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EDITORIAL

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Wall Biosynthesis: A Possible Site of Action for New Antimycobacterial Drugs

Bacterial walls form a sort of exoskeleton for the majority of bacteria, including mycobacteria. They provide protection from the environment and support against osmotic forces, and they determine the characteristic shapes of bacteria. There are strong structural similarities among walls from all types of bacteria. Since walls contain components and structures that do not occur in animal cells, they offer an excellent site for the operation of selective toxicity—in this case, the inhibition of wall synthesis or interference with wall function of pathogenic bacteria, without affecting the host. The spectacular success of the beta-lactam antibiotics (penicillins and cephalosporins) against bacterial infections illustrates the effectiveness of such an approach.

Clinically usable, wall-active antibiotics have not been particularly successful against mycobacteria (although cycloserine was formerly used against multiple drug-resistant *Mycobacterium tuberculosis*). Understanding the reasons for this general ineffectiveness, and investigating the possibility of developing chemicals that will interfere with mycobacterial wall synthesis, might lead to the development of new, clinically useful antimycobacterial drugs. It seems probable that the search for new wall-active drugs in general is best conducted using bacteria that are easier to handle than mycobacteria, although one may hope that promising new compounds will be tested against that genus. This editorial is concerned with mycobacteria-specific wall structures.

Common and special features of mycobacterial walls. Mycobacteria form a specialized subgroup of the Gram-positive bacteria. There is abundant information about the structure and routes of biosynthesis of Gram-positive walls[1]; the precise modes of action of several of the wall-inhibitory antibiotics are known. There is fairly detailed understanding of the wall structure common to several species of mycobacteria[2, 3, 4] but knowledge of its biosynthesis is slight.

[1] Rogers, H. J., Perkins, H. R. and Ward, J. B. *Microbial Cell Walls and Membranes.* London: Chapman and Hall, 1980.

[2] Lederer, E. The mycobacterial cell wall. Pure Appl. Chem. **25** (1971) 135–165.

[3] Lederer, E., Adam, A., Ciorbaru, R., Petit, J.-F. and Wietzerbin, J. Cell walls of mycobacteria and related organisms; chemistry and immunostimulant properties. Mol. Cell Biochem. **7** (1975) 87–104.

[4] Petit, J.-F. Structure chimique de la paroi des mycobactéries. Ann. Microbiol. (Paris) **129A** (1978) 38–48.

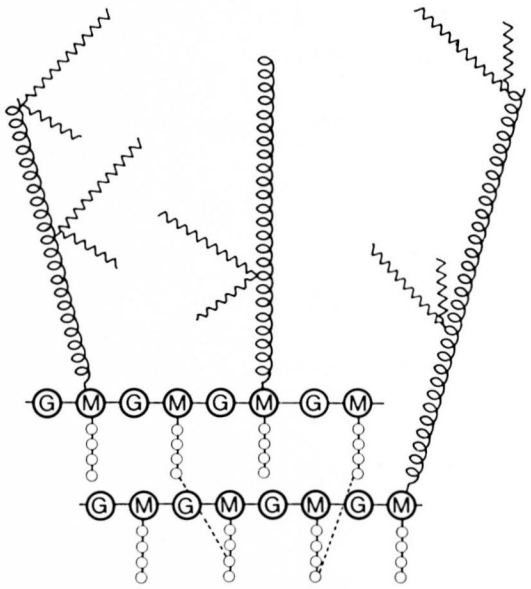
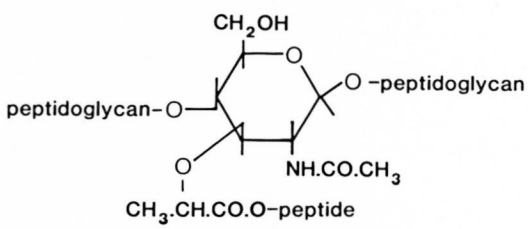


FIG. 1. "Cell-wall skeleton" of mycobacteria. The arabinogalactan is represented by spirals and the mycolic acids by zig-zag lines. Large circles are amino-sugars: M—*N*-glycolylmuramic acid; G—*N*-acetylglucosamine. Small circles are amino acid residues. Both types of cross-link are indicated but one of the peptides with DAP-DAP cross-links probably only has three amino acids. (Structures are not drawn to scale.)

