Use of Steroids To Monitor Alterations in the Outer Membrane of Pseudomonas aeruginosa

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Testosterone (a strongly hydrophobic steroid) and testosterone hemisuccinate (a negatively charged derivative) were used as probes to investigate alterations in the outer membrane of Pseudomonas aeruginosa. Diffusion rates of the steroids across the lipid bilayer were measured by coupling the influx of these compounds to their subsequent oxidation by an intracellular Δ1-dehydrogenase enzyme. Wild-type cells of P. aeruginosa (strain PAO1) were found to be 25 times more permeable to testosterone than to testosterone hemisuccinate. The uptake of the latter compound appeared to be partially dependent on the external pH, thus suggesting a preferential diffusion of the uncharged protonated form across the cell envelope. Using various PAO mutants, we showed that the permeation of steroids was not affected by overexpression of active efflux systems but was increased up to 5.5-fold when the outer membrane contained defective lipopolysaccharides or lacked the major porin OprF. Such alterations in the hydrophobic uptake pathway were not, however, associated with an enhanced permeability of the mutants to the small hydrophilic molecule N,N,N',N'-tetramethyl-p-phenylenediamine. Thirty-six agents were also assayed for their ability to damage the cell surface of strain PAO1, using testosterone as a probe. Polymyxins, rBPI, chlorhexidine, and dibromopropamidine demonstrated the strongest permeabilizing activities on a molar basis in the presence of 1 mM MgCl2. These amphiphilic polycations increased the transmembrane diffusion of testosterone up to 50-fold and sensitized the PAO1 cells to hydrophobic antibiotics. All together, these data indicated that the steroid uptake assay provides a direct and accurate measurement of the hydrophobic uptake pathway in P. aeruginosa.

Over the past decades, there have been considerable advances in the understanding of the structural and functional properties of porins, which selectively allow the passage of small hydrophilic solutes across the outer membranes of gram-negative bacteria. By contrast, the contribution of the lipid regions of the bilayers as an alternative uptake pathway for molecules showing some degree of lipophilicity or cationicity is still poorly understood. In Salmonella typhimurium and probably many other gram-negative bacilli, the outer membrane exhibits an asymmetric architecture in which an outer leaflet consisting almost entirely of lipopolysaccharides (LPS) is intimately associated with an inner leaflet of glycerophospholipids (35). Because of the extremely low fluidity of the LPS monolayer, the outer membrane is poorly permeable to moderately hydrophobic solutes that would normally partition into the interior of classical phospholipid bilayers (41, 51). Diffusion through the membrane lipids thus appears to be restricted to very hydrophobic molecular species (41) or compounds able to alter the membrane continuum by chelating or displacing the very hydrophobic molecular species (41) or compounds able to alter the membrane continuum by chelating or displacing the

action of the polycation deacetylpolymyxin B (DAPB). In the present study, we used steroids as probes to examine the effects of various mutations and agents on the outer membrane integrity of Pseudomonas aeruginosa.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains used in this study are listed in Table 1. Construction of plasmid pLE689, a derivative of the expression vector pNM185 containing the 3-oxosteroid Δ1-dehydrogenase gene from Comamonas testosteroni, was reported previously (41). This vector, which carries the xylS gene and the pm promoter of the TOI plasmid (27), enables regulated expression of the cloned gene by using m-toluate as an inducer. Another recombinant plasmid, pJR395, containing the Δ1-dehydrogenase gene was constructed by recloning a 2.2-kbp BamHI-SacI fragment from the pUC-derived plasmid pTEK22 (40), downstream of the tac promoter of the broad-host-range expression vector pMMB207 (28). Plasmid pLP5188 expresses the xylC gene that encodes phosphohamnn/glucosaminate, an enzyme required for complete LPS core synthesis and that complements the LPS defect of rough strain AK1012 (6). The P. aeruginosa strains were transformed alternatively with plasmids pLE689 and pJR395, depending on their susceptibility levels to kanamycin and chloramphenicol, the selection markers of the two vectors, respectively. Transformation was performed by electroporation according to the protocol of Smith and Iglewski (48). Transformants harboring plasmid pLE689 were isolated on Muller-Hinton (MH) (Sanofi Pasteur, Paris, France) agar plates containing kanamycin at a final concentration of 125 μg ml−1 (strain PAZ2), 250 μg ml−1 (strains PAOI, AK401, AK1012, PAO222, PAZ1, H636, and PAO-7H), or 400 μg ml−1 (strain ERYR). Chloramphenicol was added to MH agar plates at 250 μg ml−1 (PAOI) or 400 μg ml−1 (4098, 4098E, and 408ST) for the selection of cells transformed with pJR395. When necessary, expression of the cloned Δ1-dehydrogenase gene was induced by adding 0.5 mM m-toluate or 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the bacterial cultures in MH broth. All cultures were incubated at 30°C.

