ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1991, p. 1309–1314
0066-4804/91/071309-06$02.00/0

Vol. 35, No. 7

Interaction of Aminoglycosides with the Outer Membranes and Purified Lipopolysaccharide and OmpF Porin of *Escherichia coli*

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Received 11 October 1990/Accepted 15 April 1991

The mechanism of uptake of aminoglycosides across the outer membrane of *Escherichia coli* was reevaluated. Porin-deficient mutants showed no alteration in gentamicin or kanamycin susceptibility. Furthermore, the influence of kanamycin on intrinsic tryptophan fluorescence of porin OmpF (Y. Kobayashi, and T. Nakae, Eur. J. Biochem. 151:231–236, 1985) was shown to be strongly influenced by protein concentration and EDTA. This led to the hypothesis that aminoglycoside-mediated increases and decreases in intrinsic tryptophan fluorescence were due to aggregation-disaggregation of OmpF mediated by interaction at a divalent cation binding site on OmpF. Gentamicin, kanamycin, and polymyxin B increased *E. coli* outer membrane permeability to the hydrophobic fluorescent compound 1-*N*-phenyl-naphthylamine (NPN) and the peptidoglycan-degrading enzyme lysozyme. Addition of Mg^{2+} blocked these permeabilizing activities. Furthermore, gentamicin and polymyxin B bound to Mg^{2+}-binding sites on *E. coli* lipopolysaccharide, as determined in dansyl polymyxin displacement experiments. A polymyxin-resistant, lipopolysaccharide-altered *pmr* mutant of *E. coli* had a fourfold-lower MIC of gentamicin and kanamycin and was more poorly permeabilized to 1-*N*-phenylnaphthylamine than was its parent strain. These data were consistent with uptake of aminoglycosides across the *E. coli* outer membrane by the self-promoted uptake mechanism.

The outer membrane of gram-negative bacteria is a semipermeable barrier which restricts the access of all antibiotics to their targets (8, 9, 23). Hydrophilic molecules of sizes below a given exclusion limit can pass through the water-filled channels of proteins called porins. Included among these molecules are the β-lactam antibiotics. The most convincing data suggesting that β-lactams are taken up through porin channels are the increases in MICs of β-lactams in mutants with deficiencies in porins (8, 13, 15, 16, 23), although this conclusion is supported by liposome swelling data showing the uptake of β-lactams into liposomes reconstituted with porins (32).

In contrast, polycations, some of which are larger than the exclusion limit of the outer membrane, can be taken up by another mechanism, which we have termed the self-promoted uptake pathway (7, 11, 22). In this pathway, the polycations act to competitively displace divalent cations which cross-bridge adjacent lipopolysaccharide (LPS) molecules, thus disrupting these important outer membrane stabilizing sites. This, then, permeabilizes the outer membrane and is proposed to promote uptake of other molecules of the permeabilizing polycation. Mutants which interact more poorly with polymyxin B have been shown to resist killing by that polycation (11, 22, 25, 29, 30), while a mutant (*tolA*) with LPS which better interacted with polycations was supersusceptible (26). These data show that self-promoted uptake is the first stage of uptake leading to cell killing by certain polycationic antibiotics.

In the case of aminoglycosides, the mechanism of uptake across the outer membrane has been proposed to be different in two species. For *Pseudomonas aeruginosa*, mutant and in vitro interaction data have led to the conclusion that aminoglycosides are taken up via the self-promoted uptake route (11, 22). This is consistent with the polycationic nature of aminoglycosides which carry three to five positive charges. In contrast, for *Escherichia coli*, it was suggested (21) on the basis of liposome swelling data that aminoglycosides were taken up by the porin pathway. However, it should be noted that these data have been criticized for failing to take into account counterion effects (23). In addition, no differences in aminoglycoside killing of wild-type strains and their porin-deficient derivatives were observed. Despite this, it was subsequently suggested (17), on the basis of studies measuring the effects of these cations on quenching of the intrinsic fluorescence of OmpF porin, that this porin contained, within its channel, a binding site with a K_d of 10 to 18 μM for polycations, including kanamycin, and a K_d of 3 mM for Na^+. We therefore decided to reevaluate this latter finding and to seek evidence pointing to the potential self-promoted uptake of aminoglycosides in *E. coli*.

