JOURNAL OF BACTERIOLOGY, Nov. 1997, p. 7004–7010 Vol. 179, No. 22
0021-9193/97/$04.00+0

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

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Received 6 February 1997/Accepted 28 August 1997

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 Δ^1-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*-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, $rBPI_{23}$, chlorhexidine, and dibromopropamidine demonstrated the strongest permeabilizing activities on a molar basis in the presence of 1 mM $MgCl_2$. 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 divalent cations that cross-bridge the LPS molecules covering the cell surface (50).

Recently, we reported that the outer membrane of *S. typhimurium* is relatively permeable to very hydrophobic steroids (41). The permeation rates of these compounds were determined from their kinetics of oxidation by intact bacterial cells expressing the 3-oxosteroid Δ^1-dehydrogenase gene cloned from *Comamonas testosteroni* (40). We could observe that the outer membrane permeability to steroids increased markedly when the LPS layer was altered either by mutations or by the action of the polycation deacylpolymyxin 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.*

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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 Δ^1-dehydrogenase gene from *Comamonas testosteroni*, was reported previously (41). This vector, which carries the *xylS* 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 Δ^1-dehydrogenase gene was constructed by recloning a 2.2-kbp *Bam*HI-*Sac*I fragment from the pUC-derived plasmid pTEK22 (40), downstream of the *ptac* promoter of the broad-host-range expression vector pMMB207 (28). Plasmid pLPS188 expresses the *algC* gene that encodes phosphomanno/glucomutase, 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 $\mu g\ ml^{-1}$ (strain PAZ3), 250 $\mu g\ ml^{-1}$ (strains PAO1, AK1401, AK1012, PAO222, PAZ1, H636, and PAO-7H), or 400 $\mu g\ ml^{-1}$ (strain ERYR). Chloramphenicol was added to MH agar plates at 250 $\mu g\ ml^{-1}$ (PAO1) or 400 $\mu g\ ml^{-1}$ (4098, 4098E, and 4098T) for the selection of cells transformed with pJR395. When necessary, expression of the cloned Δ^1-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 Δ^1-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 (OD_{650}) 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

TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristic(s)	Source or reference
E. coli RR1	K-12 derivative with wild-type antibiotic susceptibilities	45
P. aeruginosa		
PAO1	Wild-type strain	B. W. Holloway
PAO222	*met-28 trp-6 lysA12 his-4 ilv-226 pro-82*	15
AK1401	Rough derivative of PAO1 lacking the B-band LPS	5
AK1012	Rough derivative of PAO1 lacking the A- and B-band LPS	19
PAZ1	PAO222 derivative containing the *absA* mutation from the supersusceptible strain Z61	1
PAZ3	PAO222 derivative containing the *absB* mutation from the supersusceptible strain Z61	1
H636	OprF-deficient Ω insertion derivative of PAO1	57
ERYR	Multidrug-resistant mutant of PAO1 overproducing the MexC-MexD-OprJ efflux system	16
4098	FP^- *met-9020 pro-9024 blaP9208*; PAO derivative producing a low, noninducible level of β-lactamase Id	24
4098E	Multidrug-resistant mutant of 4098 overexpressing the MexA-MexB-OprM efflux system	24
4098T	OprM-deficient Ω-Hg insertion mutant of 4098	16
PAO-7H	Multidrug-resistant mutant of PAO1 overexpressing the MexE-MexF-OprN efflux system	21

same buffer to an OD_{650} of 3.0 (0.7 ± 0.1 mg of protein ml^{-1}). Measurements of Δ^1-dehydrogenase activity were performed at 30°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 pJR395 oxidized 3-oxosteroids linearly for at least 10 min when these substrates were used at external concentrations (C_{ext}) above the K_m values of Δ^1-dehydrogenase. This allowed us to use the endpoint quantification of oxidized steroids as a convenient method to determine whole-cell Δ^1-dehydrogenase kinetics.

