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Preface

This book is based on a recent meeting of the same title sponsored by the British Society for Antimicrobial Chemotherapy at Aston University. The surface of bacteria plays a crucial role in determining sensitivity to chemotherapy and to host defence mechanisms and thus forms an integrating theme in discussing the survival of the bacterium in an infection. The distinguished authors have taken a critical look at their subject and, where relevant, considered the possible influence of chemotherapy on the various processes contributing to virulence.

It has long been known that environment plays a significant role in determining important characteristics of the bacterium. What is now much clearer are the consequences of specific nutrient depletion and of growth rate *per se*, especially for envelope structure and function. The contribution of Dr Elwyn Griffiths describes the important effects of iron availability on the capacity of a bacterium to survive in the host. The book ends with chapters which examine ways of exploiting our knowledge of the bacterial envelope in designing antibacterial agents and vaccines.

Michael Brown
August 1983

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1 The bacterial surface and drug resistance

PETER A. LAMBERT

I. INTRODUCTION

Bacteria are capable of resisting or avoiding the action of antibiotics in a number of ways, the most important of which are:

(a) production of enzymes which inactivate the antibiotics
(b) modification of the target site so that it is insensitive to the antibiotic
(c) prevention of access of the antibiotic to the target site.

Over the years mechanism (a) has provided the biggest obstacle to the effective chemotherapy of infectious diseases. The most striking example is the production of β-lactamases: enzymes which inactivate penicillins and cephalosporins by hydrolysing their vital β-lactam ring (Sykes and Matthew, 1976). Other important examples are enzymes which inactivate antibiotics by the addition of groups which destroy their antimicrobial activity. Adenylating, phosphorylating and acetylating enzymes are the most important factors responsible for resistance to aminoglycosides (Davies and Smith, 1978).

Resistance by mechanism (b) has been recognized for many years but has not posed a major therapeutic problem. An example is the resistance of pneumococci to sulphonamides, which is due to a decreased affinity of the target enzyme, tetrahydropteroic acid synthetase, for the sulphonamides (Wolf and Hotchkiss, 1963). Mechanism (c) is also familiar: the intrinsic insensitivity of Gram-negative bacteria to antibiotics which are effective against Gram-positive bacteria generally results from the inability

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of the agents to penetrate the Gram-negative outer membrane (Nikaido, 1976).

In recent years the successful development and introduction of antibiotics which are resistant to inactivating enzymes has altered the selective pressure for the emergence of resistant strains. Consequently the pattern of antibiotic resistance is beginning to change. Resistance to β-lactams due to altered penicillin binding proteins has been reported in clinical isolates of *Neisseria gonorrhoeae* (Dougherty *et al.*, 1980), *Streptococcus pneumoniae* (Hakenbeck *et al.*, 1980), and *Staphylococcus aureus* (Hayes *et al.*, 1981). Resistance caused by a reduced level of antibiotic uptake has been demonstrated in many laboratory strains of bacteria (Foulds and Chai, 1978; Harder *et al.*, 1981; Sawai *et al.*, 1982) and is now being encountered in clinical isolates (Rodriguez-Tebar *et al.*, 1982). This review is concerned with the mechanisms by which bacteria can prevent or restrict antibiotic uptake and, like an earlier review by Costerton and Cheng (1975), will concentrate upon the role of the cell envelope. It must be emphasized that even small increases in resistance can have an important bearing upon the success or failure of antimicrobial therapy. This may be particularly true in cases such as lung infections in cystic fibrosis patients, where it is difficult to achieve effective concentrations of antibiotics at the site of infection (Govan, 1976; Govan and Fyfe, 1978; Lam *et al.*, 1980; Bergogne-Berezin, 1981; Slack and Nichols, 1982).

With the exception of the β-lactams, the target sites of action of the major groups of antibiotics are intracellular. Aminoglycosides, tetracyclines, macrolides, chloramphenicol and fusidic acid all inhibit ribosome function; oxolinic and nalidixic acids inhibit DNA gyrase; rifampicin inhibits DNA-dependent RNA polymerase; trimethoprim and the sulphonamides interfere with folate metabolism; cycloserine and phosphonomycin inhibit early, cytoplasmic stages of peptidoglycan biosynthesis (Gale *et al.*, 1981). It follows that all of these agents must penetrate into the cytoplasm in order to exert their inhibitory action. In most cases they are transported across the cytoplasmic membrane by permease systems which are present to transport nutrients. The exploitation of natural transport systems to attain high intracellular concentrations of antimicrobial agents is reviewed separately in this volume by Ringrose (Chapter 8).

