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MiniReview

Mycobacterial cell wall: Structure and role in natural resistance to antibiotics

Vincent Jarlier [a] and Hiroshi Nikaido [b,*]

[a] *Laboratoire de Bactériologie-Virologie, Faculté de Médecine Pitié-Salpêtrière, F-75634 Paris, France, and* [b] *Department of Molecular and Cell Biology, 229 Stanley Hall 3206, University of California, Berkeley, CA 94720-3206, USA*

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Abstract Mycobacteria show a high degree of intrinsic resistance to most antibiotics and chemotherapeutic agents The low permeability of the mycobacterial cell wall, with its unusual structure, is now known to be a major factor in this resistance Thus hydrophilic agents cross the cell wall slowly because the mycobacterial porin is inefficient in allowing the permeation of solutes and exists in low concentration Lipophilic agents are presumably slowed down by the lipid bilayer which is of unusually low fluidity and abnormal thickness Nevertheless, the cell wall barrier alone cannot produce significant levels of drug resistance, which requires synergistic contribution from a second factor, such as the enzymatic inactivation of drugs

Key words *Mycobacterium*, Cell wall, Mycolic acid

Introduction

There are nearly 60 commonly recognized species of *Mycobacterium*, but most are saprophytic inhabitants of soil A few are major human pathogens that cause tuberculosis (*M tuberculosis, M africanum, M bovis*) and leprosy (*M leprae*), two well-documented diseases Several so-called 'atypical mycobacteria' are essentially saprophytic, yet can cause opportunistic infections, especially in immunocompromised patients (*M avium, M xenopi, M kansasii, M chelonae, M fortuitum*)

Sulfonamides and penicillin, which drastically modified the outcome of many life-threatening bacterial infections, were ineffective against tuberculosis Similarly, penicillin was ineffective and sulfonamides showed only bacteriostatic effects against leprosy [1] Among the major antibiotics, only a few showed good activity against *M tuberculosis* complex, and almost none were active against the atypical species (Table 1) This disappointing situation encouraged studies on mechanisms of intrinsic drug resistance in mycobacteria In the present review, we will concentrate on the structure and barrier properties of the mycobacterial cell wall in promoting antibiotic resistance

* Corresponding author Tel (510) 642 2027, Fax (510) 643 9290

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Table 1

Activity of major chemotherapeutic agents against mycobacteria

Agent (year introduced)	*M tuberculosis*	*M leprae*	Atypical mycobacteria
Sulfonamides (1935)	−	+	−
Penicillin G (1944)	−	−	−
Streptomycin (1944)	+	−	−
Chloramphenicol (1947)	−	−	−
Tetracyclines (1948)	−	− [a]	−
Erythromycin (1952)	−	−	− [b]
Isoniazid (1952)	+	−	−
Novobiocin (1955)	−	−	−
Vancomycin (1956)	−	−	−
Broad-spectrum β-lactams (1961)	−	−	−
Quinolones (1962)	−	−	−
Fusidic acid (1962)	−	−	−
Rifampicin (1966)	+	+	−
Fluoroquinolones (1979)	+	+	± [c]

+ and − indicate the presence and absence, respectively, of significant activity

[a] Minocycline (1961) is active against *M leprae*

[b] Clarithromycin (1984) is active against some atypical species

[c] Variable depending on species and particular compound

Mycobacteria produce cell walls of unusual structure The peptidoglycan contains *N*-glycolylmuramic acid instead of the usual *N*-acetylmuramic acid [2], but a far more distinctive feature is that up to 60% of the weight of the mycobacterial cell wall is occupied by lipids that consist mainly of unusually long-chain fatty acids containing 60 to 90 carbons, the mycolic acids [3]

The chemistry and immunology of the various components of the mycobacterial cell wall have been studied extensively, mainly with the perspective of discovering antigens that could be used for immunization and diagnosis The covalently connected structure of the cell wall is made of peptidoglycan, to which arabinogalactan is linked via a phosphodiester bridge About 10% of the arabinose residues in arabinogalactan are in turn substituted by mycolic acid [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 [3,5] The cell wall also contains several proteins [5,6]

