester mycolates, of which 60 and 64%, respectively, had trans cyclopropane (see "Results").
Mycolic Acid Structure Determines Cell Wall Fluidity

In summary, we have shown that mycolic acid structure plays a critical role in controlling the cell wall fluidity as well as contribute to the difference in transition temperature, we believe that the fraction of trans structure is likely to make a significant contribution. Particularly revealing in this respect is the comparison of M. aurum with M. chelonae: the 2 °C difference in the transition temperature is likely to reflect the difference in the content of trans double bonds, as the effective chain length appears to be identical (Table I).

Interestingly, the cell walls of M. tuberculosis and M. avium had rather high transition temperatures, in spite of the low chain length appears to be identical (Table I). Furthermore, another factor that may contribute to the rigidity of their cell walls was recently discovered. The cma2 gene, the protein product of which catalyzes the introduction of a cyclopropane at the proximal position in the meromycolate chain, was recently cloned from M. tuberculosis (13).

Expression of cma2 in M. smegmatis resulted in the cyclopropanation of the proximal double bond in the α-mycolate. DSC of cell wall materials prepared by Triton X-114 extraction and purified MAMEs from the recombinant M. smegmatis showed that cyclopropanation of the proximal position raised the melting temperature by 3 °C (13). Thus, introduction of even a cis-cyclopropane in the proximal position of meromycolate ac-

Consequences (29).

The ultimate goal of these studies is to understand the mechanisms(s) that are responsible for the extremely low permeability of mycobacterial cell wall and, consequently, for the intrinsically drug resistance of mycobacteria. When the entry of norfloxacin and chenodeoxycholate, both moderately lipophilic molecules, into intact cells of M. smegmatis grown at 30 and 45 °C was measured, the more fluid cell wall from cells grown at 30 °C indeed allowed a more rapid accumulation of these agents, in comparison with the less fluid cell wall of cells grown at 45 °C (Fig. 6). To confirm that these agents penetrate the lipid domains of the cell wall, we compared their influx using the cells from the same batch at two different temperatures. The solute permeation across lipid bilayers is known to be strongly dependent on temperature (30). The rates of entry of norfloxacin and chenodeoxycholate by M. smegmatis increased 2.5- and 3.8-fold, respectively, when assay temperature was raised by 10 °C from those temperatures used routinely for the assay (data not shown), suggesting that the major penetration pathway for these agents is the lipid domain of cell wall.

In addition, M. avium is rich in keto- and wax ester mycolates that contain 60–64% trans-cyclopropane (Fig. 4), although its α-mycolate contains only cis-cyclopropane. The M. avium cell wall containing these trans-mycolates melted at a temperature higher by 3 °C than the cell wall of M. tuberculosis H37Rv, which contained significantly lower fraction of such compounds (0, 0, and 17% trans in α-, methoxy-, and ketomycolates, respectively (Fig. 4)), as expected. When the melting temperature of individual mycolate species were compared, a striking linear relation was found between the amount of trans-cyclopropane structure and the observed melting temperature (Fig. 4). This provides direct support for the thesis that less disruptive trans structures elevate the melting temperature of the cell wall.

The comparison between the melting of cell wall and the structure of mycolic acid is more straightforward when it is made on the same organism (e.g. M. smegmatis or M. avium) grown at different temperatures. As mentioned under “Results,” earlier studies with M. smegmatis (24, 25) showed that growth at lower temperatures shortened the average chain length of mycolic acids slightly (by about 2 carbons) and drastically decreased the fraction of trans-mycolates. Cell walls from M. smegmatis grown at lower temperatures indeed melted at significantly lower temperatures (“Results”). This correlation holds true for M. avium, where a lower growth temperature decreased the fraction of mycolates containing trans-cyclopropanes and was associated with a low melting temperature (Fig. 5). This correlation between the mycolic acid structure and the melting points of the cell wall suggests that mycobacteria maintain proper cell wall fluidity by changing mycolic acid composition in response to growth temperature.

Previous studies suggested M. phlei also regulates its cell wall fluidity by decreasing the chain length of mycolates at low temperatures (25). Interestingly, M. tuberculosis, which has a very narrow temperature range for growth, apparently fails to regulate the fluidity of cell wall and also fails to synthesize mycolic acids at all at low temperature, with lethal consequences (29).

FIG. 6. Accumulation of lipophilic agents by M. smegmatis cells grown at 30 (○) or 45 °C (■). A, accumulation of [3H]norfloxacin, assayed at 23 °C; B, accumulation of [3H]chenodeoxycholate, assayed at 30 °C. Drug concentrations were 10 μM. These experiments were repeated several times, and similar differences were always observed between the cells grown at 30 and 45 °C.
the permeability of mycobacteria. We have further identified the structural motifs in the mycolic acid molecule, which contribute to the impermeability and the intrinsic drug-resistance of mycobacteria. Enzyme(s) that catalyze the introduction of these structural motifs will provide targets for the design of new agents for chemotherapy of mycobacterial infections.

