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Alteration of Susceptibility to EDTA, Polymyxin B and Gentamicin in *Pseudomonas aeruginosa* by Divalent Cation Regulation of Outer Membrane Protein H1

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Induction of outer membrane protein H1 in *Pseudomonas aeruginosa* results in decreased susceptibility to aminoglycosides, polymyxin B, and EDTA. We have previously shown that protein H1 can become the major cellular protein in cells grown in low (0·02 mM) Mg^{2+}. The induction of protein H1 was prevented by supplementation of low Mg^{2+} medium with Mg^{2+}, Ca^{2+}, Mn^{2+}, or Sr^{2+} (each at 0·5 mM), but not with Zn^{2+}, Ba^{2+}, Sn^{2+}, Al^{3+} or Na^{+} (each at 0·5 mM). Only cells grown in the presence of those cations which failed to prevent H1 induction were resistant to the cationic antibiotics, polymyxin B and gentamicin, and to chelators of divalent cations. Cells grown in Ca^{2+}, but not in Mg^{2+}, were susceptible to outer membrane permeabilization by the Ca^{2+} specific chelator EGTA, whereas both were susceptible to EDTA. In agreement with this, cells grown in Mg^{2+}, Ca^{2+}, Mn^{2+}, or Zn^{2+} showed enhanced levels of these cations respectively as their major cell envelope-associated cation. When cells were shifted from low to high Mg^{2+} medium, the time course of the decrease in the levels of protein H1 correlated well with the increase in sensitivity to EDTA and polymyxin B. These results support the hypothesis that protein H1 acts to replace divalent cations at a critical outer membrane site attacked by cationic antibiotics and chelators of divalent cations, and suggest that only a small proportion of the total divalent cation-binding sites in the outer membrane are susceptible to attack by these agents.

INTRODUCTION

It has recently become apparent that alterations in growth conditions can cause substantial changes in the protein composition of the outer membrane of *Pseudomonas aeruginosa* (Gilleland *et al.*, 1974; Gilleland & Lyle, 1979; Hancock & Carey, 1980; Nicas & Hancock, 1980; Hancock *et al.*, 1982). Some of these changes affect susceptibility to antimicrobial agents. Growth of susceptible *P. aeruginosa* in Mg^{2+}-limited medium has long been known to result in resistance to EDTA and polymyxin (Brown & Melling, 1969). We have shown that growth in low Mg^{2+} leads to a large increase in the level of outer membrane protein H1 (Nicas & Hancock, 1980). In cells grown in low Mg^{2+}, as well as in mutants which overproduce this protein, induction of protein H1 correlates with decreased levels of cell envelope Mg^{2+}. Cells overproducing protein H1 show enhanced resistance to EDTA, polymyxins, and aminoglycosides (Nicas & Hancock, 1980; Hancock *et al.*, 1981), suggesting that an outer membrane site is involved in the activity of these agents. We have hypothesized that protein H1 acts to replace divalent cations at a site on the lipopolysaccharide, and protects this site from attack by chelators of divalent cations and cationic antibiotics, such as polymyxin B and gentamicin, which may compete for this site (Nicas & Hancock, 1980; Hancock *et al.*, 1981; Hancock, 1981). In the study reported here, we have compared the effects of different divalent cations on production of protein H1, and examined the correlation between levels of protein H1 and divalent cation concentration of cell envelopes and susceptibility to polymyxin B, gentamicin and to chelators.

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METHODS

Bacterial strains. Pseudomonas aeruginosa PAO1 strain H103 was used throughout. RP1 was introduced into this strain by conjugation with *Escherichia coli* UB1636 (RP1) (kindly provided by P. M. Bennet, University of Bristol, Bristol, U.K.), using selection on 200 μg tetracycline ml^{-1} H103 (RP1) was maintained on medium containing 20 μg tetracycline ml^{-1}.

Media and growth conditions. The growth medium used was BM2 minimal medium (Gilleland *et al.*, 1974) as previously described (Nicas & Hancock, 1980), containing 20 mM-succinate. Divalent cations and sodium were added as chloride salts in the amounts specified in the text and in Tables 1 and 2. Cultures were grown at 37 °C with vigorous aeration.

