

Identification of a periplasmic protein associated with osmolarity-dependent aminoglycoside resistance in *Escherichia coli*

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ABSTRACT

A new type of osmolarity-dependent aminoglycoside resistant *Escherichia coli* mutant was isolated. Analysis of cell envelope proteins of one representative clone revealed a drastic reduction of a periplasmic protein with a relative molecular weight of 61 kDa. The involvement of this periplasmic protein in the uptake of aminoglycoside antibiotics and its possible identity with a transport protein of an oligopeptide uptake system is discussed.

INTRODUCTION

Although aminoglycoside antibiotics are known to be accumulated by an active transport system in *Escherichia coli* cells, the possibility of the uptake of these antibiotics through the cell envelope of Gram-negative cells is still an open question (Nichols and Young, 1985; Davies, 1987; Nichols, 1989). It has been shown that the bacteriocidal effects of aminoglycosides can be reduced by several compounds with diverse actions, such as inhibitors of protein synthesis or electron transport (Taber *et al.*, 1987) and polyamines (Höltje, 1978). Some reports describe a clear influence of the growth media on resistance to aminoglycoside in Gram-negative bacteria (Medeiros *et al.*, 1971; Bryan and van den Elzen, 1976; Hancock, 1981). Such effects could be associated with the ionic content (Medeiros *et al.*, 1971), presence of divalent cations (Hancock, 1981),

and osmolarity and ionic content of the growth medium (Rodriguez *et al.*, 1990).

High frequencies of aminoglycoside resistant clones of *E. coli* resulted when cultures were plated on high osmolarity medium containing kanamycin. Preliminary characterization of these mutants indicated that they have an altered uptake of these antibiotics (Rodriguez, 1991).

Our objective was to determine if cell envelope components are involved in the osmolarity-dependent aminoglycoside resistance of *E. coli*.

MATERIAL AND METHODS

Bacterial strains and growth conditions

E. coli strain J53 was used in this work (Clowes and Hayes, 1968). Osmolarity-dependent aminoglycoside resistant mutants were isolated after plating overnight cultures on a high osmolarity (448.6 mOsm) modified L medium (2% tryptone, 2% yeast extract, 1%

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NaCl, 1.5% agar) containing kanamycin at 20 µg/ml. Under such conditions the frequency of aminoglycoside resistant clones was at least 10^4 higher than the frequencies obtained in nutrient agar (NA) (Difco Laboratories, Detroit, U.S.A.), a low osmolarity medium (63.1 mOsm). Osmolarity of the nutrient broth (NB - Difco Laboratories) was increased by the addition of 1% (w/v) NaCl (347.3 mOsm) or 0.5 mol/l sorbitol (531.1 mOsm).

Isolation of outer membrane proteins and lipopolysaccharides (lps)

Cell envelopes were isolated after sonic disruption, followed by differential centrifugation. Outer membrane proteins were obtained by a modification of the procedure described by Schnaitmann (1971), based on the solubilization of cytoplasmic membrane proteins with 0.2% Triton X-100. Lipopolysaccharides (lps) were isolated from cell envelopes after digestion with proteinase K and extraction with hot phenol, as described by Darveau and Hancock (1983). Lps separated in SDS-PAGE were developed by silver staining as described by Tsai and Frash (1982).

Isolation of periplasmic proteins

Periplasmic proteins were isolated after gentle treatment of the cells with chloroform as described by Ames *et al.* (1984). Exponential phase cells from a 10 ml culture were centrifuged at 3,000 x g for 10 min. The supernatant was discarded and the cell pellet was resuspended in the residual medium, followed by addition of 40 ml of chloroform, briefly vortexed and incubated at room temperature for 25 min. Then, 200 ml of 10 mM Tris-HCl pH 8.0 was added to the tube and it was centrifuged for 25 min at 3,000 x g. The supernatant was carefully removed without disturbing the cell pellet.

Other methods

Resistance levels were determined by visual inspection of cultures grown in liquid media containing serial two-fold dilutions of kanamycin at 37°C after 48 hours. SDS-PAGE followed by silver staining were carried out as described by Lugtemberg *et al.* (1975) and Morrissey (1981). The protein content of the samples was evaluated by a modification of the procedure originally described by Lowry *et al.* (1951). The osmolarity of the growth media was measured by an osmometer (Osmometre-Precision System Inc.).

