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THE OUTER PARTS OF THE MYCOBACTERIAL ENVELOPE AS PERMEABILITY BARRIERS

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1. ABSTRACT

The permeability of mycobacteria to substances in their environment is controlled by the properties of their envelopes. Two special features are important: an outer lipid barrier based on a monolayer of characteristic mycolic acids and a capsule-like coat of polysaccharide and protein. The mycolate layer prevents entry of small hydrophilic molecules, which obtain access to the cell by way of pore-forming proteins resembling porins of Gram-negative bacteria. More lipophilic molecules can diffuse through the lipid layer. The capsule probably impedes access by macromolecules; in intracellular pathogenic species it forms the electron-transparent zone that separates the bacterium from the membrane of the host phagosome. The structure of the outer lipid barrier seems common to all mycobacteria, fast- and slow-growing, but the capsule is more abundant in slow-growing species, a group which includes all the important mycobacterial pathogens. Mycobacteria secrete proteins into their environment, which are likely to be important in the pathogenesis of mycobacterial diseases. Knowledge of how these proteins, and the polysaccharides of the capsule, cross the outer lipid barrier is minimal at present. It is likely that proper knowledge of mycobacterial permeability will enable new approaches to treatment of mycobacterial disease.

2. INTRODUCTION

Mycobacteria have conventionally been considered to be surrounded by a "thick waxy coat" of lipid. The abundance of mycobacterial lipids has been recognized for a long while and much effort has been expended on separating and identifying the various types of lipid present, many of which are unique to mycobacteria. The recent analysis of open reading frames in the genome of the tubercle bacillus (1) indicates that additional distinctive lipids remain to be found. Most of this lipid is associated with the mycobacterial envelope and has been pictured as forming an outer layer. Such a coat around mycobacterial cells could explain their limited permeability, their tendency to grow in large clumps, their physical toughness and their rather general insusceptibility to toxic substances. It is now becoming clear, though, that the real situation is much more complicated and also much more interesting: the mycobacterial envelope has a complex and unusual structure which is adapted to protect the organism from its environment. Certainly the envelope contains much lipid, but it is the detailed arrangement of the lipid which contributes to the protection, rather than the mere presence of a large amount of it. Further, it seems that the lipid itself is covered, at least in intracellular pathogenic mycobacteria, by an outer capsule-like structure of polysaccharide and protein. This review will describe the development of ideas about the way the mycobacterial envelope is arranged to control permeability and the experimental data that support these ideas. It will also discuss some of the aspects of the permeability of envelope that are still not understood. Emphasis will be on the slow-growing species because of their importance as causes of human disease, notably tuberculosis, but as far as is known at present the arrangement of the envelope is common to both slow- and fast-growing species, whether pathogens or not.

3. THE MYCOBACTERIAL ENVELOPE

The chemical structure of the envelope has been reviewed recently (2,3,4), so only a brief description will be given here. The arrangement of the various layers is shown diagrammatically in figure 1. The innermost layer of the envelope is the plasma membrane, which seems typical of bacterial membranes. Some of its important functions have been investigated, notably electron transport (5), and others may be assumed by analogy with other types of bacteria and from the increasing knowledge of the spectrum of proteins of the mycobacterial cell. One of the chief functions of the rest of the envelope must be to protect this vital, highly physiologically active structure from external influences. Outside the plasma membrane is the 'cell wall skeleton' (6), a giant macromolecule entirely surrounding the bacterial cell, and consisting of peptidoglycan (a structure of oligosaccharides formed from disaccharide units of *N*-acetylglucosamine and *N*-glycollylmuramic acid cross-linked by short peptides), arabinogalactan (a complex branched polysaccharide) and mycolic acids (long-chain, 2-alkyl-3-

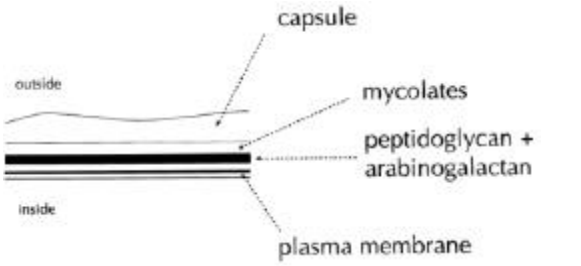

Figure 1. Arrangement of mycobacterial envelope. Thickness of layers roughly corresponds to appearance in thin sections of freeze-substituted *M.kansasii* (50).

