The Isolation and Characterization of Lipopolysaccharide-defective Mutants of *Pseudomonas aeruginosa* PAC1

By SUSAN F. KOVAL AND PAULINE M. MEADOW

Biochemistry Department, University College London, London WC1E 6BT

(Received 27 July 1976)

SUMMARY

Mutants with defective lipopolysaccharides (LPSs) were isolated from *Pseudomonas aeruginosa* PAC1R (Habs serogroup 3) by selection for resistance to aeruginocin from *P. aeruginosa* P16. Carbenicillin-sensitive mutants were isolated from *P. aeruginosa* PAC1 but not all had defective LPSs. Rough colonial morphology and resistance to bacteriophage 119X appeared to be independent of LPS composition.

The LPSs from five mutants were analysed and compared with that of the parent strain. Separation of partially-degraded polysaccharides from LPS from PAC1 on Sephadex G75 yielded two different high molecular weight fractions and a phosphorylated low molecular weight fraction (L). The mutant LPSs lacked most or all of the high molecular weight fractions but retained some low molecular weight material. That from PAC1 and two of the mutants was separated by elution from Biogel P6 into two fractions. One, L2, was the core polysaccharide while the other, L1, contained short antigenic side-chains attached to the core like the semi-rough (SR) LPSs of the Enterobacteriaceae. The two mutants which gave the L1 fraction reacted with Habs 3 and PAC1 antisera as did the parent strain. The other three mutants were unreactive and their LPSs contained core components only. One appeared to have a complete core while the other two lacked rhamnose and rhamnose plus glucose respectively. Thus there may be four types of LPS in PAC1: one contains unsubstituted core polysaccharide and yields L2 on acid hydrolysis, another has short antigenic side-chains of the SR type and yields the L1 fraction, while the two high molecular weight fractions are derived from core polysaccharides with different side-chains.

INTRODUCTION

The lipopolysaccharides (LPSs) of several strains of *Pseudomonas aeruginosa* appear to have a common core polysaccharide containing glucose, rhamnose, galactosamine, heptose, 2-keto-3-deoxyoctonate (KDO) and alanine (Meadow, 1975). Hydrolysis of the LPS with 1% acetic acid and separation of the polysaccharide fragments on Sephadex G75 or G50 yields the common core and higher molecular weight polysaccharide fractions which vary between serotypes and are responsible for O-antigenic specificity (Chester, Meadow & Pitt, 1973; Wilkinson & Galbraith, 1975). LPSs from some strains yield more than one high molecular weight fraction suggesting that in the pseudomonads, as in many other species (Nowotny, 1971), the LPS is heterogeneous. Separation of the deacylated LPS of *P. aeruginosa* PAC1 on Sephadex G200 gave four fractions each of which contained core components and it was suggested that these might be derived from different species of LPS molecules (Chester & Meadow, 1975).

In order to learn more about the structure of the pseudomonad LPS and its role in the Habs serotyping system (Habs, 1957), we wished to isolate LPS-defective mutants from

P. aeruginosa PACI. One such mutant (S-31; Fensom & Meadow, 1970) had been isolated by selection for defective glucose metabolism, but subsequent attempts to isolate more mutants using this method were unsuccessful. Few, if any, LPS-defective (R) mutants of Gram-negative bacteria other than the Enterobacteriaceae have been described. The R mutants of this family synthesize incomplete LPSs and can be recognized by their rough colonial morphology (for review see Lüderitz *et al.*, 1971). In addition, R-specific bacteriophages are available for their further characterization and isolation (Wilkinson, Gemski & Stocker, 1972). For species which do not exhibit smooth-rough transition and for which no bacteriophages specific for LPSs are available, the selection and identification of LPS-defective mutants is more difficult. The work described here was undertaken to find a method for isolating mutants of *P. aeruginosa* which partially or completely lacked the high molecular weight O-antigenic polysaccharide fractions and to use the mutants to study the structure of the LPS itself. A brief account of some of this work has been published (Koval & Meadow, 1975*a*).

METHODS

Pseudomonas aeruginosa strains. The parent strains used to isolate LPS mutants were PACI (NCIB10848), which belongs to Habs serogroup 3, and PACIR. The latter is a spontaneous mutant isolated from PACI which is resistant to bacteriophage PS1 but otherwise unchanged (Chester & Meadow, 1975). PAC556 is a LPS-defective mutant previously called S-31 (Fensom & Meadow, 1970). PAC7 is a lysine-requiring mutant previously called lys-I (Clarkson & Meadow, 1971). PAC307 (Brammar, Clarke & Skinner, 1967) and PAC366 (Brown & Clarke, 1972) have already been described.

