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Antibiotic Uptake Pathways across the Outer Membrane of *Pseudomonas aeruginosa*

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Introduction

The outer membrane of *Pseudomonas aeruginosa* serves as a permeability barrier to many conventionally used antibiotics [13]. To understand the role of the outer membrane in antibiotic resistance, we have focused our efforts towards defining and characterizing the pathways of antibiotic uptake across the outer membrane of this organism. This paper summarizes the most recent data from our laboratory.

There exist three recognized pathways for antibiotic uptake across the outer membranes of gram-negative bacteria. These are the hydrophilic pathway, the hydrophobic pathway, and the self-promoted uptake pathway [13]. Of these, only the hydrophilic and the self-promoted uptake pathways are of major importance in *P. aeruginosa* [13]. The remaining pathway, the hydrophobic pathway, involving the partitioning of amphiphilic or hydrophobic antibiotics into the membrane, is important only in a small number of gram-negative bacteria, some examples being *Haemophilus* and *Neisseria* and deep rough mutants of *Escherichia coli* and *Salmonella* [14].

Materials and Methods

The methods used in these experiments have all been described in detail elsewhere including outer membrane isolation [5], purification of protein F [18], cloning of protein F [18], functional studies using the black lipid bilayer apparatus [3], assays involving the

fluorescent probes N-phenylnapthylamine (NPN) and 1,8-anilino-1-naphthalene sulfonic acid [6, 8], assays involving dansyl-polymyxin [10], and the measurement of outer membrane permeability using nitrocefin [12].

Results

Hydrophilic Pathway

The outer membrane of *P. aeruginosa* contains at least nine different proteins which, due to their high copy number, are referred to as major outer membrane proteins [5]. One of these proteins, protein F, forms a water-filled channel that spans the outer membrane and allows the passage of water-soluble or hydrophilic compounds across the hydrophobic interior of the outer membrane [13]. Because of this ability, protein F has been categorized as a porin protein and thus serves as a major uptake route for hydrophilic antibiotics, such as β-lactams, to pass through the outer membrane. Accordingly, a porin protein F deficient mutant had a substantial decrease in outer membrane permeability to the β-lactam antibiotic nitrocefin [12]. Although protein F forms water-filled channels across the outer membrane of *P. aeruginosa*, it provides a relatively poor pathway for β-lactam antibiotics owing primarily to a functional heterogeneity which results in a low number of large-sized channels [2, 19] and a larger number of small-sized channels [18]. The functional heterogeneity of protein F was revealed by the observation that protein F cloned into *E. coli* and purified by selective solubilization and electroelution, as well as protein F from *P. aeruginosa*, was present predominantly as very small channels and to a far lesser extent as large channels (average single-channel conductances in 1 *M* KCl of 0.36 nS and around 5 nS, respectively; fig. 1). Previous studies of purified protein F had only revealed the large channels [2, 19] due to the fact that the resolution of the apparatus used to measure the large channels was incapable of detecting the smaller 0.36-nS channel. Prior to the discovery of the small-channel sizes of the majority of individual protein F molecules, low outer membrane permeability in *P. aeruginosa* was believed to be due, in part, to the small proportion of functionally active protein F molecules in the outer membrane [2, 12, 19]. However, in the light of the discovery of the small channels, our working hypothesis is that the presence of large and small channel sizes from purified preparations of protein F is a result of functional heterogeneity of channel sizes of individual protein F molecules resulting from intrinsic factors related to the assembly of active protein F channel forming units (see Discussion).

