The Effect of Lipopolysaccharide Composition on the Ultrastructure of *Pseudomonas aeruginosa*

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The surface structure of *Pseudomonas aeruginosa* PAC1 and PAC1R and of lipopolysaccharide-defective mutants derived from them was studied by negative-staining and thin-section electron microscopy and compared with that of a rough mutant with wild-type lipopolysaccharide. The rough mutant and the parent strains had fairly smooth outer layers. Negatively stained preparations of all the mutants lacking polymerized O-antigenic side-chains, including a semi-rough mutant, showed numerous blebs on the surface. In thin sections of these mutants occasional extrusions from the surface were seen. They appeared to consist of material extruded from the outer membrane, but there was no evidence to suggest they were complete unit membranes. Polymerized O-antigenic side-chains in the lipopolysaccharide appear to be required to produce the wild-type appearance of the outer membrane in *P. aeruginosa*.

INTRODUCTION

In the Enterobacteriaceae, phenotypically rough mutants have defective lipopolysaccharides (LPSs) lacking the O-antigenic side-chains and often parts of the core (for review, see Lüderitz *et al.*, 1971). Such mutants of *Salmonella typhimurium* have an unstable outer membrane, and extruded blebs of incomplete LPS can be seen by electron microscopy (Smit, Kamio & Nikaido, 1975; Irvin *et al.*, 1975). In *Pseudomonas aeruginosa*, LPS-defective mutants do not produce phenotypically rough colonies and mutants giving rough colonies on plates appear to have wild-type LPS (Koval & Meadow, 1977). It was thus interesting to see whether the ultrastructure of the surface of LPS-defective mutants of *P. aeruginosa* was different from that of the parent strain and other mutants with wild-type LPS.

METHODS

Organisms and growth conditions. *Pseudomonas aeruginosa* PAC1 (NCIB 10848) which belongs to Habs serotype 3, PAC1R which is a spontaneous mutant isolated from it as resistant to phage PS1, the LPS-defective mutants PAC605 and PAC608, and the phenotypically rough mutant PAC307 have all been described before (Koval & Meadow, 1977). *Pseudomonas aeruginosa* PAC610 is a spontaneous mutant isolated from PAC605 by T. L. Pitt (Cross Infection Reference Laboratory, Colindale, London) by selecting for resistance to dilutions of human sera. All cultures were grown at 37 °C either with shaking in flasks containing one-fifth their volume of nutrient broth (Oxoid no. 2) or on plates of nutrient agar (1.5%, w/v).

Isolation and analysis of LPS. LPS was isolated from bacterial walls, partially hydrolysed, separated on columns of Sephadex G75, and the glucose, rhamnose, heptose, phosphate and amino compounds present in each fraction were determined, all as described previously (Koval & Meadow, 1977).

Fig. 1. Fractionation of polysaccharide obtained from partially degraded LPS from *P. aeruginosa*: (a) PAC1R, (b) PAC610 (—) and PAC605 (---). LPS (30 mg) was hydrolysed with 1% (v/v) acetic acid for 1 h and the hydrolysate was eluted from a column (2.5 × 70 cm) of Sephadex G75 with pyridine/acetic acid buffer, pH 5.4. Fractions (4 ml) were analysed for total carbohydrate by the phenol/sulphuric acid method.

Electron microscopy. (i) *Negative staining.* A drop of the sample was applied to a copper grid coated with Formvar and carbon and the excess was removed. The stain [phosphotungstic acid (1%, w/v) adjusted to pH 6.5 with KOH] was applied immediately and the excess was again removed with filter paper.

(ii) *Thin sections.* Bacteria in the exponential growth phase were harvested by centrifugation (10000g). The pellets were prefixed in 0.1 M-sodium phosphate buffer (pH 7.2) containing glutaraldehyde (3%, w/v) and then washed three times in the buffer. Postfixation was for 2 h at room temperature in the same buffer containing osmium tetroxide (1%, w/v). The specimens were dehydrated through a series of concentrations of ethanol and propylene oxide and then embedded in Epon 812. Thin sections were cut with a diamond knife on a Sorvall Porter-Blum MT-2 ultramicrotome and double-stained with uranyl acetate and lead citrate. The grids were examined in a Jeol JEM 100B electron microscope at an operating voltage of 80 kV.

