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Compounds Which Increase the Permeability of the *Pseudomonas aeruginosa* Outer Membrane

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Hydrolysis of the chromogenic β-lactam nitrocefin by periplasmic β-lactamase in intact *Pseudomonas aeruginosa* cells was used to assess the influence of various compounds on the permeability of the *P. aeruginosa* outer membrane. In addition to the five previously described outer membrane-active compounds EDTA, polymyxin B, gentamicin, poly-L-lysine, and Tris, seven other compounds were shown to increase outer membrane permeability to nitrocefin by 14- to 63-fold. These other compounds included poly-L-ornithine, neomycin, cetyltrimethylammonium bromide, nitrilotriacetate, L-ascorbate, and acetylsalicylate. In each case, Mg^{2+} ions antagonized, to different extents, the enhancement of outer membrane permeability. The same compounds increased the permeability of the outer membrane to the protein lysozyme and to the hydrophobic fluorescent probe 1-*N*-phenylnaphthylamine, although L-ascorbate and acetylsalicylate showed only very weak enhancement of uptake in these assays. In this report, we discuss the possibility that these compounds act at a common outer membrane site at which divalent cations noncovalently cross-bridge adjacent lipopolysaccharide molecules.

Three mechanisms have been proposed for the uptake of antibiotics across the outer membranes of gram-negative bacteria. The hydrophilic-uptake pathway involves the passive diffusion of small hydrophilic antibiotics through the water-filled pores of porin proteins (15). An alternative hydrophobic-uptake pathway, in which hydrophobic antibiotics partition into the outer membrane bilayer, has been proposed for some *Neisseria* spp. (15). In contrast, Nikaido (14) has demonstrated that most gram-negative bacteria do not have a hydrophobic-uptake pathway and are highly resistant to hydrophobic antibiotics. We have suggested a third uptake pathway, the self-promoted pathway (4, 5, 12), in which polycationic antibiotics interact with a site on the outer membrane at which Mg^{2+} noncovalently cross-bridges adjacent lipopolysaccharide molecules. The resultant destabilized outer membrane allows enhanced passage across the outer membrane of the chromogenic β-lactam nitrocefin (5), the protein lysozyme (5), and the hydrophobic fluorophors *N*-phenylnaphthylamine (NPN) and 1-anilino-8-naphthosulfonate (B. Loh, C. Grant, and R. E. W. Hancock, submitted for publication). Consequently, we have proposed that the uptake of the polycationic antibiotic causing the outer membrane disruption is also promoted. As further evidence in favor of self-promoted uptake, EDTA, a divalent cation-chelator which removes MG^{2+} from outer membrane sites, causes similar enhancement of uptake of lysozyme and β-lactams (5, 22) as well as enhanced killing by the polycationic antibiotics (22). Furthermore, a single-point mutation of *Pseudomonas aeruginosa* renders the cells resistant to not only the polycationic antibiotics but also EDTA (11), whereas external Mg^{2+} antagonizes (i.e., competes with) both classes of agents (10, 11, 30).

The disruption of the outer membranes of a variety of gram-negative bacteria by polymyxin and EDTA has been well studied (8, 9, 23, 27; see R. E. W. Hancock, Annu. Rev. Microbiol., in press, for a review). Recently, Vaara and Vaara have provided strongly suggestive evidence that delipidated polymyxin, as well as other polycations, can also disrupt the outer membranes of *Escherichia coli* cells (25). All of these agents which disrupt outer membranes are synergistic with antibiotics (22, 24). In this paper, we have proposed that agents enhancing outer membrane permeability be given the group name permeabilizers and demonstrate that a variety of compounds have outer membrane-permeabilizing activity in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *P. aeruginosa* H309, which is a derivative of *P. aeruginosa* PAO1 strain H103 (11) and contains the plasmid RP1 (13, 16), was used for all of the nitrocefin permeation experiments described here. Strain H103 was used for all other experiments. These strains were grown on proteose peptone no. 2 medium to an optical density at 600 nm of 0.5 and centrifuged down and resuspended in 5 mM sodium HEPES (*N*-2-hydroxyethylpiperazone-*N'*-2-ethane sulfonic acid) buffer (pH 7.35) at the same optical density. Control experiments demonstrated that no leakage of β-lactamase from strain H309 occurred over a time period of 3 to 4 h after resuspension of the cells. Furthermore, no differences were observed when enhancement of NPN uptake (see below) by gentamicin or polymyxin was examined with either strain H103 or strain H309.

