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

PATRICK PLESIAT,* JULIO RAMOS AIRES, COLETTE GODARD, AND THILO KÖHLER

Laboratoire de Bactériologie, Faculté de Médecine, 25030 Besançon, France, and Département de Génétique et Microbiologie, Centre Médical Universitaire, 1211 Genève 4, Switzerland

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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 OpfR. 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′-tetramethylp-phenylene diamine. Thirty-six agents were also assayed for their ability to damage the cell surface of strain PAO1, using testosterone as a probe. Polymyxins, rBPI23, chlorhexidine, and dibromopropamide 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 indicate 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 hydrophobic solutes that would normally partition into the hydrophobic layers of the membrane lipids (50). When the LPS layer was altered either by mutations or by 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 pLE89, 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 xylA gene and the pm promoters of the TOL 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 pTEXK22 (40), downstream of the ptc promoter of the broad-host-range expression vector pMMB207 (28). Plasmid pLP518 expresses the aldC gene that encodes phosphomanno/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 pLE89 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 pLE89 were isolated on Muller-Hinton (MH) (Sanofi Pasteur, Paris, France) agar plates containing kanamycin at a final concentration of 125 μg ml⁻¹ (strain PAZ3), 250 μg ml⁻¹ (strains PAO1, AK1401, 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 (PAO1) or 400 μg ml⁻¹ (4098, 4098E, and 4098T) 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-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 pLE89) 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 (pH 7.4) and resuspended into the
same buffer to an OD$_{600}$ of 3.0 (0.7 ± 0.1 mg of protein ml$^{-1}$). Measurements of Δ$^\Delta$-dehydrogenase activity were performed at 70°C with 700-μl portions of bacterial suspension appropriately diluted in HEPES buffer. In some experiments, HEPES was replaced by 66 mM Sørensen's buffer adjusted at various pHs. Initial assays (this work and reference 41) indicated that living cells of P. aeruginosa harboring plE689 or pIR359 oxidized 3-oxosteroids linearly for at least 10 min when these substrates were used at external concentrations (C$_{\text{ext}}$) above the $K_m$ values of Δ$^\Delta$-dehydrogenase. This allowed us to use the endogenous quantification of oxidized steroids as a convenient method to determine whole-cell Δ$^\Delta$-dehydrogenase kinetics.

Typically, the enzymatic reaction was initiated by the addition of 10 μl of steroid methylmalonic solution to the cell suspension. Testosterone was assayed at 12.5 and 25 μM (final concentrations), and testosterone hemisuccinate was assayed at 100 and 200 μM. After 10 min of incubation, the bacteria were harvested in an Eppendorf centrifuge (15 s at 15,000 rpm), 500 μl 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 $\geq 95\%$ 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 μl of mobile phase consisting of methanol-water-acetonitrile-acetic acid (55:35:10:1 [vol/vol]) and submitted to high-pressure liquid chromatographic analysis through a reversed-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 Δ$^\Delta$-testosterone and Δ$^\Delta$-testosterone hemisuccinate were integrated, and the resulting values were used to calculate the Δ$^\Delta$-dehydrogenase activities expressed by intact cells, in nanomoles minute$^{-1}$ (dry weight)$^{-1}$ (FIC) indices. The minimum interaction index was defined as synergistic if $C_{\text{eq}}$ varies, at least within certain limits. To check the validity of the model, we systematically measured $C_{\text{eq}}$ at two different external concentrations (C$_{\text{ext}}$) of steroids or TMPD.

**Susceptibility testing.** 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 FIC$\leq 0.5$, additive if $>0.5$, and indifferent if $\geq 1$.

**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); cefazidine (Glaxo-Welcombe); ciprofloxacin (Bayer Pharma); colistin (Roger Bellon); chloroquine and dibromopropamino- dine isethionate (Specia, Rhône-Poulenc, Rorer); erythromycin (Abbott); ethano- nolamine fusidate (Leo); rifabutin (Pharmacia & Upjohn), trimethoprim (Roche); and vancomycin (Eli Lilly). DAPB was a gift from M. Vaara, Helsinki, Finland. N- terminal fragments of the recombinant bactericidal/permeability-increasing protein rBPI23 and XMP.30 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 or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli RR1</td>
<td>K-12 derivative with wild-type antibiotic susceptibilities</td>
<td>45</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>Wild-type strain</td>
<td>B. W. Holloway</td>
</tr>
<tr>
<td>PAO222</td>
<td>met-28 trp-b lxa12 his-t ilv-26 pro-82</td>
<td>15</td>
</tr>
<tr>
<td>AK1401</td>
<td>Rough derivative of PA01 lacking the A- and B-band LPS</td>
<td>5</td>
</tr>
<tr>
<td>AK1012</td>
<td>Rough derivative of PA01 lacking the A- and B-band LPS</td>
<td>19</td>
</tr>
<tr>
<td>PAZ1</td>
<td>PAO222 derivative containing the $absA$ mutation from the suspercesusible strain Z61</td>
<td>1</td>
</tr>
<tr>
<td>PAZ3</td>
<td>PAO222 derivative containing the $absB$ mutation from the suspercesusible strain Z61</td>
<td>1</td>
</tr>
<tr>
<td>HP2</td>
<td>OprM-deficient Ω insertion derivative of PAO1</td>
<td>57</td>
</tr>
<tr>
<td>ERYR</td>
<td>Multidrug-resistant mutant of PA01 overproducing the MexA-MexB-OprM efflux system</td>
<td>16</td>
</tr>
<tr>
<td>4098</td>
<td>FP $^{\text{met-9020 pro-9024 bla} \text{P208; PA01}}$ 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-OprM 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 PA01 overexpressing the MexE-MexF-OprN efflux system</td>
<td>21</td>
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</table>
TABLE 2. Outer membrane permeability of various PAO1 mutants to steroids and TMPD