Antibiotic and chelator susceptibility testing. All assays used have been described previously (Nicas & Hancock, 1980; Hancock *et al.*, 1981). The concentrations of antibiotics and chelators used were the optimum concentrations giving measurable killing of cells grown at all cation concentrations tested. Testing of lysis by EDTA/Tris and EGTA/Tris was carried out on cells in mid-exponential phase growth (A_{600} 0·30–0·60), which were centrifuged down at 25 °C and resuspended in 10 mM-EDTA or EGTA and 10 mM-Tris/HCl buffer (pH 8·5) at 25 °C. A_{600} was read at timed intervals. To test killing by EDTA/Tris, EGTA/Tris, and polymyxin, mid-exponential cells were centrifuged down and resuspended at 100-fold dilution in 30 mM-sodium phosphate buffer pH 7·0, with 75 μg polymyxin B ml^{-1} (Sigma; 8000 units mg^{-1}), or in either 10 mM-EDTA or 10 mM-EGTA in 10 mM-Tris/HCl, pH 8·5. After 5 min incubation at 25 °C, cells were diluted into phosphate buffer and plated for viable counts on proteose peptone no. 2 agar plates. No washing procedures were attempted due to the known competitive inhibition of polymyxin action by cations (Brown, 1975), but control experiments demonstrated that the extent of killing was related to the level of polymyxin added and the time of treatment for cells grown at given cation concentrations. To test killing by gentamicin (gentamicin sulphate, a gift from Schering Corp., Pointe Claire, Canada) the procedure was modified slightly because gentamicin is active only on respiring cells (Hancock, 1981). Centrifuged cells prepared as above were resuspended in BM2 growth medium containing 5 μg gentamicin ml^{-1} in addition to succinate and iron at normal levels but with no other cations added, and incubated at 37 °C with vigorous aeration for 5 min, at which time viable counts were carried out as above.

Chelator-mediated enhancement of nitrocefin permeability. The chromogenic β-lactam nitrocefin was kindly provided by C. O'Callaghan (Glaxo). The assay used was modified from that of O'Callaghan *et al.* (1972). H103 growing in the presence of 0·2 mg benzylpenicillin ml^{-1} or H103 (RP1) growing in the presence of 10 μg tetracycline ml^{-1} was grown to an A_{600} of 0·50 to 0·60, harvested by centrifugation at 25 °C and resuspended in 20 mM-sodium phosphate buffer pH 7·0 at an A_{600} of 1·50 to 2·0. The cells were then diluted 1 in 10 in EDTA/Tris or EGTA/Tris at a final concentration of 10 mM-EDTA or EGTA and 10 mM Tris/HCl, pH 8·5. After 2 min at 25 °C, 0·1 ml cell suspension was quickly mixed with 0·65 ml nitrocefin (12·5 μg ml^{-1}, in 20 mM-phosphate buffer pH 7·0) and the hydrolysis of nitrocefin monitored spectrophotometrically by measurement of the increase in absorbance at 540 nm. Rates of hydrolysis for untreated cells were also measured; hydrolysis rates were expressed as the ratio of hydrolysis rates in treated cells to rates in untreated cells.

Characterization of cell envelope proteins and cation levels. The methods of cell envelope preparation, SDS–gel electrophoresis, and quantification of protein H1 from gels have all been described previously (Hancock & Carey, 1979; Nicas & Hancock, 1980). Cell envelopes were extracted by the method of Kenward *et al.* (1978) and cation levels determined by atomic absorption spectroscopy carried out by Susan Jaspar and Susan Liptak (Department of Civil Engineering, University of British Columbia) and Canadian Microanalytical Corp., Vancouver, Canada.

Displacement of Mg^{2+} from the cell envelope by polymyxin B and aminoglycosides. Cells grown to an A_{600} of 0·6 were incubated with 1 mM-KCN (to prevent aminoglycoside uptake) for 15 min at 37 °C with aeration. Streptomycin, gentamicin or polymyxin was then added and the cells incubated a further 10 min. Cell envelopes were then prepared as previously described (Nicas & Hancock, 1980). Analysis of divalent cations was carried out, as described above, on lyophilized envelopes.

Shift experiments. Overnight cultures (1 ml) grown in BM2/succinate with 0·02 mM-Mg^{2+} were transferred to 200 ml of the same medium and grown to an A_{600} of 0·15 to 0·20, at which point Mg^{2+} was added to a final concentration of 0·5 mM. Twenty ml of culture was removed at this point, and at 15 min intervals, and these samples used to test polymyxin B and EDTA/Tris sensitivity, and for preparation of cell envelopes as described above.