MATERIALS AND METHODS

Strains and media. Our standard strain for investigation of aminoglycoside interactions with *E. coli* K-12 was *E. coli* UB1636 (*trp his lys rpsL lac lamB*/RP1) (11). Other *E. coli* strains utilized were MC4100 [*araD139* Δ(*lac*)*U169 rpsL relA thi* F16B], its *ompB10* mutant MH 1160 (15) received from T. Silhavy, namely, CGSC 6043 (JF699; *proC24 ompA252 his 53 purE41 ilv-277 met-65 lacY29 xyl-14 rpsL97 cycA1 cycB*? *tsx-63* λ^-) (5), its isogenic *ompC262* mutant CGSC 6047 (JF733), and isogenic *ompF254* mutant CGSC 6044 (JF700). *Salmonella typhimurium* SH6482 (*his-6116 rfaJ4041 thr-914 trpB2 xyl-404 rpsL120 flaA66*) and its polymyxin-resistant *pmrA* mutant SH6497 were kindly provided by M. Vaara. *E. coli* SC9251 and its polymyxin-resistant *pmrA* mutant SC9252 (25) were kindly provided by E. McGroarty. Our standard growth medium was LB medium (1% Bacto-Tryptone, 0.5% yeast extract, 0.5% sodium chloride). MIC de-

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TABLE 1. Influence of porin deficiency and polymyxin-resistant (*pmr*) mutation on antibiotic susceptibility

E. coli	Characteristics	MIC (μg/ml)			
		Gentamicin	Kanamycin	Polymyxin	Ceftazidime
UB1636	WT[a]	0.5	4	0.5	0.25
CGSC 6043	WT	0.5	4	0.5	0.25
CGSC 6044	$OmpF^-$	0.5	4	0.5	1.0
CGSC 6047	$OmpC^-$	0.5	4	0.5	0.5
MC4100	WT	0.125	4	0.125	0.125
MH 1160	$OmpF^-$ $OmpC^-$	0.125	2	0.125	0.5
SC9251	WT	1	1	0.5	0.125
SC9252	*pmr*	4	4	32	0.25

[a] WT, wild type.

terminations were performed by the broth microdilution method and assessed after 24 h at 37°C.

Porin fluorescence studies. OmpF porin and its electro-eluted LPS-free derivative were purified from strain CGSC 6047 as described previously (24). Measurements of fluorescence quenching or enhancement were performed as described by Kobayashi and Nakae (17) (excitation wavelength, 280 nm; emission wavelength, 324 nm), except that the OmpF porin was resuspended in 0.1% octyl glucoside-1 mM MES buffer (pH 6.0). The spectrofluorimeter utilized was a Perkin-Elmer 640-10S apparatus.

Permeabilization of outer membranes. *E. coli* K-12 strain UB1636 was grown to an optical density at 600 nm of 0.5, centrifuged, resuspended in 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.2), containing 5 mM glucose and 1 mM potassium cyanide, and held at room temperature until use. Thereafter, measurement of permeabilization of the outer membrane to 1-*N*-phenylnaphthylamine (NPN) or lysozyme was done as described previously (11, 12). NPN uptake was measured as the total increase in fluorescence (in arbitrary units) as a result of antibiotic addition. Control experiments demonstrated that background fluorescence did not increase in the absence of antibiotic addition, whereas titration of residual NPN in the supernatant, by addition of 3% Triton X-100 to the supernatant after removal of cells (19), indicated that the fluorescence enhancement after antibiotic addition was due to uptake of NPN.

LPS isolation and dansyl polymyxin displacement experiments. LPS was isolated from strain UB1636 by the method of Darveau and Hancock (4). Dansyl polymyxin displacement experiments were performed as described previously (20).

RESULTS

Reevaluation of the role of porins in aminoglycoside uptake. We confirmed the general conclusions of Nakae and Nakae (21) and of other authors (1, 16) that the OmpF- and OmpC-deficient, *ompB* mutant strain MH 1160, the OmpF-deficient strain CGSC6044, and the OmpC-deficient strain CGSC 6047 showed no differences in MIC for gentamicin or kanamycin when compared with their OmpF, OmpC porin-sufficient parent strains (Table 1). In contrast, MIC differences consistent with values in the literature (1, 13, 15) were seen for ceftazidime. These MIC differences usually vary between 2- and 16-fold for individual β-lactam antibiotics (1, 13, 15, 17).