RESULTS AND DISCUSSION

Characterization of the mutants

The serum-resistant mutant PAC610 appeared to have a defective LPS since it did not react with Habs type 3 antiserum. Separation of its partially degraded polysaccharide on Sephadex G75 yielded a single peak as detected by the phenol/sulphuric acid reagent. The polysaccharide appeared to be similar to that from PAC605 from which PAC610 had been derived except that there was more carbohydrate (Fig. 1*b*). As expected from the lack of O-antigenicity, the degraded polysaccharide contained neither the high molecular weight peaks derived from the O-antigenic side-chains (H1 and H2) nor the leading shoulder (L1) on the low molecular weight fraction, all of which are produced from the wild-type LPS (Fig. 1*a*). Analysis of the L2 peak obtained from PAC610 showed that it contained (molar ratios in parentheses) glucose (4.5), rhamnose (1.8), heptose (2.4), galactosamine (1.0) and alanine (1.3). It thus appeared to contain the core components found in the wild-type LPS (Koval & Meadow, 1977) in approximately the same molar ratios except for glucose (wild-type 5.5).

The LPS composition and some other properties of the mutants studied are summarized in Table 1.

Electron microscopy

Negative staining. The LPS-defective mutants were clearly distinguishable from the wild type by negative-staining electron microscopy (Fig. 2). The surface of the parent strain appeared fairly smooth. Many preparations showed extraneous globular material of

Table 1. *Properties of strains used*

Strain	PAC1	PAC1R	PAC307	PAC608	PAC610	PAC605
Parent strain	PAC1	PAC1	PAC1	PAC1R	PAC605	PAC1R
Phenotype selected		Resistance to PS1	Amidase ⁻	Resistance to pyocin P16	Serum-resistant	Resistance to pyocin P16
Colony type	Smooth	Smooth	Rough	Smooth	Smooth	Smooth
Habs serotype	3	3	3	3	NT	NT
LPS fractions present*						
H1, H2	+	+	+	0	0	0
L1	+	+	+	+	0	0
L2	+	+	+	+	+	+

NT, Not typable; +, present; 0, absent.

* The fractions are those shown in Fig. 1.

approximately 20 nm diameter attached to the surface of the bacteria, the flagella and the pili. This material may have been fragments of the outer membrane which were extruded from the bacterial surface and which, because of their lipophilicity, attached to the hydrophobic surfaces of the organism. Such material has been seen in several different species, and in *Escherichia coli* has been identified chemically as fragments of outer membrane (Hoekstra *et al.*, 1976). The large amounts present in some of our preparations may reflect the relatively fragile walls of *P. aeruginosa*, which in turn may depend in part on the small amounts of lipoprotein linking the outer membrane to the peptidoglycan as compared with the amounts found in *E. coli* (for a discussion of wall structure in *Pseudomonas*, see Meadow, 1975).

The parent strain PAC1, PAC1R and PAC307 as well as other mutants (not listed) known to have complete wild-type LPS, all appeared similar by negative staining (see Fig. 2*a*). In contrast all mutants lacking polymerized O-antigenic side-chains showed typical extrusions from the wall (see Fig. 2*b*). The extrusions resembled those found in rough strains of *S. typhimurium* LT2 (Irvin *et al.*, 1975; Smit *et al.*, 1975). Similar blebs can be induced by polymyxin treatment in *P. aeruginosa* p29 (Koike, Iida & Matsuo, 1969) and in smooth strains of *S. typhimurium* (Lounatmaa, Mäkelä & Sarvas, 1976). In *P. aeruginosa* PAC1 and mutants derived from it, the blebs were always present in mutants lacking the polymerized O-antigenic side-chains of the LPS. We could detect no differences either in appearance or in the number of blebs in mutants with additional core defects. The mutant shown in Fig. 2*b*), PAC610, apparently contained an almost complete core, yet its appearance was indistinguishable both from the most defective mutant PAC605 and the least defective PAC608. The latter contains not only a complete LPS core, but more of the L1 fraction (core polysaccharide with a single unit of antigenic side-chain) than the wild type (Koval & Meadow, 1977). It is thus similar to the semi-rough strains found in *Salmonella* (Naide *et al.*, 1965). Although it had enough O-antigenic material to agglutinate with Habs type 3 antiserum (Koval & Meadow, 1977), there was apparently not enough polymerization to prevent the formation of the characteristic surface blebs of the LPS-defective mutants.