RESULTS

Induction of outer membrane protein H1 in the presence of various divalent cations

Earlier studies had shown that outer membrane protein H1 is produced in very large amounts, becoming the most abundant protein in cells grown in low (0·02 mM) Mg^{2+} (Nicas & Hancock, 1980). Results of supplementation of low Mg^{2+} medium with other divalent cations are shown in Fig. 1. As shown previously (Nicas & Hancock, 1980), in the presence of 0·5 mM-Mg^{2+}, H1 was

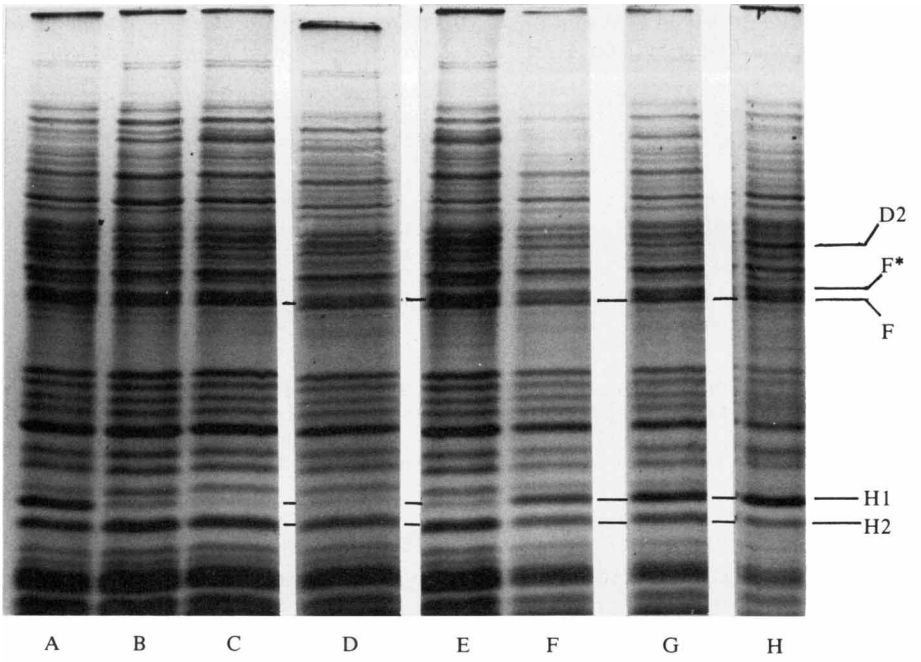

Fig. 1. Effect of growth in different divalent cations on induction of protein H1. SDS–polyacrylamide gel of cell envelopes from cells grown in the presence of different divalent cations. Lane A, 0·02 mM-Mg^{2+}; B, 0·5 mM-Mg^{2+}; C–H, 0·02 mM-Mg^{2+} plus 0·5 mM-Ca^{2+} (C), 0·5 mM-Mn^{2+} (D), 0·5 mM-Sr^{2+} (E), 0·5 mM-Ba^{2+} (F), 0·5 mM-Sn^{2+} (G), or 0·5 mM-Zn^{2+} (H). In order to ensure that all the protein H1 ran at the heat-modified position, solubilization was carried out at 100 °C for 10 min (as described by Hancock & Carey, 1979). This results in partial heat modification of protein F to run with a lower relative mobility at the position F* (Hancock & Carey, 1979). The molecular weights of the indicated proteins have been published (Hancock & Carey, 1979).

present only at very low levels. Induction of protein H1 was also prevented in medium supplemented with Ca^{2+}, Mn^{2+}, or Sr^{2+} (each at 0·5 mM), but not by Zn^{2+}, Sn^{2+}, Ba^{2+} (Fig. 1), Al^{3+} (each at 0·5 mM) or 1·0 mM-Na^{+} (data not shown).

As discussed previously (Nicas & Hancock, 1980), the increase in protein H1 under various growth conditions led to an apparent decrease in the levels of two major outer membrane polypeptides G and F. However, since loss of proteins G (Nicas & Hancock, 1980) and F (Nicas & Hancock, 1983), respectively, from the outer membrane does not result in enhanced resistance to aminoglycosides or polymyxin, we believe these lesser alterations to be unrelated to the antibiotic susceptibility alterations discussed below. It should be noted that other authors have demonstrated similar compensatory alterations in outer membrane protein profiles after strong induction of a specific polypeptide (see e.g. Diedrich & Fralick, 1982).