Figure 2. Alpha-mycolate of *M.tuberculosis* (8). This species contains two cyclopropyl rings. The figure shows the two unequal alkyl chains of the molecule. The attachment through an ester link to the envelope arabinogalactan is indicated as **ag**.

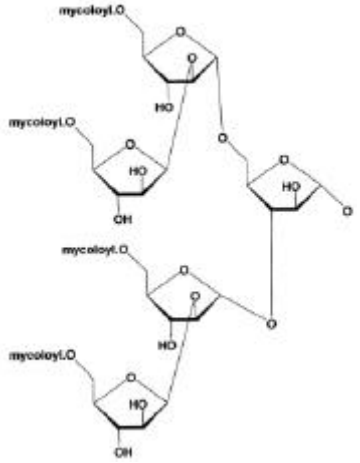

Figure 3. Arrangement of mycolates on the arabinogalactan. Four mycolate residues are attached to an arabinose pentamer, which is the non-reducing terminal motif of the arabinogalactan (18).

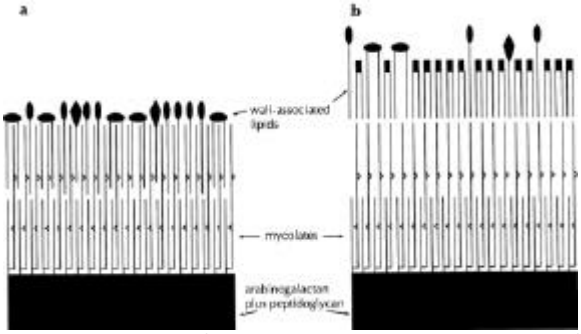

Figure 4. Models of the outer lipid permeability barrier of the mycobacterial envelope. (4a) Model of Minnikin (8), with the longer alkyl chains of the mycolates intercalated with alkyl chains of envelope-associated lipids. (4b) Model of Rastogi (10), in which the envelope-associated lipids form a bilayer with the mycolates. The variety of head-groups in the wall-associated lipids is indicated schematically; in (b) the majority are phoshoplipids. For convenience all the mycolate residues are shown as alpha-mycolates, though in reality a variety of mycolate species is present. The various alkyl chains are roughly to scale.

hydroxy fatty acids). A typical mycolate residue is shown in figure 2, and its mode of attachment to the arabinogalactan in figure 3. Peptidoglycan is found, with variants, in almost all eubacteria, and an attached polysaccharide is common in Gram-positive bacteria, but the presence of massive amounts (about half the weight of the cell wall skeleton) of covalently bound lipid is distinctive of the actinomycetales, which include the mycobacteria and corynebacteria plus several other genera. Associated with the cell-wall skeleton but not covalently attached to it are a large variety of other lipids. The surface of the envelope consists of a more-or-less loosely attached capsule-like layer of polysaccharide and protein, with rather little lipid (7). In static cultures, and (presumably) inside phagocytic cells, this layer is quite thick and is associated with the bacteria, but in shaken cultures it tends to be shed into the medium so that relatively little may be recovered with the bacteria. In their natural environment mycobacteria, whether in the soil or in water or in a host, are unlikely to be subjected to much agitation, so it seems to follow that the 'attached' situation of the capsule is the natural one.

3.1. The arrangement of mycolates

The original hypothesis, which forms the basis of current understanding of the arrangement of the components of the mycobacterial envelope, with its consequences for permeability, was put forward by Minnikin (8) in a review of mycobacterial lipids. The hypothesis depended mainly on a consideration of the detailed chemical structure of the major wall lipids, the mycolic acids. It proposed that these were arranged as a monolayer, with their long alkyl chains parallel to one another and perpendicular to the bacterial surface. A simplified version of the proposed arrangement is shown in figure 4a. Since each mycolate has two alkyl chains of very unequal length (figure 2) this would create spaces through part of the thickness of the monolayer into which alkyl chains of associated lipids would fit. The head groups of the associated lipids, many of which are quite polar, would then form a hydrophilic surface; the whole structure would be an analogue of a normal biological bilayer membrane and would form a barrier to hydrophilic molecules.