Physical organization of the lipids in the mycobacterial cell wall

Knowledge of the chemistry of mycobacterial lipids is unfortunately insufficient to understand the barrier properties of the cell wall, because these properties depend on the physical organization of the lipids More than ten years ago, Minnikin proposed a model for this physical organization, in which mycolic acid chains are packed side by side in a direction perpendicular to the plane of the cell surface [3] It was also proposed that this mycolic acid-containing inner leaflet is covered by an outer leaflet composed of extractable lipids, the whole structure thus producing an asymmetric lipid bilayer (Fig 1) This model has been recently updated [4,7], and has received support from several recent studies X-ray diffraction data of purified *M chelonae* cell wall showed that a large part of the hydrocarbon chains in the cell wall are tightly packed in a parallel array in a direction perpendicular to the cell surface [8] The second line of support came from improved understanding of the arabinogalactan structure In this polysaccharide, both the galactan main chain and the arabinan side branches are constructed in a manner that would ensure maximum freedom of movement between

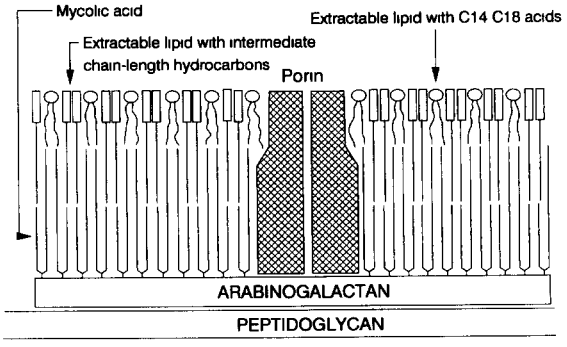

Fig 1 Modified Minnikin model for the structure of mycobacterial cell wall

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sugar residues Thus all of the sugars are in furanose form, and arabinose and galactose residues are connected through 1,5 and 1,6 linkages, respectively [4] Finally, mycolic acid residues are linked to the tips of branches of this highly branched polysaccharide [4] This structure suggests that the parallel packing of the mycolic acid chains may be achieved by the flexible movement of arabinogalactan Such packing would otherwise be difficult since all mycolic acid residues are covalently connected to this macromolecule The third line of evidence was generated by a quantitative reevaluation of existing results [8] Data on the amount of mycolic acid present in a known amount of Bacillus Calmette-Guérin (BCG) cells indicated that a monolayer of mycolic acid, the presumptive inner leaflet of the bilayer structure, can indeed cover the whole surface of the bacterial cells Finally, although it has been often argued that atypical species did not contain much extractable lipid, analysis of *M chelonae* cell wall showed the existence of sufficient amounts to produce the outer leaflet covering the entire cell surface (E Y Rosenberg and H Nikaido, unpublished results) Moreover, various lines of evidence indicate that extractable lipids are located close to the outer surface for example, glycopeptidolipids (mycosides C) function as phage receptors [9]

All these pieces of evidence favour the bilayer model of Minnikin Mycolic acid is a branched fatty acid with a long branch (40 to 64 carbons) and a short branch (22 to 24 carbons) In addition to their extraordinary lengths, mycolic acids contain very few double bonds or cyclopropane groups (none in the shorter branch and at most two in the longer branch) It is thus predicted that the inner leaflet will have very low fluidity, indeed a nearly crystalline structure Since the diffusion of lipophilic solutes through a lipid bilayer requires a fluid interior, this construction is expected to act as an effective barrier for the penetration of lipophilic antibiotics This asymmetric bilayer structure is reminiscent of that of the Gram-negative outer membrane, where the low fluidity of the lipopolysaccharide leaflet is known to impede the influx of lipophilic solutes [10]

Permeability of the mycobacterial cell wall

Mycobacterial cell wall has long been suspected to act as a permeation barrier for antibiotics Some indirect data were available For example, the antibiotic efficacy increased when detergents such as Tween were added to culture media [11], or when the cell wall structure became defective due to mutations [12] Furthermore, aminoglycosides were shown to be more active on ribosomes in cell extracts than on intact mycobacterial cells, suggesting the role of a cell surface barrier [11] In species producing intrinsic β-lactamase, the enzyme is cryptic, and the hydrolysis of β-lactams measured with disrupted cells is far faster than that measured in intact cells [13] Recent data also show a synergistic effect between various agents and those agents that are known to inhibit the synthesis of cell wall components, such as ethambutol [14]