We previously postulated that protein H1 decreases access of cationic antibiotics to a Mg^{2+}-binding site on the lipopolysaccharide thus resulting in resistance. Such a site was proposed by Brown (1975) and more recently by ourselves (Nicas & Hancock, 1980; Hancock, 1981) to be a lipopolysaccharide polyphosphate site. In order to test whether the amount of lipopolysaccharide phosphate was influenced by the various growth conditions, we extracted cell envelopes with chloroform/methanol (2 : 1, v/v) to remove phospholipids and measured the ratios of residual phosphate to the lipopolysaccharide-specific sugar, 2-keto-3-deoxyoctanate, as previously described (Kropinski *et al.*, 1982). We were unable to demonstrate a significant difference ($P >$ 0·5 by the unpaired Student *t*-test) between the ratios (μg phosphate per μg 2-keto-3-deoxyoctanate) of cells grown under conditions resulting in induction of protein H1 (1·47 ± 0·48) and cells grown under conditions where protein H1 induction was suppressed (1·57 ± 0·39).

Table 1. *Effect of growth in various divalent cations on induction of outer-membrane protein H1, lysis and killing by EDTA/Tris, and killing by polymyxin B and gentamicin*

Mg^{2+} during growth (mM)	Other cations during growth (mM)	Induction of protein H1*	Percentage lysis by EDTA/Tris†	Percentage survivors‡		
				Polymyxin B	EDTA/Tris	Gentamicin
0·02	–	+	11·5	15·0	65·0	11·0
0·02	Ba^{2+} (0·5)	+	6·0	20·3	14·6	21·5
0·02	Sn^{2+} (0·5)	+	16·0	20·5	11·7	10·5
0·02	Zn^{2+} (0·5)	+	14·0	20·2	13·0	19·5
0·02	Ca^{2+} (0·5)	–	51·5	<0·01	<0·1	1·56
0·02	Mn^{2+} (0·5)	–	53·5	<0·01	<0·1	1·65
0·02	Sr^{2+} (0·5)	–	51·7	<0·01	<0·1	0·68
0·5	–	–	52·3	<0·01	<0·1	2·01

* Induction of protein H1 as judged from SDS–polyacrylamide gels of cell envelope proteins, see Fig. 1.
† Lysis was measured as decrease in A_{600} after 15 min in 10 mM-EDTA/10 mM-Tris/HCl pH 8·5.
‡ Cells were treated for 5 min with 75 μg polymyxin B ml^{-1} in phosphate buffer, 10 mM-EDTA in 10 mM-Tris/HCl, or 5 μg gentamicin ml^{-1} in growth medium without added divalent cations.

Resistance to polymyxin B, EDTA/Tris and gentamicin

We have previously reported that resistance to polymyxin B, EDTA, and gentamicin correlates with the presence of protein H1 where susceptibility is measured in a common assay medium (Nicas & Hancock, 1980; Hancock *et al.*, 1981). This correlation remained valid when other cations were substituted for Mg^{2+}. The results in Table 1 confirmed and extended the findings of Boggis *et al.* (1979) with respect to the effects of different metal cations on susceptibility to polymyxin and EDTA/Tris. Cells grown with Mg^{2+}, Ca^{2+}, Mn^{2+} or Sr^{2+}, the cations which prevented protein H1 induction, were at least 1000 times more sensitive to polymyxin B, 100 times more sensitive to EDTA/Tris and 10 times more sensitive to gentamicin than were cells grown in low Mg^{2+}; susceptibility to lysis in EDTA/Tris was also much enhanced in cells with low levels of protein H1. In contrast, cells grown with the cations which failed to prevent protein H1 induction (Ba^{2+}, Zn^{2+}, Sb^{2+}, Al^{3+}, Na^{+}) were indistinguishable from cells grown in low Mg^{2+} in their relatively low susceptibility to polymyxin B, EDTA/Tris and gentamicin, providing that a common assay medium was used.