Although no sign of a second lipid bilayer had ever been (or has since been) reported in electron micrographs of sectioned mycobacterial cells, there was some support for the mycolate monolayer hypothesis from the then rather new technique of freeze fracture. In this process biological samples are frozen very rapidly in dilute aqueous media; the water solidifies too quickly to crystallize and the ice forms an amorphous glass. When such ice is fractured the plane of fracture is smooth and, in general, cuts across any biological structure in its path but with an important exception: if the fracture meets a biological membrane its plane is diverted briefly to split the two parts of the bilayer. Freeze-fractured samples of mycobacteria showed that these organisms had two such planes of weakness in their envelopes (9): one the plasma membrane, as expected, the other external to this in the outer part of the envelope. Minnikin (8) pointed out that the additional plane of weakness could represent the interface between the mycolate layer and the associated lipids which, like the junction between the two layers of fatty acyl chains in a biological membrane, is held together only by rather weak hydrophobic interactions.

An alternative to the original Minnikin hypothesis has been proposed by Rastogi (10), and is shown in figure 4b. The chief difference is that while in the original version the acyl chains of the mycolates and of the associated lipids are supposed to be intercalated, in the alternative the associated lipids are imagined as a separate monolayer, so that the structure is a more precise analogue of a normal biological membrane. Phospholipids were considered to make a major contribution to the structure. There is, at present, no obvious way of distinguishing between these hypotheses. However, their common ground, the arrangement of the mycolate in a monolayer, seems more important than the precise arrangement of the associated lipids, whose chemistry in any case differs considerably among the various mycobacterial species.

3.2. Evidence for the outer lipid permeability barrier

Substantial evidence for the existence of a mycolate monolayer in the mycobacterial envelope has been found as a result of a deliberate search, notably by Nikaido and his several collaborators. X-ray diffraction measurements on purified mycobacterial envelopes, free of plasma membranes (<2% contamination or better), discovered a strong reflection at 4.2 Å and a weaker and more diffuse one at 4.5 Å (11). Such reflections are distinctive of ordered fatty acyl chains, and were interpreted as indicating the presence of highly ordered and less ordered regions, respectively. The possibility that the reflections were caused by wall-associated protein was ruled out. Measurements on a partly oriented sample of walls obtained by centrifuging on to a flat surface showed that the acyl chains were aligned perpendicular to the plane of the wall, consistent with the mycolate monolayer hypothesis. Importantly, it was clear that in a species where the amount of mycolate present has been carefully measured, *Mycobacterium bovis* bacille Calmette-Guérin (BCG), there is sufficient in the envelope to form a compact monolayer.

Purified mycobacterial walls have been examined by differential scanning calorimetry, which measures phase changes (12). The lipids of the walls possess a 'melting point' well above the highest temperature at which the bacteria are viable and very much higher than that for normal biological membranes. This indicates that in the living organisms the mycolates exist as a compact and well-ordered monolayer. That the molecules involved really are mycolates is well demonstrated by modifying the types of mycolate present by molecular biological techniques. Introduction of tuberculosis-type mycolates into the walls of *Mycobacterium smegmatis* by cloning into that organism a gene from *Mycobacterium tuberculosis* which converts double bonds to cyclopropyl groups raises the 'melting point' by 3 °C (13). By studying purified walls, walls with most of their associated lipids removed with the detergent Triton X-114 and whole cells it has been clearly shown that the nature of the mycolic acids is the main determinant of the high-temperature phase change (14). Chain length is one important factor, and corynebacteria, whose mycolic acids are much shorter than those of mycobacteria, have a lower-temperature phase-change. Also important seems to be the configuration of the double bond or cyclopropyl group proximal to the carboxyl group of the mycolate, since a higher temperature phase change correlates with a higher proportion of *trans* configuration. It was also clear that some environmental mycobacteria could adjust the composition of their mycolates according to temperature, presumably to achieve the required behaviour of their outer permeability barrier. So *M.smegmatis* became less permeable to two lipophilic drugs when grown at higher temperatures.

Measurements by electron-spin resonance of lipophilic probes – spin-labelled fatty acids – 'dissolved' in purified walls or whole bacteria showed that these entered only a less ordered and more fluid region (12). This second region may either be interpreted as the alkyl chains of associated lipids that intercalate into that part of the mycolate monolayer where only the longer of the two alkyl chains of each mycolate is present or as the region occupied by the alkyl chains of the associated lipids forming the other (outer) half of a bilayer (figure 4). The measured mobility varied with depth of insertion of the spin label into the layer (determined by the position of carbon atom in the fatty acids that was substituted with the spin label). This effect had already been observed with conventional bilayers, but the change of mobility with depth was different in the case of mycobacterial walls, underlining the unusual nature of the mycobacterial outer permeability barrier. Electron-spin resonance spectra obtained with whole cells were similar to those obtained with highly purified walls, confirming that the labelled fatty acids entered only the outer part of the barrier.