Previous data demonstrated that the OmpF porin of *E. coli* K-12 contained a cation binding site and that interaction of aminoglycosides and other polycations with this site caused up to 5% quenching of intrinsic tryptophan fluorescence recorded after excitation at 280 nm (17). This was interpreted as demonstrating aminoglycoside binding inside the OmpF channel. We reexamined this question by using one of the same aminoglycosides, kanamycin, as was utilized in the earlier study (17). As shown in Fig. 1 and 2, these results were more consistent with an aggregation phenomena, since it has been shown that self-aggregation of protein molecules can cause changes in intrinsic protein fluorescence (18). Interaction of kanamycin with OmpF resulted in changes in intrinsic fluorescence, but the results obtained were quite concentration dependent, with the highest quenching (13%) observed at low protein concentrations and the lowest quenching (5%) observed at higher concentrations (Fig. 1A). From these quenching data, a K_d for kanamycin (5 to 15 μM) in the same range as that reported previously (10.5 μM; 12) could be calculated. However, at the highest concentration of protein utilized, fluorescence enhancement rather than quenching was observed. If OmpF samples were first treated

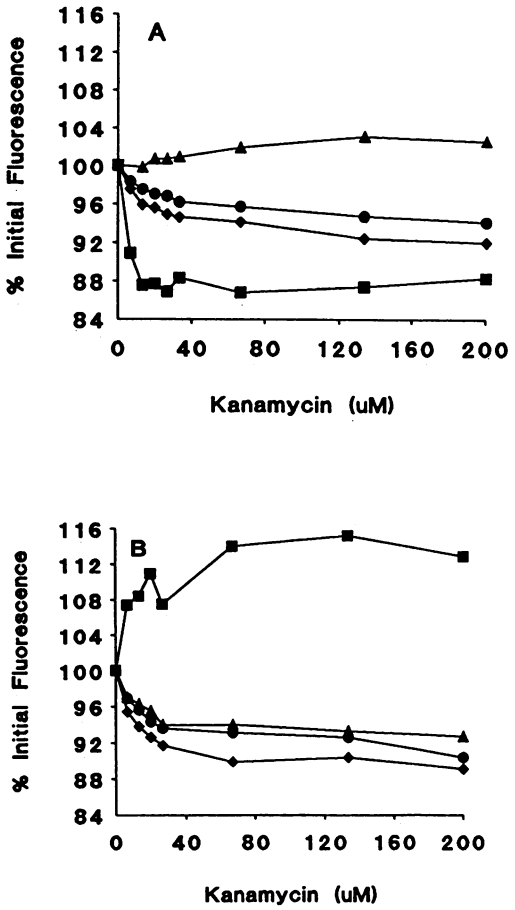

FIG. 1. Changes in intrinsic tryptophan fluorescence of purified OmpF porin of *E. coli* K-12 upon addition of increasing concentrations of kanamycin. Concentrations used (expressed in units of A_{280}) were 0.001 (■), 0.002 (◆), 0.005 (●), and 0.010 (▲). (A) Untreated porin; (B) porin pretreated with 50 mM EDTA (see also Fig. 2). The results shown are representative of four independent experiments. The trends shown were observed in all experiments performed, and the data points varied by less than 5%.

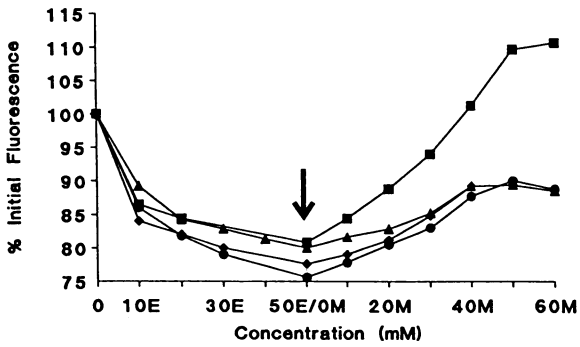

FIG. 2. Changes in intrinsic tryptophan fluorescence of purified OmpF porin of *E. coli* K-12 upon sequential addition of 0 to 50 mM EDTA (shown as 0 to 50E on the *x* axis) followed in the same cuvette by 0 to 60 mM $MgCl_2$ (shown as 0M to 60M on the *x* axis). The arrow indicates the finish of the EDTA titrations and the start of the $MgCl_2$ titrations. The symbols represent different protein concentrations as described in the legend to Fig. 1. The results shown are representative of six independent experiments, all of which demonstrated the same trends, and data points varied by less than 5%.