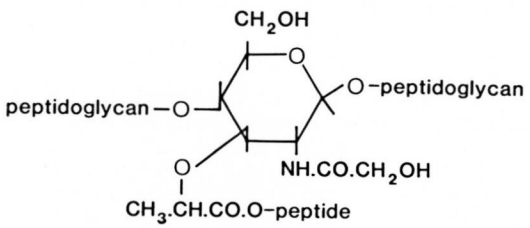
It is believed that "common" structures shared with other bacteria are synthesized in the conventional manner, on the basis of the observed accumulation of wall precursors in the presence of wall-active drugs and of the effectiveness of such drugs. In the particular case of *M. leprae,* the structure is known only in outline and the biosynthesis is uninvestigated.

It seems a safe working hypothesis that the structures and routes of biosynthesis of walls in *M. leprae* resemble those in other mycobacteria which, in turn, resemble those of Gram-positive bacteria in general. However, mycobacteria contain some special wall features not found in other microorganisms. It may be useful to review these and to consider the possibility that they may be selectively inhibited. This will, at the same time, indicate aspects of the structure of mycobacterial walls, and particularly those of *M. leprae,* which are still obscure.

Mycobacterial walls, as isolated by breaking mycobacteria and removing membrane components from the particulate fraction, contain much lipid which may be removed by extraction with solvents. What remains is a covalently bonded peptidoglycolipid which has been called the "cell wall skeleton." Figure 1 shows the overall structure of the skeleton as determined for about five species. The wall consists of a series of polysaccharide chains composed of *N*-acetyl-D-glucosamine alternating with muramic acid (3-O-lactyl-D-glucosamine). Short (usually tetra-) peptides are attached to the carboxyl groups of the muramic acid units; the peptides are cross-linked so that the carbohydrate chains are incorporated into a macromolecular network. This part of the wall, the peptidoglycan, is common to most bacteria. Among bacterial species there are many variants of the tetrapeptide and of the system of cross-links, but the mycobacterial type is a common variant, shared, for example, with *Escherichia coli.* A special feature is that the amino group of the muramic acid is glycolylated; whereas in most bacteria it is acetylated (Fig. 2). A second special feature is the cross-linking. In addition to joining terminal D-alanine with *meso*-diaminopimelic acid (DAP), as in many other bacteria, mycobacterial cross-links



N-acetyl muramic acid



N-glycolyl muramic acid

FIG. 2. *N*-acetyl- and *N*-glycolylmuramic acid. The glycolyl derivative is found only in mycobacteria and a few related organisms.

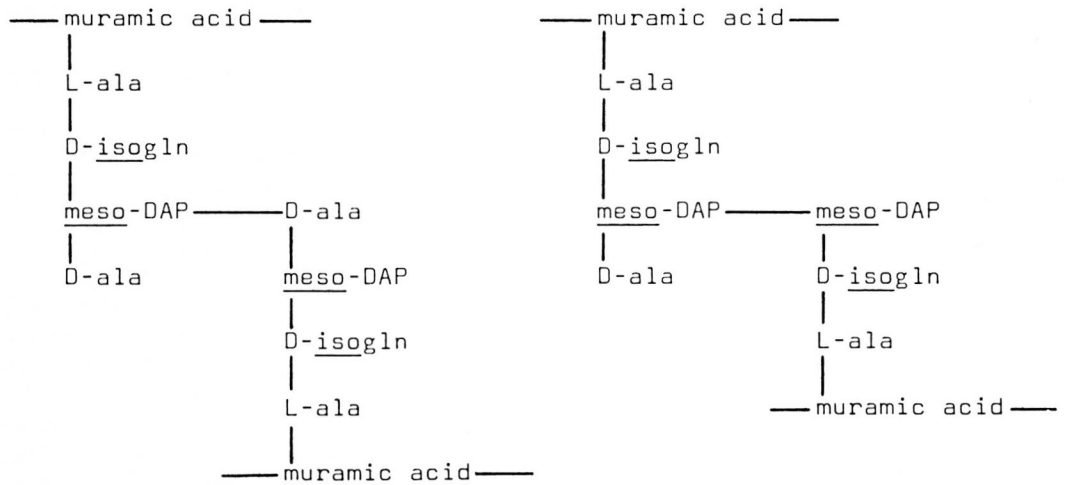


FIG. 3. Cross-links of mycobacterial peptidoglycan. The link between *meso*-DAP and D-ala (left) is common to many species of bacteria. The DAP-DAP link (right) was discovered in mycobacteria, although there is some evidence that it occurs also in other species (see text).

also exist which join two units of DAP (Fig. 3). It should be emphasized that neither of these two special features is unique to mycobacteria: *N*-glycolylmuramic acid occurs also in some corynebacteria,[5] while evidence suggesting the presence of DAP-DAP links has been found in some other bacteria (Prof. J. M. Ghuysen, personal communication and [6]).

