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The hydrophobic uptake pathway across the outer membrane of the antibiotic supersusceptible *Pseudomonas aeruginosa* mutant Z61

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1. SUMMARY

The *Pseudomonas aeruginosa* antibiotic supersusceptible mutant Z61 was 50–400-fold more susceptible than its wild-type parent K799 to 5 hydrophobic antibiotics. The strain Z61 outer membrane also demonstrated enhanced permeability towards a hydrophobic fluorescent probe. Strain Z61 cells had an altered cell surface, as revealed by phase-partitioning experiments, a lower amount of Lipid A phosphate, and a reduction in the number of Mg^{2+} binding sites in Lipid A, as demonstrated by dansyl polymyxin competition experiments. An antibiotic permation pathway directly through the outer membrane bilayer, rather than through porin proteins, is proposed for strain Z61.

2. INTRODUCTION

Three mechanisms have been described whereby antibiotics can pass across the outher membranes of Gram-negative bacteria, the hydrophilic pathway (mediated by porin proteins) [1,2], the hydrophobic pathway [2,3] and the self-promoted uptake pathway [1,4]. Most wild-type Gram-negative bacteria, with the exceptions of *Neisseria* and *Haemophilus* do not show a high degree of permeability to hydrophobic or amphiphilic molecules [2]. *P. aeruginosa* wild-type strains also show very limited hydrophobic uptake as judged by their resistance to hydrophobic antibiotics [4] and their inability to take hydrophobic fluorescent dyes [5]. In this paper we examined a multiple antibiotic supersusceptible mutant strain Z61 for an increase in permeability to hydrophobic compounds.

The mutant Z61 was isolated from the wild-type parent strain K799 by five mutagenesis steps followed by selection for supersusceptibility to cephalosporin C or gentamicin [6]. Penicillin binding proteins were shown to be unaltered in the mutant strain [7]. In contrast, mutant cells were more permeable to a chromogenic cephalosporin, nitrocefin, than cells of the wild-type parent [8].

Detailed characterization of lipopolysaccharide (LPS) by chemical and physical methods indicated that the antibiotic supersusceptible mutant Z61 had altered LPS compared to its parent strain

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K799 or single-step revertants [9]. In contrast, the porin protein F was apparently unaltered. Therefore, we hypothesized that the LPS of strain Z61 increased porin activity by passively affecting porin structure and consequently increasing the number of large, open channels in the strain Z61 outer membrane (as compared to its parent K799) [9]. However, more recently we have been unable to find substantial support for this hypothesis through model membrane studies of the function of porin protein F purified from strain Z61 and K799 (Darveau, R.P., and Hancock, R.E.W., unpublished observations). We demonstrate here a major defect in the barrier function of the strain Z61 outer membrane toward hydrophobic compounds, and an alteration in Mg^2 binding sites on Lipid A.

3. MATERIALS AND METHODS

Mutant Z61 and its parent K799 were described previously [6,8]. For determination of minimal inhibitory concentrations, overnight cultures, grown in 1% protease peptone No. 2 medium, were diluted 1/100 when 10 μl spots (approx. 2×10^5 cells) were applied to protease peptone No. 2 agar plates containing various concentrations of antibiotic. Minimal inhibitory concentrations were the lowest concentrations of antibiotic at which growth was inhibited. 1-*N*-phenylnaphthylamine (NPN) assays were performed using the methods of Loh et al. [5]. The phase partitioning of strains K799 and Z61 was done using the 2-phase polymer (dextran : polyethylene glycol) system of Magnusson et al. [10].

Lipid A was prepared from strain Z61 and K799 LPS (isolated by the method of Darveau and Hancock [12]) using sodium acetate buffer, pH 3.0, as described by Rietschel et al. [13] to minimize losses of labile phosphates. Lipid A precipitates were washed 3 times in deionized water to remove sodium acetate, lyophilized and then resuspended in deionized water at a fixed concentration. Both Lipid A samples were completely free of the LPS rough core sugar 2-keto-3-deoxyoctulosonic acid. The LPS and Lipid A samples were assayed for phosphate content by the method of Ames and Dubin [14]. Dansyl polymyxin binding and competitive displacement by Mg^{2+} were performed as described previously [11].