Permeability assays with steroids. The experimental protocol initially developed for measuring the Δ1-dehydrogenase activity expressed by intact gram-negative bacteria was modified as follows to yield more-reproducible data with P. aeruginosa (41). Strains of P. aeruginosa were grown exponentially at 30°C in 40 ml of MH broth supplemented with m-toluate and kanamycin (plasmid pLE689) or with IPTG and chloramphenicol (plasmid pJR395), until they reached an optical density at 650 nm (OD650) of 1 ± 0.1. The cells were then centrifuged at 25°C for 10 min at 3,000 × g, washed briefly with 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonate, Na+−) (pH 7.4) and resuspended into the

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same buffer to an OD

\[ \text{OD}_{600} = 3.0 \] of \(0.7 \pm 0.1 \text{ mg of protein mL}^{-1}\). Measurements of \(\Delta^{n}\)-dehydrogenase activity were performed at 300 \(\text{nm}\) with \(700 \mu\text{L}\) portions of bacterial suspension appropriately diluted in HEPES buffer. In some experiments, HEPES was replaced by \(66 \text{ mM}\) Sorbese buffer adjusted at various pHS. Initial assays (this work and reference 41) indicated that living cells of \(P. \text{aeruginosa}\) harboring pEB689 or pIR395 oxidized 3-oxosteroids linearly for at least 10 min when these substrates were used at external concentrations \(\left(2 \mu\text{M}\right)\) above the \(K_{m}\) values of \(\Delta^{n}\)-dehydrogenase. This allowed us to use the endogenous quantification of oxidized steroids as a convenient method to determine whole-cell \(\Delta^{n}\)-dehydrogenase kinetics.

Typically, the enzymatic reaction was initiated by the addition of \(10 \mu\text{L}\) of steroid in methanol to cell suspension. Testosterone was assayed at 12.5 and 25 \(\mu\text{M}\) (final concentrations), and testosterone hemisuccinate was assayed at 100 and 200 \(\mu\text{M}\). After 10 min of incubation, the bacteria were harvested in an Eppendorf centrifuge (15 s at 15,000 rpm), 500 \(\mu\text{L}\) of the supernatant was transferred to a new microtube and mixed to 1 volume of ethyl acetate–50 mM HCl. The upper organic phase containing the steroid (in oxidized and nonoxidized forms) was removed from the aqueous phase by centrifugation. This extraction step was repeated once to achieve a recovery of \(<95%\) of the initial amount of the steroid. Both organic fractions were pooled and desiccated at 55°C. The steroid mixture resulting from the enzymatic oxidation was dissolved in 500 \(\mu\text{L}\) of mobile phase consisting of methanol-water-acetonitrile-acetic acid (55:35:10:2 [vol/vol]) and submitted to high-pressure liquid chromatographic analysis through a reverse-phase column (µBondapack Phenyl; Waters) (300 by 3.9 mm [inner diameter]) at a flow rate of 1 ml min\(^{-1}\), with a UV detector set at 240 nm. The elution peaks corresponding to \(\Delta^{n}\)-testosterone and \(\Delta^{n}\)-testosterone hemisuccinate were integrated, and the resulting values were used to calculate the \(\Delta^{n}\)-dehydrogenase activities expressed by intact cells, in nanomoles minute\(^{-1}\) milligram \([\text{dry weight}]^{-1}\) \((I_{\text{cell}})\). Controls using cell supernatants were routinely performed to check that there was no significant leakage of \(\Delta^{n}\)-dehydrogenase; such leakage could lead to overestimating the outer membrane permeability. Experiments were done at least in duplicate for each concentration of steroid.

Permeabilization experiments. Minor changes were introduced into the above protocol to study the effects of 36 agents on the outer membrane permeability of strain PAO1. When not specified in the text, the cell suspension (initially adjusted to 3 \(\mu\text{L}\) of \(\text{OD}_{600}\) was diluted 1:300 in HEPES buffer and enzymatic measurements were performed with testosterone at a final concentration of 50 \(\mu\text{M}\). In some experiments, HEPES buffer was supplemented with 1 \(\text{mM}\) MgCl\(_2\). Agents examined for their potential cell surface-damaging activities were added to the bacterial suspension 1 min prior to the introduction of the steroid. Results used for the calculation of coefficient \(P\) were the means of at least three assays.

