Expression of H1 outer-membrane protein of *Pseudomonas aeruginosa* in relation to sensitivity to EDTA and polymyxin B

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Summary. During growth in magnesium (Mg^{++})-deficient mineral media, *Pseudomonas aeruginosa* cells synthesise large amounts of H1 outer-membrane protein and are resistant to polymyxins and EDTA. It has been suggested that H1 protein replaces Mg^{++} as an outer-membrane-stabilising component in Mg^{++} -deprived cells, thereby removing the EDTA target and blocking an adsorption site for polymyxins. Induction of H1 protein synthesis also occurred in *P. aeruginosa* cells grown in Antibiotic No. 3 Broth (Ab3B), although this medium is not Mg^{++} -deficient. Generally, significant induction of H1 protein did not occur in *P. aeruginosa* cultures grown in other complex media such as Proteose Peptone and Nutrient Broth, which contained less Mg^{++} than Ab3B, nor in Isosensitest Broth or Mueller Hinton Broth, which contained higher Mg^{++} concentrations. H1-protein-induced *P. aeruginosa* cells from Ab3B cultures, unlike those from Mg^{++} -deficient mineral-broth culture, remained fully sensitive to polymyxin B and, with one exception, to EDTA. It is concluded that induction of H1 protein does not itself confer resistance to polymyxin B, and has no more than a minor role in EDTA resistance. Other cell-wall changes, such as phospholipid modifications and the absence of Mg^{++} , probably account for the resistance of Mg^{++} -deprived cells.

Introduction

Pseudomonas aeruginosa cells from “magnesium (Mg^{++})-sufficient” (0.5–1 mM Mg^{++}) cultures are lysed readily by EDTA and polymyxins, whereas those from Mg^{++} -deficient (0.02 mM Mg^{++}) cultures are resistant (Brown, 1975). These observations probably relate to outer-membrane structure. The outer membranes of cells grown in Mg^{++} -sufficient media contain large amounts of Mg^{++} , and it is suggested that these ions form bridges between adjacent lipopolysaccharide molecules (Brown, 1975). Other divalent cations such as calcium (Ca^{++}), or manganese (Mn^{++}), can replace Mg^{++} in this role if they are present in large amounts (Kenward *et al.*, 1979). EDTA attacks the cation bridges directly, chelating the Mg^{++} (or other) ions, and disrupting the membrane structure. Polymyxins are thought to bind to the Mg^{++} -filled sites as an initial stage in uptake (Schindler and Osborn, 1979; Nicas and Hancock, 1980). The outer membranes of cells grown in Mg^{++} -deficient media contain greatly reduced amounts of Mg^{++} (Kenward *et al.*, 1979); thus

resistance to EDTA and polymyxins may be explained simply by the absence of the target site for these agents.

Recently it has been noted that *P. aeruginosa* cells from Mg^{++} -deficient cultures contain large amounts of an outer-membrane protein of mol. wt 21 000, designated H1, which is largely absent from cells grown in Mg^{++} -sufficient media. It has been postulated that this polypeptide directly replaces Mg^{++} in Mg^{++} -starved cells, thus causing their resistance. This hypothesis is supported by the observations of Nicas and Hancock (1980) that two *P. aeruginosa* PAO1 mutants that produced large amounts of H1 protein independently of Mg^{++} deficiency remained resistant to EDTA and polymyxins in Mg^{++} -sufficient media. However, outer-membrane phospholipid changes were detected subsequently in these mutants and the basis of their resistance is disputed (Gilleland and Conrad, 1982).

In the present study we attempted to clarify the role of H1 protein in resistance to EDTA and polymyxins in *P. aeruginosa*. Induction of this protein was demonstrated in Mg^{++} -sufficient complex media and the EDTA- and polymyxin-susceptibility of *P. aeruginosa* strains grown in these media was compared with that of the same strains

grown in H1 protein non-inducing complex and mineral media.

Materials and methods

Bacterial strains

P. aeruginosa PAO1 was described by Holloway *et al.* (1979); the other *P. aeruginosa* strains were clinical isolates obtained during a survey of antibiotic resistance in this species (Williams *et al.*, 1984).

