Characterization of Polyagglutinating and Surface Antigens in 
*Pseudomonas aeruginosa*

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Mutants of *Pseudomonas aeruginosa* PAC1R (serotype O:3) which were resistant to bacteriophage D were isolated and shown to react with O:5d, O:9 and O:13 antisera as well as O:3. Antisera to the parent strain and to the three polyagglutinating (PA) mutants also showed cross-reactions. The mutants differed from the parent strain in their lipopolysaccharide (LPS) composition. The LPS from two of the three mutants yielded high molecular weight polysaccharide fractions. Although the high molecular weight fraction from one of the mutants contained the amino sugars and other components characteristic of the O:3 serotype strains, its mobility on Sephadex G75 was different from that of the parent strain. The high molecular weight material from the second mutant lacked the O-antigenic determinants but these were present in a semi-rough LPS fraction. The third mutant appeared rough and completely lacked the O-antigenic components. These three mutants were compared with the parent strain and with a non-agglutinating LPS-defective mutant which lacked both O-antigenic side chains and all neutral sugars in the outer core. Agglutination with absorbed sera and haemagglutination using purified LPS and ELISA detection suggested that wall components other than LPS were responsible for some of the cross-reactions observed. The components responsible were detected after SDS-PAGE of crude outer membrane fractions by a combination of Coomassie blue and silver-staining and antigenic components were detected by immunoelectrophoresis and ELISA-linked immunoblotting of the gels. The main antigenic determinants detected by antiserum to the parent strain were in the high molecular weight O-polysaccharide fractions and in the semi-rough fractions of the LPS, with some activity due to the H protein of the outer membrane. O:5d antiserum reacted with unidentified high molecular weight polysaccharide fractions. Cross-reactions with the O:9 antiserum appeared to be due mainly to the F porin and, to a lesser extent, to the G and E proteins of the outer membrane. O:13 antiserum reacted with high molecular weight polysaccharide fractions but also with the rough core and F and H protein. Cross-reactivity of the other three mutant antisera could largely be interpreted in terms of the outer membrane components exposed in each strain. One reacted strongly with the F porin and the rough core, while the others reacted with a number of protein and LPS-derived fractions. It is suggested that, in these PA mutants, changes in LPS composition modify their O-antigenicity and also expose other outer membrane components to varying extents. It is these protein and rough core components which are responsible for the cross-reactivity with heterologous O-antisera.

**INTRODUCTION**

The typing of *Pseudomonas aeruginosa*, an opportunist pathogen, is generally based on the identification of the heat-stable O-antigen by means of a set of antisera. The typing strains were
originally isolated and defined by Habs (1957) and this set of 12 strains has now been increased by the addition of further groups in the current International Antigen Typing Scheme (Lanyi & Bergan, 1978). The heat-stable antigens mainly responsible for this antigenicity are the LPS components of the outer membrane and in particular the polymerized O-polysaccharide side-chain (Chester et al., 1973). In many strains, particularly those described as semi-rough, O-antigenicity is also determined by short O-chains (probably not more than one or two units long) attached to the LPS core (Koval & Meadow, 1977).

About 4% of all isolates of P. aeruginosa and up to 30% of those isolated from environmental sources or long term infections such as cystic fibrosis, cross-react with more than one O-serotype (Holby, 1982). In some strains the underlying serotype can be detected after absorption with antisera prepared against a polyagglutinating (PA) strain to reveal the underlying antigen, but in others such treatment removes all typability (Pitt & Erdman, 1977). One of the difficulties in characterizing PA strains has been that most if not all of those isolated have been of unknown provenance. An immunochemical and biochemical solution depends on the need to identify the parent strain and to compare it with its PA mutant.

In the course of studies on LPS structure and function in P. aeruginosa we have isolated a number of spontaneous LPS-defective mutants of P. aeruginosa PACIR, some of which could not be typed but which were not PA (Koval & Meadow, 1977). In seeking a new method of selecting LPS-defective mutants we used a bacteriophage D which appeared to be smooth-specific. Among the mutants which we isolated as resistant to this bacteriophage were some which reacted with O:3, O:5d, O:9 and O:13 antisera, which characterize some PA strains encountered in clinical laboratories. The work described in this paper was undertaken to try to identify the factor or factors responsible for the cross-reactivity of our mutants and to use them to study their outer membrane structure.