Permeability of intact cells to TMMP. \(\text{N}_{2}\text{N}_{2}\text{N}_{2}\text{N}_{2}\text{-tetramethylpiperidinediimine (TMMP)}\) is a small hydrophilic molecule of 164 Da, widely used as a chromogenic substrate for the characterization of organisms, such as \(P. \text{aeruginosa}\), that produce cytochrome \(c\) oxidase enzymes (49). Several pieces of evidence strongly suggest that enzymes of the heme-oxidase superfamily bind cytochrome \(c\) at a site located in the periplasmic carbohydrate domain of subunit II, a transmembrane peptide involved in the enzyme complex (11). In an attempt to measure the rate of penetration of TMMP across the outer membrane, we postulated that, in \(P. \text{aeruginosa}\), oxidation of this redox reagent by the cytochrome \(c\) oxidase complex (58) also occurs in the periplasm. We also assumed that the enzymatic oxidation of TMMP in \(P. \text{aeruginosa}\) cells follows Michaelis-Menten kinetics \((K_{m} = 720 \mu\text{M according to reference 58}) and is rate limited by the influx of the substrate through the bacterial outer membrane. In a typical experiment, 100 \(\mu\text{L}\) of freshly centrifuged cells resuspended in 100 \(\mu\text{L}\) HEPES (pH 7.4) \((\text{OD}_{600} \text{ of } 0.1)\) was introduced into a 1-cm light-path cuvette. The enzymatic reaction was initiated in the cell sample by the addition of TMMP dihydrochloride at final concentrations ranging from 50 to 800 \(\mu\text{M}\). Initial velocities of TMMP oxidation by the intact bacteria were measured spectrophotometrically at 600 nm by recording the appearance of Wurster’s blue, the oxidized form of TMMP. Enzyme kinetics were linear for at least 1 min and varied proportionally with TMMP concentration. All measurements were performed against a blank containing only TMMP in HEPES buffer to correct for the low rate of oxidation of the substrate by the oxygen in air. Specific enzymatic activities were calculated by using a molar absorption coefficient of \(9,000 \text{ M}^{-1} \text{ cm}^{-1}\) for Wurster’s blue (14). In all experiments, cell supernatants exhibited TMMP oxidase activities that were <<5% of that measured in the bacterial suspensions. This result indicated minimal leakage of cytochrome \(c\) oxidase.

Calculation of permeability coefficient \(P\). The rates of diffusion of steroids or TMMP through the outer membrane were calculated by the method developed for \(\beta\)-lactams by Zimmermann and Rosselet (59). As stated previously (41), it was postulated that steroids diffuse passively across bacterial membranes following Fick’s first law, and that at steady state, the net influx of these molecules equals the kinetics of their intracellular oxidation by the \(\Delta^{n}\)-dehydrogenase enzymes. This same hypothesis was made regarding TMMP and cytochrome \(c\) oxidase. Details for the calculation of the permeability coefficient \(P\) (expressed in nanometers second\(^{-1}\)) have been presented already (33, 41). Briefly, \(P\) was determined by using the equation \(P = (V_{\text{cell}}/60) \times A \times C_{\text{ext}}\), where \(V_{\text{cell}}\) expresses values of different concentrations \(C_{\text{ext}}\) of steroids or TMMP.

Sustaining susceptibility. MICs of antibiotics were determined by the twofold microbroth dilution method according to the recommendations of the National Committee for Clinical Laboratory Standards (2). Synergistic interactions between antibiotics and permeabilizers were studied by the microbroth checkerboard technique; results were expressed as fractional inhibitory concentration (FIC) indices. The minimum interaction index was defined as synergistic if \(\leq 0.5\), additive if \(>0.5\) and indifferent if \(>1\).

Chemical and biological products. Chemicals were purchased from Sigma (St. Quentin, France) with the exception of polymyxin B nonapeptide (PMBN) obtained from Boehringer Mannheim (Meylan, France). The following products were kindly provided by the manufacturers: amiloride, imipenem, and norfloxa- cin (Merck, Sharp & Dohme-Chibret); amikacin and kanamycin (Bristol-Myers Squibb); azithromycin (Pfizer); cefazidime (Glaxo-Welchome); ciprofloxacin (Bayer Pharma); colimycin (Roger Bellon); chloroquine and dibromopropami- dine isethionate (Specia, Rhone-Poulenc, Rorer); erythromycin (Abbott); ethyl- nalamine fusidate (Lai); rifabutin (Pharmacia & Upjohn), tetracycline hydro- chloride (Roussel & Diamant); trimethoprim (Roche); and vancomycin (Eli Lilly). DAPB was a gift from M. Vaara, Helsinki, Finland. N-terminal fragments of the recombinant bacterial/permeability-increasing protein (RIP) and XMP30 were kindly provided by Xoma Corporation (Berkeley, Calif.). Enzymes used in molecular biology experiments were purchased from Boehringer Mannheim, Human defensin HNP-2 was of high-pressure liquid chromatography grade (Sigma).

### Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristic(s)</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli RR1</strong></td>
<td>K-12 derivative with wild-type antibiotic susceptibilities</td>
<td>45</td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td>Wild-type strain</td>
<td>B. W. Holloway</td>
</tr>
<tr>
<td>PAO1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO222</td>
<td>met-28 trp-b lysA-12 his-4 ilv-26 pro-82</td>
<td>15</td>
</tr>
<tr>
<td>AK1401</td>
<td>Rough derivative of PAO1 lacking the A- and B-band LPS</td>
<td>5</td>
</tr>
<tr>
<td>AK1012</td>
<td>Rough derivative of PAO1 lacking the A- and B-band LPS</td>
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</tr>
<tr>
<td>PZA1</td>
<td>PZA222 derivative containing the absA mutation from the susceptibility strain Z61</td>
<td>1</td>
</tr>
<tr>
<td>PZA3</td>
<td>PZA222 derivative containing the absB mutation from the susceptibility strain Z61</td>
<td>1</td>
</tr>
<tr>
<td>H636</td>
<td>OprM-deficient Ω insertion derivative of PAO1</td>
<td>57</td>
</tr>
<tr>
<td>ERYR</td>
<td>Multidrug-resistant mutant of PAO1 overproducing the MexC-MexD-OprN efflux system</td>
<td>16</td>
</tr>
<tr>
<td>4098</td>
<td>FP met-9020 pro-9024 blaP208; PAO1 derivative producing a low, noninducible level of β-lactamase</td>
<td>24</td>
</tr>
<tr>
<td>4098E</td>
<td>Multidrug-resistant mutant of 4098 overexpressing the MexA-MexB-OpmR efflux system</td>
<td>24</td>
</tr>
<tr>
<td>4098T</td>
<td>OprM-deficient Ω-Hg insertion mutant of 4098</td>
<td>16</td>
</tr>
<tr>
<td>PAO-7H</td>
<td>Multidrug-resistant mutant of PAO1 overexpressing the MexE-MexF-OprN efflux system</td>
<td>21</td>
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</table>

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ALTERATIONS OF *PSEUDOMONAS AERUGINOSA* OUTER MEMBRANE

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### RESULTS

**Outer membrane permeability of strain PAO1 to steroids.**

In a previous study, we demonstrated that steroids diffuse passively across the outer membrane of gram-negative bacteria via a nonsaturable pathway that presumably involves the lipidic part of the bilayer (41). Permeability of the cell envelope to these very lipophilic solutes was expressed as a coefficient, $P$ (in nanometers second$^{-1}$), calculated from equations based on Fick’s first law of diffusion. Results presented in Table 2 confirm our preliminary data with *P. aeruginosa* (41), showing that wild-type cells are relatively permeable to the highly lipophilic, neutral, steroid testosterone ($P = 100$ to $130$ nm s$^{-1}$) but are much more resistant to the penetration of its amphiphilic, negatively charged derivative testosterone hemisuccinate ($4$ nm s$^{-1}$). The outer membrane permeability of *Escherichia coli* RR1, measured under the same conditions, was comparable to that of strain PAO1 (testosterone, $80$ nm s$^{-1}$; testosterone hemisuccinate, $2$ nm s$^{-1}$).

It has been suggested that amphiphilic solutes cross biological membranes mostly as uncharged protonated forms (34). To see whether this applies to testosterone hemisuccinate, we performed permeability experiments with PAO1 cells resuspended in phosphate buffer at various pHs. The transmembrane diffusion rate of this probe was clearly dependent on the pH of the medium, with a fourfold increase of coefficient $P$ from pH 8 to pH 5. The influx of testosterone remained unchanged over the range of pHs.

