

## Involvement of the Outer Membrane in Gentamicin and Streptomycin Uptake and Killing in *Pseudomonas aeruginosa*

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Received 24 December 1980/Accepted 6 March 1981

Induction of a major outer membrane protein, H1, in *Pseudomonas aeruginosa* resulted in decreased susceptibility to gentamicin and streptomycin. Mutants which overproduce protein H1 and cells in which H1 is induced in response to growth conditions had altered kinetics of uptake and killing. It was further demonstrated that gentamicin and streptomycin interact with the outer membrane to permeabilize it to lysozyme and to increase the permeation of a chromogenic  $\beta$ -lactam, nitrocefin. Experiments with inhibitors of aminoglycoside uptake showed that uptake was not required to increase permeability.  $Mg^{2+}$  at 1 mM totally inhibited aminoglycoside-mediated outer membrane permeabilization. We propose that the uptake and killing by these aminoglycosides requires interaction with an  $Mg^{2+}$  binding site at the outer membrane, permitting aminoglycoside uptake into the periplasm.

Aminoglycosides are currently considered to be among the few antibiotics useful in the treatment of infections caused by *Pseudomonas aeruginosa*. The discrepancy between in vitro and in vivo susceptibility is a major problem in aminoglycoside therapy (6, 8). The well-documented antagonism of aminoglycosides by divalent cations (18, 25) is one probable cause, and development of adaptive (nonmutational) resistance in vivo may also play a part. We recently showed that, in *P. aeruginosa*, induction of a major outer membrane protein, H1, results in resistance to polymyxins and ethylenediaminetetraacetate (EDTA) (21). The increase in outer membrane protein H1 was accompanied by a decrease in cell envelope  $Mg^{2+}$ . Mutants which overproduce outer membrane protein H1 also show enhanced resistance to aminoglycosides (21), suggesting the involvement of an outer membrane site, which could be protected by outer membrane protein H1, in the activity of these antibiotics. We report here that *P. aeruginosa* induced for outer membrane protein H1, either as a result of adaptation to growth in low  $Mg^{2+}$  or in mutants, has altered kinetics of aminoglycoside killing and uptake. Furthermore, we demonstrate that aminoglycosides can permeabilize outer membranes to lysozyme and a  $\beta$ -lactam antibiotic by a mechanism which is extremely rapid and independent of aminoglycoside killing and energized uptake but is inhibited by  $Mg^{2+}$ . Our results suggest a mechanism of uptake across the outer membrane different from the previously characterized hydrophilic

(porin-mediated) and hydrophobic pathways (22).

### MATERIALS AND METHODS

**Bacterial strains.** *P. aeruginosa* PAO1 strain H103 and its derivatives H181 and H185 were previously described (21). Strains H181 and H185 were isolated as polymyxin-resistant mutants and were previously shown to demonstrate EDTA resistance and low-level aminoglycoside resistance (21). The *strA* mutant used was PAO1264, obtained from D. Bradley (Memorial University, St. John's, Canada).

**Media and growth conditions.** Liquid growth media used were nutrient broth (Difco Laboratories, Detroit, Mich.) and modified nutrient broth with defined  $Mg^{2+}$  content. Modified nutrient broth was prepared by passing nutrient broth or nutrient broth with 0.5% (wt/vol) NaCl over Chelex-100 columns (Bio-Rad Laboratories, Mississauga, Canada) to extract divalent cations. The Chelex-treated medium was adjusted to pH 6.95 before autoclaving and subsequently supplemented with 1.0 ml of trace ion mixture (1.78 mM FeCl<sub>3</sub>, 1.62 mM MnCl<sub>2</sub>, 2.45 mM CaCl<sub>2</sub>, 13.91 mM ZnCl<sub>2</sub>, 4.69 mM H<sub>3</sub>BO<sub>3</sub>, 0.67 mM CoSO<sub>4</sub>; sterilized by shaking with chloroform) per liter and with MgSO<sub>4</sub> to the desired level. Before the addition of  $Mg^{2+}$ , the medium was virtually unable to support growth. Medium supplemented with 0.5 mM  $Mg^{2+}$  allowed growth to levels as high as those obtained in untreated nutrient broth and was termed  $Mg^{2+}$  sufficient.  $Mg^{2+}$ -deficient medium contained 0.02 mM  $Mg^{2+}$ , which allowed growth to 50 to 60% of the growth yield in  $Mg^{2+}$ -sufficient medium. The growth rate was not affected by the  $Mg^{2+}$  levels used. The addition of NaCl to nutrient broth before Chelex treatment decreased the batch-to-batch variation of growth rate in the modified medium. Cultures were grown at 37°C with vigorous

shaking. Growth curves were determined in each medium, and it was established that an optical density at 600 nm ( $OD_{600}$ ) of 0.40 to 0.60 represented mid-logarithmic-phase growth. Viable counts were performed by plate counts on proteose peptone no. 2 agar in 3-ml overlays of proteose peptone no. 2 containing 1.5% agar.

**Antibiotics.** Gentamicin sulfate was a gift from Schering Corp. (Pointe Claire, Canada). Streptomycin sulfate, tetracycline hydrochloride, chloramphenicol, and benzyl penicillin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Nitrocefin was kindly provided by C. O'Callaghan (Glaxo Group Research Ltd., Middlesex, U.K.). Immobilized streptomycin was prepared by following the method used by La Porte et al. to attach polymyxin to agarose beads (16).