Outer membrane permeability estimation with nitrocefin. The rates of hydrolysis of nitrocefin in intact cells were determined exactly as described previously (16). The permeability parameter *C* was calculated by the method of Zimmerman and Rosselet (31). Nitrocefin was the kind gift of Glaxo Ltd., Middlesex, England.

Lysozyme lysis. One milliliter of cells in HEPES buffer was placed in a cuvette at 23°C, and lysozyme from a 1-mg/ml stock solution in water was added to a final concentration of 20 μg/ml. A permeabilizer compound was then added to a final concentration (see Table 2), the cuvette was placed in the sample beam of a Perkin-Elmer Lambda 3 dual-beam spectrophotometer, and the decrease in optical density at 600 nm was continuously recorded on a Perkin-Elmer 561 strip chart recorder. There was no visible evidence of cell clumping during the experiment.

Fluorescence experiments. NPN was dissolved in acetone at a concentration of 500 μM and added to cells in HEPES

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buffer at a final concentration of 10 μM. Control experiments showed no effects on outer membrane permeability of the added acetone. Fluorescence spectra and emission intensities were measured with a Perkin-Elmer 650-10S fluorescence spectrophotometer equipped with a circulating-water bath to maintain the cuvette holding chamber at 30°C. Excitation and emission wavelengths were set at 350 and 420 nm, respectively, with slit widths of 5 nm. Enhancer compounds were added at the indicated concentrations, and NPN fluorescence intensity was continuously monitored on a Perkin-Elmer Coleman 165 strip chart recorder.

Chemicals. All chemicals were of the highest quality commercially available and were obtained from Sigma Chemical Co., St. Louis, Mo., with the exception of Tris (Ultra Pure; Canadian Scientific Products, London, Canada); nitrilotriacetate (Eastman Kodak Co., Rochester, N.Y); acetylsalicylate (BDH Chemicals, Vancouver, British Columbia, Canada); succinate and citrate (Canlab, Vancouver); acetate (Fisher Scientific Ltd., Vancouver); and benzalkonium chloride, α-mercaptopropionyl glycine, TES [*N*-Tris (hydroxymethyl)methyl-2-aminoethane sulfonate], magnesidin, and HEPES buffer (Calbiochem-Behring Corp., La Jolla, Calif.). All anions were in sodium salt form.

RESULTS

Measurement of outer membrane permeability with nitrocefin. Nitrocefin is a chromogenic β-lactam which undergoes a color change when hydrolyzed by β-lactamase (1). When the β-lactamase is free in solution, only the concentration of nitrocefin and the kinetic parameters of the enzyme determine the rate of conversion of nitrocefin to nitrocefoic acid. In whole cells, however, when the β-lactamase is largely periplasmic, the rate of hydrolysis at low-to-moderate nitrocefin concentrations is limited by the rate of diffusion across the semipermeable outer membrane and thus provides a measure of outer membrane permeability (1, 13, 16).

In the experiments performed here, the periplasmic β-lactamase was encoded by the resistance transfer plasmid RP1. We confirmed our previous data (13) that, under the conditons utilized here, the rate of hydrolysis of nitrocefin by the RP1-encoded β-lactamase of intact cells was less than 2% of the hydrolysis rate when these cells were disrupted by passage through a French pressure cell.

To confirm that nitrocefin hydrolysis provided a good measure of outer membrane disruption, we studied the effects of EDTA, a divalent cation chelator known to disrupt outer membranes (8). The rate of nitrocefin hydrolysis was sigmoidally related to the concentration of EDTA and approached a maximum rate of hydrolysis about 20-fold greater than that observed in the absence of EDTA (Fig. 1). Even this maximum rate of nitrocefin hydrolysis was more than twofold less than the rate of nitrocefin hydrolysis in French-pressed, broken cells, suggesting that we were not merely measuring outer membrane breakdown or loss but rather increased outer membrane permeability. In agreement with this was the fact that we could measure no β-lactamase in the supernatant after EDTA treatment and removal of cells by centrifugation. Furthermore, no apparent cell lysis occurred under these conditions (i.e., in the absence of an energy source).

