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The *Pseudomonas aeruginosa* Outer Membrane Permeability Barrier and How to Overcome It

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Introduction

Since the introduction of antibiotics into modern medical practice in the 1940s, antibiotic resistance amongst bacteria has emerged as a significant problem. Thus antibiotic treatment of bacterial infections can lead to substitution of more resistant bacteria for antibiotic-susceptible organisms. Patients whose immune system is rendered less efficient by underlying disease such as cancer, leukemia or cystic fibrosis (this latter disease appears to involve a localized immune ineffectiveness), by massive bodily lesions including severe burns or wounds or by major therapeutic interventions including radiation therapy, are particularly at risk from infections by antibiotic-resistant, gram-negative bacteria. *Pseudomonas aeruginosa* has emerged in the past two decades as a prominent cause of such infections and is now recognized as one of the three major causes of death from gram-negative septicemia in North America [12]. Because the resistance of *P. aeruginosa* to antibiotics is generalized, that is not specific to a given class of antibiotics [4], and because the incidence of resistance transfer plasmids, encoding antibiotic degrading enzymes, is relatively low [4], it was proposed some years ago that the 'intrinsic' resistance of *P. aeruginosa* to antibiotics involves a low rate of antibiotic uptake [4] into the cell.

The cell envelopes of gram-negative bacteria consist of a cytoplasmic (inner) membrane, a layer of peptidoglycan and an outer membrane. Since no antibiotic is known with certainty to have its site of action in the outer membrane (in contrast many are known to act at sites interior to the outer membrane), all antibiotics must pass through the

outer membrane on the way to their targets. We started the work described below with the hypothesis that a low rate of antibiotic permeation across the outer membrane might explain intrinsic antibiotic resistance in *P. aeruginosa.*

It is now well established that the outer membrane of gram-negative bacteria acts as a molecular filter with a defined exclusion limit for hydrophilic substances [10]. This property results largely from the presence in the outer membrane of a class of proteins called porins, which form large water filled pores through the hydrophobic core of the outer membrane [10]. Porins constitute the major route of uptake for hydrophilic compounds, including most common antibiotics [10]. The work described below strongly favors the hypothesis that the low permeability of the *P. aeruginosa* outer membrane results from a relatively low proportion (< 1%) of the major porin protein F molecules forming open functional channels in vivo.

Outer Membrane Permeability Measurements in Whole Cells

The low permeability of the *P. aeruginosa* outer membrane created major problems when we first attempted to obtain accurate measurements. We used the method of Zimmermann and Rossellet with modifications [9] to improve sensitivity (through the use of nitrocefin) and to decrease the problems of β-lactamase leakage (by using a dual beam spectrophotometric method [9]). This latter point was critical since the leakage of even 1% of the periplasmic β-lactamase out of the cell caused major errors in our measurements.

Nitrocefin is a chromogenic β-lactam which undergoes a color change when hydrolysed by β-lactamase. When the β-lactamase is free in solution, only the concentration of nitrocefin and the kinetic parameters of the enzyme determine the rate of conversion of nitrocefin to nitrocefoic acid. However, in intact cells, when the β-lactamase is largely periplasmic, the rate of hydrolysis at low to moderate nitrocefin concentrations is limited by the rate of diffusion across the semipermeable outer membrane, and thus provides a measure of outer membrane permeability [1, 9].

The results we obtained are shown in summary form in table I. The permeability of the *P. aeruginosa* outer membrane was 12-fold lower than the permeability of the *E. coli* outer membrane. When the respec-

Table I. Outer membrane permeability in vivo assessed by nitrocefin hydrolysis using the technique of Zimmermann and Rossellet

Strain	Outer membrane permeability[1]	Major porin protein (copies per cell)[2]
E. coli wild type	1.00	100,000
P. aeruginosa wild type	0.08	200,000
P. aeruginosa protein F-deficient mutant	0.01	no protein F

[1] Expressed as permeability coefficient relative to *E. coli* which is expressed as 1.00. The original data appeared in *Nicas and Hancock* [9].
[2] Data taken from *Nicas and Hancock* [9] and *Nikaido and Nakae* [10].

tive sizes of the porin pores (see below) are taken into account (using Fick's first law of diffusion and the Renkin correction for frictional and steric interactions between nitrocefin and the walls of the pore, as described previously [9]), the rate of permeation of nitrocefin can be accounted for by only 100–400 open functional protein F channels per cell being active in nitrocefin permeation. In contrast, *E. coli* is thought to have as many as 60,000 functional channels of its major outer membrane porin(s). The low permeability of the *P. aeruginosa* outer membrane was also confirmed by *Yoshimura and Nikaido* [11] using other types of measurements.