All these observations support the view of a mycobacterial outer permeability barrier as an asymmetric bilayer, with an inner leaflet of closely packed, essentially 'frozen' mycolate residues and an outer leaflet of more mobile lipids. The nature of these outer lipids is still not clarified. The original models of Minnikin (8) and Rastogi (10) assumed that they were the recognized wall-associated lipids. However, these vary in quantity and type among mycobacterial species, so provide rather unconvincing candidates for a part in a structure that is probably common to all mycobacteria. It has been pointed out (11) that there are more than sufficient fatty acyl chains of length C14 to C18 in the wall of *Mycobacterium chelonae* to provide the second leaflet of the bilayer. This material, well known to be present in samples of mycobacterial envelopes, had previously been dismissed as coming from triglyceride that had contaminated the envelope during disruption of the cells. It remains to be shown whether it comes from triglyceride or some other lipid, and whether it is really a part of the outer permeability barrier. Triglycerides and phospholipids were found in the outer layers of all species of mycobacteria examined, even though these layers were removed by gentle means which would not be expected to rearrange lipids from the interior of the cell or from the plasma membrane (15).

Some attempts have been made to model mycolate monolayers using films of the naturally-occurring lipid trehalose dimycolate. An early study (16) suggested that when the glycolipid contained long-chain mycolates of the type found in mycobacteria the monolayer was tightly packed with all the mycolate alkyl chains arranged parallel and with the high phase-change temperature characteristic of gel-phase lipid films. However, a more recent study (17) found a more open packing of the mycolates, probably with the longer alkyl chain folded back on itself. A closer packing was obtained if phosphatidylinositol was included in the layer. It was speculated that this allowed formation of an intercalated structure analogous to the layer in mycobacterial envelopes. It would clearly be advantageous if modelling could be carried out with mycolates on their natural arabinan carriers (see below), but these are not yet available.

Detailed knowledge of the structure of the arabinogalactan of the mycobacterial envelope makes it clear how a mycolate monolayer can form even though the mycolate residues are covalently attached to the polysaccharide (18). The mycolates occur as esters of terminal arabinose units on the polysaccharide, four

mycolates attached to five arabinoses (figure 3). The arabinosyl mycolate units are borne on arabinan side-chains which are attached in turn to a galactan backbone, quite close to its attachment to the peptidoglycan (19). The whole polysaccharide is composed of sugars in their furanose form, which confers additional flexibility on the chain and so presumably allows the structure to accommodate itself to the close packing of the mycolate units.

3.3. The permeability of the outer lipid barrier

The presence of an outer lipid permeability barrier obstructing access by hydrophilic substances provides an explanation of several properties of mycobacteria. The pathogenic species are susceptible only to a subset of available antibiotics; in the case of species related to *Mycobacterium avium*, an opportunist pathogen which particularly threatens immunosuppressed people, treatment is extremely difficult because this generalized drug resistance is almost complete. The barrier may also explain the ability of the pathogens to multiply inside phagocytic cells, in spite of the bactericidal properties of such cells, and also their resistance to strong chemicals such as acids, alkalis and hypochlorite. The distinctive property of acid fastness – the failure of dilute acids to decolourize bacteria stained with various basic dyes – may also be explained by the presence of the barrier. Membrane permeability of mycobacteria has been reviewed, with discussion of both the outer permeability barrier and the plasma membrane (20).

It is possible to measure the permeability of bacterial cells using various beta-lactam drugs: penicillins and cephalosporins with a range of hydrophilicities. The first mycobacterium to be investigated was *M.chelonae*, a rapid-growing species that shares with *M.avium* the property of generalized resistance to antibiotics (21). It turned out to have very low permeability indeed to hydrophilic molecules – ten times lower than *Pseudomonas aeruginosa*, whose low permeability is recognized as making it especially resistant to environmental agents, and 1000 times less permeable than *Escherichia coli*. *M.tuberculosis* is some ten times more permeable than *M.chelonae* (22), making it similar to the pseudomonad in this respect; this agrees with its lower general degree of resistance to antimycobacterial drugs. *Ps.aeruginosa*, as a Gram-negative bacillus, possesses an outer membrane, a permeability barrier composed of specialized lipids which protects the rest of the bacterial cell (23). Phylogenetically mycobacteria belong to the Gram-positive group, which mostly do not possess outer membranes. So the presence of an additional outer membrane-like barrier in the mycobacteria is probably an example of convergent evolution rather than an indication of close biological relatedness.