In spite of these largely qualitative results, there was no quantitative measurement of the permeability of mycobacterial cell wall, until our study [15] We used the method introduced for estimation of outer membrane permeability in Gram-negative bacteria [16] We measured the rate of hydrolysis of β-lactams by intact bacterial cells, and calculated the cell wall permeability by assuming that drug molecules first diffuse through the cell wall (following Fick's first law of diffusion) and then are hydrolyzed by periplasmic β-lactamase (following the Michaelis-Menten kinetics) For this method, cells should exist as unicellular dispersions in contact with the medium A strain of *M chelonae* was chosen because it produced homogeneous suspensions without the use of detergents, and because it produced a β-lactamase of sufficiently high activity which did not leak out into the medium The cell wall permeability to cephalosporins measured in this strain was indeed very low, and was about three orders of magnitude lower than that of *E coli* outer membrane, and ten times lower than the permeability of the notoriously impermeable *P aeruginosa* outer membrane (Fig 2)

Another approach was that of studying the kinetics of nutrient uptake If we assume that nutrient molecules are taken up by high-affinity

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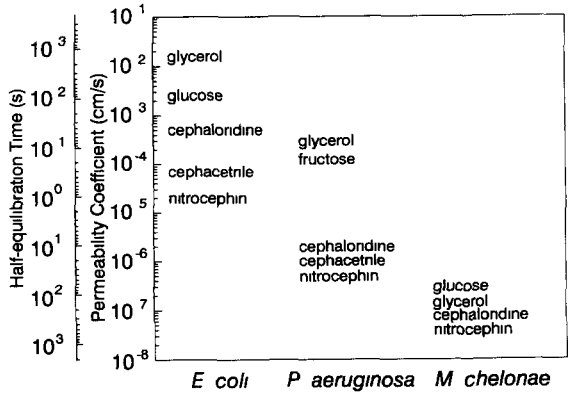

Fig 2 Permeability coefficients and half-equilibration times of nutrients and cephalosporins The permeability coefficients are those across the outer membrane or the cell wall, taken from [15] Half-equilibration times indicate the time needed for intracellular concentration to reach one-half of the external concentration, in this calculation we have disregarded the presence of a second permeability barrier (cytoplasmic membrane) and of degradative enzymes

active transport systems as soon as they cross the cell wall, we can evaluate cell wall permeability by combining the diffusion equation with the Michaelis-Menten kinetics The above assumption is probably valid, as amino acids are known to be transported across the mycobacterial cytoplasmic membrane by high affinity systems (for reference, see [15]) This approach again suggested that small nutrients penetrate through the *M chelonae* cell wall more than 10000 times

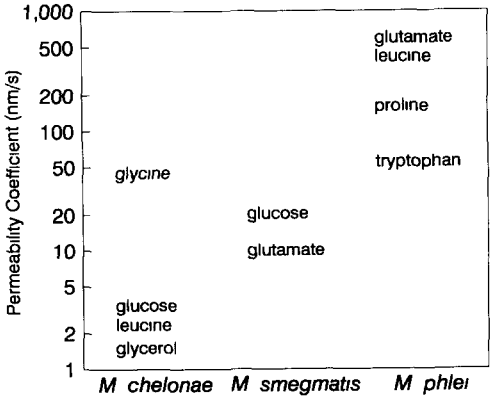

Fig 3 Estimated permeability coefficients for nutrients in *M phlei*, *M smegmatis*, and *M chelonae* Calculation was performed as in [15], utilizing the transport data from [19] and from papers cited in [18]

more slowly than through the outer membrane of *E coli* [15] (Fig 2)

M chelonae, chosen as a model because its properties were convenient for our assay, happens to be one of the most drug-resistant species among mycobacteria Thus it becomes important to study the cell wall permeability of other mycobacterial species *M smegmatis*, widely used in studies of molecular biology, was found to show about ten-fold higher permeability to β-lactams, by using an assay similar to that used for *M chelonae* [17] The assay had to be modified for *M tuberculosis*, which has a stronger tendency to aggregate Use of this modified assay showed that *M tuberculosis* H37Ra was more permeable, by a factor of nearly ten, than the *M chelonae* strain studied earlier (E Y Rosenberg and H Nikaido, unpublished results)