Effect of shift from low to high Mg^{2+}

To further confirm the relationship between the presence of outer membrane protein H1 and resistance, we carried out shift experiments. Cells growing in 0·02 mM-Mg^{2+} in early-exponential phase growth (A_{600} 0·15–0·2) were supplemented with Mg^{2+} to 0·5 mM, and levels of H1 in the cell envelope and susceptibility to polymyxin B and EDTA/Tris were followed over time. As described previously (Nicas & Hancock, 1980), 0·02 mM-Mg^{2+} allows the same growth rate as 0·5 mM up to an A_{600} of 0·6, so these cells could not be considered Mg^{2+}-limited for growth. Control experiments demonstrated that cells grown in 0·02 mM-Mg^{2+} showed increasing levels of protein H1 as the medium became depleted in Mg^{2+}. However, we performed the shift to high Mg^{2+} on early-exponential phase cells, which showed only moderately higher levels of protein H1 (two- to threefold) in order to avoid any non-specific effects of Mg^{2+} starvation, e.g. stringent response (St John & Goldberg, 1980), ribosome effects (Gestland, 1966; Schlessinger *et al.*, 1967), etc. Addition of Mg^{2+} did not alter the growth rate (about 42 min generation time), and the cells remained in exponential growth phase throughout the sampling period. The ratio of outer membrane protein H1 to protein H2, calculated from densitometer tracings of Coomassie blue-stained gels (Nicas & Hancock, 1980), was used to estimate relative levels of protein H1 in cell envelopes. Outer membrane protein H2 was used as a reference since levels of this protein vary very little with growth conditions (Nicas & Hancock, 1980). As a control, we measured the ratios of proteins H1 and H2 to total outer membrane proteins by loading SDS–polyacrylamide gels with equal amounts of outer membranes and calculating the areas under the protein H1 and H2 peaks of densitometer scans of the gels. It was

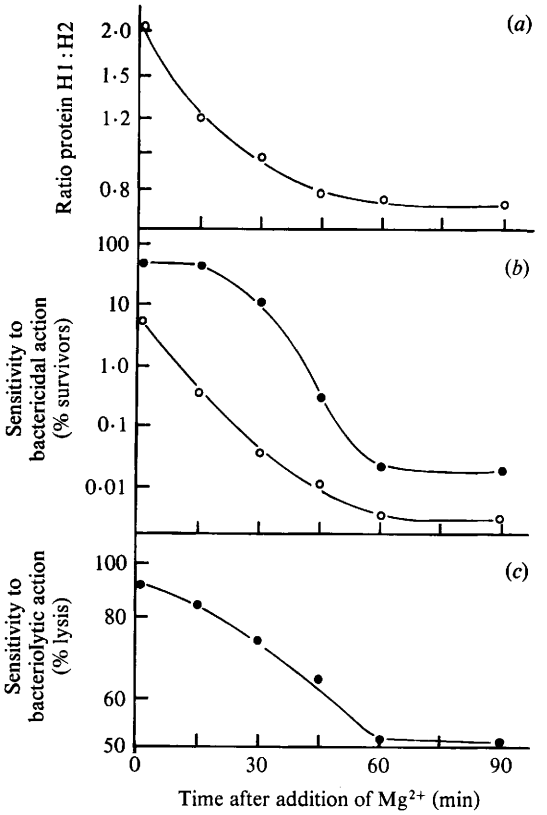

Fig. 2. Effect of shift from low to high Mg^{2+} on levels of protein H1 and sensitivity to EDTA/Tris and polymyxin B. Cells growing in 0·02 mM-Mg^{2+} received Mg^{2+} to a final concentration of 0·5 mM at time 0. Samples of cultures were subsequently removed at intervals and assayed for protein H1 levels and sensitivity. (*a*) Decrease in ratio of protein H1 : H2 measured from stained gels of cell envelopes (levels of protein H2 are constant under the conditions used). (*b*) Increase in sensitivity to bactericidal action of EDTA/Tris (●) or polymyxin B (○). Cells sampled at the given times were treated for 5 min with either 10 mM-EDTA in 10 mM-Tris/HCl pH 8·5 or 75 μg ml^{-1} polymyxin B in phosphate buffer, then plated for viable counts. (*c*) Increase in sensitivity to bacteriolytic action of EDTA/Tris. Lysis was measured as the decrease in A_{600} after 15 min treatment with 10 mM-EDTA/Tris in 10 mM-Tris/HCl pH 8·5.

demonstrated that the protein H2 peak area was constant (12% of the total area under the densitometer scan) under the growth conditions studied here, whereas the area under the protein H1 peak varied as described below. We have also studied the effects of temperature (21 °C to 42 °C), carbon source (glucose, succinate, pyruvate, etc.) and growth phase (exponential or stationary) on the level of protein H2 in the outer membrane and have seen less than 25% variation in the areas under the protein H2 densitometer peak (M. Kim & R. E. W. Hancock, unpublished observations). The time required for the relative level of protein H1 to decrease by one-half was estimated as about 38 min, close to the time for one cell division. After 45 to 60 min, the protein H1 level stabilized at the levels previously seen in cells grown in Mg^{2+}-sufficient medium. Increase in susceptibility to killing by polymyxin B and EDTA/Tris and lysis by EDTA/Tris followed a very similar time course (Fig. 2). There was a short lag before the cells increased in sensitivity to the bactericidal action of EDTA. However, sensitivity to lysis by EDTA more closely paralleled decrease in protein H1.