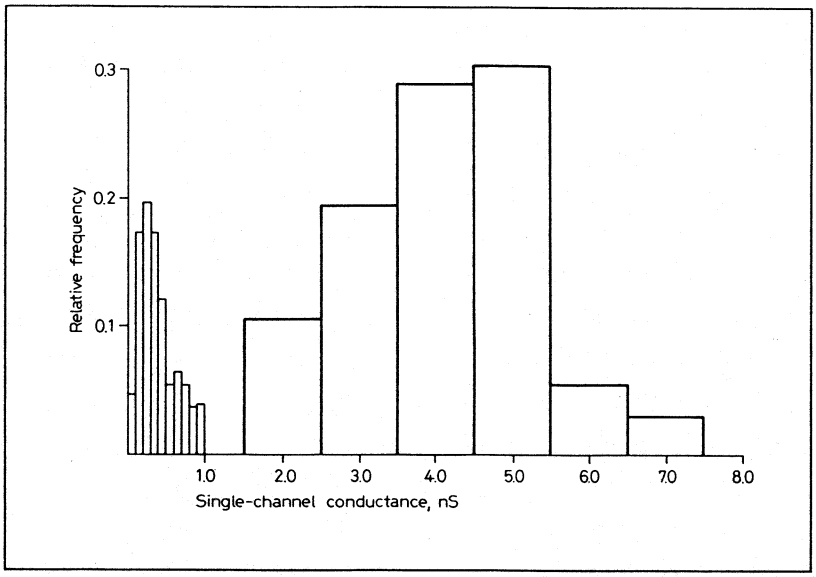

Fig. 1. Composite frequency histogram depiciting small and large channels formed by *P. aeruginosa* protein F purified from an *E. coli* strain into which the protein F gene had been cloned. Two separate experiments were performed since the two classes of channels could not be detected in a single analysis – i.e., when the instrumentation was set to examine small channels (0.1–1.0 nS), the insertion of a large channel (2–7 nS) caused the recorder to jump off scale. When the instrumentation was set to examine large channels, the small channels were below the limits of detection. The small channel was more prevalent and was seen at an input dose of 0.6 pg protein/ml. In contrast, at least 6 pg protein/ml had to be used before the large channel – analogous to those detected by Benz and Hancock [2] – was observed.

Hydrophobic Pathway

As mentioned above, the hydrophobic pathway is not a major route of antibiotic uptake across the outer membrane of *P. aeruginosa*. This is illustrated by the high resistance of *P. aeruginosa* to hydrophobic antibiotics such as erythromycin and fusidic acid [13] and by the fact that *P. aeruginosa* cells do not normally take up the hydrophobic fluorescent probes NPN or 1,8-aniline-1-naphthalene sulfonic acid [8]. Uptake of these hydrophobic probes is, however, observed when whole cells are treated with outer membrane disorganizing compounds such as the divalent cation chelator ethylenediaminetetraacetic acid (EDTA) and polycationic antibiotics like aminoglycosides and polymyxins [6, 8]. In addition, a

specific mutant, Z61, of *P. aeruginosa* will take up NPN readily and thus demonstrates a hydrophobic uptake pathway. This strain is characterized phenotypically by supersensitivity to all tested hydrophilic, hydrophobic, and aminoglycoside antibiotics [1]. The lipopolysaccharide (LPS) from strain Z61 demonstrated a modified LPS rough core region which migrated faster on an SDS-polyacrylamide gel [7] and contained alterations in the LPS phosphates as determined by ^{31}P-NMR [unpublished results] and in the Mg^{2+}-binding ability of the lipid A of LPS [15; manuscript in preparation]. Thus, it would seem that divalent cations (Mg^{2+} and Ca^{2+}) play a critical role in stabilizing the outer membrane by crossbridging adjacent polyanionic LPS molecules, thereby excluding hydrophobic compounds.

Self-Promoted Uptake Pathway

The aminoglycosides are one class of antibiotics which are relatively effective in treating *P. aeruginosa* infections. Aminoglycosides, polymyxin B, and other polycations have been shown to interact with a divalent cation-binding site in the outer membrane of *P. aeruginosa* [6, 8]. Furthermore, we recently showed that all of these compounds bind to a divalent cation-binding site located on purified LPS [10, 15]. Treatment of whole cells of *P. aeruginosa* with aminoglycosides or polymyxin B results in the disruption and enhanced permeability of the outer membrane towards the enzyme lysozyme, hydrophobic fluorescent probes, and the chromogenic β-lactam antibiotic nitrocefin [6, 8]. These observations led to the self-promoted uptake model which suggests that the polycations promote their own uptake by binding to a Mg^{2+}-binding site on the LPS and displacing Mg^{2+} [10, 15], thus destroying the stabilizing effect of the Mg^{2+} crossbridging of the adjacent LPS molecules. The consequent destablized outer membrane is then proposed to allow uptake across the outer membrane of molecules of the permeabilizing agent. The self-promoted uptake model is supported by the observation that EDTA, a divalent cation chelator, is also capable of permeabilizing the outer membrane of *P. aeruginosa* [6], presumably by removing Mg^{2+} (or Ca^{2+}) bound to LPS and by the isolation of an outer membrane altered mutant cross-resistant to the lethal effects of polymyxin B, aminoglycosides, and EDTA [11].