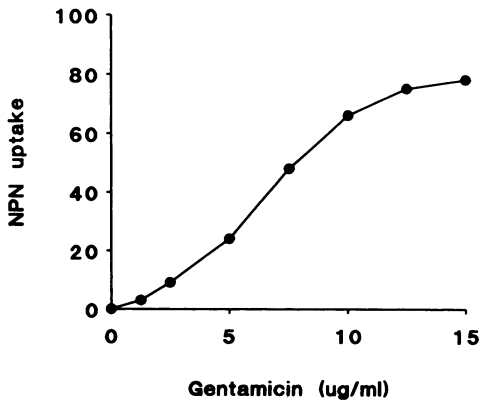

FIG. 3. Influence of gentamicin concentration on the uptake of NPN across the *E. coli* UB1636 outer membrane. The *y* axis values represent NPN uptake recorded as the fluorescence enhancement (in arbitrary units) observed as NPN entered the membrane interior. Each point represents the means of three experiments, with standard deviations of less than 10%.

with 50 mM EDTA and then with increasing concentrations of kanamycin (Fig. 1B), fluorescence enhancement occurred at the lowest concentration utilized and fluorescence quenching occurred at higher concentrations. The influence of protein concentration on the observed fluorescence quenching was consistent with the explanation of an aggregation-disaggregation phenomenon and was not consistent with a protein conformational change as previously proposed (17).

The greatest fluorescence quenching was in fact observed when OmpF was treated with the divalent cation chelator EDTA (i.e., 19 to 23% quenching; Fig. 2) rather than with cationic aminoglycosides. The subsequent addition of Mg^{2+} at least partly reversed this quenching, resulting in fluorescence enhancement (Fig. 2). These data were thus consistent with the interaction of EDTA, Mg^{2+}, and kanamycin at a divalent cation (e.g., Mg^{2+}) binding site on OmpF. We considered that this site might be on LPS since conventionally column-purified porin preparations (including this preparation) are usually associated with a molar equivalent or more of LPS molecules (24). However, electroeluted OmpF, which contained less than 1 molecule of LPS per 1,000 molecules of OmpF porin (24), also demonstrated similar fluorescence quenching and/or enhancement effects. Since interaction of cations at this divalent cation binding site on OmpF apparently influenced the aggregation-disaggregation of this protein, we hypothesized that the site lay on the surface of the porin, external to the channel. In contrast, Kobayashi and Nakae (17) proposed that the site resided inside the narrowest part of the channel and binding at this site resulted in up to a 33% decrease in liposome swelling by monovalent cationic peptides. If the binding site was actually within the channel, conductance of Mg^{2+} and Na^+ through OmpF should have reached saturation (for examples, see reference 8) as the concentration increased above the K_d (reported to be 3 mM for Na^+) and should have been blocked by binding of polyvalent cations (10). However, consistent with previous data (2), no such saturation phenomena was observed for OmpF in black lipid bilayer experiments. In addition, we saw no alteration in the average single-channel conductance of OmpF porin in 1 M KCl when 16 μM kanamycin was added.