The targets for β-lactam antibiotics, the penicillin binding proteins, are located in the cytoplasmic membrane, probably on the outer face (Spratt, 1980). Penicillins and cephalosporins therefore only need to penetrate the cell wall in order to reach their sites of action. Any changes in wall composition which affect the rate of penetration of antibiotics are likely to alter the sensitivity, not only to the β-lactams, but also to antibiotics which act at intra-cellular sites, since they must first pass across the cell wall

before reaching the permease systems in the cytoplasmic membrane. The capacity of all bacteria to vary the chemical composition of their walls in response to changes in growth rate and nutritional conditions is well documented (Ellwood and Tempest, 1972) and attention has been drawn to the implications for drug resistance and antimicrobial chemotherapy (Brown, 1977; Dean *et al.*, 1979; Brown *et al.*, 1979). In addition to wall permeability changes resulting from phenotypic variation, many envelope mutants have been shown to possess dramatically altered permeability characteristics which influence their antibiotic susceptibility (Coleman and Leive, 1979; Grundstrom *et al.*, 1980).

II. THE STRUCTURE OF BACTERIAL CELL WALLS

Figure 1 shows how the structure of the walls of Gram-positive bacteria differs from the envelope of Gram-negative bacteria; a detailed account has been given by Rogers *et al.* (1980). The Gram-positive wall is a relatively simple structure composed of roughly equal proportions of

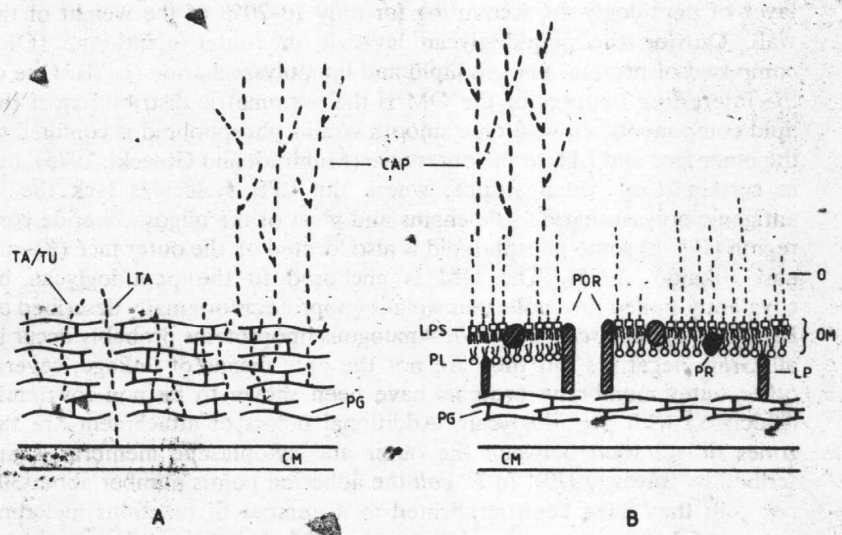

Fig. 1 Cross-section of the envelopes of typical Gram-positive (A) and Gram-negative (B) bacteria. CM, cytoplasmic membrane; PG, peptidoglycan; TA/TU, teichoic or teichuronic acid; LTA, lipoteichoic acid; CAP, capsule; OM, outer membrane; LP, lipoprotein; PR, protein; POR, porin protein; PL, phospholipid; LPS, lipopolysaccharide; O, O antigenic polysaccharide of LPS.