These results are consistent with our knowledge that atypical species, such as *M chelonae*, are usually more resistant to a number of agents than *M tuberculosis* (Table 1) It is also consistent with the earlier observation that nutrients are accumulated much more rapidly by *M smegmatis* and *M phlei* than by *M chelonae* (Fig 3) It appears that the cell wall of the former species is about one or two magnitudes more permeable than that of *M chelonae* For amino acids, *M tuberculosis* appeared to be intermediate between the highly impermeable *M chelonae* and the more permeable *M phlei* [19]

These results indicate that, although mycobacteria in general have low permeability cell wall, the precise values of permeability may differ among different species by a factor of perhaps up to 100 As seen among Gram-negative rods, those species that are obligate parasites such as *M tuberculosis* appear to have a relatively high permeability, whereas some soil inhabitants such as *M chelonae* seem to protect themselves by producing a very low permeability wall

Mechanism of diffusion across cell wall

Hydrophilic pathway

The Gram-negative bacteria, whose outer membrane acts as a permeability barrier, have

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developed porin channels that allow the diffusion of small, hydrophilic nutrient molecules [10] The penetration rate of cephalosporins across *M chelonae* cell wall is not very dependent on the lipophilicity of the drug or temperature [15], suggesting that these hydrophilic solutes traverse an aqueous pathway, perhaps through similar pore-forming proteins

When detergent extracts of *M chelonae* cell wall were reconstituted into proteoliposomes, a channel-forming activity that was destroyed by proteases was detected [20] Purification of this channel-forming activity resulted in the identification of a 59 kDa cell wall protein that allows the diffusion of small, hydrophilic solutes The channel diameter was estimated at about 2 nm [20] The purified protein also produced slightly cation-selective channels of a defined size on reconstitution into planar bilayers [20] Most importantly, the *M chelonae* porin is a minor protein of the cell wall, unlike enterobacterial porins that are the most abundant proteins in the cell Furthermore, the *M chelonae* porin produced permeability far lower than that produced by an equal weight of *E coli* porin [20] These observations, therefore, explain why the permeability of the *M chelonae* cell wall to hydrophilic solutes is so low

Lipophilic pathway

In principle, lipophilic solutes should be able to traverse any biological membrane by dissolving into the hydrocarbon interior of the lipid bilayer On the one hand, the low fluidity of the mycolic acid leaflet and the unusual thickness of the bilayer would slow down such a process in mycobacterial cell wall On the other hand, this pathway may play a relatively prominent role in solute transport because of the extreme inefficiency of the porin pathway in mycobacteria

The evidence suggesting the contribution of a lipophilic pathway to lipophilic solute transport comes from the observation that the more lipophilic derivatives of chemotherapeutic agents are often more active against mycobacteria Thus the addition of a C_{16} fatty acyl chain to isoniazid led to higher activity against *M avium* [21] Correlation with lipophilicity was seen also with tetracyclines, the more hydrophobic derivatives such as doxycycline and minocycline being more active against *M chelonae*, *M fortuitum* and *M marinum* [22,23] than the less hydrophobic forms Moreover, minocycline is efficacious in the treatment of leprosy [24] These comparisons are usually carried out by using many isolates, so that the clinical usefulness of the drugs can be estimated However, for our purpose, the heterogeneity of the strains sometimes complicates the interpretation of data Thus one study of tetracyclines [22] provided the minimal inhibitory concentration (MIC) values of individual strains, and it was clear that among relatively susceptible strains, minocycline was most active, followed by doxycycline with the least active form being tetracycline But the traditional indicators such as MIC_{50} or MIC_{90} do not give any suggestion of the differences in efficacy, because these values are biased so strongly by the presence of a large number of highly resistant strains (for example, see [23]) Clarithromycin, which is slightly more hydrophobic than erythromycin, is more active against atypical species [25,26] and *M tuberculosis* [27] Among rifamycins, more hydrophobic derivatives are more active against *M tuberculosis* and *M avium* [28] Some of them were reported to be inactive against *M fortuitum* and *M chelonae*, but again this is likely to be due to the generally high levels of resistance in these species and the problem of heterogeneity mentioned above

Among fluoroquinolones, the more hydrophobic sparfloxacin is more active than the reference fluoroquinolones against many mycobacteria including *M avium* [29] Ciprofloxacin, when made more hydrophobic by the addition of alkyl substituents, becomes more active against *M tuberculosis* and *M avium* [30] With *M leprae*, a good positive correlation was seen between the lipophilicity of fluoroquinolone and its efficacy [31, the data are analyzed in 18] However, in comparison to various agents, other factors such as the affinity to the target are expected to come into play, and a perfect correlation with just one parameter cannot be expected Indeed, PD-127391, which should be as hydrophilic as ciprofloxacin, showed a remarkably strong activity [31]