Thus it seems apparent that the interaction of aminoglycosides and polymyxin B with the outer membrane of *P. aeruginosa* and other gram-negative bacteria results in disruption of the outer membrane such that compounds previously impeded by the intact membrane are able to cross

Table I. Increase in NPN fluorescence as a result of treatment of whole cells with polymyxin B, aminoglycosides, or EDTA

Antibiotic	Concentration	Increase in fluorescence (arbitrary units)[a]		
		P. aeruginosa	*P. cepacia*	*S. marcescens*
None		0	0	0
Polymyxin B	0.83 μ*M*	120	< 1	56[b]
	8 m*M*	3,700	< 1	ND
Gentamicin	2 m*M*	245	< 1	17
Tobramycin	8 m*M*	> 9,000	22	10[c]
EDTA	5 m*M*	> 9,000	< 1	ND

ND = Not determined.
[a] Increase in fluorescence after addition of antibiotic to a cuvette containing whole cells of the appropriate organism. [For details of this method, see reference 8.]
[b] 0.45 μ*M* polymixin B.
[c] 2.0 m*M* tobramycin.

the outer membrane and reach target sites inside the cell. Since low outer membrane permeability is a problem in antibiotic therapy of *P. aeruginosa*, and since a variety of compounds termed permeabilizers can be demonstrated to open up the outer membrane [6], we have suggested these compounds may have value in combination therapy to improve the efficacy of another antibiotic.

Although the interactions of polymyxin B and polycations (but not aminoglycosides) with the outer membrane and LPS have been observed in a variety of gram-negative bacteria, including *E. coli* and *Serratia marcescens* (table I) [14], the self-promoted uptake system does not appear to be universal to all gram-negative bacteria. In the case of *Pseudomonas cepacia*, exposure of whole cells to aminoglycosides, polymxyin, or EDTA did not permeabilize the cells to either the hydrophobic fluorescent probe NPN (table I) or to the chromogenic β-lactam antibiotic nitrocefin (data not shown). In addition, whole cells of *P. cepacia* did not bind significant amounts of a fluorescent derivative of polymyxin B, dansyl-polymyxin (fig. 2). In contrast, purified LPS was able to bind dansyl-polymyxin with approximately the same affinity as LPS from *P. aeruginosa*. Furthermore,

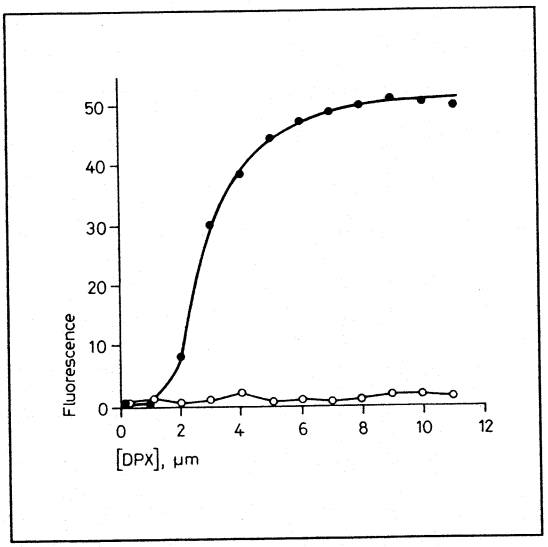

Fig. 2. Binding of dansyl-polymyxin (DPX) to whole cells of *P. aeruginosa* (●) and *P. cepacia* (○) assessed by increasing fluorescence as DPX became bound.

Mg^{2+} and aminoglycoside antibiotics competed with dansyl-polymyxin for binding to the LPS molecule. It appears, therefore, that the LPS from *P. cepacia* is capable of binding aminoglycosides and polymyxin B; however, the sites involved in the binding of these compounds are not accessible on the surface of the outer membranes of this organism. As a result, whole cells of *P. cepacia* are resistant to not only the outer membrane disorganizing effects, but also to the killing action [4] of these polycationic antibiotics.

Discussion

It is now well established that the major uptake route across the outer membrane for hydrophilic antibiotics is through the water-filled channels of a group of proteins called porins [14]. In the past 5 years, model membrane studies of porin function have contributed much to our understanding of channel size and selectivity of porin proteins [13]. However, in most cases, the data derived from these studies describe the behaviour of the

average porin channel. In contrast, black lipid bilayer studies of individual channels have provided data which suggest up to a two-fold variation in the sizes of the individual porin channels produced from a single gene, even for the well-studied *E. coli* porins [3]. The implications and basis of this modest variability are at present unclear. However, if our preliminary conclusions are borne out, the heterogeneity in the channel sizes of individual *P. aeruginosa* protein F porin channels is far more extreme, with a greater than 10-fold difference in the conductances of the predominant small channels and the rarer large channels. It should be noted that we have not entirely eliminated the possibility that the large channel is a contaminating *P. aeruginosa* porin with a similar molecular weight to protein F and mapping within approximately 10 kb of the protein F gene. However, we feel that the evidence for large channels in the *P. aeruginosa* outer membrane is strong [13].