Table 2. *Divalent cations of cell envelopes after growth in the presence of different cations*

Mg^{2+} present during growth (mM)	Other cations present during growth (mM)	Divalent cations in cell envelope* [nmol (mg dry wt)$^{-1}$] Mg^{2+}	Ca^{2+}	Mn^{2+}	Zn^{2+}	Total † [nmol (mg dry wt)$^{-1}$]
0·02	–	54·7	<18	<0·4	<1	54·7
0·02	Ba^{2+} (0·5)	48·5	<18	ND	ND	ND
0·02	Sn^{2+} (0·5)	53·0	<18	ND	ND	ND
0·02	Zn^{2+} (0·5)	19·5	<18	ND	95	114·5
0·02	Ca^{2+} (0·5)	26·3	147	ND	ND	173·3
0·02	Mn^{2+} (0·5)	25·0	<18	111	ND	136
0·02	Sr^{2+} (0·5)	26·0	<18	ND	ND	ND
0·5	–	122·5	<18	<0·4	<1	122·5
0·5	Ca^{2+} (0·5)	76·5	115·5	ND	ND	192

ND, Not determined.

* Determined by atomic absorption spectroscopy; means of up to five separate determinations on two or three separate samples.

† Mg^{2+} plus other cations present during growth.

Table 3. *Effects of EGTA/Tris and EDTA/Tris on cells grown in Mg^{2+} and Ca^{2+}*

Cations present during growth (mM)	Lysis (%) EGTA	EDTA	Killing (%)* EGTA	EDTA	Increase in nitrocefin hydrolysis† EGTA	EDTA
Mg^{2+} (0·5)	0	51	0	>99·99	1	32
Mg^{2+} (0·02); Ca^{2+} (0·5)	40	36	37	>99·99	30	29

* See Table 1.

† Ratio of rate of nitrocefin hydrolysis in cells treated for 2 min with 10 mM-EGTA or EDTA in 10 mM-Tris/HCl pH 8·5 to rate of hydrolysis in untreated cells.

Divalent cation concentration of cell envelopes and displacement of cations by aminoglycosides and polymyxin B

We previously reported that a decrease in levels of protein H1 was accompanied by an increase in cell envelope Mg^{2+} content, suggesting that H1 exerts its effect by replacing Mg^{2+} at a site susceptible to chelators and antibiotics. This led us to examine whether polymyxin or aminoglycoside could act to displace Mg^{2+} in whole cells. Cells were pre-treated with KCN to prevent inner membrane uptake of aminoglycosides. When cells grown in 0·5 mM-Mg^{2+} were treated with 50 μg polymyxin B ml^{-1}, cell envelope Mg^{2+} content was reduced by about 10%. Treatment with 25 μg gentamicin ml^{-1} or 50 μg streptomycin ml^{-1} reduced Mg^{2+} levels by about 3·5 to 5%. This suggests that these agents can act to displace Mg^{2+}, but that a relatively small number of sites are involved.

Table 2 shows that when cells were grown with Ca^{2+}, Mn^{2+}, or Zn^{2+} as the major divalent cation, high levels of the major divalent cation were incorporated into the cell envelope. The levels of Zn^{2+} incorporated in cells grown with 0·5 mM-Zn^{2+} were significantly lower than the levels of Ca^{2+} or Mg^{2+} ($P < 0·05$ by Student's unpaired *t*-test) in cells grown with 0·5 mM-Ca^{2+} and 0·5 mM-Mg^{2+}, respectively.