4. RESULTS

Previous results for antibiotic susceptibility [6,8] had shown that the mutant Z61 was 2.5- to 10 000-fold more susceptible than its wild-type parent K799 to a wide variety of hydrophilic antibiotics. In this study we tested susceptibility to a group of antibiotics which were suspected to cross the outer membrane through the LPS/phospholipid bilayer [3], including novobiocin, nalidixin acid, rifampicin, trimethoprim and the dye crystal violet.

It was confirmed that mutant Z61 had enhanced susceptibility to both carbenicillin and gentamicin (Table 1), as previously described [8]. Between 50- and 400-fold greater susceptibility was also observed, in strain Z61, for all 5 hydrophobic antibacterial agents tested (Table 1). The use of crystal violet as an indicator of outer membrane permeability to hydrophobic compounds has been described previously for *S. typhimurium* [11]. Mutant Z61 was 100-fold more susceptible to this agent than was its wild-type parent, K799. These data indicated that the outer membrane of mutant strain Z61 was considerably more permeable to hydrophobic compounds than that of its parent K799.

Permeability though the LPS-phospholipid bilayer (hydrophobic outer membrane permeability) does not occur readily in wild-type Gram-negative bacterial cells which possess smooth LPS [3]. However, since the antibiotic supersusceptible mutant Z61 was shown to be quite susceptible to hydrophobic agents (Table 1), this permeation pathway was examined using the hydrophobic fluorescent probe, NPN. This probe, which is normally unable to penetrate wild-type cells, fluoresces weakly in an aqueous environment but strongly in a hydrophobic environment, such as the interior of a membrane, and thus can be used to assess hydrophobic permeability [5]. As seen in Fig. 1, NPN reacted only weakly with wild-type K799 cells, but demonstrated nearly 6-fold-enhanced

Table 1

Minimal inhibitory concentrations of (MIC) selected antibiotics for strain K799 and its antibiotic supersusceptible derivative Z61

Strain	MIC (μg/ml)						
	Carbenicillin	Gentamicin	Trimethoprim	Crystal Violet	Nalidixic acid	Novobiocin	Rifampicin
K799	20	5	100	200	50	20	50
Z61	0.25	0.05	2	2	0.5	0.05	0.2

fluorescence emission intensity when added to mutant Z61 cells. NPN uptake by strain Z61 was shown to be significantly ($P < 0.02$ by Student's t test) greater than that by strain K799. The outer membrane of strain Z61 therefore appeared to present a lower barrier to hydrophobic substances in the environment, as judged by uptake of the hydrophobic probe and the correlated supersusceptibility to hydrophobic substances.