**METHODS**

Pseudomonas aeruginosa strains. The parent strain PACIR (O:3), derived from PAC1 (NCIB 10848), and the LPS-defective mutants PAC605, PAC609 and PAC608 have all been described previously (Koval & Meadow, 1977; Meadow & Wells, 1978). The three PA mutants PAC601, PAC700 and PAC701 were isolated as mutants resistant to bacteriophage D by plating 0.1 ml of an exponential phase culture of PAC1R together with 0.1 ml phage lysate (10$^9$ p.f.u. ml$^{-1}$) on nutrient agar. Colonies detected after overnight incubation at 37°C were purified by replating and tested to confirm their resistance. Bacteriophage D was obtained from Dr S. B. Primrose, University of Warwick, UK.

The bacteria were grown in nutrient broth or on nutrient agar at 37°C and their pyocin and bacteriophage sensitivities were determined as previously described (Koval & Meadow, 1977).

Antisera. Standard O: sera, prepared using whole killed bacterial cells, were obtained from Dr T. L. Pitt, Cross Infection Reference Laboratory, PHLS, Colindale, London, UK. Dr Pitt also prepared antisera to strains PAC1R, PAC601, PAC700 and PAC701 as previously described (Chester et al., 1973).

LPS-absorbed sera were prepared by adding freeze-dried LPS (100 μg) suspended in 5 μl PBS (containing, g l$^{-1}$: NaCl 7.2; Na$_2$HPO$_4$ 1.48; KH$_2$PO$_4$ 0.43; pH 7.2) to 500 μl undiluted serum. After thorough mixing the serum was stored at 4°C for 48 h with occasional shaking. The resulting precipitate was removed by centrifugation in an Eppendorf Microfuge and the supernatant fluid retained as LPS-absorbed serum. The absence of excess LPS in the serum was checked by its inability to cause haemagglutination of red blood cells without prior sensitization.

Sera were absorbed with whole bacteria by a modification of the method of Dankert & Hofstra (1978). Stationary-phase bacteria were pelleted and washed with PBS before adding serum. The mixtures were incubated for 48 h at 4°C.

Agglutination reactions. Slide agglutination tests were carried out using fresh overnight cultures of bacteria grown on nutrient agar. Antisera were previously absorbed with bacteria or LPS as described by Dankert & Hofstra (1978).

Passive haemagglutination was carried out essentially as described by Hammerling & Westphal (1967) using sheep red blood cells sensitized with LPS. A sample (0.5 ml) of suspension of red blood cells (2%, v/v, in PBS) was incubated with 0.1 ml LPS suspension in PBS (50 μg ml$^{-1}$) at 37°C for 30 min. The cells were washed three times to remove excess antigen and finally resuspended in PBS to give a concentration of 0.5%. Agglutination was recorded after incubation with serial dilutions of antisera at 4°C overnight.

Enzyme-linked immunosorbent assay (ELISA) was done as described by Hofstra & Dankert (1980a). Immunodiffusion reactions were investigated as described by Ouchterlony (1949).
Isolation and analysis of LPS fractions. LPS was isolated from bacterial walls by phenol extraction, hydrolysed with acetic acid and fractionated on Sephadex G75 and Biogel P6 as previously described (Rowe & Meadow, 1983). It contained no detectable protein as measured by automatic amino compound analysis and Coomassie blue staining of SDS-PAGE gels. The methods for estimating glucose, rhamnose, heptose, and amino compounds have all been described previously (Koval & Meadow, 1977).

Isolation of crude outer membranes. The method was essentially that of Hofstra & Dankert (1980b) except that a French pressure cell was used to disrupt the bacteria and the unbroken bacteria were then removed by centrifugation at 6000 g for 10 min at 4 °C.

Polyacrylamide gel electrophoresis (SDS-PAGE). Slab gel electrophoresis using a discontinuous buffer system was as described by Mizuno & Kageyama (1978) with the following modifications. In all gels the ratio of acrylamide to bis-acrylamide was 12:0-33 (w/w) and they were supplemented with 0-07% ammonium persulphate. The Tris/HCl buffer used for the stacking gel was 62·5 mm, pH 6·8.

The freeze-dried crude outer membrane fractions (2 to 3 mg) were solubilized in a mixture containing Tris/HCl (0·05 M, pH 6·8), SDS (1%, w/v) and glycerol (10%, w/v) with β-mercaptoethanol (1%, v/v) where indicated, by heating at 100 °C for 15 min to give a final concentration of 10 mg crude membrane per ml. Bromophenol blue (0·05% final concentration) was added as a marker. This solution (10 μl) was then separated by electrophoresis at a constant current of 24 mA per gel until the marker reached the bottom of the gel. Proteins were detected by staining overnight with acetic acid (10%, v/v), methanol (25%, v/v) and Coomassie brilliant blue (0·1%). Carbohydrates were detected by the silver stain described by Tsai & Frasch (1982).