peptidoglycan and an anionic polymer which is usually a teichoic or a teichuronic acid. The peptidoglycan is responsible for the strength and shape of the cell wall. The teichoic and teichuronic acids are long, flexible polymers containing either acidic phosphodiester groups (teichoic acids) or acidic carboxyl groups on uronic acids (teichuronic acids). Both types of polymer are linked covalently at one end to muramic acid residues on the peptidoglycan network. The basic structure of the Gram-positive wall is an open matrix in which the anionic polymers are interwoven with the peptidoglycan (Costerton and Cheng, 1975). The anionic groups have a strong affinity for metal ions, especially magnesium, which is usually tightly bound by the walls. Neutral polysaccharides and proteins are also associated with the walls of some Gram-positive bacteria. The streptococci in particular contain a range of type-specific polymers linked to the wall (Campbell *et al.*, 1978) and some strains of *S. aureus* produce protein A, part of which is released into the medium and part remains linked to peptidoglycan and protrudes from the wall (Sjodahl, 1977). Finally, some Gram-positives produce capsules, normally polysaccharides which are loosely associated with the wall; over 80 different, antigenically-distinct types have been described in *Strep. pneumoniae* (Sutherland, 1977).

The Gram-negative envelope is far more complex. It comprises a thin layer of peptidoglycan accounting for only 10–20% of the weight of the wall. Outside the peptidoglycan layer is the outer membrane (OM) composed of protein, phospholipid and lipopolysaccharide (LPS). One of the interesting features of the OM is the asymmetric distribution of the lipid components. In wild type smooth strains phospholipid is confined to the inner face and LPS to the outer face (Muhlradt and Golecki, 1975), but in certain deep rough strains, where the LPS molecules lack the O antigenic polysaccharide side chains and most of the oligosaccharide core region (Fig. 2) some phospholipid is also located on the outer face (Kamio and Nikaido, 1976). The OM is anchored to the peptidoglycan by covalently-linked low molecular weight lipoproteins originally described by Braun (1975) in *Escherchia coli*. Analogous lipoproteins probably occur in all Gram-negatives but they are not the only means of linkage; several other outer membrane proteins have been shown to be non-covalently associated with peptidoglycan. Additional points of attachment are the zones of adhesion between the outer and cytoplasmic membranes described by Bayer (1979). In *E. coli* the adhesion points number about 300 per cell; they have been implicated in a number of functions including export of LPS to the outer face of the OM, but not in the uptake of nutrients or antibiotics.

Permeability of the OM towards low molecular weight hydrophilic nutrients is due to the presence of a special group of proteins called porins

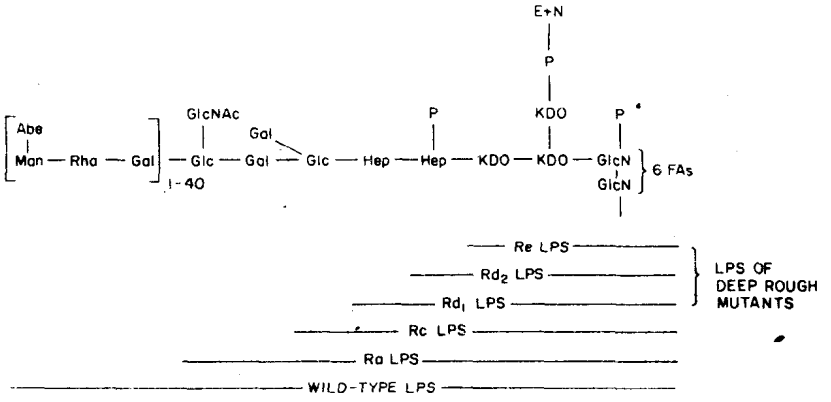

Fig. 2 Structure of the lipoplysaccharide (LPS) in various mutants of *Salmonella typhimurium* and the wild-type strain. Abe, abequose; Man, D-Mannose; Rha, L-rhamnose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; Glc, D-glucose; Hep, L-glycero-D-mannoheptose; KDO, 2-keto-3-deoxyoctonic acid (3-deoxy-D-mannooctulosonic acid); GlcN, D-glucosamine; FA, fatty acids; P, phosphate; EtN, ethanolamine.