**Killing by aminoglycosides.** Bacteria were grown in  $Mg^{2+}$ -deficient or -sufficient medium to an  $OD_{600}$  of 0.5 to 0.6. Aminoglycoside treatment was carried out by diluting the cells 100-fold into prewarmed (37°C) assay medium (modified nutrient broth with additives as described below for individual experiments) containing streptomycin or gentamicin. The assay medium was held at 37°C with shaking, and at defined intervals, samples were removed, diluted in assay medium, and plated for viable counts.

**Streptomycin uptake assays.** [ $^3H$ ]dihydrostreptomycin (1.2 Ci/mmol; Amersham Searle, Oakville, Canada) was diluted by the addition of nonradioactive streptomycin to a specific activity of 50  $\mu$ Ci/mg of streptomycin. Cells were grown by shaking at 37°C to an  $OD_{600}$  of 0.5 to 0.6 in unmodified nutrient broth, and the uptake assay was started by the addition, to the growing cells, of streptomycin to the desired final concentrations. At defined times, 1.0-ml samples were removed, and the cells were collected by filtration onto nitrocellulose filters (0.45  $\mu$ m; Millipore Corp., Bedford, Mass.) which had been presoaked in 0.1 M LiCl before use. The filtered cells were then washed twice with 3 ml of 0.1 M LiCl, dried at 60°C for 1 h, and then assayed for radioactivity in a toluene-based scintillant. This method was based on those of Høltje (12) and Bryan and Van Den Elzen (4) for streptomycin uptake assays. Trial experiments demonstrated that the filter preparation and washing techniques were critical, as previously suggested (4, 12), but that either of the above-referenced methods was satisfactory. Nutrient broth rather than modified nutrient broth was the growth medium of choice since use of the latter medium resulted in extremely variable binding of streptomycin to filters.

**Aminoglycoside-promoted lysozyme lysis.** Bacteria were grown to an  $OD_{600}$  of 0.4 to 0.5 in  $Mg^{2+}$ -sufficient modified nutrient broth, harvested by centrifugation at 25°C, resuspended in prewarmed modified nutrient broth without added  $Mg^{2+}$  at an  $OD_{600}$  of about 0.8, and maintained at 37°C with shaking. Lysozyme (Sigma Chemical Co.; final concentration, 20  $\mu$ g/ml) and streptomycin (final concentration, 50 to 200  $\mu$ g/ml) or gentamicin (final concentration, 2 to 50  $\mu$ g/ml) were added simultaneously to cells, and 1.0-ml samples were withdrawn at frequent intervals for  $OD_{600}$  estimations in a Perkin-Elmer 124 spectrophotometer. During the experiment there was no evidence of cell clumping. In experiments in which KCN, dini-

trophenol, or sodium azide (all from Sigma Chemical Co. and used at final concentrations of 1 mM, 5 mM, or 0.1% [wt/vol], respectively) was used, the addition was made 2 min before the other additions. Chloramphenicol (to 500  $\mu$ g/ml), tetracycline hydrochloride (to 100  $\mu$ g/ml), or  $MgSO_4$  (1 mM) was added, when necessary, at the same time as lysozyme and the aminoglycosides.

**Aminoglycoside-mediated enhancement of nitrocefin permeability.** Bacteria were grown to an  $OD_{600}$  of 0.5 to 0.6 on  $Mg^{2+}$ -sufficient medium in the presence of 0.2 mg of benzyl penicillin per ml to induce chromosomal  $\beta$ -lactamase production. Cells were harvested as above, washed in sodium phosphate buffer (0.1 M, pH 7.0), and resuspended in the same buffer at an  $OD_{600}$  of about 1.0. To 0.1 ml of cell suspension, 1  $\mu$ l of a gentamicin solution was added to give a final concentration of 10 to 100  $\mu$ g/ml. After 2 min at 25°C, 0.6 ml of nitrocefin (250  $\mu$ g/ml) was added, and hydrolysis of the nitrocefin was monitored spectrophotometrically by measurement of the increase in absorbance at 540 nm.

**Characterization of outer membrane proteins.** Separation of outer and inner membranes and characterization of outer membrane proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out as previously described (10, 21).

## RESULTS

**Characterization of Chelex-treated nutrient broth.** A complex medium with defined  $Mg^{2+}$  content was devised to allow measurement of the effects of  $Mg^{2+}$  levels during growth on subsequent aminoglycoside activity. Phosphate-buffered minimal medium was judged unsuitable, as high phosphate was found to inhibit aminoglycoside activity. Tris(hydroxymethyl)aminomethane (Tris)-hydrochloride-buffered medium was unsuitable because Tris itself can act on the outer membrane in a manner similar to aminoglycosides (see below). Thus, nutrient broth was chosen as a starting medium because of its relatively low  $Mg^{2+}$  content (20) and prior use in aminoglycoside uptake experiments (4). Extraction of nutrient broth was effective in reducing the level of divalent cations to quantities below those required for measurable growth. After the replacement of divalent cations other than  $Mg^{2+}$  by addition of a trace ions mixture, growth yields were dependent on the level of  $Mg^{2+}$  added, up to 0.2 mM  $Mg^{2+}$ . The levels of  $Mg^{2+}$  required were comparable to those of the phosphate-buffered minimal medium used previously (21). Sodium dodecyl sulfate-gel electrophoresis showed that the outer membrane protein patterns in modified nutrient broth were similar to those seen in minimal medium (21). Thus, wild-type cells (H103) grown in  $Mg^{2+}$ -deficient nutrient broth had outer membrane protein H1 induced to levels up to 116-fold higher than cells grown in  $Mg^{2+}$ -sufficient medium. The