The following standard proteins were used to calibrate the molecular weights: Brøchner Mannheim protein calibration kit, consisting of bovine serum albumin (mol. wt 68000), DNA polymerase (β 165000, β 155000, α 39000) and trypsin inhibitor (21500), together with horse heart cytochrome c type III (12384) and ovalbumin (Sigma) (45000). The molecular weights quoted are the average derived from at least three runs.

Two-dimensional immunoelectrophoresis. The method described by Converse & Papermaster (1975) was used except that the second agarose layer contained antiserum at a final concentration of 4·8% (v/v), and the immunoprecipitates were stained with Coomassie brilliant blue for 10 min.

Electrophoretic transfer and immunoblotting of outer membrane proteins from SDS-PAGE (Western blotting). The transfer of separated components from SDS-PAGE gels to nitrocellulose paper (0·45 μm BASS, Schleicher & Schüll GmbH, D-3354 Dassel, FRG), was adapted from the method described by Towbin et al. (1979). After electrophoresis in the first dimension the SDS-PAGE gels were suspended in a Bio-Rad Trans-Blot electrophoretic transfer cell containing 25 mm-Tris/HCl, 192 mm-glycine and methanol (20%, v/v) pH 8·3. The nitrocellulose sheet was placed nearest to the anode and electrophoresis was carried out at 30 V, 100 mA for 16 h. After electrophoresis sufficient protein remained on the SDS-PAGE gel to enable it to be detected by Coomassie brilliant blue staining as before. The nitrocellulose sheet was then treated with primary (O-antiserum) and secondary (goat anti-rabbit IgG conjugated to horse radish peroxidase; Miles Laboratories, Slough, UK) antibodies as described by Towbin et al. (1979) and modified by E. Smith & K. Roberts, John Innes Research Institute, Norwich, UK (personal communication). The nitrocellulose sheet was initially incubated at 20 °C for 15 min in a mixture of bovine serum albumin (3%, w/v), NaCl (0·9%, w/v) and Tris/HCl (10 mm, pH 7·4). The primary antibody (100 μl) was then added to give a final dilution of 1:1000 and the incubation continued for a further 2 h. The nitrocellulose sheet was then washed five times (300 ml per wash) in Tris-buffered saline (TBS) containing NaCl, 0·9% (w/v) and Tris/HCl (10 mm, pH 7·4) for 30 min at 4 °C. The sheet was then incubated for 2 h at 20 °C in secondary antibody (100 μl) diluted 1:1000 in TBS supplemented with bovine serum albumin (3%, w/v) and Nonidet P-40 (0·05%). It was washed again in TBS as described above and the antibody complexes were detected by incubating in a solution (100 ml) of 4-chloro-1-naphthol and hydrogen peroxide for 10 min at 20 °C as described by Hawkes et al. (1982). Each experiment was performed at least three times with essentially the same results. Runs were also repeated using different concentrations of antiserum and membranes.

RESULTS

Isolation and characterization of polyagglutinating (PA) mutants

Bacteriophage D was originally selected for use because it appeared to be smooth-specific for strains of PAC1R. Both the parent strain PAC1R and PAC609, a mutant which retained the polymerized O-side-chains but lacked a glucose unit in the core (Rowe & Meadow, 1983), were sensitive to this bacteriophage. Mutants lacking the O-polymer, including the semi-rough strain PAC608 and those with defective cores to their LPS, were all resistant. We therefore isolated a number of mutants which were resistant to bacteriophage D. They fell into two distinct classes. The majority resembled our most LPS-defective mutant PAC605, which lacks O-antigenic material (although it is slightly leaky), contains no glucose or rhamnose in the core, is not
0.5

Fig. 1. Profile of polysaccharide obtained from partially degraded LPS from *P. aeruginosa*: (a) PAC1R, (b) PAC601, (c) PAC701 and (d) PAC700. LPS (30 mg) was hydrolysed with 1% (v/v) acetic acid for 1 h and the hydrolysate was eluted from a column (2.5 x 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, measuring $A_{490}$.

Table 1. *O*-antigenicity and bacteriophage sensitivity of PAC1R and mutants derived from it

<table>
<thead>
<tr>
<th>Strain</th>
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<th>3</th>
<th>5d</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>13</th>
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<td>-</td>
<td>-</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
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<td>+</td>
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<td>+</td>
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<tr>
<td>PAC605</td>
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</table>

Response to *O*-serum

<table>
<thead>
<tr>
<th>Response to bacteriophage D</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
</tr>
<tr>
<td>R</td>
</tr>
</tbody>
</table>

+, Agglutinated; -, not agglutinated; S, sensitive; R, resistant.

agglutinated by any of the typing sera and is resistant to the R-type pyocins and bacteriophages to which the wild-type is sensitive (Meadow & Wells, 1978). However, some of the mutants isolated as resistant to bacteriophage D retained the agglutinating properties of the parent strain with O:3 antisera, and were also agglutinated by some of the other typing sera. They thus resembled one of the classes of PA strains isolated from clinical material. Their LPS was isolated and analysed and three of these PA mutants, named PAC601, PAC701 and PAC700, with apparently different types of LPS structures, were selected for further study. Table 1 compares their O-type and bacteriophage-sensitivity with that of the parent strain and the LPS-defective mutant PAC605.