Influence of polycations on outer membrane permeability in *E. coli*. The outer membranes of gram-negative bacteria like *P. aeruginosa* and *E. coli* exclude hydrophobic compounds, including hydrophobic antibiotics (23) and the fluorescent probe NPN (13, 19), as well as proteins like the peptidoglycan-attacking enzyme lysozyme (11). Thus, these probe molecules were utilized to observe the influence of gentamicin on their uptake. As shown in Fig. 3, gentamicin substantially enhanced the uptake of NPN in a manner sigmoidally dependent on concentration. Analogous results were also obtained for polymyxin B (data not shown) and for kanamycin (Fig. 4). Cells to which either no antibiotic was added or to which 500 μg of carbenicillin per ml or 128 μg of imipenem, ceftazidime, or cefpirome per ml was added demonstrated no enhancement of fluorescence over time, indicating that these β-lactam antibiotics were unable to permeabilize *E. coli* at concentrations 50- to 150-fold the minimal effective concentration of gentamicin. Addition of high concentrations of NaCl (150 mM), which significantly influence gentamicin MIC and the kinetics and extent of gentamicin uptake in *E. coli* (14), led to a depression in the enhancement by gentamicin- and polymyxin-enhanced NPN fluorescence of *E. coli*. However, even at these high concentrations, gentamicin could still act as a permeabilizer.

Addition of gentamicin, polymyxin (Fig. 5), or kanamycin to lysozyme-treated *E. coli* cells showed that these antibiotics stimulated lysozyme-mediated lysis in a manner sigmoidally dependent on antibiotic concentration. Control experiments showed that this was due to uptake of lysozyme across the outer membrane to its target, the peptidoglycan, since neither lysozyme alone nor gentamicin alone caused cell lysis.

The data in Fig. 3 to 5 could be redrawn as Hill plots with slopes greater than 1, suggesting that gentamicin (and polymyxin) interacted cooperatively with the outer membrane to permeabilize it to the probe molecules. From these Hill plots, an estimate of the affinity of gentamicin and polymyxin for the outer membrane (i.e., $S_{0.5}$, the antibiotic concentration resulting in half-maximal permeabilization of the outer membrane) was obtained and found to be somewhat dependent on the probe molecule. Use of the most sensitive probe,

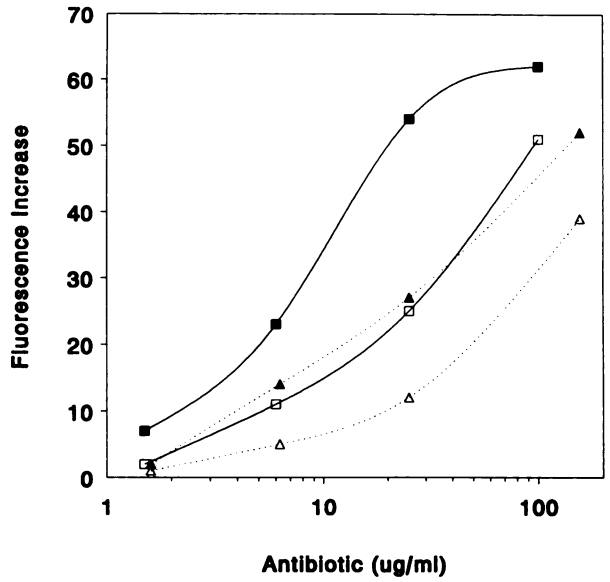

FIG. 4. Influence of the E. coli *pmr* mutation on permeabilization of the outer membrane to NPN (measured as NPN fluorescence increase). Data for kanamycin (dotted lines) and gentamicin (solid lines) as the permeabilizers are given. Symbols: ■ and ▲, wild-type strain SC9251; □ and △, *pmr* mutant strain SC9252. These data are representative of three independent experiments.