Growth vessels

Disposable plastic screw-capped 7 ml (bijou) and 28 ml (universal) containers (Sterilin Ltd, Feltham) were used when small volumes of culture (up to 10 ml) were required. Larger volumes of culture were grown in glass conical flasks, which were cleaned overnight with nitric acid 20% v/v then rinsed with multiple changes of deionised water before sterilisation and use.

Media and conditions of culture

Antibiotic No. 3 Broth (Ab3B) and Proteose Peptone No. 2 (PP) were from Difco Ltd, Detroit, MI, USA; Isosensitest Broth (ISTB), Mueller Hinton Broth (MHB), Nutrient Broth No. 2 (NB) and Nutrient Agar were from Oxoid Ltd, Basingstoke, Hants. These media were reconstituted according to the manufacturers' directions, but with deionised instead of distilled water as the diluent. Mineral Broth was prepared in deionised water to the formulation of Kelly and Clarke (1962), with the magnesium sulphate concentration adjusted to 20–30 μM (Mg^{++} -deficient Mineral Broth; MgDMB) or to 1 mM (Mg^{++} -sufficient Mineral Broth; MgSMB). After sterilisation at 121°C for 15 min, the mineral media were supplemented with filter-sterilised D-glucose (final concentration 0.5% w/v) as the sole carbon and energy source.

All cultures were grown on an orbital shaker at 37°C and broths were warmed to this temperature before inoculation. To obtain sufficient numbers of cells to undertake the procedures described below the bacteria were grown to late-exponential phase. This was done by adding a 10% v/v inoculum of overnight culture to fresh, identical broth, and incubating for 5–6 h (complex media) or for 7 h (mineral media). Cells were harvested by centrifugation for 15 min at 5000 g and 37°C.

Isolation of outer membranes

Late-exponential phase cells were harvested from 1-L volumes of culture and the outer membranes were isolated by sucrose-gradient centrifugation (Anwar *et al.*, 1983). Membrane preparations were stored at –20°C before analysis.

Electrophoresis of outer membranes and estimation of H1 protein

Outer-membrane preparations were adjusted to a protein concentration of 1 mg/ml by adding distilled water, and mixing with an equal amount of freshly-prepared "solubilisation buffer"— β -mercaptoethanol 10% v/v, glycerol 20% v/v, sodium dodecyl sulphate 4% w/v, bromophenol blue 0.01% w/v in 0.25 M Tris (hydroxymethylmethylamine) HCl buffer, pH 6.8. Usually the mixtures were heated to 90°C for 10 min in an oil bath before electrophoresis, although these conditions were varied during preliminary experiments designed to identify H1 protein (see *Results*). Samples (30 μl) of the mixtures were loaded on to discontinuous sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gels. The gel dimensions were 150 mm wide $\times$ 120 mm long $\times$ 1.5 mm thick. The acrylamide and *NN* methylene bisacrylamide concentrations in the running gel were adjusted to 14 and 0.255%, respectively, and to 6 and 0.06%, respectively, in the stacking gel. Sodium chloride (70 mM) was included in the running gel, and the buffer system of Lugtenburg *et al.* (1975) was used. Electrophoresis was performed at a constant current of 60 mA per gel whilst the samples passed through the stacking gel, and this was increased to 100 mA per gel as they migrated through the running gel. Protein bands were visualised by staining the gels overnight with Coomassie Blue 0.1% w/v in methanol:acetic acid:water (50:10:40) followed by repeated destaining in water:acetic acid:methanol (85:10:5). H1 protein was measured as a proportion of total outer-membrane proteins by scanning densitometry, with a Bio-Rad Densitometer, (Bio-Rad Ltd, Watford, Herts) linked to a Shimadzu CR-1-B peak integrator (Dyson Instruments, Houghton, Tyne and Wear).

Estimation of divalent cation concentrations in different media

Concentrations of Mg^{++} and Ca^{++} in the various media were measured with an Instrumentation Laboratory Model 151 Atomic Absorption Spectrophotometer.