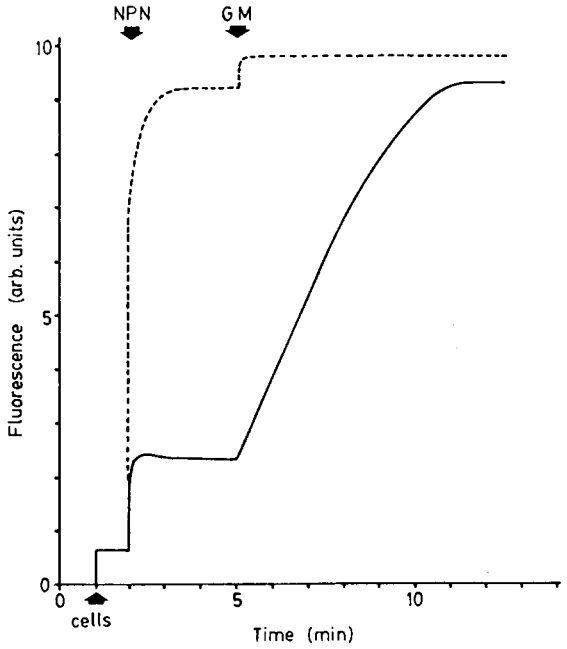

Fig. 1. NPN uptake by strains K799 and Z61 with or without gentamicin treatment. Cells were suspended to a final absorbance at 600 nm of 0.5. NPN was added at the indicated time to give a final concentration of 10 μM, then fluorescence emission intensity was measured at 420 nm. Gentamicin was added, at the time indicated, to give a final concentration of 4 μg/ml. These data represent chart tracings from a single, representative experiment. NPN, 1-*N*-phenylnaphthylamine; ———, K799; - - - - - -, Z61.

It has been previously demonstrated that a variety of compounds including gentamicin and EDTA, interacted at Mg^{2+}-binding sites on the cell surface, displacing Mg^{2+} and thereby disorganising the outer membrane to allow uptake of the hydrophobic probe, NPN. The rate of NPN uptake was found to be kinetically dependent upon the concentration of the interacting compound (called a permeabiliser), however, the final extent of NPN uptake after permeabilisation was nearly identical, regardless of the concentration of permeabiliser [5]. In agreement with these results, addition of 4 μg/ml gentamicin to wild-type strain K799 caused an increase in NPN uptake to approx. 9 arbitrary units, similar to the value obtained for untreated strain Z61 (Fig. 1). In contrast, similar treatment of strain Z61 resulted in only a small increase in NPN fluorescence. This suggested that Z61 demonstrated maximum possible uptake of NPN in the absence of permeabilisers.

The NPN uptake experimenta suggested potential alterations to the Mg^{2+}-binding sites of strain Z61. Therefore, we investigated these sites by dansyl polymyxin titration and Mg^{2+} competition experiments. Previous data indicated that dansyl polymyxin binds to whole cells, purified LPS or the Lipid A portion of LPS at polymyxin-binding sites, a subset of which sites also bind divalent cations with a reasonably high affinity, as revealed by competitive displacement of dansyl polymyxin by Mg^{2+} [11]. Since LPS-bound dansyl polymyxin is highly fluorescent but unbound dansyl polymyxin is weakly fluorescent these titrations can be simply followed by fluorescence spectrophotometry. In the experiments reported below Lipid A was used rather than LPS since the reduced num-

ber of dansyl polymyxin-binding sites (2 rather than 4.4; see [11]) allowed easier visualization of the Z61 defect.

Titration of dansyl polymyxin binding to the Lipid A of strains Z61 and K799 revealed similar binding constants ($S_{0.5}$ approx. 0.64 μM) but an 18% decrease in the number of dansyl polymyxin-binding sites (Fig. 2). Despite this difference, addition of Mg^{2+} resulted in competitive displacement of dansyl polymyxin until the same level of dansyl polymyxin remained bound (Fig. 1). This was confirmed by measuring the unbound dansyl polymyxin in the supernatant by addition of excess LPS as previously described [11]. These data can be explained if the reduction in dansyl polymyxin binding to strain Z61 Lipid A occurred preferentially at the Mg^{2+}-binding sites of Lipid A. Thus one can estimate a reduction of approx. 25% in the Mg^{2+}-binding sites of strain Z61 compared to strain K799 Lipid A. While this is less than a stoichiometric loss, it could be explained if the phosphates of the *P. aeruginosa* Lipid A demonstrate heterogeneity, as observed in preliminary ^{31}P-NMR experiments (unpublished results).