Strains PAOI and PATI were provided by Professor B. W. Holloway, Monash University, Melbourne, Australia, and strain PI6 by Dr N. Suzuki, Institute of Medical Science, University of Tokyo, Japan. Other *P. aeruginosa* strains used were ATCC7700 and type strains for the original Habs types (Chester *et al.*, 1973).

All cultures were grown at 37 °C either in flasks containing one-fifth their volume of nutrient broth (Oxoid no. 2) with shaking, or on plates of nutrient agar (1.2 %, w/v) or minimal medium with succinate, citrate or glucose as carbon source (Clarkson & Meadow, 1971).

Isolation, degradation and fractionation of lipopolysaccharides. LPSs were isolated from bacterial walls by extraction with 45 % (w/v) phenol, hydrolysed for 1 h with 1 % (v/v) acetic acid and fractionated by elution from a column of Sephadex G75 as previously described (Chester *et al.*, 1973). A column (2.5 × 70 cm) of Biogel P6 (Bio-Rad) was also used and eluted with the same pyridine/acetic acid buffer (pH 5.4; 0.05 M). Fractions (4 ml) were collected and analysed for carbohydrate (Dubois *et al.*, 1956), 2-amino sugars (Rondle & Morgan, 1955) and phosphorus (Bartlett, 1959).

Analytical methods. Methods for the determination of glucose, rhamnose and heptose and for the estimation of amino compounds using the Locarte analyser were described previously (Chester *et al.*, 1973; Koval & Meadow, 1975*b*). The amount of 2-amino-2-deoxygalacturonic acid was calculated assuming it had the same colour yield to reaction with ninhydrin as norleucine, the internal standard. Diaminopimelic acid was detected by ninhydrin (0.2 %, w/v, in acetone) after paper chromatography (Whatman 3MM) of hydrolysed (6.1 M-HCl, 18 h, 105 °C) LPS (5 mg) using methanol/water/pyridine/HCl (Hoare & Work, 1955) as solvent.

Measurement of carbenicillin sensitivity. Overnight broth cultures of organisms were diluted 1:100 in dilution buffer (Clarkson & Meadow, 1971). Samples (0.05 ml) were

inoculated into 2 ml nutrient broth containing dilutions of carbenicillin (Beecham Pharmaceutical Co.) and shaken for 24 h at 37 °C. The minimal inhibitory concentration (m.i.c.) was the lowest concentration of carbenicillin which completely inhibited growth.

Isolation of carbenicillin-sensitive mutants. A 4 h culture of PAC1 was collected by centrifugation and resuspended in 0.1 M-citrate buffer pH 6.0. A freshly prepared solution of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was added to a concentration of 0.1 mg ml⁻¹. The culture was incubated for 30 min at 37 °C, washed in dilution buffer and 0.1 ml was inoculated into 5 ml nutrient broth. After overnight incubation, the culture was diluted 1:10⁶ and 0.1 ml was plated on a nutrient agar plate to obtain single colonies. After overnight incubation, the master plate was replicated on to nutrient agar plates containing carbenicillin (5 µg ml⁻¹). These were incubated overnight and examined for colonies which did not grow with carbenicillin. These colonies were picked off and restreaked to single colonies on nutrient agar with carbenicillin. Their sensitivity to carbenicillin was measured in liquid medium.

Aeruginocin preparation and sensitivity. The methods used were described by Koval & Meadow (1975*b*). Aeruginocins were prepared from strains PI6, PAT1, PAO1, ATCC7700, and the Habs type strains 2B, 3, 5C and 10. The crude lysate resulting from induction with mitomycin C was used as the aeruginocin preparation.

Isolation of aeruginocin-resistant mutants. An overnight broth culture of the test organism was diluted 1:50 in dilution buffer and 0.1 ml was plated on nutrient agar. The crude undiluted aeruginocin preparation (0.2 ml) was spread on the same plate which was incubated overnight. Aeruginocin-insensitive colonies could be seen in the clear zone of lysis caused by the aeruginocin. These colonies were streaked to obtain single colonies and their aeruginocin insensitivity was confirmed by retesting. They were classified as resistant rather than tolerant (Holloway & Krishnapillai, 1974) if they did not absorb the aeruginocin and hence change the titre when tested as follows. A 20 ml culture of the mutant to be tested was grown to mid-exponential phase ($E_{670}^{1.0\text{ cm}} = 1.0$), collected by centrifugation in sterile bottles and resuspended in 4 ml NaCl (0.9 %, w/v). One ml of this suspension was mixed with 1 ml of the undiluted aeruginocin preparation in a sterile bottle and incubated without shaking for 20 min at 37 °C. The bottle was centrifuged (10 min, 4000 *g*) and the supernatant fluid was removed to make doubling dilutions in sterile saline to determine the aeruginocin titre.