Bacterial susceptibility to EDTA and polymyxin B

Late-exponential-phase cells from 100 ml of culture were resuspended at a density of 1 mg (wet weight)/ml in warm (37°C) 50 mM Tris HCl buffer, pH 8.5, for EDTA susceptibility tests, or in warm (37°C) 0.1 M phosphate buffer, pH 7.4, containing sodium chloride 0.9% w/v for polymyxin susceptibility tests. EDTA (10 mM final concentration) or polymyxin B (75 mg/L) was then added. Lysis by EDTA was monitored by spectrophotometry at 450 nm and 37°C in a Pye Unicam SP1700 spectrophotometer (Pye Unicam, Cambridge). Killing by polymyxin B was monitored by sequential viable counts as follows: the polymyxin-containing cell suspensions were maintained at 37°C on an orbital shaker, and 1-ml samples were withdrawn at timed intervals. The cells in these samples were harvested by centrifugation at 12 000 g for

1 min, and re-suspended to their original density in antibiotic-free 0.1 M phosphate buffer, pH 7.4, containing sodium chloride 0.9% w/v. Viable counts were performed by the method of Miles, Misra and Irwin (1938) on nutrient agar.

Protein assays

Protein was estimated by the method of Lowry *et al.* (1951).

Results

Recognition of H1 protein

Outer-membrane proteins were identified by their apparent molecular weights, as derived from SDS-PAGE, after solubilisation at various temperatures. The nomenclature follows the scheme proposed by Hancock and Carey (1979). H1 protein was characterised as a species that migrated more rapidly than H2 protein when solubilisation was performed below 60°C, but more slowly than H2 protein when solubilisation was performed at higher temperatures. In the latter conditions, H1 protein had an apparent mol. wt of 21 000. H2 protein had

an apparent mol. wt of 20 000 regardless of the solubilisation temperature.

Expression of H1 protein in different media

H1 protein production by twelve *P. aeruginosa* strains was estimated for cultures grown in Ab3B, MgSMB and MgDMB (table I). All the strains produced measurable amounts of the protein during growth in these media. Cells grown in Ab3B usually synthesised 2–3 times more H1 protein than those grown in MgSMB, but slightly less than those grown in MgDMB. Three strains, however, synthesised more H1 protein in Ab3B than in MgDMB.

H1 protein production by four strains (M1221, M1573, M1805 and PAO1) was examined also after growth in NB, MHB, PP and ISTB (table II). Strains M1221, M1573 and M1805 synthesised negligible amounts of H1 protein in these media, but strain PAO1 produced significant amounts of H1 protein (>5% of the total outer-membrane protein) in PP and NB. Outer-membrane-protein profiles of strains M1805 and PAO1, after growth in the various media, are illustrated in figs 1a and b.

Table I. Expression of H1 protein by twelve *P. aeruginosa* strains after growth in various media

Medium	H1 as percentage of total outer-membrane protein	
	Mean for 12 strains	SD
Ab3B	17.8	4.5
MgSMB	7.6	4.1
MgDMB	20.4	5.7

Divalent cation concentrations in different media

The Mg^{++} and Ca^{++} concentrations in the various media are shown in table II. MgSMB, ISTB and MHB all had total divalent cation concentrations >1 mM, whereas those of Ab3B, NB, PP and MgDMB were ≤ 0.25 mM.

Effect of EDTA and polymyxin B on cells grown in different media

The four strains listed in table II were examined for susceptibility to 10 mM EDTA and polymyxin B

Table II. Expression of H1 protein by *P. aeruginosa* strains PAO1, M1221, M1573 and M1805 in various media

Medium	H1 as percentage of total outer-membrane protein in strain				Divalent cation concentration (mM) of medium		
	PAO1	M1221	M1573	M1805	Mg^{++}	Ca^{++}	Total ($Mg^{++} + Ca^{++}$)
1% PP	9.8	<1	<1	<1	0.05	<0.01	0.06
NB	6.6	<1	<1	<1	0.07	0.04	0.11
ISTB	<1	<1	<1	<1	1.04	0.07	1.11
Ab3B	20.2	12.9	13.3	30.6	0.14	0.11	0.25
MHB	<1	<1	<1	<1	0.71	1.65	2.36
MgSMB	13.6	3.9	4.8	10	1.25	<0.01	1.26
MgDMB	24.1	14.9	30.2	30.6	0.02	<0.01	0.03