RESULTS

Several independent aminoglycoside-resistant mutants were isolated as clones derived from the *E. coli* strain J53. They were able to grow in 20 µg/ml of kanamycin, but only in media of high osmolarity. The susceptibility levels of one representative aminoglycoside resistant clone, (A31), was further evaluated. The osmolarity and ionic strength of the growth medium had a marked physiological influence on the susceptibility to aminoglycosides in both parental and mutant strains (Table I). Nonetheless, the resistance levels of the A31 mutant to kanamycin and other aminoglycoside antibiotics was always approximately 100 times higher than the levels detected for the parental strain (Table I and data not shown).

Table I - Evaluation of the kanamycin resistance expressed by *Escherichia coli* J53 and the A31 aminoglycoside resistant mutant strains.

Strain	Resistance level ¹ (µg/ml)		
	NB	NB + 1% NaCl	NB + 0.5 M sorbitol
J53	0.2	2	1
A31	20	200	100

¹ Resistance levels are represented by the highest kanamycin concentration which allowed full growth of the strains after 48 h at 37°C.

The protein and lps contents of the outer membrane of the A31 strain did not reveal any consistent differences when compared to the wild type strain. The osmoregulated behaviour of the porins as well as the total porins content after growth in NB, NB + 1% NaCl or NB + 0.5 mol/l sorbitol were exactly the same in the susceptible and resistant strains. No significant difference in the lps composition of both strains was detected, based on electrophoretic profiles, after growth in the three different media used (data not shown).

There was a striking difference in the periplasmic proteins of the parental strain and the A31 mutant grown in NB, represented by a major 61 kDa protein in the periplasm of the J53 strain, which was almost undetectable in the A31 strain. The disappearance of a less abundant protein with an Mr of approximately 57 kDa from the periplasm of the A31 strain was apparently not relevant since it was not observed in other mutants or after cultivation of the A31 strain in the other media (unpublished observations). The expression of the 61

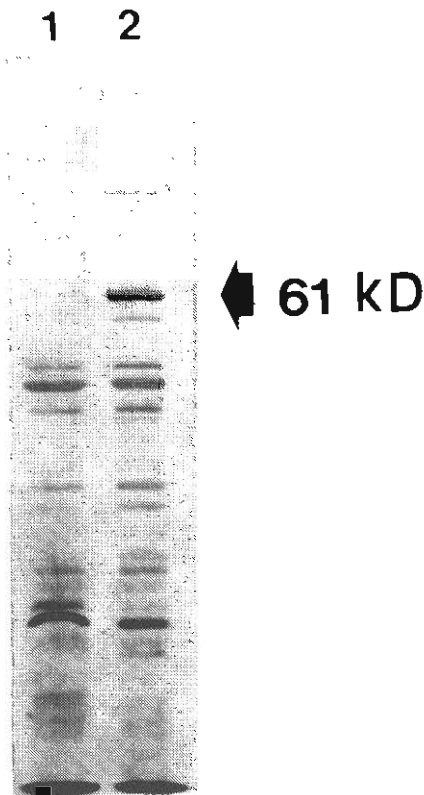


Figure 1 - Silver-stained electrophoretic profile of periplasmic proteins of *Escherichia coli* J53 (lane 2) and A31 (lane 1) strains cultivated in nutrient broth. The position of the 61 kDa protein is indicated by an arrow on the right side.

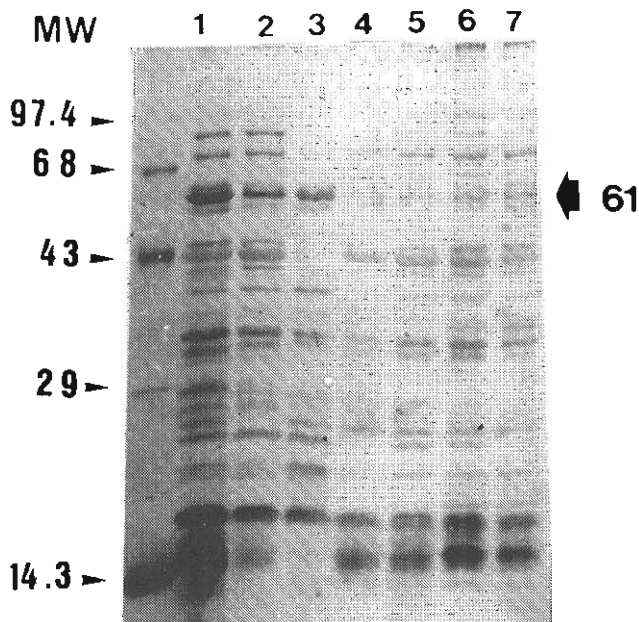


Figure 2 - Periplasmic proteins isolated from the aminoglycoside resistant mutant strain A31 and the wild type strain J53 after growth in media of different osmolarities. Periplasmic proteins were isolated from exponential phase cultures of the J53 (1, 2, and 3) and A31 (4, 5, 6, and 7) strains cultivated in nutrient broth (1 and 4) including 1% NaCl (2 and 5), 0.5 mol/l sorbitol (3 and 6), or sorbitol plus 50 µg/ml kanamycin (7). The arrow indicates the position of the 61 kDa protein. Molecular weight markers are indicated on the left.