polymyxin B-resistant mutants H181 and H185 produced high levels of protein H1 in both media. In both the mutants and the induced bacteria, outer membrane protein H1 appeared to be the cellular protein present in the largest quantity.

**Effects of growth conditions and outer membrane protein H1 induction on resistance to killing by aminoglycosides.** We previously demonstrated (21) that the polymyxin-resistant isolates H181 and H185 had minimal inhibitory concentrations of gentamicin and streptomycin fourfold higher than the parent strain H103. It was suggested that the high levels of outer membrane protein H1 in H181 and H185 were responsible for the enhanced resistance to aminoglycosides. To confirm this, H103 and its derivatives were grown in either  $Mg^{2+}$ -sufficient or  $Mg^{2+}$ -deficient modified nutrient broth and subsequently resuspended and tested for aminoglycoside killing in a common assay medium (Fig. 1 and 2). The parent strain H103 grown in  $Mg^{2+}$ -sufficient medium was susceptible to a wide range of concentrations of gentamicin (Fig. 1). Growth of H103 in  $Mg^{2+}$ -deficient medium, which induces outer membrane protein H1, resulted in a marked decrease in sensitivity to killing at all gentamicin concentrations. The level of resistance achieved was similar to that seen for the polymyxin-resistant isolates H181 (Fig. 1) and H185 (data not shown) grown under  $Mg^{2+}$ -sufficient conditions.

Altered killing kinetics were also observed.

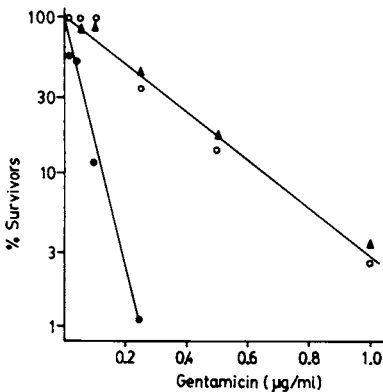


FIG. 1. Comparison of gentamicin killing in the wild-type strain H103 and in the outer membrane protein H1-overproducing mutant H181 after growth in  $Mg^{2+}$ -sufficient or -deficient medium. The common assay medium was modified nutrient broth without added NaCl or  $Mg^{2+}$ . Viable counts were performed after 30-min exposure to the given gentamicin concentrations. Symbols:  $\blacktriangle$ , H181 grown in 0.5 mM  $Mg^{2+}$ ;  $\bullet$ , H103 grown in 0.5 mM  $Mg^{2+}$ ;  $\circ$ , H103 grown in 0.02 mM  $Mg^{2+}$ .

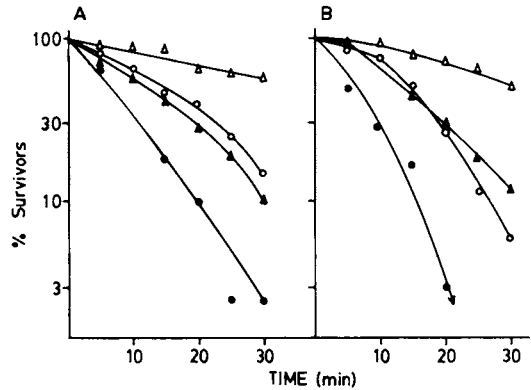


FIG. 2. Kinetics of killing by gentamicin (A) and streptomycin (B). Assay medium was modified nutrient broth (A) with 0.5 mM  $Mg^{2+}$ , containing 0.5  $\mu$ g of gentamicin per ml, and (B) with 0.5% (wt/vol) NaCl and no added  $Mg^{2+}$ , containing 8  $\mu$ g of streptomycin per ml. Symbols:  $\circ$ , H103 grown in 0.02 mM  $Mg^{2+}$ ;  $\bullet$ , H103 grown in 0.5 mM  $Mg^{2+}$ ;  $\Delta$ , H181 grown in 0.02 mM  $Mg^{2+}$ ;  $\blacktriangle$ , H181 grown in 0.5 mM  $Mg^{2+}$ .