Fig. 1A. Outer-membrane-protein profiles of strain PAO1 after growth in PP (track 2), NB (3), ISTB (4), Ab3B (5), MHB (6), MgSMB (7) and MgDMB (8). Track 1 contains mol. wt markers: phosphorylase B (mol. wt 92 500), bovine serum albumen (68 000), ovalalbumen (45 000), carbonic anhydrase (30 000), soyabean trypsin inhibitor (21 500) and lysozyme (14 400).

Fig. 1B. Outer-membrane-protein profiles of strain M1805 after growth in PP (track 1), NB (2), ISTB (3), Ab3B (4), MHB (6), MgSMB (7) and MgDMB (8). Track 5 contains mol. wt markers as in track 1 of fig. 1a.

75 mg/L after growth in the various media. Limited killing or lysis was observed when cells grown in MgDMB were exposed to these agents, but a substantial proportion of the cells appeared resistant. Cultures grown in the other media contained much larger proportions of susceptible cells (figs 2a-d and 3a-d). In the case of strain M1805 only, cultures in Ab3B were partially resistant to lysis by EDTA, but remained fully susceptible to polymyxin B (figs 2d and 3d). Otherwise, Ab3B-grown cultures were as sensitive as those grown in ISTB or PP 1% w/v. Cells of strain M1573 from MgSMB were more resistant to EDTA and polymyxin B than were those from Ab3B, ISTB or PP 1% w/v, but remained more susceptible than those from MgDMB.

Discussion

In common with previous workers (e.g., Brown and Melling, 1969; Nicas and Hancock, 1980 and 1983) we found that *P. aeruginosa* cultures grown in MgDMB produced more H1 protein and were more resistant to EDTA and polymyxin B than were those grown in MgSMB. Such findings have led Nicas and Hancock (1980) to propose that H1 protein directly replaces Mg^{++} as an outer-membrane-stabilising component in Mg^{++} -deprived *P. aeruginosa*. They suggested that this replacement removes the target for EDTA and blocks an adsorption site for polymyxin B.

Furthermore, we found that growth in Ab3B

induced synthesis of large amounts of H1 protein by *P. aeruginosa* (tables I and II). Despite this induction, Ab3B-grown cultures of *P. aeruginosa* were generally as susceptible to EDTA and polymyxin B as were those grown in H1-non-inducing complex media such as ISTB and PP (figs 2 and 3). The sole exception was strain M1805; cultures grown in Ab3B were incompletely lysed by EDTA. Perhaps significantly, strain M1805 synthesised larger amounts of H1 protein during growth in Ab3B than did any other strain tested. This observation seems to support the hypothesis that overproduction of H1 protein, of itself, may protect against lysis by EDTA. However, the EDTA-resistance of Ab3B-grown cultures of strain M1805 remained less complete than that of MgDMB-grown cultures, despite induction of similar amounts of H1 protein during growth in both media. Our results thus suggest that induction of H1 protein makes, at most, a minor contribution to EDTA resistance in *P. aeruginosa*, and that induction of the protein does not protect against polymyxin B.

We conclude, therefore, that other factors must largely cause the EDTA- and polymyxin-resistance that is observed in *P. aeruginosa* grown in Mg^{++} -deficient media. Numerous cell-wall changes have been reported previously in *P. aeruginosa* cells which were deprived of Mg^{++} and other divalent cations. In particular, Kenward *et al.* (1979) found that the walls of Mg^{++} -deprived *P. aeruginosa* contained less Mg^{++} and phosphorus, but more

Fig. 2a-d. Lysis of strains PAO1 (a), M1221 (b), M1573 (c) and M1805 (d) by 10 mM EDTA after growth in Ab3B ■, ISTB △, MgDMB ●, MgSMB ○ or 1%PP ▲.