Isolation of bacteriophage-resistant mutants. Bacteriophage 119X was obtained from T. L. Pitt (Cross Infection Reference Laboratory, Colindale, London) who tested the strains for their sensitivity to other bacteriophages. Resistant mutants were isolated after plating together 0.1 ml of an exponential-phase culture of PAC1 with 0.1 ml phage lysate (10⁹ plaque forming units ml⁻¹) on nutrient agar. Colonies detected after overnight incubation were purified by replating and tested to confirm their resistance.

Serological tests. The preparation of antisera and the measurement of serological reactivity by quantitative agglutination tests has been described previously (Chester *et al.*, 1973).

RESULTS

Isolation of mutants

Pseudomonas aeruginosa does not normally show smooth-rough transition in colonial morphology but a few rough strains (type 3; Wahba & Darrell, 1965) have been isolated from clinical material and Martin (1971) showed that such strains retained a type-specific O-antigen. PAC1 produced smooth colonies on all media tested, but after mutagen treat-

ment occasionally gave rise to rough, lacy type 3 colonies. These colonies appeared rough only on minimal medium, reverting to a smooth appearance on nutrient agar. Four such mutants were selected, their LPSs were isolated, partially degraded by acid hydrolysis and fractionated by elution from a column of Sephadex G75 as described in Methods. All retained the high molecular weight polysaccharide fractions (H1 and H2) characteristic of PAC1 (see Fig. 1*a*) and their elution profiles were identical. Similarly we could detect no differences between the LPS of PAC1 and that of another colonially rough mutant PAC307. Moreover, PAC556 whose LPS lacks the high molecular weight fractions (Fensom & Meadow, 1970) produced smooth colonies on all media tested. Therefore in *P. aeruginosa* rough colonial morphology appears to be independent of LPS composition.

No bacteriophages specific for *P. aeruginosa* LPS have so far been described. However PAC556 unlike PAC1 was resistant to bacteriophage 119X. A spontaneous mutant of PAC1 resistant to this bacteriophage was therefore isolated but its partially degraded LPS was again indistinguishable from that of PAC1.

Some LPS-defective mutants of the Enterobacteriaceae are more sensitive to deoxycholate (Sanderson, MacAlister & Costerton, 1974; Sanderson *et al.*, 1974) and take up more crystal violet (Gustaffson, Nordstrom & Normark, 1973) than their parent strains. In preliminary experiments comparing these properties in PAC556 and PAC1 we could detect no differences and alternative methods of isolating LPS-defective mutants were therefore sought.

Isolation of mutants sensitive to carbenicillin. In *Escherichia coli* K12 (Tamaki, Sato & Matsuhashi, 1971; Tamaki & Matsuhashi, 1973) and *Salmonella minnesota* (Schlect & Westphal, 1968) sensitivity to some antibiotics is correlated with progressive defects in the core part of the LPS. We therefore compared the sensitivities of PAC556 and PAC1 to antibiotics. The defective mutant PAC556 was more sensitive than PAC1 to several antibiotics and 10 times more sensitive to carbenicillin. Seven carbenicillin-sensitive mutants were therefore isolated from PAC1 after mutagen treatment as described in Methods. Two mutants from the laboratory collection (PAC7 and PAC366) were also found to be more sensitive to carbenicillin than PAC1. The m.i.c. of carbenicillin for these nine mutants range from 10 to 40 $\mu\text{g ml}^{-1}$, compared with 100 $\mu\text{g ml}^{-1}$ for PAC1. Their LPSs were isolated and analysed after partial degradation. One of the new mutants, named PAC557, contained less rhamnose than PAC1 and also lacked the high molecular weight polysaccharide fractions. The LPS from PAC7 was also defective but that from each of the other carbenicillin-sensitive mutants appeared similar to that of the parent strain and they were not investigated further.