<table>
<thead>
<tr>
<th>Strain (plasmid)</th>
<th>Genotype or phenotype</th>
<th>Permeability coefficient P (nm s⁻¹)²</th>
<th>Testosterone</th>
<th>Testosterone hemisuccinate</th>
<th>TMPD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1(pLE689)</td>
<td>Wild type</td>
<td>100</td>
<td>4</td>
<td>4,750</td>
<td></td>
</tr>
<tr>
<td>PAO1(pJR395)</td>
<td>Wild type</td>
<td>130</td>
<td>4</td>
<td>4,750</td>
<td></td>
</tr>
<tr>
<td>AK1041(pLE689)</td>
<td>A`B’ band LPS</td>
<td>180</td>
<td>4</td>
<td>4,550</td>
<td></td>
</tr>
<tr>
<td>AK1012(pLE689)</td>
<td>A `B band LPS</td>
<td>550</td>
<td>12</td>
<td>4,000</td>
<td></td>
</tr>
<tr>
<td>AK1012(pLE689, pLPS188)</td>
<td>Wild-type LPS</td>
<td>100</td>
<td>2</td>
<td>4,900</td>
<td></td>
</tr>
<tr>
<td>PAO222(pLE689)</td>
<td>Wild-type LPS</td>
<td>70</td>
<td>2</td>
<td>5,400</td>
<td></td>
</tr>
<tr>
<td>PAZ3(pLE689)</td>
<td>absD</td>
<td>430</td>
<td>9</td>
<td>7,500</td>
<td></td>
</tr>
<tr>
<td></td>
<td>absB</td>
<td>90</td>
<td>3</td>
<td>5,450</td>
<td></td>
</tr>
<tr>
<td>H636(pLE689)</td>
<td>OprF¹</td>
<td>630</td>
<td>25</td>
<td>5,950</td>
<td></td>
</tr>
<tr>
<td>4098(pJR395)</td>
<td>Wild-type LPS</td>
<td>130</td>
<td>3</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>4098E(pJR395)</td>
<td>MexA-B-OprM+²</td>
<td>80</td>
<td>2</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>4098T(pJR395)</td>
<td>OprM</td>
<td>120</td>
<td>3</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>PAO-7H(pLE689)</td>
<td>MexE-F-OprN+²</td>
<td>90</td>
<td>2</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ERYR(pLE689)</td>
<td>MexC-D-OprJ+²</td>
<td>120</td>
<td>3</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

* Values are means of three to five independent assays, with variations from experiment to experiment of <20%. Data in bold type are at least four times greater than those of the wild-type strains.

1 Measurements were performed on strains not transformed with plasmid pLE689 or pJR395. ND, not determined.
2 A`B’ band LPS, has A-band LPS and lacks B-band LPS.
3 MexA-B-OprM+, overexpressing the MexA-MexB-OprM system.

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 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, 2 nm s⁻¹ (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-chlorophenylhydrazide 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 nonsaturable uptake pathway (at least up to 800 μM). 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 coliminycin (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 penetration 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 colicin (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 no 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 predictably resulted in higher antagonism by Mg2+.

To evaluate the effects of polycations on the penetration of hydrophilic molecules, we measured the permeability of PAO1 cells to TMPD in the presence of increasing concentrations of polymyxin B (C), PMBN (A), or DAPB (B) alone or with 1 mM Mg2+ added (C, D, and E, respectively) (see Materials and Methods for details). coef., coefficient.

Recombinant peptide rBPI12, 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 rBPI12, like the holo-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-stabilizing activity. Two polycationic antibiotics, 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), cefuzidine, ceftriumide (18), ciprofloxacin, crystal violet, erythromycin, fosfomycin, imipenem, nalidixic acid, p-amino benzoate (43), pefloxicin, 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 respective intrinsic activities (nonpermeabilizers are listed below).

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 hydrophobic antibiotics

<table>
<thead>
<tr>
<th>Permeabilizer (MIC [mg liter(^{-1}))</th>
<th>FIC index(^a)</th>
<th>Novobiocin</th>
<th>Erythromycin</th>
<th>Fusidic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymyxin B (0.125)</td>
<td>0.375</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>DAPB (1.25)</td>
<td>0.31</td>
<td>0.375</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Chlorhexidine (128)</td>
<td>0.25</td>
<td>0.256</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Dibromopropamidine (256)</td>
<td>0.125</td>
<td>0.25</td>
<td>0.375</td>
<td></td>
</tr>
<tr>
<td>Chlorpromazine (&gt;128(^b))</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Amikacin (2)</td>
<td>0.625</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Triphosphosphate (&gt;1,024(^b))</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Tetracaine (&gt;1,024(^b))</td>
<td>0.125</td>
<td>0.5</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Data in bold type indicate synergism (FIC ≤ 0.5).
\(^b\) Synergism was assayed at the highest concentration of the compound achievable in MH broth before precipitation.
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. aeruginosa* 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 membrane-stabilizing activity in *P. aeruginosa* (see “Permeabilizing 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 exerted by divalent cations at 1 mM. However, the electrostatic interaction between these products and LPS, as evidenced by

### Acknowledgments

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### References

26. Little, R. Personal communication.

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