(Nikaido, 1979; Nikaido and Nakae, 1979). These proteins have molecular weights ranging from 32 000 to 41 000 daltons and are present in large numbers, at least 10^5 per cell. They span the OM forming aqueous channels or pores and are usually associated non-covalently with peptidoglycan. The pores only permit the passage of hydrophilic molecules up to a certain size: the exclusion limit is low in enteric bacteria, of the order of 600–700 daltons in *E. coli* and *Salmonella typhimurium* (Decad and Nikaido, 1976), but is thought to be much higher in other Gram-negatives, the limit in *Pseudomonas aeruginosa* being 4 000–6 000 daltons (Hancock and Nikaido, 1978; Hancock *et al.*, 1979; Benz and Hancock, 1981). Whilst the pore size is a property of the porin proteins themselves, little is yet known about factors which control their functional state. It is possible that other OM proteins and LPS molecules influence the ability of porins to adopt conformations in the OM which enable them to function as pores.

Important advances have been made in recent years on extraction and separation techniques used to study LPS. It has been shown that the LPS from many Gram-negative bacteria is heterogeneous with respect to size (Goldman and Leive, 1980; Palva and Makela, 1980; Munford *et al.*, 1980; Kropinski *et al.*, 1982; Tsai and Frasch, 1982). The total LPS population comprises a range of molecules differing in the number of O antigen subunits from 0 to approximately 40.

The outer surface of the OM is therefore made up from exposed regions of proteins (mainly the porins) surrounded by protruding polysaccharide chains (the O antigens) of varying lengths. Many Gram-negatives also have capsular polysaccharides (the K antigens) loosely associated with the envelope. The polysaccharide chains are often branched and contain negatively charged carboxyl residues (Sutherland, 1977). Capsules provide a highly hydrated gel-like coat around the cell which protects them from phagocytosis *in vivo* and from desiccation in habitats prone to periodic drying (Dudman, 1977). Costerton *et al.* (1981) describe the capsule and other exopolysaccharides as forming a glycocalyx around cells which figures prominently when cells are grown *in vivo* or isolated from natural environments and viewed under the electron microscope. The glycocalyx is presumed to help the cells to adhere, colonize and survive under adverse conditions. Capsular polysaccharides and expolysaccharides probably do not present a physical barrier against the penetration of low molecular weight hydrophilic species, since encapsulated strains have no difficulty in taking up nutrients and are capable of growing as rapidly as unencapsulated strains. In certain cases the negatively charged groups might interfere with the passage of positively charged antibiotics by acting like an ion-exchange resin and immobilizing the antibiotics (Slack and Nichols, 1981). On the other hand, trapping of antibiotics in the capsule would lead to a high local concentration around the cells, which would then be available for transport into the cells if any subsequent dissociation of bound antibiotic occurred (Slack and Nichols, 1982).

III. BARRIER PROPERTIES OF THE GRAM-POSITIVE CELL WALL

The Gram-positive cell wall contains no receptor molecules or permease enzymes to assist the penetration of antibiotics to the underlying cytoplasmic membrane. However, it does not prevent access of antibiotics either; the exclusion limit of the *Bacillus magaterium* wall, which contains a teichuronic acid, is between 30000 and 57000 daltons (Scherrer and Gerhardt, 1971). The strong negative charge of the wall, and of capuslar components when present, might be expected to exert some ion-exchange effect upon the diffusion of charged antibiotics across the wall. In fact Gram-positives are sensitive to anionic, cationic and zwitterionic antibiotics, resistance being generally due to other factors such as inactivation, insensitivity of targets or failure of cytoplasmic membrane transport systems. Take for example *S. aureus* which contains a teichoic acid with a highly crosslinked peptidoglycan and, in some strains, additional components like protein A or a capsule made up of aminosugars. *S. aureus* strains

are usually sensitive to fusidic acid (anionic), β-lactamase stable penicillins such as cloxacillin and flucloxacillin (anionic), and the aminoglycosides (cationic).

Attempts have been made to correlate the lipid composition of staphylococci with resistance to agents such as fusidic acid (Chopra, 1976), penicillins (Hugo and Stretton, 1966), and disinfectants which act upon the cytoplasmic membrane (Hugo and Davidson, 1973). Resistance to fusidic acid was related to an increase in the ratio of the phospholipids lysylphosphatidylglycerol : phosphatidylglycerol whilst with penicillin and phenolic disinfectants cells containing high levels of phospholipid were more resistant than cells depleted of lipid by growth under biotin-deficient conditions (Hugo and Davidson, 1973). Lipids are not recognized as components of the Gram-positive wall, they are associated exclusively with the cytoplasmic membrane. Therefore the changes in drug resistance most likely reflect alterations in membrane composition which affect the transport of fusidic acid, accessibility of the penicillin binding proteins to penicillin and susceptibility to damage by phenols.