The sigmoidal relationship between the EDTA concentration and the rate of nitrocefin hydrolysis strongly suggests that EDTA interacted cooperatively with outer membranes. Redrawing the data as a Hill plot (Fig. 1 inset) confirmed that the permeabilization of outer membranes by EDTA exhibited positive cooperativity. The slope of the Hill plot, which

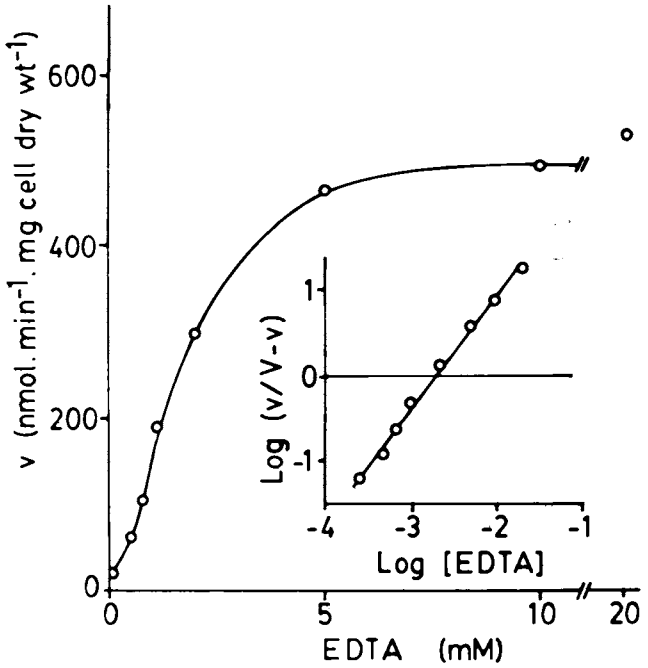

FIG. 1. Effect of varying the concentration of EDTA on the rate of nitrocefin hydrolysis (expressed as nanomoles of nitrocefin hydrolyzed per minute per milligram of cells [dry weight]) by periplasmic β-lactamase of strain H309 (RP1). In the inset, the data is redrawn as a Hill plot. *v*, Rate of nitrocefin hydrolysis; *V*, maximal rate of nitrocefin hydrolysis extrapolated from the data.

provides a measure of this cooperativity, was 1.38, suggesting at least two interaction sites. The substrate concentration at one-half the V_{max}, which provides an average measurement of the affinity of the two sites for EDTA, was 1.88 mM EDTA.

The enhancement of outer membrane permeability to nitrocefin by EDTA was found to be strongly antagonized by Mg^{2+} (Table 1) and almost pH independent between pH 6 and 9 (data not shown). EDTA action on *E. coli* requires an organic cation such as Tris to promote its effects (19), and HEPES buffer does not promote EDTA action (R. E. W. Hancock and V. J. Raffle, unpublished results). In contrast, as shown by Goldschmidt and Weiss (3) and confirmed here (Fig. 1), EDTA action on *P. aeruginosa* is independent of the presence of Tris and occurs readily in the presence of HEPES buffer. Tris itself was capable of increasing nitrocefin uptake in *P. aeruginosa*. We were able to demonstrate a marked effect of pH on the ability of Tris to permeabilize *P. aeruginosa* outer membranes to nitrocefin (e.g., Table 1).

Other compounds with well-known abilities to permeabilize outer membranes are the polycationic antibiotics gentamicin (in *P. aeruginosa* only [5]) and polymyxin B (20, 23, 27). We were able to confirm that both of these antibiotics increased nitrocefin hydrolysis by periplasmic β-lactamase (Table 1).

Screening for enhancers by use of nitrocefin hydrolysis as a tool. In the above experiments, we defined three groups of compounds capable of permeabilizing outer membranes: chelators (like EDTA), polycations (like polymyxin B and gentamicin), and large organic monovalent cations (like Tris). Using this as a basis for the selection of potential permeabilizers of the outer membrane, we screened 20 other compounds for their effects on nitrocefin hydrolysis by intact cells (Table 1). Of these, eight increased the perme-

TABLE 1. Effect of different compounds and Mg^{2+} on outer membrane permeability as measured by the rate of nitrocefin hydrolysis by periplasmic β-lactamase