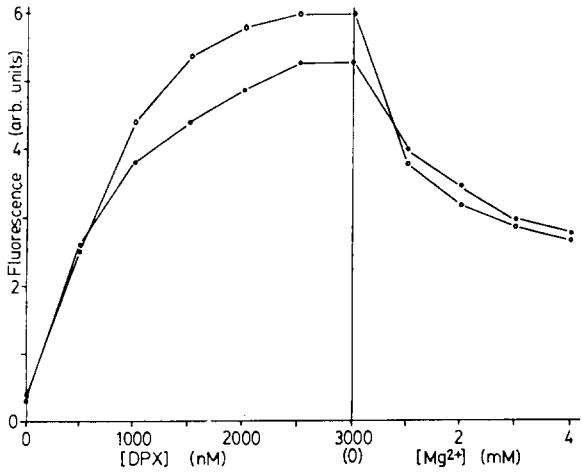

Fig. 2. Binding of dansyl polymyxin to Lipid A and competitive displacement by Mg^{2+}. Binding of dansyl polymyxin to Lipid A was measured as the increase in fluorescence emission at 485 nm. Mg^{2+} was added to dansyl polymyxin-saturated lipid A at the indicated concentrations, and competition for dansyl polymyxin-binding sites was measured by loss of fluorescence emission intensity. Three independent trials were performed with similar results. A representative experiment is shown. ○, K799; ●, Z61.

Table 2

Phase partitioning of strains K799 and Z61

Strain	Ratio of cells in upper phase/lower phase [a]
K799	0.175
Z61	2.80

[a] Ratios of bacteria in the different phases of the 2-phase (polyethylene glycol as the upper phase and dextran as the lower phase) partitioning system utilized [10], are present as the mean of 3 separate trials. Partitioning of mutant strain Z61 cells into the upper (polyethylene glycol) phase was found to be significantly different from partitioning of K799 cells by Student's t test ($P < 0.05$).

Strain Z61 had 21% less phosphate in its LPS and 38% less phosphate in its Lipid A than the wild-type strain K799. These LPS phosphate estimates agreed with those from independent experiments performed by Dr. E. McGroarty (personal communication) by inductively coupled plasma emission spectroscopy. These data suggest that the reduction in phosphate content occurred preferentially in the Lipid A phosphates.

The surface properties of the mutant strain Z61 were examined by partitioning in an aqueous, 2-phase polymer system. Mutant strain Z61 (Table 2) separated preferentially into the uncharged, polyethyleneglycol-rich top phase, whereas the wild-type strain K799 partitioned into the somewhat negatively charged dextran-rich bottom phase. These results indicated that the mutant cell surface was altered in its presentation to the environment, although the exact nature of the alteration could not be determined by this method since this system separates cells on the bases of both charge and hydrophobicity [10].

5. DISCUSSION

The results presented here suggest that the antibiotic supersusceptible mutant Z61 is also supersusceptible to hydrophobic antibiotics, demonstrates loss of its outer membrane permeability barrier to the hydrophobic fluorophor NPN, and

has an altered Lipid A and cell surface. Since we have been unable to confirm that mutant Z61 is altered in its porin pathway, it is possible that the increased presence of a hydrophobic uptake pathway somehow makes the outer membrane more permeable to β-lactams, aminoglycosides and other hydrophilic antibiotics as well as to hydrophobic antibiotics. Previous results have demonstrated that strain Z61 LPS has a reduced affinity and binding capacity for polycations, suggesting an alteration in Mg^{2+} binding sites [15]. We confirm here that strain Z61 has a reduction in Mg^{2+}-binding sites associated with the Lipid A portion of its LPS. This is probably related to a reduction in assayable Lipid A phosphates. It is known from experiments similar to those shown in Fig. 1 (in which gentamicin was used as a permeabiliser) that divalent cation crossbridging of adjacent LPS molecules is important for maintaining the barrier properties of the *P. aeruginosa* outer membrane to NPN [5] as well as to the β-lactam nitrocefin [1]. Therefore, it is our working hypothesis that the primary defect at the cell surface of mutant Z61, resulting in enhanced uptake of antibiotics across the outer membrane, is a decrease in the number of membrane-stabilising, divalent cation-binding sites at the cell surface.

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