Strain H103 grown in  $Mg^{2+}$ -sufficient medium showed a rapid loss of viability when exposed to gentamicin in assay medium with 0.5 mM  $Mg^{2+}$  (Fig. 2A). The same strain tested in the same assay medium was more resistant to killing by gentamicin when grown before the killing assay in  $Mg^{2+}$ -deficient medium. Strains H181 and H185 were quite resistant regardless of growth conditions, although their resistance could be augmented by growth in the  $Mg^{2+}$ -deficient medium. Comparable results were obtained with streptomycin (Fig. 2B), although higher levels of antibiotic were required. The presence or absence of  $Mg^{2+}$  in the assay medium did not affect the relative resistance of cells grown in  $Mg^{2+}$ -sufficient or -deficient medium. However, addition of  $Mg^{2+}$  to the assay medium did increase resistance in all strains irrespective of the growth conditions. Addition of 0.5% (wt/vol) NaCl to the assay medium also had a slight protective effect, but did not affect the above differences in susceptibility (data not shown). In previous studies (21), and using these media, the parent strain H103 grown on  $Mg^{2+}$ -deficient medium and its mutant H181 grown on  $Mg^{2+}$ -sufficient medium were shown to have very similar outer membrane protein H1 levels. The data presented in Fig. 1 and 2 demonstrate that these strains under the above conditions were killed at the same rate by aminoglycosides, strongly suggesting that the effects described in Fig. 1 and 2 were related to the relative levels of outer membrane protein H1 and correspondingly, inversely related to the cell envelope  $Mg^{2+}$  levels [21]. In addition, growth of the mutant strain H181 un-

der  $Mg^{2+}$ -deficient conditions led to a moderate (twofold) increase in protein H1 levels (21) (compared with the same strain grown under  $Mg^{2+}$ -sufficient conditions). Correspondingly, strain H181 pregrown under  $Mg^{2+}$ -deficient conditions was more resistant to killing by gentamicin and streptomycin (Fig. 2).

**Streptomycin uptake in susceptible and resistant strains.** The pattern of streptomycin uptake in both strains H103 and H181 (illustrated by a typical experiment in Fig. 3) followed three-phase kinetics, as described by Bryan and Van Den Elzen for other strains (4): an instantaneous binding phase, an early slow uptake phase (EDP-I), and a later rapid uptake phase (EDP-II). We performed an extensive series of experiments in an attempt to demonstrate differences in the apparent amount of streptomycin binding to cyanide-treated i.e., non-streptomycin-transporting [5] or untreated wild-type strain H103 or mutant H181 cells. For five separate experiments done at eight different concentrations of streptomycin (data not shown), statistical analysis of the data suggested that there was no significant difference ( $P > 0.5$ ) in aminoglycoside binding to the two strains. Thus, any apparent differences in streptomycin binding to the two strains (e.g., as seen at 10  $\mu\text{g}/\text{ml}$

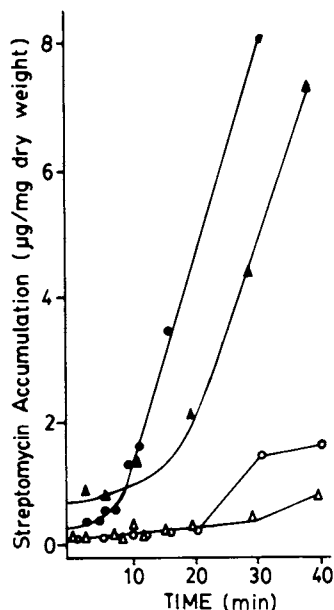


FIG. 3. Uptake of [ $^3\text{H}$ ]streptomycin at two concentrations by the wild-type strain H103 and the outer membrane protein H1-overproducing strain H181. Symbols:  $\circ$ , H103, 2  $\mu\text{g}$  of streptomycin per ml;  $\bullet$ , H103, 10  $\mu\text{g}$  of streptomycin per ml;  $\Delta$ , H181, 2  $\mu\text{g}$  of streptomycin per ml;  $\blacktriangle$ , H181, 10  $\mu\text{g}$  of streptomycin per ml.

in Fig. 3) were shown by more careful analysis not to be significant. Scatchard analysis of the data from one experiment suggested on the order of  $2 \times 10^7$  to  $5 \times 10^7$  potential binding sites for streptomycin per cell. We consider that the large number of nonspecific binding sites on the cell (since there are only about  $2 \times 10^6$  to  $4 \times 10^6$  molecules of lipopolysaccharide per *P. aeruginosa* cell) and the high-background filter adsorption of streptomycin even under the stringent washing procedures used (see Materials and Methods) together obscured expected differences in aminoglycoside binding.

The major alteration in the kinetics of streptomycin uptake seen in the resistant strain, H181, was that at all concentrations of antibiotic used transition from the early slow phase of uptake (EDP-I) to the later rapid phase (EDP-II) was delayed in the resistant strain (Fig. 4). This difference was consistently observed in seven separate experiments, each using several levels of antibiotic.

**Permeabilization of the outer membrane by aminoglycosides.** The ability of aminoglycosides and Tris-hydrochloride to alter outer membrane permeability was investigated by examining the effect of these agents in promoting lysis by lysozyme (Fig. 5 and 6). Lysozyme is normally unable to penetrate the outer membrane of gram-negative bacteria to reach its site of activity, the peptidoglycan. As a control, we confirmed that lysozyme alone did not cause lysis of our strains. Treatment with gentamicin alone resulted in a slow decrease in  $\text{OD}_{600}$ , but only after a prolonged delay (Fig. 5A). The combination of lysozyme and gentamicin however, led to very rapid cell lysis. At high levels of gentamicin (10 to 25  $\mu\text{g}/\text{ml}$ ), there was a 90% drop in absorbance within 1 min.