Compound (concn)	Outer membrane permeability coefficient C^a	
	No added Mg^{2+} (fold increase over control)	With 1 mM Mg^{2+}
None (control)	5.1 (1)	3.9
EDTA (0.18 mM)	140 (27)	2.8
Tris-hydrochloride, pH 7.4 (35 mM)	133 (26)	3.1
Tris-hydrochloride, pH 9 (35 mM)	4.1 (<1)	
Polymyxin B (6 μM)	281 (55)	180
Gentamicin (0.2 mM)	290 (57)	4.4
Poly-L-ornithine (0.04 μM)	92 (18)	31
Poly-L-lysine (31 μM)	296 (58)	5.9
Neomycin (0.15 mM)	320 (63)	6.4
Gramicidin S (12 μM)	173 (34)	17
Cetrimide (1.2 mM)	81 (16)	55
Nitrilotriacetate (0.8 mM)	73 (14)	50
L-Ascorbate (1.2 mM)	163 (32)	29
Acetylsalicylate (1.2 mM)	135 (26)	55
Succinate (1.2 mM)	3.7 (<1)	
Acetate (1.2 mM)	5.2 (1)	
Citrate (1.2 mM)[b]	4.2 (<1)	
Deoxycholate (1.2 mM)	4.5 (<1)	
TES (1.2 mM)	5.2 (1)	
Putrescine (1.2 mM)	5.2 (1)	
L-Penicillamine (1.2 mM)	10 (2)	
Procaine-hydrochloride (1.2 mM)	4.6 (<1)	
Benzalkonium chloride (20 mg/ml)	13 (3)	
α-Mercaptopropionylglycine (6 mM)	4.6 (<1)	
Magnesidin (0.4 mM)	3.3 (<1)	

[a] C is expressed as per seconds per milligram of cells and calculated by the diffusion law of Fick, as expressed by Zimmermann and Rosselet (31): $V_I = C(S_0 - S_e)$, where V_I is the rate of nitrocefin hydrolysis by intact cells, S_0 is the concentration of nitrocefin added to cells, and S_e is the apparent concentration of nitrocefin in the periplasm calculated by the Michaelis-Menten equation. The data presented are usually the averages of three independent experiments.

[b] Citrate at 10 mM permeabilized the outer membrane to give a permeability coefficient of 21.

ability of outer membranes by 8- to 37-fold. These enhancers included the polycations poly-L-ornithine, poly-L-lysine, neomycin and gramicidin S, the monovalent cation cetrimide, and the chelator nitrilotriacetate, as well as ascorbate and acetylsalicylate. None of these compounds caused the release of β-lactamase into the supernatant, as measured after removal of cells by centrifugation. The action of each of these enhancers on outer membrane permeability was antagonized (inhibited) by 1 mM Mg^{2+} (Table 1). In the cases of poly-L-lysine, gramicidin S, EDTA, neomycin, gentamicin, and Tris at the concentrations used, 90% or greater antagonism was observed. For the other compounds, greater antagonism was demonstrated when the concentration of Mg^{2+} was increased at fixed concentrations of permeabilizer (date not shown).

Permeabilization of outer membranes to lysozyme. Lysozyme is a 14,000-dalton protein which cleaves the sugar backbone of peptidoglycan. In gram-negative cells, the outer membrane prevents the access of lysozyme to the peptidoglycan. When passage of lysozyme across the outer membrane is enhanced, lysozyme destroys the peptidoglycan, which normally maintains the osmotic stability of bacterial cells; consequently, the cells lyse. Addition of polymyxin B, gentamicin, or EDTA to cells in the presence of lysozyme caused over 80% lysis of cells (as measured by decrease in optical density at 600 nm) in less than 1 min, confirming previously published results (5). Similarly, nitrilotriacetate, poly-L-ornithine, and poly-L-lysine caused substantial cell lysis (Table 2). Neomycin had substantially smaller effects at the concentration used, whereas two compounds, L-ascorbate and acetylsalicylate, caused very little enhancement of cell lysis by lysozyme (Table 2). For compounds causing substantial enhancement of cell lysis by lysozyme, 1 mM Mg^{2+} was antagonistic (Table 2). Since lysozyme is a basic protein, however, at least some of the effects of Mg^{2+} might be due to direct effects on lysozyme uptake rather than antagonism of the permeabilizing compounds.