kDa protein in the periplasm of the J53 strain was greater in NB and NB + 0.5 M sorbitol than in NB + 1% NaCl (Figure 2, lanes 1-3). On the other hand, the mutant A31 showed very low levels of this protein when cultivated in NB and NB + 1% NaCl, while growth in NB + 0.5 mol/l sorbitol resulted in an expression of the 61 kDa corresponding to approximately 10% of the levels found in the parental strain (Figure 1 and Figure 2, lanes 4, 5, and 6). Growth in the presence of kanamycin did not affect the level of the 61 kDa protein in the periplasm of the A31 strain (Figure 2, lane 7). The fate of this periplasmic protein after cultivation in NB was also investigated in five other aminoglycoside resistant mutants, isolated in high osmolarity medium, and all of them proved to be deficient in the expression of this protein (data not shown).

DISCUSSION

It has already been shown that resistance to aminoglycosides can be affected by several growth medium factors (Medeiros *et al.*, 1971; Höltje, 1978, 1979; Hancock, 1981). In a previous work we observed that the susceptibility of different Gram-negative bacteria to aminoglycosides can be drastically altered depending on the growth media used. Increasing osmolarity resulted in a decreased susceptibility to several aminoglycoside antibiotics (Rodriguez *et al.*, 1990). Aminoglycoside resistant clones selected in a high osmolarity medium also showed the same variation in susceptibility to kanamycin when assayed on other media. All aminoglycoside resistant mutants isolated in high osmolarity medium were approximately 100-fold more resistant to kanamycin as well as to some other aminoglycoside antibiotics in any media when compared to the wild type strain (Table I and unpublished observations). The decrease in levels of a non osmoregulated 61 kDa periplasmic protein in the A31 strain as well as in other mutants selected in high osmolarity medium, strongly suggest that it may be involved in the uptake of the aminoglycoside in these mutants.

The induction of two periplasmic proteins is usually observed after growth in high osmolarity medium. One protein (35 kDa), the product of the *proU* gene, is involved with the uptake of glycine betaine while the other one (22 kDa), coded by the *osmY* gene, does not have any known physiological function (May *et al.* 1986; Yim and Villarejo, 1992). However, no significant difference in the electrophoretic profile of periplasmic proteins extracted from the A31 and J53 strains could be observed in the range between 35 and

20 kDa. Moreover, the expression of the 61 kDa protein was not osmoregulated in the wild type strain as could be seen with SDS-PAGE (Figures 1 and 2).

The oligopeptide transport pathway is controlled by an operon composed by five genes named *oppA*, *B*, *C*, *D* and *F*. The products of the *oppB*, *C*, *D*, and *F* genes are cytoplasmic membrane proteins while the one coded by *oppA*, the most abundant product of the operon, is a major periplasmic protein with a reported Mr ranging from 59 to 62 kDa in SDS-PAGE (Mitsui et al., 1984; Guyer et al., 1985; Hiles et al., 1987). Curiously, it has been shown that the OppA protein can be induced by adding polyamines to the growth medium even though it is not a component of the ordinary polyamine transport system (Mitsui et al., 1984; Kashiwagi et al., 1990). The construction of *E. coli* strains with insertions in the *oppA* gene and overexpression of the wild type OppA protein disclosed the relevant role of this periplasmic protein in the uptake of several amino-glycosides (Kashiwagi et al., 1992). Based on the relative abundance in the periplasm and similar molecular weights and reduced levels in the periplasm of our aminoglycoside resistant mutants an identity between the OppA protein and the 61 kDa protein is possible.

If the osmodependent aminoglycoside resistant mutants are indeed affected in the expression of the OppA protein, some important conclusions could be drawn about the regulatory mechanisms disturbed in these strains.

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RESUMO

Uma nova classe de mutante de *Escherichia coli* resistente aos antibióticos aminoglicosídios, dependente de osmolaridade, foi isolada. A análise de proteínas do envoltório celular de um clone representativo revelou uma drástica redução de uma proteína periplasmática (61 kDa). O envolvimento dessa proteína na incorporação dos aminoglicosídios e sua possível identidade com uma proteína do sistema de transporte de oligopeptídios é discutido.

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