Attached to a proportion of the muramic acid units, by a phosphodiester link to carbon 6, is a branched-chain arabinogalactan (Fig. 4) whose basic structure is apparently common to mycobacteria, corynebacteria, and nocardias, on the basis of immunological crossreactivity. It has recently been shown[7] that despite the similarity of the antigenic determinants, variations may occur in the sugar composition of the polysaccharides among these genera. The distal ends—arabinose units—of the arabinogalactan are esterified with mycolic acids, which are complex fatty acids of high molecular weight. Analogous acids occur in corynebacteria, nocardias and some related organisms, but these have smaller chain lengths.

Thus, there are at least four special features in mycobacterial walls that might be suitable sites for interference by novel drugs: a) mycolic acid esters, b) arabinogalactan, c) *N*-glycolylmuramic acid, and d) DAP-DAP cross-links. These will now be discussed in more detail.

Mycolic acids. Mycolic acids are α-branched, β-hydroxy fatty acids with long hydrocarbon chains[8]. The branch usually contains about 24 carbon atoms and is a simple linear alkyl chain; the main chain contains about 54 carbon atoms and includes a variety of special structures—one or two cyclopropane rings, double bonds, epoxy groups, methyl branches, methoxyl or keto functions, for example. Since mycolic acids comprise up to 50% by weight of the wall, it seems likely that interference with their synthesis might be deleterious to mycobacteria. Indeed, it has been suggested

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[6] Glauner, B. and Schwarz, U. The analysis of murein composition with high-pressure-liquid chromatography. In: *The Target of Penicillin.* Hakenbeck, R., Hoeltje, J. V. and Labischinski, H., eds. Berlin: Walter de Gruyter & Co., 1983, pp. 29–34.

[7] Abou-Zeid, C., Voiland, A., Michel, G. and Cocito, C. Structure of the wall polysaccharide isolated from a group of corynebacteria. Eur. J. Biochem. **128** (1982) 363–370.

[8] Minnikin, D. E. Lipids: complex lipids, their chemistry, biosynthesis and roles. In: *The Biology of the Mycobacteria. Vol. 1. Physiology, Identification and Classification.* Ratledge, C. and Stanford, J., eds. London: Academic Press, 1982, pp. 95–184.

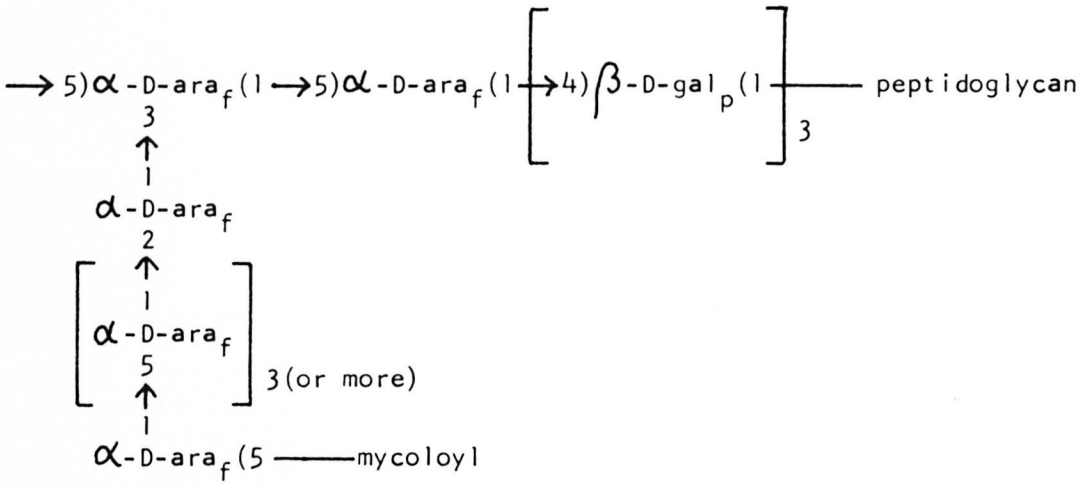

FIG. 4. Structure of the mycobacterial arabinogalactan. The structure shown is the repeating unit[14]. Only the terminal unit is attached to the peptidoglycan, in the position shown. The site of the mycolate ester is also shown. "araf" is arabinofuranose; "galp" is galactopyranose.

that isoniazid works in this way[9, 10]. (The experiments show clearly that mycolic acid synthesis is inhibited, but it is not yet clear that this is the primary site of action of the drug. Its apparent ineffectiveness against *M. leprae* is also puzzling if it indeed works by inhibiting mycolic acid synthesis.)