Permeabilization of outer membranes to NPN. NPN fluoresces weakly in aqueous environments and strongly in hydrophobic environments. Wild-type *E. coli* and *P. aeruginosa* cells take up little or no NPN (20; Loh et al., submitted for publication). Addition of EDTA (6; Table 3) or aminoglycosides (Loh et al., submitted for publication) to cells causes enhancement of NPN uptake, as measured by increased NPN fluorescence, owing to NPN partitioning into the hydrophobic membrane interior (6; Loh et al., submitted for publication). Since NPN is hydrophobic, it provides a direct measurement of enhancement of outer membrane permeability to hydrophobic compounds. Each of the permeabilizer compounds tested caused increased NPN uptake, although L-ascorbate and acetylsalicylate had extremely small effects (Table 3). The enhancement of NPN uptake into cells was strongly antagonized by 1 mM Mg^{2+} when EDTA, gentamicin, neomycin, or gramicidin S were used as permeabilizers (Table 3). In the cases of poly-L-ornithine, cetrimide, and nitrilotriacetate, however, Mg^{2+} was poorly antagonistic at the concentrations used.

DISCUSSION

In this paper we have demonstrated that 11 different compounds were capable of permeabilizing the *P. aeruginosa* outer membrane to nitrocefin. We propose that these compounds, which have a variety of different chemical compositions, be given the general name permeabilizers. Some of these permeabilizers, including EDTA (5), polymyxin B (10), Tris (5), and gentamicin (5, 12), are well known to increase outer membrane permeability in *P. aeruginosa*. All except gentamicin also act on other bacteria (7, 8, 23; see Hancock, in press, for a review). In addition, indirect evidence has implicated poly-L-lysine as an outer mem-

TABLE 2. Effect of permeabilizer compounds on lysozyme-mediated cell lysis

Compound (concn)	% Cell lysis[a]	
	With lysozyme alone	With lysozyme + 1 mM Mg^{2+}
None (buffer only)	0	0
EDTA (0.1 mM)	83	0
Polymyxin B (2.5 μM)	80	27
Gentamicin (33 μM)	78	0
Poly-L-ornithine (0.2 μM)	83	48
Poly-L-lysine (1.3 μM)	76	21
Neomycin (23 μM)	22	ND[b]
Gramicidin S (5 μM)	24	2
Nitrilotriacetate (0.8 mM)	90	10
L-Ascorbate (1.2 mM)	4	0
Acetylsalicylate (1.2 mM)	8	0

[a] Percent cell lysis was measured as the percent decrease in optical density at 600 nm at 3 min after the addition of the permeabilizer compound to cells resuspended in 5 mM sodium HEPES buffer (pH 7.2). The addition of cetrimide at 1.2 mM caused precipitation in this assay.

[b] ND, Not determined.

TABLE 3. Effect of permeabilizers on NPN fluorescence of *P. aeruginosa* cells

Compound (concn)	Increase in NPN fluorescence[a]	
	Without Mg^{2+}	With 1 mM Mg^{2+}
None (buffer only)	0	0
EDTA (0.3 mM)	43	0
Polymyxin B (2.5 μM)	43	ND[b]
Gentamicin (33 μM)	43	2
Poly-L-ornithine (0.2 μM)	18	17
Neomycin (23 μM)	43	1
Gramicidin S (5 μM)	23	2
Cetrimide (1.2 mM)	41	32
Nitrilotriacetate (0.8 mM)	15	12
L-Ascorbate (1.2 mM)	2	1
Acetylsalicylate (1.2 mM)	4	2

[a] NPN fluorescence was measured 2 min after the addition of the permeabilizer compound to cells resuspended in 5 mM sodium HEPES buffer (pH 7.2) in the presence of 10 μM NPN. Results are expressed in arbitrary units. Background fluorescence in the absence of enhancer (6 arbitrary units) was subtracted.

[b] ND, Not determined.

brane-active compound in *E. coli* (25). In this study, we have identified seven new compounds which increased outer membrane permeability to the β-lactam nitrocefin.

The 11 permeabilizer compounds identified can be partially grouped according to their chemical characteristics. Polymyxin B, poly-L-lysine, poly-L-ornithine, gentamicin, and neomycin are all polycations, whereas gramicidin S carries two positive charges at neutral pH. Each of these increased outer membrane permeability to nitrocefin as well as to the 14,000-dalton protein lysozyme (Table 2) and the hydrophobic fluorescent compound NPN (Table 3). We have also demonstrated that six other polycationic aminoglycoside antibiotics, streptomycin, tobramycin, sisomycin, netilmycin, amikacin, and kanamycin, enhanced the uptake of NPN in *P. aeruginosa* (Loh et al., submitted for publication) and thus should be classed as permeabilizers.