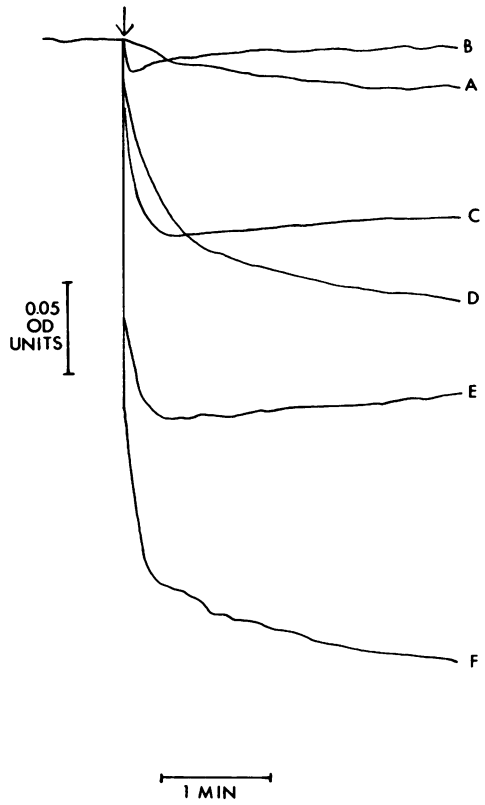

FIG. 5. Influence of gentamicin and polymyxin B on lysozyme-mediated lysis of *E. coli* UB1636. Cell lysis is indicated by a decrease in A_{600} over time. At 10 s prior to antibiotic addition (indicated by arrow), lysozyme was added to cells to a final concentration of 50 μg/ml. (A) No antibiotic added (note: antibiotic added, but no lysozyme gave a similar curve); (B) gentamicin (10 μg/ml) plus 3 mM $MgCl_2$ added; (C) polymycin B (6.4 μg/ml) plus 3 mM $MgCl_2$ added; (D) polymycin B (1.6 μg/ml) added; (E) gentamicin (10 μg/ml) added; (F) polymyxin B (6.4 μg/ml) added.

NPN, revealed that the $S_{0.5}$ for gentamicin (6.5 μg/ml) was 4.3-fold that for polymyxin B (1.5 μg/ml). Use of lysozyme revealed an $S_{0.5}$ for gentamicin of 38 μg/ml and an $S_{0.5}$ for polymyxin B of 2.5 μg/ml. For both probe molecules, it was shown that Mg^{2+} could inhibit the ability of gentamicin, polymyxin, and kanamycin to permeabilize the *E. coli* outer membrane (see, e.g., Fig. 5), a result consistent with action of these polycationic antibiotics at a divalent cation binding site.

In *P. aeruginosa* it has been shown that the ability of polycations to permeabilize outer membranes reflects their ability to bind to LPS with higher affinity than the native cross-bridging cation Mg^{2+} (20). To investigate this, LPS was purified from *E. coli* UB1636 and utilized in a dansyl polymyxin displacement experiment (Table 2). In the present study, LPS was titrated with dansyl polymyxin until maximal binding occurred, as judged by maximal fluorescence enhancement due to dansyl polymyxin binding to LPS. At this stage (i.e., in the presence of 8 μM dansyl polymyxin), other competitive cations were titrated in to displace dansyl polymyxin. (Since nonbound dansyl polymyxin was only marginally fluorescent [20], this resulted in a decrease in fluorescence.) The results demonstrated that gentamicin had an affinity for LPS that was somewhat lower than that of polymyxin B but superior to that of the native cation Mg^{2+}.

Influence of gentamicin and polymyxin B on outer membrane permeability in *E. coli*. Peterson et al. (25) have shown that a polymyxin-resistant mutant strain, *E. coli* SC9252, has a substantial increase in MIC of polymyxin and enhanced esterification of phosphate monoesters in its LPS. As a result, this mutant demonstrated decreased binding of polymyxin B and other cations (including gentamicin) to its LPS when compared with its parent SC9251 (25). We confirmed a 64-fold-reduced MIC to polymyxin, and found that SC9252 also had a 4-fold-reduced MIC to gentamicin and kanamycin (Table 1). Consistent with this, higher gentamicin and kanamycin concentrations were required to permeabilize, to NPN, the outer membrane of mutant strain SC9252 than that of its parent strain SC9251 (Fig. 4). In contrast, a polymyxin B-resistant *pmrA* mutant of *S. typhimurium* (29, 30) showed no difference in gentamicin MIC (data not shown). This may indicate that either self-promoted uptake of gentamicin is not rate limiting for killing in *S. typhimurium* or may reflect the fact that this mutant is milder, causing only an 8-fold increase in MIC to polymyxin (cf. strain SC9252, which had a 64-fold increase).

TABLE 2. Kinetics of displacement of 8 μM dansyl polymyxin from *E. coli* K-12 strain UB1636 LPS

Displacing cation	I_{50}[a] (μM)	I_{max}[b] (%)
Polymyxin	12.5	100
Gentamicin	159	74
Mg^{2+}	3,500	55

[a] I_{50}, concentration of cation resulting in half-maximal displacement of dansyl polymyxin from LPS derived from a Lineweaver-Burke plot of cation concentration versus fluorescence decrease due to displacement of LPS-bound dansyl polymyxin.