4. PORINS IN MYCOBACTERIA

Comparison of rates of permeation of the envelope of *M.chelonae* by more- or less-hydrophilic beta-lactams led to a further conclusion: permeation by hydrophilic molecules has properties consistent with the presence of water-filled pores in the permeability barrier (21). This sort of pore was already well recognized and studied in Gram-negative organisms, where it is formed by specialized outer-membrane proteins known as porins (23,24). Though there are examples of porins with considerable specificity for particular types of molecule, the common sort is relatively non-specific and allows passage of small hydrophilic molecules up to 5-600 Da in mass. Thus Gram-negative bacteria are protected by their outer membranes from large noxious molecules – enzymes, antibodies or toxins – but still have access to the small molecules needed for nutrition. A search was made for analogous pore-forming proteins in mycobacteria, and a protein with appropriate properties was isolated from highly purified walls, devoid of plasma membrane, of *M.chelonae* (25). This was able to form pores in artificial membranes in the form of liposomes or planar bilayers. The pores were larger than those produced by the most abundant porins of, for example, *E.coli*, but specific activity of the protein was lower. In these respects the mycobacterial protein resembled the porin of *Ps.aeruginosa*, OprF.

Protein with pore-forming ability was subsequently extracted from envelopes of *M.smegmatis* (26). This formed even larger pores than the protein from *M.chelonae*, which it resembled in having some specificity for cations. A pore-forming protein has since been purified from *M.smegmatis* and its size and amino-terminal sequence determined (27). Its reported pore size differs from that noted in (26), though the methods of measurement were different, and it is not clear whether these are the same or different proteins. The material investigated in (26) included only one active species, as did that from *M.chelonae* (28). A mutant strain of *M.smegmatis* has recently been obtained with modified permeability (29). The exact nature of the defect is not yet known, but one possibility is that this strain has a mutated porin gene.

4.1. Porin-like protein of M.tuberculosis

Both *M.chelonae* and *M.smegmatis* are rapid-growing species which have rarely been associated with infection in humans, so the question remained whether the slow-growing species possessed similar pore-forming proteins, and particularly what sort of porin would occur in the major human pathogen *M.tuberculosis*. An investigation of wall associated proteins of *M.tuberculosis* detected a protein with some sequence resemblance to outer membrane proteins of Gram-negative bacteria (30), but nothing was known of the physico-chemical properties of this protein. We attempted to extract pore-forming proteins from purified envelopes of *Mycobacterium microti*, a species very closely related to *M.tuberculosis* but not regarded as a human pathogen. Extracts were able to form pores in liposomes, but we were not able, using cultures grown on a practicable scale, to obtain sufficient active protein to be visible as a stained band on sodium dodecyl sulphate-polyacrylamide electrophoresis (31).

Interim results from the sequencing of the genome of *M.tuberculosis* H37Rv at the Sanger Centre, Cambridge, UK, and preliminary analyses of the data obtained were regularly released on the World Wide Web, and an open reading frame MTCY31.27 was provisionally identified as having homology with 'outer membrane proteins'. We cloned and expressed this gene in *E.coli* (32). The protein obtained was able for form pores in liposomes and planar lipid bilayers, and it was possible to measure several relevant physical properties; the pore size was apparently a little smaller than that of the *M.chelonae* porin. The gene contained a possible signal sequence at its amino-terminus, with a length and a putative cleavage site rather similar to those of known secreted mycobacterial proteins (the antigen 85 group, which are mycoloyl transferases) (33). Expression of the gene lacking the proposed signal sequence produced an inactive protein; it is not yet known whether the sequence is cleaved from the protein when it is expressed normally by *M.tuberculosis*.

Comparison of the *M.tuberculosis* gene with sequences in the published database showed that it possessed a region at its carboxyl-terminus with strong homology to the corresponding region of a number of

Table 1. Comparison of mycobacterial and other porins.