For the large channel we can determine the diameter by applying a classic equation which suggests that the conductance through a conduit is proportional to the area per unit length. This suggests a channel diameter of 2.0 nm, in good agreement with other model studies of *P. aeruginosa* protein F [2, 13]. Such a channel should allow relatively easy passage of β-lactam antibiotics. However, due to the small number of these channels – approximately 100–400 per *P. aeruginosa* outer membrane [12], cf. the 200,000 molecules of protein F per cell – the permeability across the outer membrane remains low [12, 19]. In the small-channel conformation, a low ion conductance (0.36 nS in 1 *M* KCl) is observed. Unfortunately, it is not possible to directly relate this conductance to a given channel size, and it will probably be extremely difficult to study this channel by vesicle exclusion or swelling methods. However, we can conclude that this channel will probably either exclude β-lactam antibiotics or considerably impede their movement.

We feel that there is at least one possible way in which channel heterogeneity for the protein F porin could be generated during synthesis, given that there is only one copy of the gene per *P. aeruginosa* chromosome [18]. Protein F has four cysteines per polypeptide [9], and on the basis of the effect of graded concentrations of 2-mercaptoethanol on the electrophoretic mobility of protein F, it was proposed that these cysteines formed two disulphide bonds [5]. One possible method of gene rating channel size heterogeneity for protein F would be, if there were two possible arrangements of the four cysteines in these two cystine disulphides (fig. 3). Thus, in this scheme, the most common arrangement would generate the small

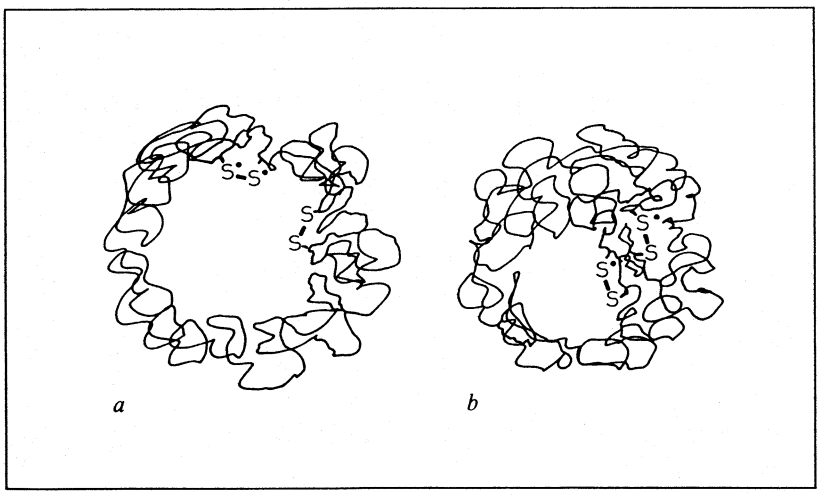

Fig. 3. Speculative model for the way in which two different sized protein F channels could be generated by rearrangement of its four cysteines into two different cystine disulphide pairings. A section through the channel, parallel to the membrane, is shown. Although protein F is arranged as a trimer, evidence has been presented suggesting that each monomer is capable of forming a channel [13, 18]. *a* The molecule represents the rarer large-channel [2, 18] conformation. *b* This molecule represents the more common small-channel [18] conformation.

channel greater than 99% of the time. Alternatively, if the cysteines paired in a different but less thermodynamically favourable fashion, it would generate the large channel. At present, we have only one line of data consistent with such a possibility. The treatment of outer membranes with the reducing agents ascorbate or acetylsalicylate was found to dramatically increase the permeability of the outer membrane to the β-lactam nitrocefin, but unlike the other permeabilizing compounds (polycations and EDTA) referred to above did not increase outer membrane permeability to the hydrophobic dye NPN or the enzyme lysozyme [6]. Thus, ascorbate and acetylsalicylate could be reducing one or more of the cystine disulphides and causing protein F to undergo a conformational shift to the large-channel form. We are currently testing this hypothesis by model membrane and directed mutagenesis studies.

This report, therefore, provides two hints at possible methods for overcoming the outer membrane permeability barrier. The first, described above, would utilize, e.g., ascorbate to overcome the *P. aeruginosa* outer

membrane permeability barrier. The synergy of ascorbate and antibiotics has been described under both in vitro and in vivo conditions [16]. The second method would be to use a 'permeabilizer' (polycation, divalent cation chelator or organic monovalent cation) to increase outer membrane permeability [6] by interacting at and disrupting the sites on the surface of the outer membrane, at which divalent cations crossbridge adjacent LPS molecules. This synergy of 'permeabilizers' like aminoglycosides, polymyxin B, or EDTA with antibiotics is also well described [17]. Unfortunately, neither of these methodologies is of general value. Most porins do not have internal cystine disulphides, and certain bacteria, e.g., *P. cepacia* and *Serratia marcescens*, have divalent cation-binding sites that are inaccessible to many permeabilizers [unpublished data]. Nonetheless, we feel that this general approach to combination therapy is of great interest.

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