Typically, the enzymatic reaction was initiated by the addition of 10 μl of steroid in methanolic 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 >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 (55:35:10:2 [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^1-testosterone and Δ^1-testosterone hemisuccinate were integrated, and the resulting values were used to calculate the Δ^1-dehydrogenase activities expressed by intact cells, in nanomoles $minute^{-1}$ milligram (dry weight)$^{-1}$ (V_{cells}). Controls using cell supernatants were routinely performed to check that there was no significant leakage of Δ^1-dehydrogenase; such a 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 U of OD_{650}) was diluted 1:30 in HEPES buffer and enzymatic measurements were performed with testosterone at a final concentration of 50 μM. In some experiments, HEPES buffer was supplemented with 1 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 TMPD. *N,N,N′,N′*-tetramethyl-*p*-paraphenylenediamine (TMPD) is a small hydrophilic molecule of 164 Da, widely used as a chromogenic substrate for the characterization of organisms, such as *P. aeruginosa*, that produce cytochrome *c* oxidase enzymes (49). Several pieces of evidence strongly suggest that enzymes of the heme-copper oxidase superfamily bind cytochrome *c* at a site located in the periplasmic carboxy domain of subunit II, a transmembrane peptide involved in the enzyme complex (11). In an attempt to measure the rate of penetration of TMPD across the outer membrane, we postulated that, in *P. aeruginosa*, oxidation of this redox reagent by the cytochrome c_{554} complex (58) also occurs in the periplasm. We also assumed that the enzymatic oxidation of TMPD in *P. aeruginosa* cells follows Michaelis-Menten kinetics (K_m = 720 μM according to reference 58) and is rate limited by the influx of the substrate through the bacterial outer membrane. In a typical experiment, 1 ml of freshly centrifuged cells resuspended in 100 mM HEPES (pH 7.4) (OD_{650} 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 TMPD dihydrochloride at final concentrations ranging from 50 to 800 μM. Initial velocities of TMPD oxidation by the intact bacteria were measured spectrophotometrically at 600 nm by recording the appearance of Wurster's blue, the oxidized form of TMPD. Enzyme kinetics were linear for at least 1 min and varied proportionally with TMPD concentration. All measurements were performed against a blank containing only TMPD 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 M^{-1} cm^{-1} for Wurster's blue (14). In all experiments, cell supernatants exhibited TMPD 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 TMPD through the outer membrane were calculated by the method developed for β-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 Δ^1-dehydrogenase enzyme. The same hypothesis was made regarding TMPD 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 $P = V_{cells}/(60 \times A \times C_{ext})$, where V_{cells} represents the rate of oxidation of the solute (in nanomoles $minute^{-1}$ milligram [dry $weight^{-1}$]) by the intact cells in the 0.5-cm^3 (steroids) or 1-cm^3 (TMPD) reaction mixture, *A* corresponds to the cell surface arbitrarily set at 132 cm^2 mg (dry weight)$^{-1}$ for *P. aeruginosa*, and C_{ext} is the external concentration of the solute (in nanomoles 0.5 or 1 cm^{-3}). According to Fick's first law, *P* is predicted to remain constant when C_{ext} varies, at least within certain limits. To check the validity of the model, we systematically measured V_{cells} at two different external concentrations (C_{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 ≤0.5, additive if >0.5, and indifferent if >1.

Chemicals 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 norfloxacin (Merck, Sharp & Dohme-Chibret); amikacin and kanamycin (Bristol-Myers Squibb); azithromycin (Pfizer); ceftazidime (Glaxo-Wellcome); ciprofloxacin (Bayer Pharma); colimycin (Roger Bellon); chloroquine and dibromopropamidine isethionate (Specia, Rhône-Poulenc, Rorer); erythromycin (Abbott); ethanolamine fusidate (Leo); rifabutin (Pharmacia & Upjohn), tetracycline hydrochloride (Roussel & Diamant); 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 $rBPI_{23}$ 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 2. Outer membrane permeability of various PAO mutants to steroids and TMPD

