OUTER SURFACE CHANGES OF *PSEUDOMONAS AERUGINOSA* IN RELATION TO RESISTANCE TO GENTAMICIN AND CARBENICILLIN

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SUMMARY. The outer surface structure of clinical isolates of *Pseudomonas aeruginosa* resistant to carbenicillin or gentamicin or both was studied by thin-section electronmicroscopy and compared with that of sensitive isolates. The latter had a fairly smooth outer layer. Strains resistant to both antibiotics were characterised by extrusions that appeared to constitute extensions of the outer membrane. The outer membrane appeared wavy with distortion of its tripartite structure. The latter findings were also present in isolates resistant to only one of the two antibiotics. The disorganisation of the outer membrane might contribute to the expression of resistance.

INTRODUCTION

The importance of the cell envelope and particularly the outer membrane (OM) of *Pseudomonas aeruginosa* in the expression of resistance to antimicrobial drugs by an exclusion mechanism has been well documented (Nakae and Nikaido, 1975; Osborn and Wu, 1980).

In strains of *P. aeruginosa* that are resistant to carbenicillin (Barrett and Asscher, 1972) or to polymyxin (Gilleland and Lyle, 1979) because of a permeability defect, a correlation between the chemistry of the OM and resistance has been indicated. Alterations of OM protein have also been reported for *P. aeruginosa* mutants resistant to polymyxin, EDTA and gentamicin (Nicas and Hancock, 1980).

It has also been shown that variants of *P. aeruginosa* habituated to increased concentrations of carbenicillin or gentamicin are characterised by increased adherence, evidently due to an altered surface as detected by electronmicroscopy (Lianou *et al.*, 1980). The morphological responses of *P. aeruginosa* to N-formimidoyl thienamycin, azthreonam, ceftazidime and cefoperazone were studied by transmission electronmicroscopy (Elliot and Greenwood, 1984).

In this investigation we have studied the fine structure of *P. aeruginosa* clinical isolates that were resistant to carbenicillin or gentamicin or both.

MATERIAL AND METHODS

Organisms. Ten fresh isolates of *P. aeruginosa* derived from various clinical sources were used. They were identified by previously published criteria (Legakis and Papavassiliou, 1975) and were chosen to differ only in their susceptibility to gentamicin and carbenicillin (table). Resistant isolates had an inhibition zone of not greater than 8 mm with gentamicin disks and 10 mm with carbenicillin disks. All strains were grown, with aeration and stirring, at 37°C in Mueller Hinton Broth (Difco) with inocula taken from an 18-h broth culture.

Susceptibility testing. The single-disk diffusion method was used as described by Bauer *et al.* (1966). Zones of inhibition were measured with a ruler to the nearest millimeter. All disks were obtained from Bio-Merieux, Paris, France.

Electronmicroscopy. Cultures in the exponential phase were harvested by centrifugation at 3000 *g* for 20 min. The pellets were prefixed in 0.1M sodium cacodylate containing glutaraldehyde 3% w/v and formaldehyde 2% v/v. Postfixation was for 30 min at 4°C in 2% w/v OsO₄. Thin sections were cut on a MT-2 ultramicrotome with glass knives. They were collected on copper grids, double stained with uranyl acetate and lead citrate and then examined with a Phillips EM 200 electron microscope operating at 80 kV.

Serotyping. Unabsorbed antisera, sixteen (1–16) monovalent and four pools from Institut Pasteur, Paris, were used for serotyping the strains. Cultures grown on Mueller-Hinton Agar (MHA) (Difco) at 37°C for 18–24 h were used as antigens. Typing was done by the slide-agglutination technique (Brokopp *et al.*, 1977; Legakis *et al.*, 1982).

Carbenicillinase activity. Hydrolysis of carbenicillin was detected in intact cells, disrupted cells and supernates in MHA containing carbenicillin at a twofold concentration of that required to inhibit the sensitive strain (NCTC10490) incorporated into the medium at a density of about 10⁴ cfu/ml. Strain PA401, producing the PSE-4 enzyme, kindly provided by Dr G. Rolinson (Beecham Research Laboratories, Surrey) was used as a positive control.