Five mutants of PAC1 whose LPSs contain polymerized O-antigenic side-chains and six LPS-defective mutants have now been examined. All those yielding H1 fractions after partial hydrolysis of the LPS looked like PAC1 (Fig. 2*a*) whereas all those lacking this fraction looked like PAC610 (Fig. 2*b*). PAC307, the mutant with rough colonial morphology but wild-type LPS, had a smooth outer layer like PAC1, but the cells were often longer and the rough phenotype may have been caused by some defect in cell division.

Thin sections. Thin sections of PAC1 and PAC1R (Fig. 3*a*) showed the typical unit membrane appearance of Gram-negative bacteria with no additional extrusions. The phenotypically rough mutant PAC307 appeared similar except that there was some evidence for

Fig. 2. Surface structure of *P. aeruginosa*: (a) PAC1, (b) PAC610. The preparations were stained with phosphotungstate. Bar markers represent 200 nm.

Fig. 3. Thin sections of *P. aeruginosa*: (a) PAC1R, (b) PAC605. The bacteria were prefixed with glutaraldehyde and postfixed with osmium tetroxide. The sections were double-stained with uranyl acetate and lead citrate. Bar markers represent 100 nm.

a delay in cell separation, supporting the tentative conclusion that this mutant is defective in cell division. In thin sections of each of the LPS-defective mutants there were a few small vesicles varying in size from about 20 to 40 nm. They appeared to extrude from the outer membrane (Fig. 3b). They were fewer in number and considerably smaller than the blebs seen in negatively stained preparations, but their existence was limited to those mutants in which blebs had been seen. The small number of vesicles visible in thin sections may result from their small size so that the bleb seldom fitted into the plane of the section. In addition the washing procedures involved in preparing samples for thin sections might be expected to lead to loss of loosely associated material and to extraction of lipid therefrom. The latter together with the poor staining of polysaccharide material by the procedure used would explain their small size. We could see no sign of unit membrane structure in the vesicles

although this was visible in a few sections where the outer membrane had detached from the bacteria completely and had curled into the characteristic cylinders found in other species (Nakamura & Mizushima, 1975; Costerton, Ingram & Cheng, 1974).

We conclude therefore that in *P. aeruginosa* a rough colonial morphology does not necessarily reflect changes in outer membrane surface structure, but that defects in LPS synthesis preventing synthesis or attachment of polymeric O-antigenic side-chains result in a rough outer membrane surface and the formation of lipophilic blebs. Undulation is sometimes seen on the surface of wild-type *S. typhimurium* and *P. aeruginosa* PAO if acrolein is present in the fixative (Gilleland & Murray, 1976; Lounatmaa, 1977). Even after glutaraldehyde fixation, wild-type *Salmonella* sometimes show undulation of the surface after growth on solid media rich in salts (K. Lounatmaa, personal communication). Strains of *P. aeruginosa* PAC with wild-type LPS had the type of appearance shown in Fig. 2(a) whether grown on solid or in liquid media.

It is perhaps surprising that despite the gross alterations in surface structure seen in the LPS-defective mutants of *P. aeruginosa* PAC1 described here, none seemed to be affected in their general stability, growth rate or metabolism and only the most defective mutant PAC605 showed increased sensitivity to any antibiotics (Koval & Meadow, unpublished).

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