Strain (plasmid)	Genotype or phenotype	Permeability coefficient P (nm s^{-1})[a]		
		Testosterone	Testosterone hemisuccinate	TMPD[b]
PAO1(pLE689)	Wild type	100	4	4,750
PAO1(pJR395)	Wild type	130	4	
AK1401(pLE689)	A^+B^- band LPS[c]	180	4	4,550
AK1012(pLE689)	A^-B^- band LPS	**550**	**12**	4,000
AK1012(pLE689, pLPS188)	Wild-type LPS	100	2	4,900
PAO222(pLE689)	Wild-type LPS	70	2	5,400
PAZ1(pLE689)	*absA*	**430**	**9**	7,300
PAZ3(pLE689)	*absB*	90	3	5,450
H636(pLE689)	$OprF^-$	**630**	**25**	5,950
4098(pJR395)	Wild-type LPS	130	3	ND
4098E(pJR395)	MexA,B-$OprM^{++}$[d]	80	2	ND
4098T(pJR395)	$OprM^-$	120	3	ND
PAO-7H(pLE689)	MexE,F-$OprN^{++}$	90	2	ND
ERYR(pLE689)	MexC,D-$OprJ^{++}$	120	3	ND

[a] 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.
[b] Measurements were performed on strains not transformed with plasmid pLE689 or pJR395. ND, not determined.
[c] A^+ B^- band LPS, has A-band LPS and lacks B-band LPS.
[d] 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 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-OprJ (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 Δ^1-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*-chlorophenylhydrazone 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 aspecifically 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 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 penetration of testosterone through the bilayer,

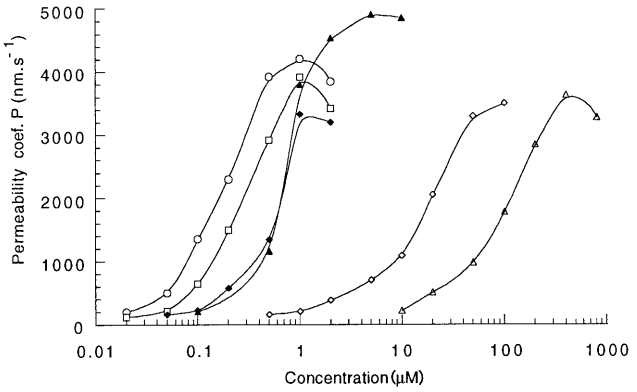

FIG. 1. Outer membrane-permeabilizing effect of polymyxins on strain PAO1. Bacterial cells in mid-log phase were centrifuged and resuspended to an OD_{650} of 0.1 in 50 mM HEPES buffer (pH 7.4) alone or supplemented with 1 mM Mg^{2+}. Penetration rates of testosterone across the outer membrane were measured in the presence of increasing concentrations of polymyxin B (○), PMBN (▲), or DAPB (◆) alone or with 1 mM Mg^{2+} added (□, △, and ◇, respectively) (see Materials and Methods for details). coef., coefficient.

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 Δ^1-dehydrogenase enzyme. The effect on the outer membrane was relatively insensitive to competitive inhibition by divalent cations (Mg^{2+} [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 predictably resulted in higher antagonism by Mg^{2+}.

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 PMBN (polymyxin B could not be assayed because, at concentrations above 0.5 μM, this compound partially inhibited the TMPD oxidase activity of the cells). Penetration rates of the redox reagent at 100 μM PMBN were actually 3.5-fold less than at 0.125 μM (data not presented), thus giving no evidence for an enhanced passage of TMPD by the LPS route in the treated cells. Using a cell lysate of PAO1, we verified that higher concentrations of PMBN did not inhibit cytochrome *c* oxidase activity.