Assay for aminoglycoside-modifying enzymes. Extracts of the strains were examined for the presence of aminoglycoside-adenylylating, -acetylating and -phosphorylating enzymes as described by Holmes *et al.* (1974).

RESULTS

Thin sections of sensitive strains (fig. 1) showed the typical trilaminar unit-membrane appearance of gram-negative bacteria with a fairly smooth surface. Strains resistant to carbenicillin and gentamicin (fig. 2) were characterised by the presence of well defined globular bulges, appearing to be extensions of the OM, which showed undulations and distortion of its tripartite structure. The undulations and altered stratification characterised also the strains resistant only to gentamicin (fig. 3). The membrane of the carbenicillin-resistant strains showed the same features.

Although the number of isolates was limited, it seems that resistant strains were differentiated from their sensitive counterparts in terms of the O-antigen reaction with specific antisera. As shown in the table, serotype O6 included the three sensitive strains, while resistant strains were expressed mainly by serotype O "PME", agglutinating in pooled antisera but not in the respective individual antisera. It is noteworthy that no resistant strain belonged to serotype O6, confirming previous results (Legakis *et al.*, 1982).

Most of the carbenicillin-resistant strains did not produce β -lactamase and most strains resistant to aminoglycosides, excepting amikacin, did produce aminoglycoside-modifying enzymes (table).

FIG. 1—Thin section of *P. aeruginosa* strain PA428 sensitive to carbenicillin and gentamicin. Bar = 0.2 μ m.

FIG. 2—Thin section of a clinical isolate of *P. aeruginosa* (strain PA388) resistant to carbenicillin and gentamicin. Bar = 0.2 μ m.

FIG. 3—Thin section of a clinical isolate of *P. aeruginosa* (strain PA391) resistant to gentamicin but sensitive to carbenicillin. Bar = 0.2 μ m.

TABLE

Resistance phenotype, O serotype, carbenicillinase and aminoglycoside-modifying enzyme production in P. aeruginosa

Strain no.	Resistance phenotype	O serotype	Hydrolysis of carbenicillin	Aminoglycoside-modifying enzyme
Pa28-0	Na, C, Te	6	NT	ND
Pa388	Na, C, Te, Cb, Gm, Tm, Si	PME	—	ANT-2''
Pa389	Na, C, Te, Cb, Gm, Tm, Si	PME	—	AAC(6)-II
Pa390	Na, C, Te, Cb, Gm, Tm, Si	PME	—	ANT-2''
Pa391	Na, C, Te, Gm, Tm, Si	4	NT	ANT-2''
Pa400	Na, C, Te, Cb	5	+	NT
Pa402	Na, C, Te, Cb	NT	+	NT
Pa403	Na, C, Te	6	NT	NT
Pa405	Na, C, Te, Gm, Tm, Si	PME	NT	ANT-2''
Pa428	Na, C, Te	6	NT	NT

Na = nalidixic acid; C = chloramphenicol; Tc = tetracycline; Cb = carbenicillin; Gm = gentamicin; Tm = tobramycin; Si = sisomycin. All strains were sensitive to polymyxin and amikacin.

PME = agglutinating in pooled antisera (2, 5, 15, 16) but not in the individual antisera.

NT = not tested. ANT-2'' = aminoglycoside 2''-0 nucleotidyl-transferase; ANT(6)-II = aminoglycoside 6'-N-acetyltransferase-II.

DISCUSSION

Evidence has been produced that lipopolysaccharides (LPS) linked with proteins in a protein-LPS complex (Barrett and Asscher, 1972; Stinnett *et al.*, 1973) occupies, as in the Enterobacteriaceae (Nikaido and Nakae, 1979) the outer half of the OM in *P. aeruginosa* (Eagon, 1974; Gilleland and Murray, 1976). The supramolecular organisation of these molecules results in the formation of transmembrane pores for the entrance of hydrophilic substances, including polar antibiotics, into the cells (Nakae and Nikaido, 1975).