IV. BARRIER PROPERTIES OF THE GRAM-NEGATIVE CELL WALL

Our current understanding of the diffusion of nutrients and antibiotics across the wall of Gram-negative bacteria has developed rapidly since the recognition, some twelve years ago, of the OM as a vital envelope component. The structure, composition and properties of the OM have been extensively reviewed (Inouye, 1979; Nikaido and Nakae, 1979; Nikaido, 1979). Without doubt, a major contribution has been made by Nikaido and his associates, who introduced the concept of pore-forming proteins and established their fundamental properties (Nakae and Nikaido, 1975). Nikaido has also attempted to distinguish between the uptake of hydrophilic antibiotics by passage through the aqueous porin channels and the uptake of hydrophobic antibiotics by diffusion across hydrophobic regions of the OM.

A. The outer membrane as a permeability barrier

Even before the recognition of the OM as an envelope component it was generally believed that the intrinsic resistance of Gram-negative bacteria towards antimicrobial agents was due to the permeability barrier presented by the cell envelope. In particular, the high lipid content of the envelope was considered to impede access of antimicrobial agents to the cells,

although Gram-negatives clearly were able to take up nutrients efficiently. The effectiveness of the barrier was known to depend upon the growth conditions: for example, cells of *P. aeruginosa* grown under conditions of magnesium limitation were shown to be more resistant to agents such as polymyxin and EDTA than cells grown in media containing ample magnesium (for review see Brown, 1975). Leive showed that brief exposure of *Salm. typhimurium* or *E. coli* to EDTA did not kill the cells but released up to 50% of the LPS, together with some protein. The result was a dramatic increase in sensitivity towards agents such as actinomycin D, novobiocin and rifampicin which were ineffective against untreated cells because they could not penetrate the wall (Leive, 1974). As LPS is only found in the OM, it was concluded that the penetration barrier of the OM was impaired by removal of the LPS and that divalent metal ions play an important part in maintaining the integrity of the OM, probably by binding LPS molecules together on the outer face (Nikaido, 1973).

With the discovery of porins the ability of low molecular weight hydrophilic nutrients to penetrate the OM permeability barrier was explained; small hydrophilic antibiotics were presumed to cross the wall by the same route. Nikaido (1976) investigated the uptake mechanisms of a wide range of antimicrobials, including antibiotics, dyes and disinfectants, by considering data on their activity against a series of *Salm. typhimurium* mutants which differed only in the amount of polysaccharide contained in the core and O antigen region of the LPS (Fig. 2). The antimicrobials were chosen to cover a range of molecular weights and hydrophobicities, measured in terms of their partition coefficients between octanol and 0·05 M sodium phosphate buffer, pH 7·0 at 24°C (Table 1). His conclusions are summarized in Fig. 3 (Nikaido, 1979). Firstly, a large group of small, hydrophilic antibiotics, with partition coefficients of 0·02 or less and molecular weights below 650 daltons were equally active against wild type, smooth strains with complete O antigens and the rough mutants containing LPS with varying degrees of truncated polysaccharides. A second group of agents regarded as hydrophobic, with partition coefficients greater than 0·02, were only active against the rough strains. The conclusion reached by Nikaido was that there are two pathways by which agents can cross the OM: a hydrophilic pathway via the aqueous pores, and a hydrophobic pathway involving diffusion across the OM bilayer. The porin-mediated pathway for small hydrophilic molecules is available in rough and smooth strains, the complete LPS O side chains do not impede access of such molecules to the hydrophilic pores. The hydrophobic pathway is not available in wild type smooth strains, either because the LPS O side chains prevent access of the hydrophobic molecules to the outer face of the OM, or because of a lack of hydrophobic patches on the OM which could act as

Table 1 Hydrophobicity, size and activity of some agents against LPS mutants of *Salmonella typhimurium*

Agent	Partition[a] coefficient	Molecular weight	Activity against *Salm. typhimurium*
Actinomycin D	>20	1255	
Novobiocin	>20	613	
Phenol	>20	94	Active against deep
Crystal violet	14	408	rough mutants. Very
Rifamycin SV	9	698	weak or no activity against
Nafcillin	0·3	414	smooth wild-type
Oxacillin	0·07	418	strain.
Pencillin G	0·02	334	
Ampicillin	<0·01	349	
Cephalothin	<0·01	395	Similar activity
Carbenicillin	<0·01	378	against deep rough
Neomycin	<0·01	615	mutants and smooth
Cycloserine	<0·01	102	wild-type strain.