The major *O*-antigenic determinants in *P. aeruginosa* are the polymeric side-chains of the LPS which yield characteristic high molecular weight fractions (H1 and H2) after hydrolysis and separation on Sephadex G75 (Chester et al., 1973). Further separation of partially degraded polysaccharide fractions of the LPS on Biogel P6 yields two lower molecular weight fractions (L1 and L2). The L2 fraction is derived from the core (Rowe & Meadow, 1983) while the L1 fraction appears to consist of core components with at least one unit of *O*-antigenic side-chain. In semi-rough mutants which over-produce the L1 fraction but lack the polymeric high molecular weight fractions, high O-titres are obtained due to the large amounts of L1 (Koval & Meadow, 1977). Two of the three PA mutants, PAC601 and PAC701, yielded high molecular weight fractions after separation of their partially degraded LPS on Sephadex G75. However, that from PAC601 (Fig. 1b) was eluted slightly more slowly than the main *O*-antigenic material from PAC1R (H1,
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Table 2. Types of LPS isolated from *P. aeruginosa* PAC1R and its mutants

LPS was isolated by phenol extraction of isolated walls and hydrolysed by acetic acid, and its partially degraded polysaccharide fractions were separated and analysed as described in Methods. The H1 and H2 fractions were separated by chromatography on Sephadex G75 (see Fig. 1). Biogel P6 was used to separate the L1 and L2 fractions. Core components were present in all fractions. In addition H1 and L1 fractions contained glucosamine, aminogalacturonic acid, bacillosamine and more rhamnose. The H2 fraction contained mannosse and additional rhamnose and glucose.

<table>
<thead>
<tr>
<th>Type of LPS isolated</th>
<th>Smooth LPS</th>
<th>Secondary smooth LPS</th>
<th>Semi-rough LPS</th>
<th>Rough LPS</th>
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<tbody>
<tr>
<td>Structure</td>
<td>O-polymer-core-lipid A</td>
<td>Secondary polymer-core-lipid A</td>
<td>O-unit-core-lipid A</td>
<td>Core-lipid A</td>
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<tr>
<th>Degraded polysaccharide fraction</th>
<th>H1</th>
<th>H2</th>
<th>L1</th>
<th>L2</th>
</tr>
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<tr>
<td>Isolated from:</td>
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<tr>
<td>PAC1R</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PAC601</td>
<td>+ *</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>PAC701</td>
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<td>PAC700</td>
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<td>PAC605</td>
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+, Present; -, not detected; *, lower molecular weight and less rhamnose than PAC1R; †, lacks glucose and rhamnose.

Fig. 1(a) and that from PAC701 (Fig. 1(c)) appeared to correspond to the H2 polysaccharide from PAC1R. Analysis of the isolated fractions confirmed this view. The high molecular weight material from PAC601 contained rhamnose and the amino sugars glucosamine, bacillosamine and aminogalacturonic acid, which comprise the repeating O-unit of type-3 strains (Tahara & Wilkinson, 1983). However, it contained less rhamnose than the equivalent fraction from PAC1R (H1) and this, together with its behaviour on Sephadex G75 and Biogel P6 columns, suggested an alteration in structure despite the presence of the main O-antigenic determinants. The high molecular weight fractions from PAC701 contained mainly glucose and rhamnose, like the secondary (H2) polymer in PAC1R. All three PA mutants yielded low molecular weight fractions which differed from those of the parent strain either in apparent molecular size or in amount. The sole peak detected in PAC700 LPS (Fig. 1(d)) contained only the core components found in the L2 fraction of PAC1R. The low molecular weight material from PAC701 (Fig. 1(c)) was eluted in the same position as PAC1R and could be resolved by chromatography on Biogel P6 into a major fraction containing both side-chain and core components (L1), and a smaller amount of a fraction (L2) containing only core components. The two low molecular weight fractions from PAC601 appeared similar to the L1 and L2 fractions from the parent strain but were eluted faster. Elution from Biogel P6 yielded a lower molecular weight fraction which appeared to consist solely of core components in the same molar ratios as the L2 fraction from PAC1R, while the slightly heavier fraction contained in addition the characteristic O-antigenic components. Table 2 summarizes the LPS components detected by these analyses. It was thus established that the LPS from each of the PA mutants was different and was also different from that of the parent strain. Their PA behaviour could not therefore be explained solely in terms of LPS structure.