This architecture appears to be disturbed in LPS-defective mutants of *P. aeruginosa* lacking polymerised O-antigenic side chains, in the sense of redistribution of membrane components so that more hydrophobic molecules (phospholipids) are exposed to the surface of the membrane (Nakae and Nikaido, 1975; Meadow *et al.*, 1978). These changes were similar to those observed in our resistant isolates and to those found after treatment with polymyxin (Koike *et al.*, 1969). The bulges observed in isolates resistant to carbenicillin or gentamicin or both, as in LPS-deficient mutants (Meadow *et al.*, 1978), may have been fragments of the outer membrane, as are found in *Escherichia coli* (Hoekstra *et al.*, 1976), which are extruded from the bacterial surface and which, because of their lipophilicity, are attached to the hydrophobic surface of the OM. LPS appears to be responsible for the heat-stable O-antigenic specificity (Chester *et al.*, 1973).

If it is assumed that LPS alterations are present in the resistant isolates, the data of the present paper might provide an explanation for the differential distribution of O serotypes among sensitive and resistant isolates as reported by Legakis *et al.* (1982) and Penketh *et al.* (1983). This hypothesis is supported by previous reports on the loss of the specific O-serotyping reaction in *P. aeruginosa* mutants characterised by defective structure of the O-antigenic region of LPS (Meadow, 1975).

The fact that the sensitivity of the LPS-defective mutants was greater (Meadow, 1975; Koval and Meadow, 1977) or equal to (Meadow *et al.*, 1978) that of their parent strains, is not apparently relevant to the above consideration. However alterations of the OM-proteins responsible for an increase of the sensitivity could most probably also exist as occurs in the heptose-deficient mutants of *E. coli* (Koplow and Goldfine, 1974) and *Salmonella typhimurium* (Ames *et al.*, 1974; Roantree *et al.*, 1977) and in the supersensitive strains of *P. aeruginosa* isolated from patients with cystic fibrosis (Irvin *et al.*, 1981).

At present it is difficult to assign any role to OM-surface alterations for the expression of resistance. However it has been suggested that impaired cellular permeability might reflect differences in the detailed organisation of the OM (Meadow, 1975). The present results provide indirect evidence for the involvement of impaired permeability as the expression of resistance in the clinical isolates confirming previous reports (Bryan and Van Den Elzen, 1977; Richmond, 1978; Davies 1979; Legakis *et al.*, 1979).

The presence of carbenicillinase and aminoglycoside-modifying enzymes in some of our isolates does not necessarily exclude the importance of impaired permeability as the expression of resistance. Studies with β -lactam antibiotics (Richmond and Curtis, 1974) have demonstrated that β lactamases, even though strategically located in the periplasmic space, provide poor protection against β -lactam attack when the antibiotic

can penetrate the OM freely. In connection with the production of aminoglycoside-modifying enzymes, it has been demonstrated that where inactivation accompanies modification of the drug, the rate of loss of antibiotic potency is usually extremely low (Diedrichsen *et al.*, 1977). Thus, although a certain amount of drug modification or inactivation or both, by the above enzymes, may occur, it is unlikely that this leads wholly to the expression of the resistance phenotype. It seems more likely that the impermeability of the OM, accompanied in some strains by the production of drug-inactivating enzymes as an additional resistance function, leads to the expression of resistance.

The data obtained, the fact that the LPS constitutes an exclusion barrier for carbenicillin and has a great affinity for gentamicin (Day, 1980), and the role of OM proteins in the function of the OM (Nakae and Nicaido, 1975; Nicas and Hancock, 1980; Osborn and Wu, 1980) should stimulate further interest in the molecular organisation of the OM and its role in the expression of the resistance phenotype. Work is in progress to study the OM proteins and to analyse the lipopolysaccharides of these strains.

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