Comparison of EGTA and EDTA susceptibility of Ca^{2+}- and Mg^{2+}-grown cells

Since cells were always provided with some Mg^{2+} as a growth factor, and cell envelopes all contained significant levels of Mg^{2+} (Table 2), we sought to determine whether EDTA was exerting its effect on Ca^{2+}-grown cells by removal of the small amounts of Mg^{2+} or by removal of the major cation Ca^{2+} (Table 3). Cells grown in Ca^{2+} were equally susceptible to lysis by EGTA (which can be regarded as a Ca^{2+}-specific chelator) and EDTA (which chelates both Ca^{2+} and

Mg^{2+} at high efficiency). EGTA had no measurable effect on cells grown on 0·5 mM-Mg^{2+} as the sole divalent cation. EGTA/Tris had some bactericidal activity on Ca^{2+}-grown cells, but none on Mg^{2+}-grown cells. However, the bactericidal activity of EGTA was several orders of magnitude lower than that of EDTA. We also measured permeabilization of the outer membrane as a result of chelator treatment, by examining hydrolysis of a chromogenic β-lactam nitrocefin. An increase in the rate of hydrolysis indicates increased permeation of the β-lactam through the outer membrane to the periplasmic β-lactamase (Angus *et al.*, 1982), and thus provides a sensitive and specific technique for demonstrating disruption of the outer membrane permeability barrier. EGTA/Tris and EDTA/Tris treatment were of similar efficiency in permeabilizing Ca^{2+}-grown cells to nitrocefin, producing hydrolysis rates about 30 times higher than those seen in unbroken cells. Mg^{2+}-grown cells were similarly affected by EDTA, but were not affected by EGTA. Furthermore, if Ca^{2+}-grown cells which had been treated with EGTA were subsequently treated with EDTA, only a small increase (5–10%) in the rate of hydrolysis was observed. A similar increase was seen in EDTA-treated Ca^{2+}-grown cells subsequently treated with EGTA. All of the above experiments were repeated using an RP1 plasmid-encoded β-lactamase, with essentially identical results.

DISCUSSION

This study demonstrates close correlation between levels of outer membrane protein H1 and susceptibility to chelators, polymyxin B, and gentamicin in *P. aeruginosa* under a variety of different growth conditions. In shift experiments, the decrease in protein H1 brought about by increasing Mg^{2+} in the growth medium closely paralleled increase in susceptibility to EDTA/Tris and polymyxin B. When other divalent metal cations were substituted for Mg^{2+}, only cells grown in those cations which prevented induction of protein H1 were susceptible to EDTA, polymyxin B and gentamicin.

It has been suggested that polymyxins, chelators (Brown, 1975; Nicas & Hancock, 1980), and aminoglycosides (Hancock, 1981; Hancock *et al.*, 1981) act at a common site on the outer membrane, possibly a phosphate site on the lipopolysaccharide. We have proposed that divalent cations bound at this site would be required for the stability of the outer membrane. Thus EDTA would act to remove divalent cations by chelation, while the cationic antibiotics, polymyxins and aminoglycosides, would act by competing for the lipopolysaccharide-binding site. Protein H1 is proposed to act by replacing divalent cations at this site on the lipopolysaccharide, thus protecting it from attack by these agents (Nicas & Hancock, 1980; Hancock *et al.*, 1981; Hancock, 1981). Kenward *et al.* (1979) have shown that there is no clear correlation between the response of bacteria, grown under a variety of different conditions, to EDTA and polymyxin and the levels of individual or total phospholipids, readily extractable lipids, amino sugars or carbohydrates. In agreement with their conclusions, we observed no alteration in the phosphate to 2-keto-3-deoxyoctanate ratios of the chloroform/methanol-extracted cell envelopes. Thus, it seems unlikely that our results can be explained on the basis of lipopolysaccharide or phospholipid alterations. In addition, although we noted small compensatory alterations in the levels of porin protein F upon H1 induction, we have recently measured outer membrane permeability to a hydrophilic antibiotic nitrocefin in our H1-overproducing mutant strain H181 (Nicas & Hancock, 1982). Although this mutant had similar outer membrane alterations to cells grown in divalent cation-deficient medium (i.e. high H1, lowered F, Nicas & Hancock, 1980), it was as permeable to nitrocefin as the wild-type strain H103 grown in Mg^{2+}-sufficient medium ($P < 0{\cdot}5$ by unpaired Student *t*-test). Other evidence against a significant alteration in the hydrophilic uptake pathway for antibiotics was presented previously (Hancock *et al.*, 1981).