Conditions which are known to block transport of aminoglycosides (5, 24) did not interfere with the permeabilization of the outer membrane to lysozyme (Fig. 5). Lysis by gentamicin and lysozyme was very similar in the presence or absence of cyanide, which is known to inhibit aminoglycoside uptake, probably at the level of the cytoplasmic membrane. In contrast, the lysis mediated by gentamicin in the absence of lysozyme was 100% inhibited by cyanide (Fig. 5A). The uncouplers dinitrophenol and sodium azide also had no effect on gentamicin-lysozyme lysis (data not shown), indicating that the process did not require an energized membrane. Chloramphenicol, which is known to prevent aminoglycoside killing (13, 14, 24) and eliminate the late rapid phase of aminoglycoside uptake (5), also failed to inhibit lysis by gentamicin and lysozyme (Fig. 5B). Because H103 is quite resistant to chloramphenicol compared with most wild-

type *P. aeruginosa* strains, we also examined the effect of tetracycline as an alternative inhibitor of protein synthesis and aminoglycoside killing (24; Fig. 5C). Tetracycline in combination

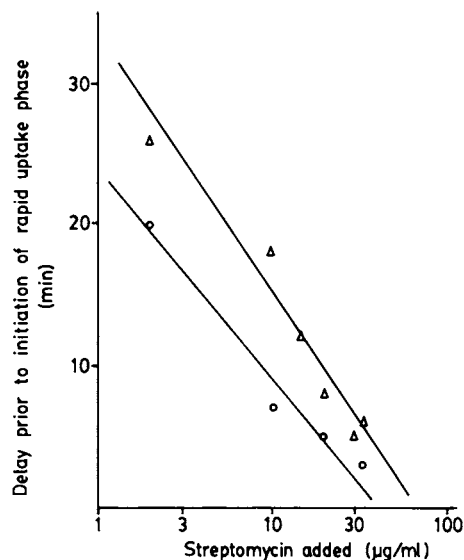


FIG. 4. Time required for initiation of rapid uptake of streptomycin (EDP-II) in the wild-type strain H103 (○) and the outer membrane protein H1-over-producing strain H181 (△). The points represent the means of three experiments; the given lines were drawn by linear regression analysis of the points with correlation coefficients ( $r^2$ ) of 0.98 for H103 and 0.97 for H181.

with lysozyme caused some lysis, but at a relatively low rate and after a slight delay. However, it clearly did not interfere with the rapid gentamicin-promoted lysozyme lysis. Ribosomally altered *strA* mutants are resistant to very high levels of streptomycin and do not show the late rapid uptake phase of streptomycin (4). Streptomycin-mediated lysozyme lysis was indistinguishable in the wild-type PAO1 strain H103 and a *strA* mutant of PAO1 (data not shown).

As cyanide prevents aminoglycoside uptake (4, 5) but did not prevent its effect on outer membrane permeability, 1 mM KCN was used in subsequent experiments. Figure 6 shows the lytic activity of aminoglycosides and Tris in the presence of cyanide. With gentamicin, lysis occurred very rapidly and the percentage of cells lysed was proportional to the amount of aminoglycoside added. The same effect could be obtained with streptomycin, but as in the killing experiment, much higher levels of this antibiotic were required. High concentrations of Tris (0.1 to 0.3 M) were also able to promote lysis by lysozyme, although Tris-lysozyme lysis was relatively slow. We also tried high levels of the polyamines spermine tetrahydrochloride (300 µg/ml) or spermidine trihydrochloride (500 µg/ml) with lysozyme. These combinations did not result in lysis. We were unable to obtain promotion of lysozyme lysis with streptomycin bound to agarose beads through a 1-nm spacer, although such immobilized streptomycin did somewhat inhibit cell growth.

Aminoglycoside-promoted lysozyme lysis

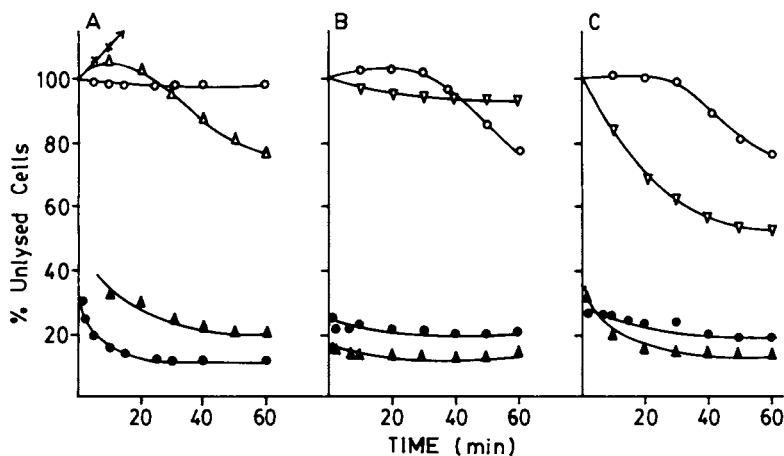


FIG. 5. Effects of inhibitors of gentamicin uptake on gentamicin (25 µg/ml)-promoted, lysozyme (20 µg/ml)-mediated lysis of the wild-type strain H103. (A) Effect of cyanide (1 mM): ×, no additions; △, gentamicin only; ○, gentamicin and KCN; ▲, gentamicin and lysozyme; ●, gentamicin, KCN and lysozyme. (B) Effect of chloramphenicol (500 µg/ml): ▽, chloramphenicol and lysozyme; ○, gentamicin and chloramphenicol; ▲, gentamicin and lysozyme; ●, gentamicin, chloramphenicol, and lysozyme. (C) Effect of tetracycline (100 µg/ml): ▽, tetracycline and lysozyme; ○, gentamicin and tetracycline; ▲, gentamicin and lysozyme; ●, gentamicin, tetracycline, and lysozyme.