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In view of the complexity of the correlation, we clearly need studies of drug penetration into the cell, but few such studies exist Preliminary data suggest that clarithromycin indeed penetrates more rapidly into *M avium* cells than does erythromycin (F Doucet-Populaire and V Jarlier, unpublished results)

Cell wall barrier is a necessary, but not a sufficient, factor for resistance

Although mycobacterial cell wall is a formidable permeation barrier, production of clinically significant levels of resistance usually requires the participation of an additional resistance mechanism A similar situation exists with the outer membrane barrier of Gram-negative bacteria, where a second factor synergistically and dramatically increases resistance, by removing the inflowing antibiotic molecules either by chemical modification (notably by hydrolysis in the case of β-lactams [32]) or by active efflux into the medium [33]

At least the first mechanism was shown to be operating in mycobacteria, most species of which produce β-lactamase [13] In fact, quantitative analysis with *M chelonae* showed that the synergism between the cell wall barrier and the periplasmic β-lactamase decreased the β-lactam concentration at the target precisely to the level sufficient for inhibition of the most susceptible PBP (2 mg ml^{-1} for cephalothin, for example), when the external concentration of the drug was equal to MIC (about 3000 mg ml^{-1} for cephalothin) [34] We emphasize that the low permeability of the mycobacterial cell wall is essential for this high degree of resistance Thus, although the activity of β-lactamase found in *M chelonae* is only 20% of that in TEM-plasmid-containing *E coli*, the synergistic effect of the cell wall barrier is able to decrease the periplasmic concentration of cephaloridine to 0 2% of the external concentration, in contrast, the TEM-containing *E coli* with its high outer membrane permeability can lower the cephaloridine concentration in the periplasm only to about 80% of the external concentration

Although the cell wall barrier exerts a powerful influence in the presence of a synergistic factor such as β-lactamase, half-equilibration across the cell wall takes place in several minutes (see Fig 2), a much shorter time in comparison with the generation time of these organisms The barrier alone should not thus produce significant resistance Mathematical analysis along the lines presented in [35] shows that even the extremely low level of cephalosporin permeability observed in *M chelonae* would lower the periplasmic drug concentration by less than 0 5% in comparison with the external concentration, if it acted alone in the total absence of β-lactamase activity Thus a second synergistic factor must obviously exist for most other antibiotics to which mycobacteria are resistant, this could be degradation or active efflux (Aminoglycoside-inactivating enzyme has been reported in some mycobacteria [36]) In any case, this analysis gives us some hope that an effective therapy may become possible if we could eliminate the second synergistic factor Indeed, *M tuberculosis*, which has a relatively high permeability cell wall (see above), becomes susceptible to ampicillin if its β-lactamase is inhibited by clavulanate [37] Similar approaches are difficult with atypical species, because even a very small amount of residual β-lactamase activity will be able to cope with the few molecules of β-lactam that penetrate through their low permeability cell wall [34] Nevertheless, cefamycins (cefoxitin and cefmetazole), which are nearly completely stable to the class A β-lactamases, are active against *M fortuitum* [38]

Finally, scattered evidence suggests that, in mycobacteria, the targets of antibiotic action often have a lower affinity than in many common bacteria This is true with PBPs [34] In wild-type strains of *M tuberculosis*, *M avium*, and *M smegmatis*, the GyrA protein, the target for fluoroquinolones, harbours a characteristic alteration found in quinolone-resistant mutants of *E coli* [39,40] The ribosomes of *M avium* and *M smegmatis* also appear to have a lower affinity to macrolides than those of *Staphylococcus aureus* (F Doucet-Populaire and V Jarlier, unpublished results), and RpoB, the rifampicin-sensitive subunit of RNA polymerase appears to have a se-

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quence found in resistant mutants of other bacteria [41] The lower susceptibility of targets described above may also contribute as a synergistic factor to the drug resistance of mycobacteria, especially that of the atypical species It seems that mycobacteria, as the ultimate soil bacteria, have learned to combine all possible resistance mechanisms This makes it difficult to treat mycobacterial diseases in the age of antibiotics, once we become infected by these well-protected bacteria

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