To conclusively demonstrate that porin protein F was responsible for nitrocefin permeation across the outer membrane, a mutant lacking protein F was isolated [9]. Classical techniques for the isolation of *E. coli* porin-deficient mutants did not work for *P. aeruginosa*, possibly due to the already low permeability of wild type *P. aeruginosa* membranes (table I). Therefore *P. aeruginosa* was mutagenized with NTG to 0.04% survival; we assumed these survivors would have many individual mutations. The cell envelopes of 500 survivors were screened by SDS polyacrylamide gel electrophoresis for the presence of protein F. One, strain H283, lacked protein F. This strain had a sixfold lower permeability to nitrocefin than wild type strain H103 demonstrating that protein F is the major outer membrane porin in *P. aeruginosa*. Since protein F is present in 100,000–300,000 copies per cell, and by the above calculations only 100–400 pores are active, then only about

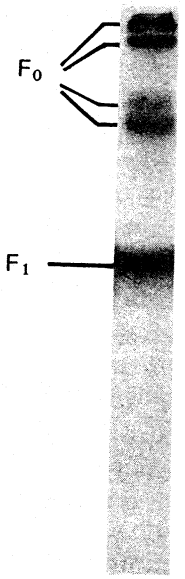

Fig. 1. Oligomer formation by protein F assessed using monoclonal antibody MA5-8 [8]. Purified protein F was separated by SDS polyacrylamide gel electrophoresis and then transferred to nitrocellulose by the Western blotting method. The blot was then interacted with protein F-specific monoclonal antibody MA5-8 and the position of binding of the antibody revealed as described in *Mutharia and Hancock* [8]. F_1 refers to the monomer form of protein F, F_o refers to the oligomer forms.

0.2% of the protein F in the outer membrane must form open functional channels.

Our major efforts now are directed to understanding why 99.8% of the protein F molecules are nonfunctional as entry ports for hydrophilic molecules. As a working hypothesis, we have proposed that the chemical nature of the lipopolysaccharide (LPS) molecules which interact with individual protein F trimers determines the number of open, functional channels, i.e. cellular LPS is heterogeneous and some LPS species favor open channels and some favor closed channels [1]. In support of this hypothesis we have demonstrated that protein F is strongly noncovalently associated with LPS [5, 6] and have characterized an antibiotic supersusceptible mutant Z61 with an LPS alteration which is associated with a higher rate of permeation of nitrocefin through the outer membrane [1]. However, our working hypothesis is far from proven.

Porin Protein F – Physicochemical Properties

Protein F is present as around 15% of the mass of *P. aeruginosa* outer membrane proteins. SDS polyacrylamide gel electrophoresis in the presence or absence of 2-mercaptoethanol has provided evidence that the monomer unit of the protein has two internal disulphides [5]. Protein F is noncovalently associated with both the LPS [5, 6] and the peptidoglycan [6] and in addition self-associates to form trimers as evidenced by chemical cross-linking studies [2]. Unlike the porins of many other bacteria [10], *P. aeruginosa* protein F does not readily form oligomers in the presence of SDS [5, 8]; however, recently we were able to demonstrate such oligomeric SDS-stable forms using a Western blot technique and a monoclonal antibody to protein F [8] (fig. 1). Protein F is a transmembrane protein (as required by logic for a membrane pore-forming protein) since it interacts with the peptidoglycan [6] on the periplasmic side of the outer membrane and with monoclonal antibodies on the cell surface [8].

Reconstitution of Porin Protein F in Model Membranes

Purified protein F forms large water filled channels through lipid bilayer membranes as demonstrated in three model membrane systems. The exclusion limit of the protein F channel is about 6,000 daltons for saccharides, equivalent to a channel diameter of around 2.3 nm [3]. In contrast, *E. coli* has an exclusion limit of 600 daltons and a channel diameter of around 1.3 nm [10] [n.b. this small difference in diameter gives rise to a three fold difference in channel area]. In agreement with this, *E. coli* grows only on peptides of sizes smaller than or equal to tetrapeptides [10], whereas *P. aeruginosa* will grow adequately on heptapeptides [*Miller,* personal commun.]. Model system studies have also confirmed the low activity of the porin protein F channels [3], as implied from in vivo experiments (see above).