Agglutination reactions

Antisera to PAC1R and the three PA mutants were prepared and tested for their ability to agglutinate whole bacteria before and after absorption with the other strains as described in Methods. All of the antisera agglutinated all four strains, but they differed after preabsorption with bacteria (Table 3). The activity of PAC1R antiserum against homologous cells was abolished by absorption with PAC1R but agglutinating activity against the other three strains remained. Similarly, absorption with PAC700 removed PAC700-agglutinating activity but had no effect on the other activities. In contrast, the agglutinating activity of antisera prepared against PAC601 and PAC701 was abolished by absorption with either of these two strains or the parent strain, but not by PAC700, while agglutinating activity of PAC700 antiserum was
removal of the organisms by absorption with any of the mutants. These differences may reflect differences in the expression of surface antigens in wild-type and PA strains and were investigated further.

Table 3. Agglutination by antisera absorbed with bacteria

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Strain used for absorption</th>
<th>PAC1R</th>
<th>PAC601</th>
<th>PAC701</th>
<th>PAC700</th>
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<tbody>
<tr>
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<tr>
<td></td>
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<td></td>
<td>PAC700</td>
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+, Agglutination; −, no agglutination.

These results suggested that the LPS was only partly responsible for the antigenicity of the envelope preparations and attempts were made to quantify this by the haemagglutination of red blood cells sensitized by LPS preparations. Antiserum were tested before and after absorption with purified LPS. PAC700 LPS did not sensitize sheep red blood cells towards any of the antisera, as was expected from its lack of high molecular weight fractions. LPS preparations from other mutants lacking O-antigenic side chains have also been found to be inactive in this respect. Sensitization of sheep red blood cells by LPS from PAC1R, PAC601 and PAC701 gave high titres against these three antisera and most of the activity was removed by preabsorption with LPS. PAC700 antisera gave low titres with the other three LPS preparations. The differences between the strains could be seen more clearly by the ELISA technique. Using PAC1R antiserum, for example, high titres were obtained for envelope fractions from each strain, though those with defective LPS gave lower titres. However, when purified LPS was used, only those LPS which contained high molecular weight fractions could be titrated (Table 4). The antigenicity detected by the PAC1R antiserum in the envelope fractions from PAC700 and PAC605 was therefore not due to their LPS.

Separation and identification of outer membrane components

The work described had indicated that LPS was only partially responsible for the antigenic response of two of the three PA strains and played no part in the antigenicity of the third. The
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Fig. 2. SDS-PAGE separation of crude outer membrane fractions from *P. aeruginosa*: lanes 1 and 6, PAC700; lanes 2 and 7, PAC1R; lanes 3 and 8, PAC605; lanes 4 and 9, PAC701; lanes 5 and 10, PAC601; lane 11, LPS from PAC1R. Lanes 1 to 5 were stained with Coomassie blue and lanes 6 to 11 with the silver stain.

Table 4. Comparison between outer membranes and purified LPS from PAC1R and its mutants, using ELISA and PAC1R antiserum

<table>
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<tr>
<th>Source</th>
<th>Outer membranes</th>
<th>Purified LPS</th>
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<td>12800</td>
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<tr>
<td>PAC601</td>
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<td>3200</td>
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antisera used had all been prepared against whole bacteria and it seemed likely that other outer membrane components which were exposed on the cell surface might be the antigenic determinants. Crude outer membranes were prepared and their constituents separated by 12% (w/v) SDS-PAGE, as described in Methods, to see whether any differences could be detected between the parent strain, the LPS-defective mutant PAC605 and the three PA strains. The major proteins identified in 12% SDS-PAGE gels were similar in all strains tested (Fig. 2, lanes 1 to 5). By comparison of their mobility with molecular weight standards, their response to heat treatment and their behaviour after treatment with mercaptoethanol, they could be identified with those found by other workers in different strains of *P. aeruginosa* (Hancock & Carey, 1979). All the preparations contained major outer membrane proteins which we have named D, E, F', F and H to conform with the nomenclature of Hancock & Carey. Protein D (48000 Dal) has been shown by others to be detectable only after heat treatment, which was used routinely in our preparations. The F protein can be identified as the heat-modifiable porin (31000 Dal) and the
heat-modified F' component can also be seen. The mobility of the F porin was decreased to correspond to a molecular weight of 33000 by β-mercaptoethanol. The H protein can sometimes be resolved into doublets, but this was difficult to reproduce in 12% gels. There were indications that PAC605 may lack the larger one of the H bands, but otherwise there were apparently no major differences between the strains.