Ca^{2+}, Mn^{2+} and Sr^{2+} were able to substitute for Mg^{2+} both in preventing H1 induction and in allowing sensitivity to these agents. These four cations showed very similar effects, implying that they are equivalent in their ability to regulate protein H1. Regulation of protein H1 production by cations could be mediated by a common receptor for these cations. Similar receptor specificity has been seen for the chemotaxis receptor for Mg^{2+} in *Salmonella typhimurium* and *Escherichia coli* (Koshland, 1979). Alternatively, the expression of H1 may be

sensitive to the amounts of divalent cation in the outer membrane itself, or to cation bound at specific sites in the outer membrane. Alteration of outer membrane protein composition in response to changes in outer membrane components (Van Alphen *et al.*, 1976; DiRienzo & Inouye, 1979) is known to occur in *E. coli*. The ability to respond to conditions at the outer membrane by alteration of outer membrane protein is also seen in the modulation of levels of major outer proteins of *E. coli* in response to an osmotic pressure gradient across the outer membrane (Kawaji *et al.*, 1979).

As cell envelopes under all conditions used contained some Mg^{2+}, we considered the possibility that the site of activity of the agents used could involve this small amount of Mg^{2+} rather than a site occupied by other cations. The effect of EGTA on Ca^{2+}-grown cells, however, strongly suggested that Ca^{2+} is replacing Mg^{2+} at the target site. EGTA chelates Ca^{2+} efficiently but Mg^{2+} poorly (K_{eff} 10·4 vs 4·7, Roberts *et al.*, 1970). Ca^{2+}-grown cells appeared as sensitive to the outer membrane effects of EGTA as they are to those of EDTA, as shown both by the direct measurement of lysis and by increase in the rate of hydrolysis of nitrocefin. EGTA, however, did not have the high bactericidal activity of EDTA, suggesting that additional sites are involved in the bactericidal action of EDTA. Other workers (Boggis *et al.*, 1979) have noted that sensitivity to lysis by EGTA is dependent on the amount of Ca^{2+} present in the growth medium, but that sensitivity to EGTA is decreased if Mg^{2+} is present as well as Ca^{2+}, suggesting that either cation may occupy the site attacked by the two chelators.

The number of cation-binding sites involved in the action of EDTA, polymyxin and gentamicin on the outer membrane may well represent a small proportion of the total number of sites. Schindler & Osborn (1979) have demonstrated at least two classes of Ca^{2+}- and Mg^{2+}-binding sites on the lipopolysaccharide of *Salmonella typhimurium* with widely differing affinities. In contrast, only high affinity binding sites for polymyxin B were found. The involvement of a small number of sites is suggested by the displacement of only 3·5 to 10% of the cell envelope Mg^{2+} from cells treated with aminoglycosides or polymyxin B. A relatively small proportion of critical target sites for polymyxins, EDTA/Tris, and gentamicin, could also explain why cells grown in Zn^{2+} are not sensitive despite the high levels of Zn^{2+} in cell envelopes and the very high efficiency of Zn^{2+} chelation by EDTA. We observed that Zn^{2+}-grown cells had high levels of protein H1, and that their level of divalent cations was 15–35% lower than the level found in cells grown in Ca^{2+} or Mn^{2+} and 8% lower than cells grown in 0·5 mM-Mg^{2+}. It may be that those divalent cation-binding sites which are sensitive to attack by cationic antibiotics are protected by protein H1, and the remaining sites occupied by Zn^{2+} are not required for membrane stability. This would suggest that these critical divalent cation-binding sites can only be occupied by specific divalent cations, i.e., Mg^{2+}, Ca^{2+}, Sr^{2+} or Mn^{2+}. Alternatively, protein H1 could only displace divalent cations from these sites when induced by limitation of these divalent cations. We have previously suggested that there is a linear reciprocal relationship between the decrease in Mg^{2+} levels and increase in protein H1 in the cell envelope of wild-type cells. It now appears that resistance to chelators and cationic antibiotics correlates more closely with the presence and amounts of protein H1 than with absolute levels of divalent cations in the cell envelope. This is supported by studies of mutant strains resistant to these agents, which overproduce protein H1 to about sevenfold wild-type levels in 0·5 mM-Mg^{2+}, but show a reduction of less than twofold in their envelope Mg^{2+} levels (Nicas & Hancock, 1980; T. I. Nicas, unpublished observations). Protection of a relatively small proportion of divalent cation-binding sites would thus appear to be sufficient to confer resistance.

How then does protein H1 lead to resistance to polycationic antibiotics? It seems likely that it functions by blocking divalent cation-binding sites in the outer membrane, thus greatly decreasing the rate of permeation of aminoglycosides and polymyxin across the outer membrane. This relatively low level exposure of cells to antibiotic (despite high external concentration) may then allow cells to adapt to higher polymyxin resistance as described by Gilleland & Lyle (1979) or to aminoglycoside resistance as described by Pechey & James (1974).

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