**Penetration of steroids in cells overexpressing active efflux systems.** Because of the high hydrophobicity of steroids, one could assume that these compounds are pumped out by one of the active efflux systems known to be involved in the export of lipophilic antibiotics in *P. aeruginosa* (10, 25, 42). Such an efflux of the probes would make them unsuitable for accurate permeability measurements. Actually, we found no difference in the $P$ values for both steroids between mutants overexpressing the MexA-MexB-OprM (strain 4098E), MexC-MexD-OprF (strain ERYR), or MexE-MexF-OprN (strain PAO-7H) system and parental strains PAO1 and 4098 (Table 2). These data strongly suggest that, under the experimental conditions used, testosterone and testosterone hemisuccinate are not actively transported by the above efflux machineries. The pumps indeed failed to prevent the steroid molecules from reaching the Δ1-dehydrogenase enzyme bound to the cytoplasmic membrane (40). This interpretation is supported by the fact that deenergization of the inner membrane of PAO1, ERYR, and 4098E cells by uncouplers like carbonyl cyanide m-chlorophenylhydrazine and dinitrophenol added to the cells at a final concentration of $1$ mM had virtually no effect on the values of coefficient $P$ (data not presented).

**Outer membrane mutants of *P. aeruginosa*.** Strains of *S. typhimurium* (41) and *E. coli* (unpublished results) producing deep rough LPS are known to be hypersusceptible to a wide range of hydrophobic inhibitors and to have bilayer regions highly permeable to steroids. To determine whether some alterations in the bilayer of *P. aeruginosa* produce similar effects, we measured the permeation rates of testosterone and testosterone hemisuccinate across the outer membranes of a series of mutants and their parents (Table 2). Expression of LPS defective in the outer core region (strain AK1012) or displaying altered electrophoretic profiles (strain PAZ1) rendered the outer membrane up to 5.5-fold more permeable to both probes and was associated with an increased susceptibility (8 to 64 times) of the mutants to hydrophobic antibiotics, such as nalidixic acid, chloramphenicol, trimethoprim, and erythromycin (data not shown). Lack of the major porin OprF, a protein known to stabilize the lipid bilayer (13), also enhanced the penetration of steroids but had no impact on the cell sensitivity to the above antibiotics (strain H636).

It could be argued that the higher membrane permeability seen in the mutants is not restricted to hydrophobic solutes and that steroids specifically monitored gross changes in the permeability of *P. aeruginosa*. Strain PAZ1 is indeed hypersusceptible to both hydrophilic and hydrophobic antibiotics (1). We used TMPD, a molecule of $164$ Da, as an indicator to assess the uptake of small hydrophilic solutes into the PAO mutants. Preliminary experiments showed that TMPD diffuses passively and rapidly through the envelope of strain PAO1 following a nonsaturatable uptake pathway (at least up to 800 nm). Careful measurements demonstrated that the mutants listed in Table 2 are not more permeable to TMPD than their parents. Interestingly, the OprF-deficient strain H636 exhibited no reduced TMPD uptake, a result concordant with data obtained by other investigators with monosaccharides (molecular mass, $<200$ Da), using intact H636 cells (4) or proteoliposomes (13, 32).

**Permeabilization studies with polymyxins.** Polymyxins are polycationic antibiotics with well-known outer membrane permeability-increasing properties (50). Using the steroid permeability assay, we examined the cell surface activities of several of these agents including colimycin (polymyxin E), polymyxin B, DAPB (a polymyxin B derivative lacking the fatty acid moiety of the parent compound), and PMBN in which an additional α,γ-diaminobutyric acid residue has been removed from the delipidated side chain. Viljanen and coworkers have shown that DAPB and PMBN have lost part of the bactericidal activity of polymyxin B but have retained the cation-displacing capabilities of the original molecule, responsible for the outer membrane-disrupting action (52, 53). As demonstrated by the enhanced permeation of testosterone through the bilayer,
polymyxin B permeabilized PAO1 cells at concentrations as low as 0.05 μM (Fig. 1). At higher concentrations, the diffusion rates of the steroid across the lipid bilayer increased dramatically, nearly up to 40-fold, to reach a maximum level at 1 μM, and then paradoxically tended to decrease. This apparent decrease at lethal concentrations of polymyxin B was attributed to perturbations of the inner membrane and subsequent inhibition of Δ1-dehydrogenase enzyme. The effect on the outer membrane was relatively insensitive to competitive inhibition by divalent cations (Mg2+ [1 mM]). Similar results were obtained with colimycin (data not presented). Though slightly less efficient than the parent polymyxin B, delipidated derivatives DAPB and PMBN were also potent permeabilizers of the cell envelope. However, their action was substantially antagonized by divalent cations at 1 mM, thus giving indirect evidence for the role of the hydrophobic tail of polymyxin B in the interaction with the bacterial surface (29). In PMBN, loss of a positive charge able to interact with the negative charges of the LPS cell envelope. However, their action was substantially antagonized by divalent cations (Mg2+ [1 mM], this compound partially inhibited the membrane-damaging effects of chlorpromazine (net charge of +2) (22), human defensin HNP-2 (+3), and amikacin (+5) (54). Finally, the cation chelators EDTA (18) and tripolyphosphate, as well as the dications chloroquine (22), tetracaine (22), and sulfadiazine (43), showed extremely low permeabilizing activities compared with polymyxins.