REFERENCES

1. Bloom, B. R., and Murray, C. J. L. (1992) Science 257, 1055–1064
2. Jarlier, V., and Nikaido, H. (1994) FEMS Microbiol. Lett. 123, 1–8
3. Frieden, T. R., Sterling, T., Pablos-Mendez, A., Kilburn, J. O., Cauthen, G. M., and Dooley, S. W. (1993) N. Engl. J. Med. 328, 521–526
4. Goble, M., Isemann, M. D., Madsen, L. A., Waite, D., Ackerson, L., and Horshburg, C. R., Jr. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 5021–5025
5. Trias, J., Jarlier, V., and Benz, R. (1992) Science 258, 1479–1481
6. Trias, J., and Benz, R. (1994) Mol. Microbiol. 14, 283–290
7. Jarlier, V., and Nikaido, H. (1990) J. Bacteriol. 172, 1418–1423
8. Minnikin, D. E. (1982) in The Biology of the Mycobacteria (Ratledge, C., and Stanford, J., eds) pp. 95–184, Academic Press, San Diego, CA
9. Brennan, P. J., and Nikaido, H. (1995) Annu. Rev. Biochem. 64, 29–63
10. Brennan, P. J. (1995) Rev. Infect. Dis. 11, S420–S430
11. Nikaido, H., Kim, S.-H., and Rosenberg, E. Y. (1993) Mol. Microbiol. 8, 1025–1030
12. Liu, J., Rosenberg, E. Y., and Nikaido, H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11294–11298
13. George, K. M., Yuan, Y., Sherman, D. R., and Barry, C. E., III (1995) J. Biol. Chem. 270, 27272–27278
14. Snyder, F., and Stephens, N. (1959) Biochim. Biophys. Acta 34, 244–245
15. Yuan, Y., Lee, R. E., Besra, G. S., Belisle, J. T., and Barry, C. E., III (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6630–6634
16. Hamid, M. E., Minnikin, D. E., and Goodfellow, M. (1993) J. Gen. Microbiol. 139, 2203–2213
17. Minnikin, D. E. (1988) in Bacterial Cell Surface Techniques (Hancock, I. C., and Porton, I. R., eds) pp. 125–135, John Wiley & Sons, Chichester, United Kingdom
18. Liu, J., Takiff, H. E., and Nikaido, H. (1996) J. Bacteriol. 178, 3791–3795
19. Lutten, E. S. (1967) in Fatty Acids (Markley, K. S., ed) 2nd Ed., pp. 2588–2593, Interscience, New York
20. Lewis, R. N. A. H., and McElhaney, R. N. (1991) in The Structure of Biological Membranes (Veague, P., ed) pp. 73–115, CRC Press, Boca Raton, FL
21. Nikaido, H. (1990) in Membrane Transport and Information Storage (Aloia, R. C., Curtin, C. C., and Gordon, L. M., eds) pp. 165–190, Wiley-Liss, New York
22. Dewa, T., Vignond, S. J., and Regen, S. L. (1996) J. Am. Chem. Soc. 118, 3435–3440
23. Kaneda, K., Imaizumi, S., and Yano, I. (1995) Microbiol. Immunol. 39, 563–570
24. Baba, T., Kaneda, K., Kusunose, E., Kusunose, M., and Yano, I. (1989) J. Biochem. 106, 81–86
25. Toriyama, S., Yano, I., Matsui, M., Kusunose, M., and Kusunose, E. (1978) FEMS Lett. 9, 111–115
26. Minnikin, D. E., and Goodfellow, M. (1980) in Microbiological Classification and Identification (Goodfellow, M., and Board, R. G., eds) pp. 189–256, Academic Press, London
27. Kaneda, K., Imaizumi, S., Mizuno, S., Baba, T., Tsukamura, M., and Yano, I. (1988) J. Gen. Microbiol. 134, 2213–2229
28. Dobson, G., Minnikin, D. E., Minnikin, S. M., Parlett, J. H., Goodfellow, M., Ridell, M., and Magnusson, M. (1985) in Chemical Methods in Bacterial Systematics (Goodfellow, M., and Minnikin, D. E., eds) pp. 237–265, Academic Press, London
29. Takayama, K. Armstrong, E. L., Davidson, E. L., Kunugi, K. A., and Kilburn, J. O. (1978) Am. Rev. Respir. Dis. 118, 113–117
30. Gale, W. R., Owen, J. D., and Solomon, A. K. (1973) J. Gen. Physiol. 61, 727–746