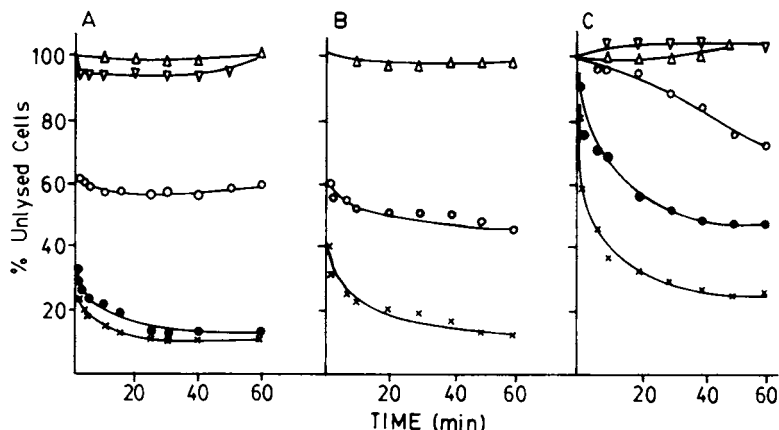


FIG. 6. Promotion of wild-type strain H103 lysis by lysozyme (20 µg/ml) using gentamicin (A), streptomycin (B), or Tris-hydrochloride (C) in the presence of 1 mM KCN. Symbols: (A) Δ, controls (25 µg of gentamicin per ml in the absence of lysozyme or lysozyme in the absence of gentamicin); ∇, 2 µg of gentamicin per ml; ○, 5 µg of gentamicin per ml; ●, 10 µg of gentamicin per ml; ×, 25 µg of gentamicin per ml. (B) Δ, Controls (150 µg of streptomycin per ml in the absence of lysozyme or lysozyme in the absence of streptomycin); ○, 75 µg of streptomycin per ml; ×, 150 µg of streptomycin per ml. (C) ∇, 500 mM Tris-hydrochloride in the absence of lysozyme, as a control; Δ, lysozyme but no Tris-hydrochloride; ○, 200 mM Tris-hydrochloride; ●, 300 mM Tris-hydrochloride; ×, 500 mM Tris-hydrochloride.

could also be demonstrated for cells resuspended in 10 mM Tris-hydrochloride, pH 7.4, or 10 mM HEPES (*N*-2-hydroxyethylpiperazine-2-ethanesulfonic acid) buffer, pH 7.2, in the absence of a carbon source. This amount of Tris-hydrochloride was insufficient to promote lysozyme lysis in the absence of aminoglycoside (Fig. 6C and 7). The addition of 1 mM  $Mg^{2+}$  to such cells completely inhibited gentamicin-mediated lysozyme lysis of cells (Fig. 7), although some osmotic effects remained, as demonstrated by the slight swelling of cells. The extent of the inhibition observed with 1 mM  $Mg^{2+}$  was similar to the inhibition of aminoglycoside uptake observed by Bryan and Van Den Elzen after addition of divalent cations during transport experiments (4).

Permeabilization of the outer membrane by gentamicin in strain H103 was also examined by measuring the hydrolysis of a chromogenic  $\beta$ -lactam, nitrocefin. An increased rate of hydrolysis in intact nongrowing cells would indicate increased permeation of the  $\beta$ -lactam through the outer membrane to the periplasmic  $\beta$ -lactamase. The rate of hydrolysis could be increased 3.5-fold over that of untreated strain H103 cells by pretreatment with 100 µg of gentamicin per ml and 1.7-fold with 10 µg of gentamicin per ml (the actual concentrations of gentamicin present during the assay were 15 and 1.5 µg/ml, respectively). Treatment with 50 mM EDTA increased the rate of hydrolysis 10-fold.

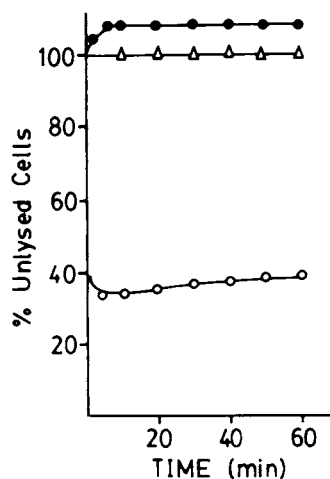


FIG. 7. Inhibition by  $Mg^{2+}$  of gentamicin-promoted, lysozyme-mediated lysis of wild-type strain H103. Cells were resuspended in 10 mM Tris-hydrochloride (pH 7.4) with the noted additions. Symbols: Δ, control with 1 mM  $MgSO_4$ , no lysozyme or gentamicin added; ○, 15 µg of gentamicin and 20 µg of lysozyme per ml in the presence or absence of 1 mM KCN; ●, as for ○ with the addition of 1 mM  $MgSO_4$ .