It is believed that mycolic acids are synthesized by condensation of preformed fatty acid units[11], but no details of this condensation process are known. Cell-free extracts of mycobacteria can synthesize long-chain fatty acids that would be suitable starting materials for the condensation[12]. It is likely that these large and hydrophobic molecules are attached or complexed to carriers in the mycobacterial cells. There is evidence that 6-mycolyl-6′-acetyltrehalose is involved as a carrier in *M. tuberculosis*[13]. The mechanism of the introduction of the various oxygen functions into the chain is not properly understood, nor is the formation of the cyclopropyl groups, although it is known that the "third" carbon atoms of the cyclopropane rings (and also the methyl branches) come from methionine[14]. Finally, the mechanisms of attachment of the mycolic acids to the arabinogalactan and of the "export" of these substances into the bacterial wall (which is an extracellular structure) are unknown.

Arabinogalactan. The biosynthesis of the arabinogalactan has not been studied. The arabinose is the D-isomer[15], which is relatively unusual in nature and not simply related to other common sugars by one or a few enzymic conversions. In other bacteria, synthesis of polysaccharides is usually carried out with the growing chain attached to a lipid carrier, but the presence of such a carrier has not been investigated in mycobacteria. It also seems important to know whether, as suggested by Rogers and his colleagues[1], there is a special linkage region joining the polysaccharide to the muramic acid. (This is the case in many other bacteria having wall polysaccharide so attached.) In the original publication describing the phos-

[9] Winder, F. G. and Collins, P. B. Inhibition by isoniazid of synthesis of mycolic acids in *Mycobacterium tuberculosis*. J. Gen. Microbiol. **63** (1970) 41–48.

[10] Takayama, K., Wang, L. and David, H. L. Effect of isoniazid on the *in vivo* mycolic acid synthesis, cell growth, and viability of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **2** (1972) 29–35.

[11] Etemadi, A. H. Corrélations structurales et biogénétiques des acides mycoliques en rapport avec la phylogenèse de quelques genres d'actinomycétales. Bull. Soc. Chim. Biol. **49** (1967) 695–706.

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[13] Takayama, K. and Armstrong, E. L. Isolation, characterization, and function of 6-mycolyl-6′-acetyltrehalose in the H37Ra strain of *Mycobacterium tuberculosis*. Biochemistry **15** (1976) 441–447.

[14] Lederer, E. Some problems concerning biological C-alkylation reactions and phytosterol biosynthesis. Quart. Rev. **23** (1969) 453–481.

[15] Misaki, A., Seto, N. and Azuma, I. Structure and immunological properties of D-arabino-D-galactans isolated from cell walls of *Mycobacterium* species. J. Biochem. **76** (1974) 15–27.

phodiester linkage of the arabinogalactan to the peptidoglycan in mycobacteria[16], it was suggested that a small proportion of the attachments were through a different linkage, possibly glycosidically through glucosamine. This should, perhaps, be further investigated.

Together, the mycolic acid and the arabinogalactan comprise the major part (the glycolipid) of the mycobacterial wall. It seems likely that interference with their formation would be harmful to the organisms and that drugs acting in this way might be effective against mycobacteria. In particular, it might be expected that damage to the glycolipid might leave the underlying peptidoglycan less well protected and therefore more susceptible to damage by other drugs or by the host's own defenses.

Peptidoglycan. There is no information on whether the *N*-glycolyl groups on the muramic acid of mycobacteria are essential to the cell. Glycolyl groups are formed, at least in nocardias[17], by oxidation of the N-acetyl groups in UDP-*N*-acetylmuramic acid, which is a nucleotide precursor of peptidoglycan synthesis. The oxidation appears to involve the direct use of molecular oxygen in mycobacteria[18], and seems a possible site for selective inhibition.

Formation of cross-links between the peptide side-chains is the site of action of the beta-lactam antibiotics. A deficit of cross-links loosens the structure of the peptidoglycan, possibly allowing better access by (host) lytic enzymes, weakens the wall physically, and leads to lysis of growing bacteria. It is possible that the presence of an alternative type of cross-link in mycobacteria, between two units of DAP, accounts for their insensitivity to the beta-lactam antibiotics. Formation of the special cross-links requires two steps: removal of the terminal D-alanine and formation of the cross-link.