Black lipid bilayer model studies [3] have demonstrated two important points. Firstly, they show that protein F is not voltage gated or regulated (i.e. it does not appear to open or shut due to a voltage differential across the membrane). Secondly, they demonstrate that protein F is not strongly selective for permeating molecules on the basis of either chemical differences or charge (it is only about 2.5-fold selective

Table II. Effect of different compounds on *P. aeruginosa* outer membrane permeability towards the β-lactam nitrocefin, the peptidoglycan-degrading enzyme lysozyme and the hydrophobic fluorescent dye NPN[1]

Permeabilizer compound	Outer membrane permeability		
	nitrocefin[2]	lysozyme[3]	NPN[4]
Control	1	0	0
EDTA	27	83	43
Tris	26	ND	ND
Polymyxin B	55	80	43
Gentamicin	57	78	43
Neomycin	63	22	43
Poly-*L*-ornithine	18	83	18
Poly-*L*-lysine	58	76	ND
Gramicidin S	34	24	23
Cetrimide	16	ND	41
Nitrolotriacetate	14	90	15
L-Ascorbate	32	4	2
Acetylsalicylate	26	8	4

ND = Not done.

[1] This table was adapted from data appearing in *Hancock and Wong* [7].

[2] Expressed as the relative hydrolysis of nitrocefin by the β-lactamase of cells either untreated or treated with various permeabilizer compounds.

[3] Measured as % lysis of cells by lysozyme (which normally is unable to pass across the outer membrane) 3 min after the addition of various compounds.

[4] Measured as increase in fluorescence (in arbitrary units) of NPN partitioning into cells 2 min after the addition of permeabilizer compounds.

for cations over anions) [3]. This would seem to be strong evidence against a selective permeation mechanism (i.e. letting in nutrients and excluding the antibiotics) for protein F.

Compounds which Permeabilize Outer Membranes – 'Permeabilizers'

As mentioned above, *P. aeruginosa* infections are extremely difficult to treat due to the organism's high intrinsic resistance to antibiotics which is primarily due to the low permeability of the *P. aeruginosa* outer membrane. One possible therapeutic strategy against *P. aeruginosa*

might be to attempt to reduce the effects of low outer membrane permeability by cotreatment of cells with a hydrophilic antibiotic and a compound which permeabilizes outer membranes. We started to screen for such compounds using three separate assays: (a) an increase in the rate of nitrocefin permeation; (b) promotion of lysozyme uptake, and (c) uptake of a hydrophobic fluorescent dye NPN [7]. A total of 18 compounds were able to dramatically increase outer membrane permeability in one or all of these assay systems (table II); gentamicin and seven other aminoglycosides [7; Loh, Grant, Hancock, submitted for publ.], Tris, polymyxin B, EDTA, nitrolotriacetate, poly-*L*-ornithine, poly-*L*-lysine, gramicidin S, cetrimide, ascorbate and acetylsalicylate [7]. Five of the compounds were previously shown to increase outer membrane permeability [reviewed in ref. 7]. Because of the wide variety of chemical types represented we have proposed that these compounds be given the group name 'permeabilizers'. Since evidence exists in the literature for synergy between permeabilizers and antibiotics [reviewed in ref. 7], it is my feeling that these or similar compounds may provide a new approach to antimicrobial therapy.

Summary

The intrinsic resistance of *P. aeruginosa* to most hydrophilic antibiotics can be explained, in part, on the basis of its low outer membrane permeability. Protein F which constitutes the major outer membrane porin protein for the uptake of hydrophilic compounds, is poorly functional. We have demonstrated that less than 1% of the 200,000 or so copies of protein F per cell can form active functional channels. Our working hypothesis is that the species of LPS associated with individual protein F trimers determines whether these trimers adopt a functional conformation.

Since low outer membrane permeability constitutes a major problem for the penetration of antibiotics into *P. aeruginosa*, we have started to search for compounds which permeabilize outer membranes ('permeabilizers') and thus would be potentially synergistic with antibiotics. Eighteen permeabilizer compounds have been discovered and fall into defined chemical groupings including polycations, organic cations and divalent cation chelators.

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