Species	Mass (kDa)	Pore diameter[1] (nm, neutral)	Pore diameter[2] (nm, ion)
M.chelonae	59 (25)	2 (25)	1.9 (25)
M.smegmatis	40 (27)	2 (27)	3 (26)
M.tuberculosis (OmpATb)	38 (32)	1.6 (32)	
Ps.aeruginosa (OprF)	35 (66)	2 (66)	2.2 (67)
E.coli (OmpF, OmpC)	35[3] (23)	1.1,1.2 (23)	

Properties of the mycobacterial porins so far identified are compared with OprF (a typical porin of the OmpA family) and major porins of *E.coli*. Mass is measured by SDS-PAGE. [1]Diameters measured using diffusion of neutral hydrophilic substances into proteoliposomes. [2]Diameters measured by diffusion of ions in planar lipid bilayers. [3]Porins of this type exist, and are only active, as stable trimers.

known proteins, most of which were porins of the 'OmpA family' from Gram-negative bacteria. These proteins differ in several respects from the major porins of *E.coli*; they occur in that species, and are the major porins of *Ps.aeruginosa* (24). Typically they form larger pores but have considerably lower specific activities than the classic porins of *E.coli*, and differ from the latter by occurring as monomers instead of trimers. We have proposed the name OmpATb for the porin-like protein of *M.tuberculosis* to draw attention to the relationship. The gene is identified as *ompA* in the published complete sequence of the *M.tuberculosis* genome (1), and has been given the formal number Rv0899. From the published sequences and from the relative molecular masses it is clear that OmpATb is not the same as the wall-associated protein described in (30).

We showed by reverse transcription-polymerase chain reaction of mRNA extracted from *M.tuberculosis* that *ompA* is expressed in growing cells (32), and also that, insofar as its properties could be measured, the activity extracted from walls of *M.microti* resembled the recombinant protein. This material reacts with rabbit antiserum to OmpATb (34). It is still not clear whether *M.tuberculosis* possesses only one type of porin, or whether it has several like *E.coli*. However, the conclusion that the mycobacterial pathogen makes use of a porin-like passive transport mechanism to convey small hydrophilic molecules across its outer lipid permeability barrier is not affected by the question of whether there are several or one species of pore involved.

Meanwhile a pore-forming protein has been extracted from whole cells of *Nocardia farcinica* (35); nocardias are closely related to mycobacteria and also have mycolic acids in their envelopes, though of smaller size than those of mycobacteria. If this extraction technique is applicable to mycobacteria this is an important finding, because it greatly simplifies the preparation of porins from bacteria, making it easier to investigate them.

Properties of mycobacterial proteins so far identified are summarized in table 1, which also includes data for OprF, the major porin of *Ps.aeruginosa*, which also belongs to the OmpA family, and for OmpF and OmpC of *E.coli*, which belong to a different family operating as trimers. It is not clear that the porin activity identified in *M.smegmatis* as active in planar lipid bilayers (26) is the same as the 40 kDa polypeptide active in the liposome swelling assay (27).

4.2. Porins in other slow-growing mycobacteria

The availability of DNA probes for *ompA* of *M.tuberculosis* has permitted a search for the gene in other mycobacteria by polymerase chain reaction (PCR) (36). It was present in four members of the *M.tuberculosis* group: *M.tuberculosis*, *M.microti*, *M.bovis* and *M.bovis* BCG and also in *M.avium* and *Mycobacterium intracellulare*, but not in another slow-growing pathogen, *Mycobacterium kansasii*, nor in several rapid-growing species, including *M.smegmatis* and *M.chelonae*. The PCR reaction requires a very close match between probe and DNA to be amplified, so this data does not rule out homologies between porin genes of various mycobacteria.

4.3. Access of molecules to mycobacteria

It is probable that lipophilic molecules are able to diffuse across the outer permeability barrier of mycobacteria. Small hydrophilic substances can pass through the porin pores, whereas larger hydrophilic molecules are unable to obtain access to the inside of the mycobacterial cell. So an ideal antituberculosis drug should either be a rather small molecule or a rather lipophilic one. Surprisingly, even the small and relatively polar drug isoniazid passes so readily through lipid bilayers in liposomes that it is not possible to measure any difference in diffusion rate when OmpATb is incorporated into the liposomes (37). A bilayer of which one leaflet was mycolate would be a substantially thicker than a bilayer prepared from natural phospholipid, so rates of diffusion of lipophilic substance would be slower through the mycobacterial envelope than into liposomes. At present the nature of the 'other half' of the outer lipid permeability barrier is unknown, so calculations of the thickness of the barrier are not possible. Though monolayers of trehalose dimycolate have been prepared, these were on air-water surfaces and not suitable for measurement of diffusion (16,17). So theoretical or experimental determination of the permeability of the mycobacterial outer lipid barrier is not possible except insofar as it can be measured in whole cells. Data obtained with beta-lactam drugs of varying degrees of hydrophobicity have been mentioned above (21,22) and have been reviewed (38). An important point is that for small molecules the outer permeability barrier alone is an insufficient protection since some diffusion occurs, albeit at a low rate, and other mechanisms such as drug-metabolizing enzymes or efflux pumps are needed. The relative importance of diffusion through the outer permeability barrier and through porin channels is unknown for the majority of solutes; porin-negative mutants of mycobacteria (if viable) would be useful tools in exploring this important area.