To evaluate the effects of polycations on the penetration of hydrophobic molecules, we measured the permeability of PAO1 cells to testosterone of PAO1 cells treated with 1 mM permeabilizer and untreated cells (100 nm s⁻¹). Calculations were made by extrapolation of the P values obtained at optimal permeabilizing concentrations. As depicted in Fig. 2, agents able to perturb the cell surface of P. aeruginosa showed considerable differences, up to 5 orders of magnitude, in their respective intrinsic activities (nonpermeabilizers are listed below).

Recombinant peptide rBPI13a, a polycationic 23-kDa aminoterminal fragment from the bactericidal/permeability-increasing protein (BPI) present in the granules of human polymorphonuclear neutrophils (12), demonstrated a potent activity, close to that of polymyxin B. This result agrees with the fact that BPI13a, like the holom-BPI protein, binds avidly to the lipid A region of LPS and efficiently kills cells of P. aeruginosa at very low concentrations (12, 56). A small bactericidal 3-kDa fragment of BPI, XMP.30 (25a), was also remarkable for its strong outer membrane destabilizing activity. Two polycationic antiseptics, chlorhexidine and dibromopropamidine (44), required higher concentrations to alter the cell surface but retained some activity in the presence of 1 mM Mg2+. In contrast, physiological concentrations of divalent cations completely abolished the membrane-damaging effects of chlorpromazine (net charge of +2) (22), human defensin HNP-2 (+3), and amikacin (+5) (54). Finally, the cation chelators EDTA (18) and tripolyphosphate, as well as the dications chloroquine (22), tetracaine (22), and sulfadiazine (43), showed extremely low permeabilizing activities compared with polymyxins.

The following agents, some of which have been suspected to permeabilize the outer membrane of gram-negative bacteria, were screened for potential cell surface activity in the absence of competing cations: acetylsalicylate (18, 37), amiloride (20), ascorbate (18), azithromycin (9), cefuzadime, etrimide (18), ciprofloxacin, crystal violet, erythromycin, fosfomycin, imipenem, nalidixic acid, p-amino-benzoate (43), pefloxacin, rifabutin, rifampin, sodium azide, tetracycline, trimethoprim, and vancomycin (8). None of these agents exhibited a noticeable action on the permeability of PAO1 cells to testosterone when assayed at 100 μM (final concentration) (P ≤ 130 nm s⁻¹).

Sensitization to hydrophobic antibiotics. P. aeruginosa PAO1 is highly resistant to antibiotics that, because of their moderate lipophilicity, cannot efficiently access either the porin pathway or the LPS uptake pathway. These antibiotics include novobiocin (MIC, 512 mg liter⁻¹), erythromycin (256 mg liter⁻¹) and fusidic acid (512 mg liter⁻¹). Sensitization of PAO1 cells to these compounds by the permeabilizers was assayed by the checkerboard, twofold dilution technique. All the polycationic

![Permeabilizing actions of other compounds](http://jb.asm.org/ on August 28, 2017 by guest)

**FIG. 2.** Comparison of permeabilizers on a molar basis. The permeabilization coefficient used to compare the agents corresponds to the ratio between the outer membrane permeability to testosterone of PAO1 cells treated with 1 mM product and the permeability of untreated cells (100 nm s⁻¹). Values for permeabilized cells were extrapolated from data obtained with optimal concentrations of permeabilizer in the presence or absence (●) of 1 mM Mg2+. DBP, dibromopropamidine.
Table 3. Sensitization of P. aeruginosa PAO1 to hydrophilic antibiotics

<table>
<thead>
<tr>
<th>Permeabilizer (MIC [mg liter⁻¹])</th>
<th>FIC index*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Novobiocin</td>
</tr>
<tr>
<td>Polymyxin B (0.125)</td>
<td>0.375</td>
</tr>
<tr>
<td>DAPB (1.25)</td>
<td>0.31</td>
</tr>
<tr>
<td>Chlorhexidine (128)</td>
<td>0.25</td>
</tr>
<tr>
<td>Dibromopropamidine (256)</td>
<td>0.125</td>
</tr>
<tr>
<td>Chlorpromazine (&gt;128)³</td>
<td>1</td>
</tr>
<tr>
<td>Amikacin (2)</td>
<td>0.625</td>
</tr>
<tr>
<td>Tripolyphosphate (&gt;1,024)⁴</td>
<td>1</td>
</tr>
<tr>
<td>Tetracaine (&gt;1,024)⁴</td>
<td>0.125</td>
</tr>
</tbody>
</table>