When the gels were visualized by silver-staining, marked differences could be seen (Fig. 2, lanes 6 to 11). This procedure identifies sugar groups susceptible to periodate oxidation. Purified LPS from PAC1R (lane 11) contained two major bands (Lps1 and Lps) which have the mobility expected of peptides of molecular weight 16000 and 10000, but nothing could be detected in gels stained with Coomassie blue (not shown). These two bands are probably the fractions which give rise to the L1 (semi-rough) and L2 (core) fractions separable from partially degraded polysaccharides by Sephadex chromatography (see Table 2). Heterogeneity in this LPS preparation can be seen by several bands of slightly higher molecular weight and also by the staining of the area corresponding to peptides of molecular weights between 50000 and 100000. None of these areas stained heavily, probably because of the structure of the O:3 antigen, which is known to contain only one bond susceptible to periodate oxidation in each repeating unit (Tahara & Wilkinson, 1983). It is not therefore surprising that the high molecular weight fractions of LPS were not silver-stained in the crude outer membrane fractions from PAC1R (lane 7) and PAC701 (lane 9). In these preparations and in the outer membranes from PAC700 (lane 6) the lower molecular weight fractions are readily identified and it can be seen that in PAC605, where the core fraction is defective, the Lps band (rough LPS) has a faster mobility (lane 8). Surprisingly, the outer membranes from PAC601 (lane 10) showed a continuous ladder of silver-stained material up to molecular weights equivalent to 100000, apart from the two unstained regions where the F and F' proteins appear to interfere with the silver stain. This again supports the analyses of the LPS which suggested an altered O-antigenic polysaccharide in PAC601, presumably containing more periodate-sensitive bonds.

Crossed immunoelectrophoresis by running the gels in a second dimension into the different antisera was used to try to identify the individual components responsible for the antigenicity of the outer membrane components. However, strong immunoprecipitates were detected only in those preparations known to contain high molecular weight polysaccharides. No reaction was detected using PAC605 and PAC700 outer membranes with any of the antisera. The precipitins detected in PAC1R outer membranes by PAC1R antisera are shown in Fig. 3. There are two
clear peaks due to components with mobilities corresponding to proteins of molecular weight 100000 and 55000, named lpsO' and lpsO, which are in the regions stained generally by the silver stain in the LPS preparations. They probably correspond to smooth LPS with differing lengths of O-antigenic side chains. In addition, a series of minor components of lower molecular weight are just visible. Reaction with PAC1R antiserum was also obtained with membranes from PAC601 where precipitin lines attributable to high molecular weight O-antigenic side-chains were detected, but there were no obvious peaks. There were no precipitin lines corresponding to any of the protein bands detected by Coomassie blue staining or to any antigen common to the three PA strains. The gel conditions were varied, using from 5 to 18% polyacrylamide, but these too failed to reveal additional antigenic components and a more sensitive technique was sought.

**Antigenicity of crude outer membrane fractions**

The envelope components separated by 12% (w/v) SDS-PAGE were transferred electrophoretically on to nitrocellulose sheets, and the blots so obtained were incubated with antisera as described in Methods. The complexes formed were then detected by a modified ELISA technique. Using this method (Western blotting) it was possible to identify the individual components in the crude outer membrane fractions which were responsible for the antigenic reactions to the different antisera.

Figure 4 shows the Western blot obtained using PAC1R antiserum and crude outer membrane fractions of PAC1R and the four mutants. Purified PAC1R LPS (lane 6) was included as a control and shows pronounced laddering as expected from LPS preparations containing O-side chains of different lengths. There are also the two high molecular weight regions of strong antigenicity lpsO' and lpsO (smooth LPS) detected previously by immunoelectrophoresis (see Fig. 3). The other major antigenic component in the LPS is the band of semi-rough LPS (lpsl) previously detected by the silver stain. Surprisingly the rough LPS (band lps, see Fig. 2) was not detectable. The same three LPS bands appear to be the major antigenic determinants in PAC1R outer membranes as expected (lane 1). In addition, the first component of the smooth LPS ladder (lpsl') is clearly detectable. In outer membranes from PAC601 and PAC701 (lanes 2 and 3), whose LPS yielded large amounts of the L1 fraction (see Table 2), the lpsl component is the major determinant, confirming its identification as semi-rough LPS. Some higher molecular weight fractions corresponding to some of the ladder of smooth LPS are also visible in PAC601, as well as smaller amounts of the lpsO' and lpsO components. These fractions can also be detected in PAC605 (lane 5), which is known to be leaky, but lpsl is missing from this and from PAC700 preparations (lane 4). In these two strains the major determinants are the F and H proteins, which are also just detectable in the other three strains. A third protein, P, the 39000 porin forming anion-specific phosphate channels (Hancock et al., 1982a) is also clearly detectable in PAC701 and PAC601.