## DISCUSSION

The results of this study demonstrate that the outer membrane of *P. aeruginosa* is a major determinant of aminoglycoside activity. Re-

cently (21), we established that outer membrane protein H1 overproduction is accompanied by a reduction in cell envelope  $Mg^{2+}$  content, both in the mutants H181 and H185, which constitutively overproduce protein H1, and in wild-type cells overproducing protein H1 in response to low  $Mg^{2+}$ . The results suggested a linear reciprocal relationship between the amount of protein H1 and cell envelope  $Mg^{2+}$  levels. In both mutants and wild type, high levels of protein H1 were associated with increased resistance to killing and lysis by polymyxin and EDTA (21). We have now shown that protein H1-overproducing cells are also relatively resistant to killing by aminoglycosides (Fig. 1 and 2). Our previous data (21) suggested that EDTA and cationic antibiotics such as polymyxins and aminoglycosides act by removing or replacing  $Mg^{2+}$  at a site on the lipopolysaccharide. This suggests that protein H1 exerts its effect by replacing  $Mg^{2+}$  at this site, protecting it from these agents.

The increase in resistance to aminoglycosides conferred by protein H1 is smaller than that seen for polymyxins. This could be due to alternative binding or uptake sites for aminoglycosides, a requirement for fewer binding sites for aminoglycoside activity, or to a lower affinity of aminoglycosides for the proposed lipopolysaccharide binding site. Schindler and Osborn (23) have demonstrated that polymyxin has a higher affinity for *Salmonella typhimurium* lipopolysaccharide than does  $Mg^{2+}$ , whereas others have demonstrated that moderately high levels of  $Mg^{2+}$  are necessary to inhibit polymyxin activity (6). In the case of aminoglycosides, antagonism by  $Mg^{2+}$  occurs at quite low  $Mg^{2+}$  levels, and this competition tends to mask the protective effects of protein H1 when the protein is induced by growth in low  $Mg^{2+}$ . Thus, in minimal inhibitory concentration measurements of aminoglycoside susceptibility, *P. aeruginosa* cells in low  $Mg^{2+}$  are apparently more susceptible than cells in high  $Mg^{2+}$ . When the differential competitive effects of  $Mg^{2+}$  are eliminated by comparison of loss of viability in a common assay medium (Fig. 1 and 2), cells grown in  $Mg^{2+}$ -deficient medium are actually more resistant to aminoglycoside killing than cells grown in  $Mg^{2+}$ -sufficient medium.

Aminoglycoside uptake in both *Escherichia coli* and *P. aeruginosa* has been shown to occur in three consecutive phases (4,5): an initial rapid electrostatic binding, followed by an early slow uptake phase (EDP-I) and a later rapid uptake phase (EDP-II). The binding is energy independent, whereas the two latter phases are energy requiring and occur only in presence of an en-

ergized cytoplasmic membrane and electron transport (4, 5). EDP-II may coincide with or follow the onset of loss of viability, since both EDP-II and lethality can be inhibited with chloramphenicol (4, 13) and do not occur in ribosomally resistant (*strA*) strains (4). Alteration of ribosomal affinity in other mutants also affects uptake (1). Comparison of streptomycin uptake in wild-type strains with uptake in resistant mutants which overproduce protein H1 showed that in the resistant strains the late rapid phase EDP-II was always delayed (Fig. 3 and 4). It would thus appear that the outer membrane has a critical influence on the events required to initiate EDP-II. Somewhat similar delays in the onset of EDP-II have been observed for certain *E. coli* mutants with altered ribosomal affinity to aminoglycosides (1). However, since our mutant was selected for resistance to polymyxin and EDTA, and since ribosomal effects are usually specific to given aminoglycosides, we consider it to be unlikely that our mutants also have ribosomal alterations. Furthermore, we previously demonstrated (21) that single-step revertants of strains H181 and H185 to polymyxin susceptibility had low levels of protein H1 on  $Mg^{2+}$ -sufficient medium and had wild-type aminoglycoside and EDTA susceptibilities, suggesting that the different phenotypic alterations in the mutants had a common basis.

The increase in resistance seen in outer membrane protein H1-overproducing strains cannot be attributed to a general decrease in outer membrane permeability. The major pore-forming protein (porin or protein F) is not significantly reduced in amount in protein H1-overproducing strains (this study; 21). Furthermore, susceptibility to both carbenicillin and tetracycline (which use the so-called hydrophilic [porin-mediated] pathway in *E. coli* [22]) is not altered in these strains (21), and their growth rate on  $Mg^{2+}$ -deficient media is unaffected (this study; 21), suggesting that the porin is functionally normal. This suggests that the hydrophilic pathway (22) of passive permeation through the hydrophilic pores formed by porin (11, 22) may not be the major route taken by aminoglycoside antibiotics in *P. aeruginosa*. Although the large pore size of *P. aeruginosa* (11) would not be expected to offer any barrier to permeation of such antibiotics, there is evidence that most of the pores at any given time are not in an active, open state (R. Benz and R. E. W. Hancock, manuscript in preparation). Preliminary studies of a severely porin-deficient *P. aeruginosa* strain isolated in our laboratory has shown no decrease in aminoglycoside susceptibility. However, it is

possible that in other organisms in which a greater proportion of porins are in the active state (2) and there are less  $Mg^{2+}$  binding sites on the cell surface (3), the hydrophilic pathway of antibiotic uptake may offer an alternative, efficient means of streptomycin permeation (9). Streptomycin action in such organisms should be, and is, less affected by  $Mg^{2+}$  antagonism (18).