* Data in bold type indicate synergism (FIC ≤ 0.5).
³ Synergism was assayed at the highest concentration of the compound achievable in MH broth before precipitation.
⁴ Data for these agents tested, except amikacin, potentiates the antibacterial activities of novobiocin, erythromycin, and fusidic acid to a significant extent (FIC ≤ 0.5) (Table 3). Other compounds had only limited effects, if any, on the susceptibility levels of PAO1 to these antibiotics. Interestingly, tetracaine demonstrated strong synergism with novobiocin, a property already noted for some other local anesthetics (22, 37). These results confirm that efficient permeabilizers may produce sufficient damage to the outer membrane bilayer to increase strongly the penetration of lipophilic antibiotics via the LPS uptake route (36, 53). By contrast, polymyxin B and DAPB failed to sensitize strain PAO1 to the hydrophilic antibiotics ceftazidime and ciprofloxacin (data not shown).

Discussion

We previously demonstrated that the permeability of entero-bacterial outer membranes to steroids can be accurately measured by coupling the influx of these compounds to their subsequent oxidation by the enzyme 3-oxosteroid Δ¹-dehydrogenase (41). In the present work, we have adapted this method to P. aeruginosa and developed a new assay for measuring the outer membrane permeability of the organism to the small hydrophilic molecule TMPD.

Our results confirm that the outer membrane of P. aeruginosa is relatively permeable to strongly lipophilic probes, such as testosterone, but forms a very efficient barrier against the penetration of amphiphilic derivatives, like testosterone hemisuccinate. The 25- to 50-fold-lower penetration rates of testosterone hemisuccinate can be explained if one considers that only the uncharged form of the steroid crosses the lipid bilayer to a significant extent, while the negatively charged species mostly remain excluded from the cells. This interpretation is supported by the fact that the P values for this probe vary in parallel with the extracellular pH, at least within certain limits. However, the relationship between the diffusion rates and the fraction of steroid existing in the protonated form is not proportional. Indeed, calculations show that, with a pKₐ of 6 for testosterone hemisuccinate, the concentration of the uncharged form is 3 orders of magnitude higher at pH 5 than at pH 8, whereas the value of coefficient P increases only four times. One explanation for this poor correlation might be that, at pH below 7, most of the protonated form is sequestered in mixed micelles with the salt form, as is the case with bile acids (these steroids also possess a negatively charged group on a side chain) (47). In agreement with this, titration curves of testosterone hemisuccinate obtained upon the addition of HCl displayed the same typical plateau as bile acids, due to the “buffering” effect of micelles (47) (data not presented).

Interestingly, P. aeruginosa exhibits the same outer membrane permeability to steroids that E. coli does, despite differences in the fatty acid composition of the hydrocarbon interior and in the phosphate content of the core LPS (31). This finding could suggest that these microorganisms have the same permeability to a wider range of lipophilic agents. Recently, evidence has been obtained showing that the elevated intrinsic resistance exhibited by P. aeruginosa toward a variety of hydrophobic inhibitors (e.g., erythromycin, trimethoprim, and nalidixic acid) is mostly due to the expression of the MexA-MexB-OprM efflux system (16). The outer membrane permeability barrier is insufficient by itself to produce significant levels of resistance to these agents.

The involvement of the lipid regions of the bilayer in the uptake of steroids in P. aeruginosa is supported by observations that show that defects in the LPS or chemically induced disorganization of the packing arrangement of the LPS outer leaflet notably increase the influx of the probes. Such alterations in the hydrophobic uptake pathway are seen in mutants lacking the distal core region of the LPS (strain AK1012) (7) or having undefined LPS alterations such as PAZ1 (1), but not in AK1401, a rough mutant producing a complete oligosaccharide core capped with a single O-antigen repeat unit (5). It should be stressed that extreme deletions in LPS, equivalent to those observed in deep rough mutants of S. typhimurium referred to as chemotypes Rd1, Rd2, and Re (30) (and that determine a very high outer membrane permeability to steroids) (41) have not been reported so far in P. aeruginosa.

Permeability assays performed with bacteria lacking the major porin OprF did not allow us to rule out some participation of the porin channels to the uptake of steroids in P. aeruginosa, since the pathway for small hydrophilic molecules, as assayed with TMPD, was not reduced in the OprF-deficient cells (see also references 4, 13, and 32). Increased amounts of LPS and phospholipids in the OprF-deficient outer membrane (13) may, in contrast, account for the higher permeability of the OprF mutants to steroids and the hydrophobic fluorophore N-phenylnaphthylamine (NPN) (57).