Fig. 3a-d. Lysis of strains PAO1 (a), M1221 (b), M1573 (c) and M1805 (d) by polymyxin B 75 mg/l after growth in Ab3B ■, ISTB △, MgDMB ●, MgSMB ○ or 1%PP ▲.

amino-sugars, total carbohydrates, readily-extractable lipids, and phospholipids than did the walls of cells grown in Mg^{++} -sufficient conditions. The lack of cell-wall Mg^{++} itself provides an obvious and direct explanation for EDTA-resistance in cation-depleted media and also may contribute significantly to polymyxin-resistance. The relative amounts of different cell-wall phospholipids also vary with divalent cation availability (Kenward *et al.*, 1979) and this finding may be relevant to polymyxin resistance, because some phospholipids (e.g., phosphatidyl-ethanolamine and phosphatidyl-glycerol) bind these antibiotics (Finegold *et al.*, 1974). Moreover Mg^{++} -sufficiency or -deficiency cannot be viewed in isolation as the sole direct or indirect arbiter of EDTA and polymyxin susceptibility. Within the outer membrane of the *P. aeruginosa* cell, the Mg^{++} ions associate electrostatically with the phosphate residues attached to the lipid A and core oligosaccharide regions of the lipopolysaccharide molecules (Wilkinson, 1975). The extent of lipopolysaccharide phosphorylation, which varies with the growth conditions (Melling and Brown, 1975), therefore may influence the ease with which Mg^{++} ions may be chelated from the membrane by EDTA, or displaced by polymyxin B. It has been suggested that phosphate availability itself may influence EDTA and polymyxin susceptibility in *P. aeruginosa*, perhaps by modulating lipopolysaccharide phosphorylation, but results on this aspect are contradictory (Melling and Brown, 1975).

The basis of induction of H1 protein in Ab3B remains obscure. This medium contains larger amounts of Mg^{++} and Ca^{++} than did 1% PP or NB, both of which generally failed to induce H1 protein. It could be argued that H1-protein induction in Ab3B arose from depletion of divalent cations by the growing cells. However this seems unlikely, primarily because the Ab3B-grown cells failed to develop the resistances normally associated with Mg^{++} depletion. Moreover, depletion of Mg^{++} would be expected to occur more rapidly in media such as 1% PP and NB, which contained lower initial divalent cation concentrations than did Ab3B.

Induction of H1 protein may also protect *P.*

aeruginosa against aminoglycoside antibiotics (Nicas and Hancock, 1980 and 1983); these are thought to adsorb to the same outer membrane site as polymyxins. Because aminoglycosides are only very slowly bactericidal for *P. aeruginosa* cells resuspended in buffer, the methods used here were unsuitable for testing this hypothesis. Preliminary results (Said *et al.*, 1986) indicated that, although the aminoglycoside susceptibility of *P. aeruginosa* was strongly medium-dependent, there was no correlation between the MICs observed in different media and the H1-protein-inducing power of these media. MICs of amikacin, gentamicin and tobramycin for strains PAO1, M1221, M1573 and M1805 largely conformed to the pattern: MIC in 1% w/v PP < MIC in NB < MIC in ISTB < MIC in Ab3B < MIC in MHB < MIC in MgSMB < MIC in MgDMB, whereas H1-protein-expression was greatest in Ab3B and MgDMB. These observations argue against a role for H1 protein in aminoglycoside resistance, whilst supporting the view that Mg^{++} deficiency can promote aminoglycoside resistance. However, some caution is necessary with regard to this conclusion because the MIC values reflect the net effect of all the bacteria-drug interactions over a 16–18 h period, whereas our data on induction of H1 protein relate specifically to late-exponential phase cells, and it is possible that the amount of H1 present in the bacteria may fluctuate during the growth cycle.

In summary, our results show that induction of H1 outer-membrane protein occurs independently of divalent cation deficiency in *P. aeruginosa* cells grown in Ab3B. H1 protein-induced *P. aeruginosa* from Ab3B, unlike those from MgDMB, generally were not resistant to EDTA and polymyxin B, and we conclude that induction of this protein is not the major basis of resistance to these agents observed in *P. aeruginosa* grown in conditions of divalent cation starvation.

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