Permeabilizing actions of other compounds. The outer membrane-damaging activities of 32 other agents were assayed on PAO1 cells with testosterone as a probe. To compare these products on a molar basis, we used a permeabilization coefficient defined as the permeability ratio between cells exposed to 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 $rBPI_{23}$, a polycationic 23-kDa amino-terminal 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 $rBPI_{23}$, 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-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 Mg^{2+}. 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), ceftazidime, cetrimide (18), ciprofloxacin, crystal violet, erythromycin, fosfomycin, imipenem, nalidixic acid, *p*-aminobenzoate (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) ($P \leq 130$ 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

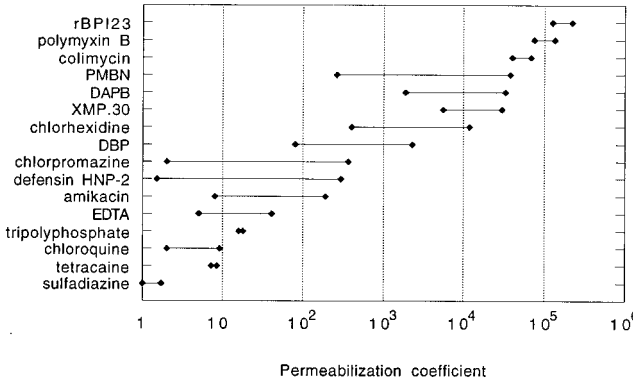

FIG. 2. Comparison of permeabilizers on a molar basis. The permeabilization coefficient used to compare the agents corresponds to the ratio between the outer membrane permeability to testosterone of PAO1 cells treated with 1 mM product and the permeability of untreated cells (100 nm s^{-1}). Values for permeabilized cells were extrapolated from data obtained with optimal concentrations of permeabilizer in the presence or absence (◆) of 1 mM Mg^{2+}. DBP, dibromopropamidine.

TABLE 3. Sensitization of *P. aeruginosa* PAO1 to hydrophobic antibiotics

Permeabilizer (MIC [mg liter^{-1}])	FIC index[a]		
	Novobiocin	Erythromycin	Fusidic acid
Polymyxin B (0.125)	**0.375**	**0.5**	**0.5**
DAPB (1.25)	**0.31**	**0.375**	**0.5**
Chlorhexidine (128)	**0.25**	**0.256**	**0.5**
Dibromopropamidine (256)	**0.125**	**0.25**	**0.375**
Chlorpromazine (>128)[b]	1	1	**0.5**
Amikacin (2)	0.625	2	1
Tripolyphosphate (>1,024)[b]	1	**0.5**	1
Tetracaine (>1,024)[b]	**0.125**	**0.5**	1

[a] Data in bold type indicate synergism (FIC $\leq$ 0.5).
[b] Synergism was assayed at the highest concentration of the compound achievable in MH broth before precipitation.

agents tested, except amikacin, potentiated the antibacterial activities of novobiocin, erythromycin, and fusidic acid to a significant extent (FIC $\leq$ 0.5) (Table 3). Other compounds had only limited effects, if any, on the susceptibility levels of PAO1 to these antibiotics. Interestingly, tetracain 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 enterobacterial outer membranes to steroids can be accurately measured by coupling the influx of these compounds to their subsequent oxidation by the enzyme 3-oxosteroid Δ^1-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 pK_a 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 extensive 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 Mg^{2+} and Ca^{2+} 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., tetracain 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 $MgCl_2$). 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. 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-destabilizing 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 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 increased penetration of testosterone (up to 50-fold) and significant sensitization of *P. aeruginosa* cells to various hydrophobic antibiotics. At higher concentrations of permeabilizer, ultimately the outer membrane became as permeable as the inner membrane (41). Our results conclusively show the greater ability of amphipathic polycations such as polymyxin B and $rBPI_{23}$ 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 contains two apolar pockets that each interact with the acyl chain of a molecule of phosphatidylcholine or LPS through hydrophobic 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 permeabilizing 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 remain 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 (cecropins), or amphibian skin (magainins) (17) should give new insights into the structural determinants necessary for outer membrane permeabilization and bacterial killing. Because 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.*

ACKNOWLEDGMENTS

We are grateful to H. Nikaido for critically reading the manuscript. We thank M. Vaara, R. E. W. Hancock, J. Goldberg, and D. Haas for providing us with useful strains or chemicals.

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