A second group of compounds, organic monovalent cations such as Tris and cetrimide, were able to increase outer membrane permeability to nitrocefin at high concentrations. Cetrimide was also effective in enhancing outer membrane permeability to NPN, whereas we previously demonstrated that Tris at very high concentrations (≥200 mM) caused enhanced permeability to lysozyme (5). Interestingly, both cetrimide and Tris are capable of potentiating the action of EDTA in *E. coli* and other organisms(26). Possibly some or all of the 32 organic cations shown by Voss (26) to individually promote EDTA action against *E. coli* may also have permeabilizer activity in *P. aeruginosa*.

A third group of outer membrane-permeabilizing compounds were the divalent cation chelators EDTA and nitrilotriacetate. These chelators caused increased permeation of nitrocefin, lysozyme, and NPN across the outer membrane. The mechanism of action of the other two permeabilizers identified in this study, ascorbate and acetylsalicylate, is unknown. both of these compounds caused enhanced uptake of nitrocefin but had only a minor effect on lysozyme and NPN uptake. The inhibition (antagonism) by Mg^{2+} of enhanced nitrocefin uptake, a feature demonstrated for all other enhancers, suggested a similar mode of action. Rawal has suggested that ascorbate may be a weak chelator of Mg^{2+} (17), although no specific Mg^{2+}-ascorbate chelate could be demonstrated. Alternatively, the property of ascorbate and acetylsalicylate as reducing compounds (electron donors) may be important in their action on outer membranes.

We have previously proposed (4, 5, 12) that aminoglycosides such as polymyxin B and EDTA interact with an outer membrane site at which Mg^{2+} stabilizes outer membranes by noncovalently cross-bridging adjacent lipopolysaccharide molecules. Thus aminoglycosides and polymyxin G, being polycations, would displace Mg^{2+} by competition, whereas EDTA would remove Mg^{2+} by chelation, causing outer membrane disruption. In favor of this hypothesis is the demonstration by us and others of antagonism by Mg^{2+} of the action of these compounds on cells (10, 11, 30; Tables 1–3); disruption by the compounds of outer membranes (4, 5, 19; Tables 1–3), making them permeable to nitrocefin, lysozyme, and NPN; and interaction of gentamicin and polymyxin B with a cation binding site on purified lipopolysaccharide (A. A. Patterson, E. J. McGroarty, and R. E. W. Hancock, manuscript in preparation). The similar action on outer membranes of the other enhancer compounds described here and the comparable antagonism by Mg^{2+} in most cases suggested that these enhancers may act at the same type of lipopolysaccharide site. This proposal is not without problems, however. For example, although 1 mM Mg^{2+} caused a 50% inhibition of poly-L-ornithine-stimulated nitrocefin and lysozyme uptake, it had no effect on poly-L-ornithine-stimulated uptake of NPN. Similar differential effects of Mg^{2+} on the action of cetrimide and poly-L-lysine were also noted. We believe that the different levels of permeabilizer compounds required to increase outer membrane permeability and the observed differential antagonism by 1 mM Mg^{2+} can be explained in part by consideration of the relative affinities of this lipopolysaccharide site for each enhancer and for Mg^{2+}. Alternatively, the compounds may act at more than one type of outer membrane site.

P. aeruginosa infections are extremely difficult to treat, owing to the high intrinsic resistance of the organism to antibiotics. The primary cause of this high antibiotic resistance is reduced outer membrane permeability (1, 2, 13, 29). It has been estimated that the rate of permeation of β-lactam antibiotics across the *P. aeruginosa* outer membrane is about 12-fold less than that across *E. coli* outer membranes (13). This low rate of permeation results in a relatively lower rate of uptake of hydrophilic β-lactams into the periplasm and consequently decreases the effective exposure of cells to these antibiotics. One possible therapeutic strategy against *P. aeruginosa*, therefore, might be to attempt to reduce the effect of low outer membrane permeability by cotreatment with permeabilizers, thus allowing increased uptake of hydrophilic β-lactams across the outer membrane. Although we have not specifically addressed this question in this report, there is ample evidence in the literature for synergy between five of the outer membrane-permeabilizing compounds described here and a variety of antibiotics (e.g., 17, 18, 20–22, 24). Furthermore, reports on the use of ascorbate and EDTA together with antibiotics in patient and animal model studies have appeared (18, 28). In this respect, it should be noted that many of the permeabilizers described here are currently used in medical practice. By use of the three relatively simple screening tests described here, it should be easy to identify other compounds capable of increasing outer membrane permeability.

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