5. A MYCOBACTERIAL CAPSULE

Mycobacteria in laboratory cultures are commonly grown in the presence of detergents (to control clumping) and with shaking. Consequently the accumulation of a capsule-like outer layer of hydrophilic molecules, polysaccharide and protein, is not evident. However, some such structure was evident in intracellular pathogenic mycobacteria in the form of the 'electron transparent zone' which was described in thin sections of cells infected with *Mycobacterium lepraemurium* (39) and, subsequently,

Table 2. Composition of capsule of *M.tuberculosis*

Component		
Polysaccharide	30-55	
❑ Glucan		70[1]
❑ Arabinomannan & mannan		28[1]
❑ Xylan		2[1]
Protein	55-40	
Lipid	5-8	

Data taken from (7). Amounts are given as percent of the total recovered capsule, which amounted to 2-3% of the dry mass of the bacteria.[1] Individual components as percent of total polysaccharide. The protein consists of a large number of molecular species.

other intracellular mycobacteria. This was a zone around the apparent margin of the conventionally understood envelope, separating it from the vacuolar membrane of the phagocytic cell. Hanks and colleagues argued that this material was of bacterial origin (40), and also demonstrated, by light microscopy, the presence of capsular material on the outside of mycobacteria obtained from tissues (41). In some cases, including *Mycobacterium leprae* (42) and *M.lepraemurium* (43), the zone consists partly of defined glycolipids (phenolic glycolipid and mycoside C, respectively) which are produced in massive amounts by these species. There is evidence that such lipids are also part of the superficial layer in other species (44,45), but the amounts are smaller. In other cases such as *M.tuberculosis* no equivalent lipids are produced, while strains of *M.avium* and *M.intracellulare* unable to produce glycolipids of this type still form an electron transparent zone inside infected cells (46). So the presence of specific glycolipids cannot be a general explanation of the zone. Fréhel and her colleagues showed that formation of the zone around intracellular *M.avium* depended on viability: a smaller proportion of killed bacteria were surrounded by a zone, and the proportion fell with time (47).

Rastogi noted that several special stains showed up the presence of a layer of the mycobacterial envelope external to the recognized structures (48); the nature of the stains suggested that this was carbohydrate. The layer was at first interpreted as being the outer, hydrophilic part of the glycolipids associated with the mycolate layer and forming a bilayer analogue (10). The layer was found on all mycobacterial species, though it varied considerably in thickness. Beveridge and Paul applied the technique of freeze substitution, which avoids the shrinkage of hydrated structures that normally occurs during preparation for electron microscopy and minimizes extraction of soluble lipids during processing, to several species of mycobacteria. They observed a more-or-less extended layer, with variable electron density, on the external surface of several rapid-growing species (49). It was very conspicuous in *Mycobacterium phlei* and *Mycobacterium thermoresistibile*, but harder to detect (though definitely present) in *Mycobacterium aurum* and *Mycobacterium fortuitum*. The slow-growing opportunist pathogen *M.kansasii* was subsequently investigated (50). Here the outer layer was conspicuous, and there was evidence that it was at least partly composed of species-specific glycolipid, which agrees with the data for *M.leprae* and *M.lepraemurium* discussed above and also with the detection of such specific lipids in the outer part of the capsule (15). The material identified by Rastogi was presumably the same, but in a collapsed, dehydrated form. The chemical nature of the material has been established (7,51): it is a mixture of glucan and arabinomannan, with some protein. The approximate composition of the capsule of *M.tuberculosis* is shown in table 2. In static cultures it remains associated with the mycobacteria, and can be removed by gentle treatment with glass beads. In shaken cultures most of it is shed from the cells and corresponds to the well known polysaccharides of culture filtrates of mycobacteria (52). The capsule-like layer has rather little lipid, and most of this is present in its inner part which requires rather more vigorous treatment to remove it from the cells. What may be similar material has been identified in cultures of *M.bovis* BCG (53); these authors equate the material with the electron-transparent layer of the wall. The structure of the capsular glucan obtained from the Tice strain of BCG (54) is different from the glucan identified by the Toulouse group (7); this may represent a strain difference. It may also be noted that the material in BCG cultures can be released by treatment with protease (55), suggesting that protein may be a more major constituent in BCG than it is in other species; in this connection the reported presence of a crystalline protein layer on the outside of BCG cells may be relevant (56). The protein is apparently a well-known mycobacterial extracellular antigen, MPB70 (57).