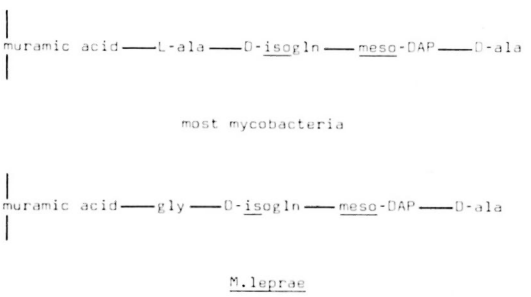

FIG. 5. Peptides of mycobacterial peptidoglycan. Most mycobacteria contain both D- and L-alanine. In *M. leprae* the L-alanine appears to be replaced by glycine.

In other bacteria the analogous steps, resulting in the formation of D-alanine-DAP links, are carried out by the same enzyme, which is sensitive to inhibition by penicillin. New drugs to inhibit both types of cross-linking in mycobacteria might be valuable.

Glycine in *M. leprae* walls. *M. leprae* seems, as far as is known, to share most of the features of the mycobacterial wall, but there is one unusual structure. The tetrapeptide contains glycine (Fig. 5) in place of L-alanine (personal communications, Dr. A. D'Abre and Prof. O. Kandler, and [19]). Essentially, all the alanine in the walls of *M. leprae* is the D-isomer. (It should be noted that the measurement requires substantial amounts of walls and has so far only been made on organisms isolated from infected armadillos.) As with all of the special features discussed in this editorial, there is no experimental evidence that this feature is essential for bacterial survival. It has been found in only one other group of organisms, *Micromonospora*[20], which is only remotely related to the mycobacteria (although, interestingly, it also contains *N*-glycolyl muramic acid). Experimentally, substitution by glycine (or other amino acids) of the normal amino acids in the wall may be produced in many types of bacteria by growing them in high concentrations of the "wrong" amino acid; in these cases the substitution is not usually position specific. It often leads to morphological aberrations and to weak-

[16] Kanetsuna, F. and San Blas, G. Chemical analysis of a mycolic acid-arabinogalactan-mucopeptide complex of mycobacterial cell wall. Biochim. Biophys. Acta **208** (1970) 434–443.

[17] Gateau, O., Bordet, C. and Michel, G. Étude de la formation de l'acide N-glycolylmuramique de peptidoglycane de *Nocardia asteroides*. Biochim. Biophys. Acta **421** (1976) 395–405.

[18] Essers, L. and Schoop, H. J. Evidence for the incorporation of molecular oxygen, a pathway in biosynthesis of N-glycolylmuramic acid in *Mycobacterium phlei*. Biochim. Biophys. Acta **544** (1978) 180–184.

[19] Draper, P. Cell walls of *Mycobacterium leprae*. Int. J. Lepr. **44** (1976) 95–98.

[20] Kawamoto, I., Oka, T. and Nara, T. Cell wall composition of *Micromonospora olivasterospora, Micromonospora sagamiensis*, and related organisms. J. Bacteriol. **146** (1981) 527–534.

ening of the walls. The significance of the specific substitution in *M. leprae* (assuming that it also occurs in human-derived bacteria, which needs to be checked if possible) should be evaluated.

Tactics for developing wall-active antimycobacterial drugs. Although there has been progress toward the goal, the process of designing drugs to inhibit specific enzymes is not yet generally practicable. However, it does seem worthwhile to obtain a full understanding of the steps in biochemical pathways that one would like to be able to inhibit, in order to exploit progress in drug design as it occurs. Some tactical considerations arise, particularly concerning the use of model systems. The relative difficulty of growing and handling mycobacteria, and the paucity of experimental (e.g., genetic) techniques applicable to them, make them not "experimental animals" of choice for the study of bacterial wall synthesis or its inhibition. Thus pathways and processes shared with other bacteria may be better studied in those bacteria. A similar argument would apply to the use of rapid-growing rather than slow-growing mycobacteria for the study of mycobacteria-specific processes, and to the use of mycobacteria other than *M. leprae,* except for processes unique to that organism. It is important to consider carefully what organism is a suitable model in each instance; it is unlikely that there is a "universal" model for *M. leprae,* for example. Results obtained with the model will need to be checked in the target organism but, even allowing for this complication, much time and effort is likely to be saved by the use of appropriate models.

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