It has long been known that compounds able to displace or chelate the divalent cations Mg²⁺ and Ca²⁺ that stabilize adjacent LPS molecules at the surfaces of gram-negative bacteria may alter the outer membrane barrier (50). In P. aeruginosa, the permeabilization of the lipid bilayer was studied in assays involving either the crypticity of the periplasmic β-lactamase, the partition of NPN into the membrane interior, or the sensitization to noxious products normally excluded from the bacterial cells (hydrophobic antibiotics, anionic detergents, or lysozyme) (18, 26, 39, 53). However, these methods do not provide a direct and accurate measurement of outer membrane permeability. In addition, they may produce results difficult to interpret when performed under nonphysiological conditions. For example, NPN has been used in extremely dilute buffers (5 mM) with deenergized bacterial cells in order to prevent the efflux of the probe by active transport systems (18, 26). The test based on cell sensitization to lysozyme suffers from a lack of sensitivity (39, 50). Finally, synergistic antibiotic interactions may result from mechanisms other than facilitated entry of drugs across the bacterial outer membrane (e.g., tetracaine combined with novobiocin).

Permeability experiments with testosterone proved to be very sensitive to monitor and compare the outer membrane-damaging activities of a number of compounds, under conditions compatible with those met in body fluids (100 mM HEPES, 1 mM MgCl₂). The steroid specifically monitored
changes in the hydrophobic uptake pathway and not gross alterations in the cell envelope. To support this, we showed that PMBN, a potent permeabilizer that does not induce lesions in LPS-phospholipid bilayers (46), rendered the P. aerugi-
osa outer membrane more permeable to testosterone but not to small hydrophilic molecules such as TMPD. Several agents reported elsewhere to induce perturbations of the lipid bilayer were actually revealed to have no or very limited outer mem-
brane-stabilizing activity in P. aeruginosa (see “Permeabiliz-
ing actions of other compounds” above). In view of our results, it seems very unlikely that antibiotics such as amikacin and sulfadiazine possess an outer membrane-permeabilizing effect on P. aeruginosa in vivo because of the strong antagonism exerted by divalent cations at 1 mM. However, the electrostatic interaction between these products and LPS, as evidenced by the higher influx of testosterone in the absence of Mg$$^{2+}$$, could be sufficient to initiate the uptake of the antibiotic molecules through the lipid bilayer (26).

Polycations produced concentration-dependent damages to the outer membrane permeability barrier, leading to an in-
creased penetration of testosterone (up to 50-fold) and signif-
icant sensitization of P. aeruginosa to various hydrophobic antibiotics. At higher concentrations of permeabilizer, ulti-

mately the outer membrane became as permeable as the inner membrane (41). Our results conclusively show the greater abil-
ity of amphipathic polycations such as polymyxin B and rBPI23 to disrupt the outer membrane of P. aeruginosa. Such a high permeabilizing efficiency is correlated with the abilities of the compounds to bind avidly the cation-binding sites of LPS (38, 55) and to have hydrophobic interactions with the lipid A region of LPS (12, 29). Recent crystallographic studies on BPI have revealed that this boomerang-shaped polypeptide con-
tains two apolar pockets that each interact with the acyl chain of a molecule of phosphatidylcholine or LPS through hydro-
phobic interactions (3). As pointed out by Vaara (50) and clearly evidenced in this study, polycationicity is not the sole determinant required for efficient cell surface alteration. For example, DAPB and amikacin, two products that both carry net charges of +5 were found to differ in their intrinsic per-
meabilizing activities by a factor of 1,000 (in the absence of Mg$$^{2+}$$). This result indicates that the positive charges must have an appropriate spatial arrangement to allow an optimal binding of the permeabilizer molecule to LPS.

Although extensively studied, the mechanisms by which polycations interact with bacterial outer membranes still re-
main poorly understood at the molecular level. The growing interest for the defense peptides present in the leukocytes of mammals (e.g., BPI, lactoferrin, and defensins [23]), lymph of insects (cercopins), or amphibian skin (magainins) (17) should give new insights into the structural determinants necessary for outer membrane permeabilization and bacterial killing. Be-
cause it provides a direct and accurate measurement of outer membrane permeability, the model based on the oxidation of steroids by intact cells may be useful to assess the effect of natural or synthetic cationic agents on the cell surface of P. aeruginosa.

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