An alternative explanation for our results might be that protein H1 is a magnesium-binding outer membrane protein which also binds gentamicin and streptomycin and thus specifically limits access of aminoglycosides to porin. However, as described in Results, we could demonstrate no significant differences in the binding of streptomycin to our mutant strain H181 with high protein H1 levels when compared with our wild-type strain H103. In addition, at a streptomycin concentration with which we could demonstrate a large difference in killing of strains H181 and H103 (Fig. 2B) less than 0.5% of the added streptomycin became bound to cyanide-treated cells, suggesting that binding did not significantly alter the effective concentration of streptomycin in the medium. Also, we previously demonstrated that mutant strains have less  $Mg^{2+}$  in their outer membranes, suggesting that protein H1 is probably not a specific  $Mg^{2+}$ -binding protein (21). Finally, the above alternative does not explain aminoglycoside-mediated permeabilization of outer membranes or the unusually high  $Mg^{2+}$  antagonism of aminoglycosides in wild-type *P. aeruginosa* (18). Thus, the above alternative model seems unlikely, although we cannot rigorously exclude that binding of aminoglycosides to protein H1 contributes to the phenotype of the mutants.

The ability of aminoglycosides to interact with the outer membrane and promote a significant alteration in its permeability was shown by both cell lysis in the presence of lysozyme and aminoglycosides and increased hydrolysis of nitrocefin in the presence of gentamicin. Lysozyme is normally inactive on gram-negative bacteria as it is unable to penetrate the outer membrane to reach its site of activity, the peptidoglycan. Previous studies have demonstrated that either polymyxin or EDTA can permeabilize the outer membrane to lysozyme (3, 15, 16). In this study we have shown that gentamicin, streptomycin, and, to a lesser extent, Tris-hydrochloride overcame this penetration barrier, allowing lysozyme to attack the peptidoglycan and lyse the cells. Lysozyme itself is known to bind to outer membranes (7), and this ability may contribute to the efficiency of its permeation in aminoglycoside-treated cells. This is further suggested by the relatively inefficient gentamicin-mediated per-

meabilization of outer membranes to nitrocefin, a chromogenic  $\beta$ -lactam. In fact, this implies that aminoglycoside-mediated permeabilization has some specificity for cationic substrates such as lysozyme and aminoglycosides (see below).

The permeabilization we observe cannot be attributed to aminoglycoside killing as we have shown that it occurs under conditions in which aminoglycosides are known not to be transported or lethal (5, 14), such as in chloramphenicol- or KCN-treated cells, and can occur even in ribosomally resistant *strA* strains. This differentiates the effects we describe from the extreme outer membrane disruptions in aminoglycoside-treated cells, as has been seen in electron microscope studies (15). Such disruptions occur only under conditions resulting in cell death.

Aminoglycosides are clearly able to promote the passage of other molecules through the outer membrane, and it seems likely that they are also capable of promoting their own transport. We have observed that the amounts of gentamicin and streptomycin required to cause permeabilization of outer membranes to lysozyme (Fig. 6) are 10- to 20-fold higher than the concentrations resulting in rapid cell killing (Fig. 2). This may be explained by the different sizes of the molecules involved, since killing would involve permeabilization to the aminoglycosides themselves, which are molecules of 500 to 600 daltons, whereas aminoglycoside-promoted lysozyme lysis would involve permeabilization to lysozyme, a protein of 14,000 daltons. In the latter case, a more extreme disruption of the outer membrane would be required. We propose that aminoglycoside uptake and killing requires interaction with an  $Mg^{2+}$  binding site at the outer membrane. This interaction promotes uptake of the antibiotic into the periplasm, permitting further transport at the level of the cytoplasmic membrane. We observed that  $Mg^{2+}$  was able to completely inhibit aminoglycoside-mediated permeabilization to lysozyme. This inhibition, coupled with the results of killing experiments with protein H1-overproducing strains, strongly suggests that the site of aminoglycoside activity at the outer membrane is an  $Mg^{2+}$  binding site. Competition at the level of the outer membrane may well explain the unusually high antagonism of aminoglycosides by divalent cations (18, 25) in *P. aeruginosa*, although it is probable that other sites of competition also exist. Our results also suggest that the ability of aminoglycosides to promote permeabilization of the outer membrane may in fact be a major determinant in their activity. We have shown that gentamicin is more active than streptomycin in inducing permeability to lysozyme. This difference is



closely reflected by their relative effectiveness as measured in killing assays or minimal inhibitory concentration determinations. In this light, it is interesting that some of the new antipseudomonal aminoglycosides differ from their parent compounds largely in their efficiency of transport (17) rather than in their inhibition of ribosomal function. Improved ability to interact with the outer membrane could, in part, account for their increased transport.

#### ACKNOWLEDGMENT

We acknowledge the financial assistance of the Canadian Cystic Fibrosis Foundation.

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