Though there is at present no direct proof of the identity of the capsule-like layer and the electron-transparent zone, it seems highly probable that the latter represents accumulated secreted polysaccharide and protein, mixed with specific lipids in the particular special cases noted above, retained by the phagocytic vacuole. In cells heavily infected with *M.lepraemurium* material of evident capsular origin is found in vacuoles not containing bacteria (43), a phenomenon corresponding to the shedding of capsular materials by mycobacteria in culture.

5.1. Permeability and the capsule

The polysaccharide/protein capsule is strategically placed to protect the intracellular mycobacterium, but whether or how it achieves this is completely unknown. Some examples of bacterial capsules seem to be anti-phagocytosis devices (58), but pathogenic mycobacteria possess a variety of mechanisms to ensure that phagocytosis occurs (59,60), since they thrive inside phagocytic cells. The polysaccharides may be part of one of these mechanisms, or they may modify the behaviour of the host cell in some way beneficial to the bacterium (61). Their primary function may be concerned with permeability. Inside the phagosome they will clearly impede diffusion of both potentially harmful host-derived substances, particularly macromolecules, towards the bacterium and of bacterial material into the phagocytic cell, where it might be recognized as 'foreign' and trigger off antibacterial mechanisms. Electron microscope images suggest that potentially lytic lysosomal material is held away from intracellular mycobacteria by the capsule (62,63), though it is not clear whether lysosomal enzymes are important in controlling mycobacterial growth. There is clearly a need for further study of the role of the capsule in mycobacterial diseases.

The macromolecules of the capsule must themselves pass through the outer lipid permeability barrier, and there is no indication at present of how this occurs. Several mechanisms are known by which such substances can be exported through the outer membrane of Gram-negative bacteria (64,65), and homologous processes may occur in mycobacteria. The rapidly accumulating library of sequences of open reading frames in mycobacterial genomes should allow a search for mycobacterial transport

proteins similar to those involved in the Gram-negative organisms.

6. PERSPECTIVE

Mycobacteria are primarily soil and water dwellers, and their well-developed machinery for controlling access by environmental molecules probably reflects this. Some, like *M.avium*, are opportunist pathogens which can multiply intracellularly in a host when conditions are suitable but normally occur in the environment. Presumably the same permeability-controlling mechanisms are efficacious in both situations. A few, notably *M.tuberculosis*, *M.bovis* and *M.leprae*, are found only in animal hosts, and *M.leprae* has never been coaxed to grow in any medium. In these cases the mechanisms originally appropriate for growth in the environment are likely to have been modified to facilitate survival in the host. Thus it would not be surprising if there were detailed differences in the operation of the mechanisms in the various species, even pathogenic ones. At the moment knowledge is very limited, yet is seems essential to understand permeability in mycobacteria if rational ways are to be found of controlling the grave diseases they cause.

The complete structure of the outer lipid permeability barrier needs to be understood, which will involve determining whether the associated lipids are intercalated or form a separate leaflet and also what these lipids actually are. It would also be useful to know, since it affects the choice of models for investigating permeability, whether the outer permeability barrier is identical in the rapid- and slow-growing species, or even among the various slow-growing species. Porins penetrate the barrier (though their location in the envelope has not been fully determined) and are likely to control access by hydrophilic drugs. Yet at present only one mycobacterial porin has a known primary structure, while the conformation of these proteins in the envelope is unknown. It is not clear whether there are several types of porin present in a given species, as is the case with *E.coli*, nor is there any information on how (or if) the expression of the porin genes is regulated. It is a plausible hypothesis that some sort of adjustment of amounts or types of porin might be an important part of adaptation to intracellular growth.

Knowledge of the capsule-like structure as the outermost part of the mycobacterial envelope is even newer than that of porins. At present the bulk composition is known, and there is data about the distribution of various lipids at different levels within the capsule; though lipids are minor components they may be structurally important, especially at the interface between the capsule and the outer lipid permeability barrier. It is not clear whether the major constituents are arranged in any distinctive manner, nor what is the interaction between polysaccharide, protein and lipid. Yet the capsule is visibly the structure that separates the components of the host cell from the pathogenic mycobacterium, and its ability to maintain that separation and to prevent access to the mycobacterial cell by macromolecules seems highly relevant to understanding the success of mycobacterial pathogens.

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