The experiments were repeated using antisera to each of the three PA mutants and to other Habs serotypes. Each of the antisera which agglutinated whole cells gave a characteristic pattern of protein and/or LPS bands in the Western blots. No antigenicity could be detected using typing sera with which whole cells gave no reaction. The pattern obtained with O:9 antiserum is shown in Fig. 6. There was no reaction with purified LPS (track 6) and there are no bands attributable to lps in the membrane blots. The main antigenic determinant in all
preparations was the F porin, with some activity also associated with the E protein, which is particularly obvious in the PA and LPS-defective strains. This antiserum also reacted with a band corresponding to a protein of molecular weight 27000 which is probably the heat-modified G protein described by Hancock & Carey (1979). This protein was not present in sufficient amounts to be detected by Coomassie blue staining. Table 5 summarizes the results obtained using the various antisera.

DISCUSSION

The results presented provide strong evidence that most if not all of the observed cross-reactivity between the PA mutants and antisera prepared against PAC strains and other serotypes is due either to LPS components or to outer membrane proteins common to all strains of *P. aeruginosa*.

The outer membranes of Gram-negative bacteria are highly asymmetric structures. LPS and some outer membrane proteins generally reside in the outer leaflet of this structure, with phospholipids and other proteins making up the inner leaflet. There is some evidence to suggest that in *P. aeruginosa* the outer membrane may contain patches in which there are phospholipids in the outer leaflet, as in the deep rough mutants of the *Enterobacteriaceae* (Nikaido, 1979). Further complexity arises from the heterogeneity of the LPS of *P. aeruginosa*. Analyses of partially-degraded polysaccharide suggested that the wild-type strain PAC1R contains at least three different forms of LPS in which the core is unsubstituted (rough) and mono- or oligo-substituted (semi-rough), as well as being substituted with polymerized O-antigen (Koval & Meadow, 1977). Furthermore, analyses of deacylated LPS showed that there were at least three fractions which contained both O-antigenic determinants and core components (Chester & Meadow, 1975). In the present work, some heterogeneity of isolated LPS was shown by silver-staining, but the O:3 polysaccharide reacts poorly with this stain. However, Western blots with PAC1R and PAC601 antisera showed a series of bands both in purified LPS and in solubilized outer membranes. The bands included those attributed to rough and semi-rough LPS, as well as LPS containing high molecular weight O-antigenic side chains. In LPS from *P. aeruginosa*
Fig. 5. Antigens detected in Western blots of outer membrane preparations by reaction with PAC601 antiserum. Samples in each lane as in Fig. 4.

Fig. 6. Antigens detected in Western blots of outer membrane preparations by reaction with O:9 antiserum. Samples as in Fig. 4.

PAO1 similar laddering has been detected using fixation with phosphotungstate, and Coomassie blue staining after separation in a linear 10 to 12% polyacrylamide gradient (Kropinski et al., 1982).

The asymmetry of the outer membrane of PAC1R strains can be seen by comparing the antigens detected in whole cells with those in solubilized outer membranes. Agglutination of whole cells of PAC1R occurred only with O:3 or O:3-derived antisera, and the main antigens detected by PAC1R antiserum (prepared against whole cells) were the high molecular weight polymerized O-antigenic fractions (lpsO and lpsO') and the semi-rough core fraction (lps1).
Table 5. Major components detected by different antisera in Western blots of crude outer membrane preparations from PAC1R and its defective mutants

Membranes were prepared and separated by 12% SDS-PAGE and then transferred on to nitrocellulose and incubated with antisera, as described in Methods. LPS components (with their apparent molecular weights) are: smooth LPS, designated lpsO' (100000) and lpsO (55000); semi-rough LPS, designated lpsl' (18000) and lpsl (16000); rough LPS, designated lps (10000). The protein components identified by their molecular weights are: F (39000), F' (33000), F (31000) and H (21000).

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<th>Antiserum prepared against:</th>
<th>Source of crude outer membranes:</th>
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<tr>
<td></td>
<td>PAC1R</td>
</tr>
<tr>
<td>PAC1R</td>
<td>lpsO'</td>
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<tr>
<td>Habs 0:5d</td>
<td>-</td>
</tr>
<tr>
<td>Habs 0:9</td>
<td>F</td>
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<tr>
<td>Habs 0:13</td>
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<td></td>
<td>lps</td>
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<td>Habs 0:10</td>
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* Material forming a ladder corresponding to proteins of molecular weight 30000 to 48000 but which was not detected in Coomassic blue stains.
- - No major bands detected.