[b] I_{max}, maximal displacement as estimated from a Lineweaver-Burke plot.

DISCUSSION

The data in this article demonstrate that aminoglycosides could interact with the outer membrane of *E. coli* to permeabilize it to the probe molecules NPN and lysozyme. In addition, dansyl polymyxin displacement experiments demonstrate that gentamicin is capable of interacting at the same Mg^{2+}-binding sites on LPS as polymyxin B, a result in keeping with earlier experiments by Peterson et al. (25). These results are consistent with the utilization, by gentamicin, of the self-promoted uptake pathway in these strains for which gentamicin, through disruption of Mg^{2+} bridges between adjacent LPS molecules in the outer membrane, would promote its own uptake across the outer membrane. In addition, the data in Table 1 and Fig. 1 and 2 appear to contradict previous conclusions (17, 21) that aminoglycosides utilize the porin pathway to cross the outer membrane. Indeed, the normal aminoglycoside susceptibility of porin-deficient mutants of *Proteus mirabilis* (28), *Enterobacter cloacae* (27, 28), *Klebsiella pneumoniae* (27), and *Aeromonas salmonicida* (31) and the lack of cross-resistance between β-lactam antibiotics and aminoglycosides in others (27) suggest that similar conclusions can be made for these other species. One possible exception is *Serratia marcescens* strain no. 2 in which mutants cross-resistant to β-lactam antibiotics and aminoglycosides were obtained at a frequency of 10^{-6} to 10^{-9} (27). However, a similar mutant in *Salmonella paratyphi* A had multiple alterations in several outer membrane proteins and in LPS (6).

It should be noted that this proposal does not negate the possibility that aminoglycosides are taken up to some extent through porin channels (especially since the mutation in strain SC9252 has a much greater influence on polymyxin than on aminoglycoside susceptibility). However, given the large size (3) of aminoglycosides (approximately 1.8 by 1.0 by 1.0 nm) in comparison with the size of OmpF porin channels (an estimated 1.16 nm in diameter), it might be predicted that aminoglycosides would at best pass very slowly through porin channels. Although the channel of porin OmpF is three- to sixfold more selective for the cation K^+ over the anion Cl^-, this selectivity and the resultant flux of ions through the channel is strongly influenced by cation size (2). Thus, we favor the hypothesis that self-promoted uptake of aminoglycosides is the major route of uptake of these antibiotics across the outer membranes of *E. coli*.

A polymyxin-resistant mutant of *E. coli* which had a mutation in LPS phosphates, which decreased the affinity of LPS for polymyxin and gentamicin (25), caused a fourfold increase in MIC to gentamicin and kanamycin (Table 1). Furthermore, this resistance to gentamicin and kanamycin reflected a reduced ability of these molecules to permeabilize the outer membrane to NPN. These data suggest that self-promoted uptake across the outer membrane is rate limiting for aminoglycoside killing and uptake in *E. coli* since alteration of the LPS interaction site in *E. coli* caused an increased MIC. Consistent with this, Mg^{2+} can increase the MIC of *E. coli* for aminoglycosides (7, 14) and block self-promoted uptake of these molecules (Fig. 5; Table 3). Analogous studies of mutants showed a similar role for self-promoted uptake in *P. aeruginosa* (7, 11, 20), although as mentioned above no such conclusion was possible on the basis of *Salmonella pmrA* mutant studies.

The results of our reevaluation of the influence of aminoglycosides on intrinsic OmpF fluorescence suggest the presence of a divalent cation binding site on the outside of the OmpF porin. Such a site could be involved in interaction of the OmpF porin with LPS through a divalent cation bridge, since porins are known to associate strongly with LPS (24). It will be of some interest to determine whether such sites are accessible to polycations, and other antibiotic compounds, on the surface of cells.

ACKNOWLEDGMENTS

This research was supported by grants to R.E.W.H. from the Canadian Cystic Fibrosis Foundation and the Canadian Bacterial Diseases Network. K.P. was a recipient of a studentship from the Natural Sciences and Engineering Research Council, Ottawa, Ontario, Canada. Z.L. was supported in part as a Visiting Scholar of the People's Republic of China Ministry of Education.

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