[a] Octan-1-ol: 0·05 M sodium phosphate buffer, pH 7·0 at 24°C.

receptor surfaces. Only in the deep rough strains can the hydrophobic molecules approach the OM, bind, and cross by a diffusion process. Apart from a lack of protecting O polysaccharide chains on the surface of rough strains, it is thought that phospholipid molecules present on the outer face (Kamio and Nikaido, 1976) might provide sites of access for hydrophobic molecules. The occurrence of phospholipids on the outer surface of rough strains has been questioned (Shales and Chopra, 1982) but it seems likely that excision of 50% of the LPS of smooth strains by EDTA must result in a reorientation of the OM lipids, and that under these conditions the cells become sensitive to the large hydrophobic molecule, actinomycin D (Leive, 1974). Direct measurement of the rate of uptake of the hydrophobic penicillin, nafcillin by the *Salm. typhimurium* mutants (Nikaido, 1976) has shown that it penetrates more rapidly into the deep rough strains (Rd, Rd_2 and Re) than into the wild type and rough strains (Ra and Rc).

There are some antibiotics which do not fit in with the general pattern described by Nikaido. Chloramphenicol is extremely hydrophobic but is quite active against smooth Gram-negative strains. Most tetracyclines are also hydrophobic but penetrate smooth strains with little difficulty. Additional factors must be involved in the mechanism by which these agents cross the OM.

The majority of antibiotics used to treat Gram-negative infections are small, hydrophilic molecules which presumably utilize the porin channels

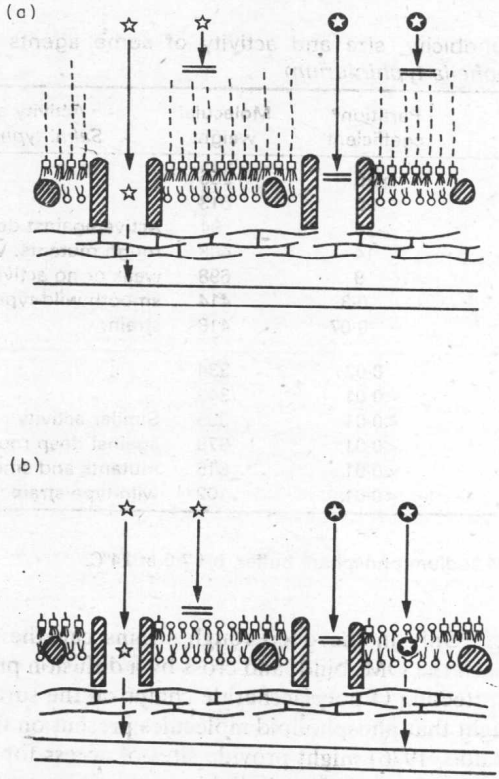

Fig. 3 Pathways for the passage of hydrophilic and hydrophobic antimicrobial agents across the outer membrane of smooth (a) and deep rough strains (b) of Gram-negative bacteria. Hydrophilic agents (☆) penetrate through the aqueous pores; hydrophobic agents (✪) can only penetrate via hydrophobic patches (phospholipid?) on the deep rough outer membrane.

to traverse the OM. Three factors control the rate of passage of molecules through the porin channels: size, hydrophobicity, and charge. The pores of *E. coli* and *Salm. typhimurium* have been 'measured' indirectly by establishing the cut-off point at which a series of oligosaccharides of increasing size are excluded (Decad and Nikaido, 1976). Sucrose and raffinose (342 and 504 daltons) penetrate easily; stachyose (666 daltons) penetrates at less than 25% of the rate of the smaller sugars; and verbascose (828 daltons) and larger oligosaccharides fail to penetrate at all. Hence the exclusion limit is around 650 daltons, which is equivalent to a diameter of about 1·2 nm. Most β-lactam antibiotics have molecular