However, the solubilized outer membranes of this strain also contained unsubstituted LPS, as shown by analysis of the partially degraded polysaccharide and by Western blotting using PAC601 antiserum. In PAC1R, the O-polymers may extend into the external medium, thus shielding both proteins on the cell surface and rough LPS. In PAC601, where the O-antigenic structure is altered in some way, the shielding effect is not so marked, and antiserum to this mutant reacted strongly not only with all the major LPS determinants (bands lps0, lpsO', lpsl', lps1 and lps) but also with a number of proteins (A, B, C, P, F and H) as well as the unidentified determinants X and Y. Using antiserum to PAC700, which contains no O-antigenic material, the rough core was the only LPS antigen. This antiserum showed strong antigenic activity against the F porin and some residual H protein activity, suggesting that in this mutant these proteins are exposed on the cell surface.

A comparison of all the Western blots suggested that in addition to the LPS components on the outer leaflet of the outer membrane, the F and H proteins are accessible and strongly antigenic, together with smaller amounts of other proteins. Using [125I]lactoperoxidase to label PAO1 in vivo, Lambert & Booth (1982) showed that protein F was the major protein labelled, with smaller amounts of D and E. Surprisingly, they did not detect the H proteins by this
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...technique, perhaps because the latter did not contain exposed tyrosine or histidine residues. However, Hancock *et al.* (1982b) have prepared monoclonal antibodies to the H2 protein from outer membranes of PA01 confirming its surface location. Lambert & Booth (1982) found a labelled band which was not detectable as a major outer membrane protein by Coomassie blue and whose mobility corresponded to a protein of molecular weight 72000. It is possible that this is related to the A, B and C cluster detected by us and by Mizuno & Kageyama (1978), but the different gel conditions used made comparison difficult.

The PA activity of the three mutants studied here can readily be attributed to the polyvalent nature of the antisera. Where the mutants retained LPS O-antigens, they reacted strongly, whether or not the latter were polymerized, as shown by the reactions with antisera to PAC1R, PAC601 and PAC701. Cross-reactivity with antiserum to PAC700 was due mainly to the F porin which appears to be the major antigenic determinant in this mutant. Cross-reaction with the heterologous antiserum O:5d, O:9 and O:13 appeared to be due to different combinations of antigens. Using O:5d, antisera bands corresponding to proteins of molecular weights 30000 to 48000 were detected and these may be related to material of 38000 detected by Lambert & Booth (1982). The dominant reacting antigen in the O:9 antiserum was clearly the F and to a lesser extent the E and G proteins, while the O:13 antiserum reacted with rough LPS, F and H proteins, but also with the high molecular weight O:3 antigen when it was present in large amounts. LPS from the type 13 strain contains aminogalacturonic acid and other amino compounds characteristic of type 3 strains (Chester *et al.*, 1973), so that cross-reaction with PAC strains is not entirely unexpected. Our work does not provide an easy solution to the identification of PA mutants. If such mutants retain residual O-antigenic side-chains, as do PAC601 and PAC701, then they could be identified with O:antisera from which the major cross-reacting protein components had been removed. However, in strains like PAC700, which entirely lacks the O-antigen, the outer membrane proteins, which then become the dominant antigen, may be common to all strains of *P. aeruginosa*. The H and F proteins were the major non-LPS antigens detected in the PAC strains and these cross-reacted with the same proteins from at least two other serotypes of *P. aeruginosa*. Using a monoclonal antibody to the H2 lipoprotein, Hancock *et al.* (1982b) have shown cross-reactivity with other strains of *Pseudomonas* and *Azotobacter* as well. However, as they suggest, the existence of common outer membrane proteins is encouraging for vaccine production and our work would suggest that such vaccines would be equally active against PA strains.

Our results do not explain the properties of the LPS-defective mutant, PAC605, which was not PA under standard test conditions and yet resembled PAC700 in the Western blots. The difference may be quantitative, but, unlike PAC700, PAC605 is leaky. Its purified LPS contains O-antigenic material which can be detected by amino compound analysis and haemagglutination, and it may be that this material shields the cell surface, making the membrane proteins inaccessible to antisera in whole cells. Nor do our results identify the antigenic determinants responsible for the reaction of PAC700 with O:5d antiserum. It seems likely that the determinant in PAC700 would be the F protein, which is exposed on the surface. However, it is possible that the determinant causing agglutination of PAC700 by O:5d antiserum is heat-or SDS-labile and is therefore destroyed in preparing the membranes used in Western blots. Further work with purified antiserum will be required to resolve these problems.

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REFERENCES