Mutants resistant to aeruginocins. Some aeruginocins appear to bind to LPS-receptors (Ikeda & Egami, 1973; Govan, 1974) and Suzuki (1974) suggested that specific amino sugars in the lipopolysaccharide may be the receptors. Possibly, therefore, mutants isolated by their resistance to aeruginocins might have defective LPSs and we tested this by comparing the response to aeruginocins of the defective mutants already isolated with that of PAC1. A crude aeruginocin preparation from PAT1 distinguished PAC1, which was sensitive, from PAC7, PAC557 and PAC556 which were resistant. Two mutants of PAC1 resistant to this aeruginocin were therefore isolated and their LPSs were analysed, but they appeared to be wild type. Crude aeruginocin preparations from six other strains of *P. aeruginosa* were also tested but did not discriminate between the defective mutants and PAC1. However, an aeruginocin preparation from strain P16 was active against PAC1 but did not appear to lyse the three defective mutants and so we tried to isolate mutants of PAC1 resistant to this aeruginocin. The crude preparation induced the lysogenic bacteriophage PS1 carried by PAC1 producing unstable sectorial colonies and we therefore used PAC1R as the parent

Table 1. Some properties of *P. aeruginosa* PAC1 and PACIR and their lipopolysaccharide-defective mutants

Strain	Parent	Mutagen treated	Phenotype selected	Habs type	Titre of PACI antiserum	Bacteriophage response					Response to PATI aeruginocin	Sensitivity to carbenicillin: m.i.c. ($\mu\text{g ml}^{-1}$)
						21	68	109	119X	PS1		
PAC1	PAC1	0	Wild type	3	1280	S	S	S	S	S	S	100
PAC7	PAC1	+	Lysine-requiring	3	1280	R	R	R	R	S	R	30
PAC556	PAC1	+	Glucose defective	NT	20	S	S	R	R	S	R	10
PAC557	PAC1	+	Carbenicillin sensitive	NT	20	R	S	S	R	S	R	20
PACIR	PAC1	0	Resistant to phage PS1	3	1280	S	S	S	S	R	S	100
PAC605	PACIR	0	Resistant to P16 aeruginocin	NT	160	R	R	R	R	R	R	100
PAC608	PACIR	0	Resistant to P16 aeruginocin	3	1280	R	R	S	R	R	R	80

o, Spontaneous; +, mutagen treated; s, sensitive; R, resistant; NT, not typable.

Table 2. *Composition of lipopolysaccharides*

LPSs were isolated from walls by extraction with aqueous phenol (45 %, w/v), hydrolysed and analysed as described in Methods. No correction has been made for losses during hydrolysis. Compound Y (Koval & Meadow, 1975*b*) and post-arg are unidentified amino compounds detected by the amino acid analyser.

Component	Strain ...	Composition (% w/w)					
		PAC1	PAC7	PAC608	PAC557	PAC556	PAC605
Glucose		6.9	5.6	6.8	10.0	11.4	0
Rhamnose		10.0	5.6	5.4	3.3	0	0
Heptose		3.9	3.6	3.5	5.3	5.1	6.7
Glucosamine phosphate		2.1	2.2	2.7	4.5	3.4	4.8
Glucosamine		5.3	3.5	5.1	5.6	4.2	5.7
Galactosamine		1.6	1.8	2.2	2.8	2.7	3.3
Alanine		1.2	1.5	2.3	2.5	2.4	2.7
Aminogalacturonic acid		1.6	0.3	0.4	0.1	0	0
Fucosamine		+	+	+	+	+	0
Compound Y		+	0	0	0	0	0
Post-arg		+	+	+	0	0	0
Phosphorus		4.5	5.9	6.9	7.3	8.3	8.3
Fatty acids		16	24	24	32	31	31

+, Present, but not estimated; 0, not detected.

strain to isolate mutants resistant to P16 aeruginocin. The LPSs from two of these mutants lacked glucose and rhamnose as well as the high molecular weight polysaccharide fractions; one, PAC605, was selected for further study. The LPS from a third mutant, PAC608, contained both glucose and rhamnose but lacked most of the high molecular weight fractions.

Some of the properties of the mutants analysed are listed in Table 1.

Analysis of the lipopolysaccharides

The same amount [approximately 100 mg (g walls)⁻¹] of LPS was isolated from the defective mutants as from the parent strains. The compositions of the mutant LPSs differed from that of PAC1 particularly in their glucose, rhamnose and amino sugar contents (Table 2). These results are expressed as percentage compositions so the increased fatty acid composition of the mutants reflects their lack of polysaccharide components. In addition to aminogalacturonic acid, fucosamine and the unknown amino compound Y previously found in the LPS of PAC1 (Koval & Meadow, 1975*b*), there was an amino compound, named post-arg, that eluted just after arginine from the amino acid analyser. A similar compound, which may be a diamino sugar, has been found in LPS from another Habs type 3 strain (S. G. Wilkinson, personal communication).

The LPSs from PAC556, PAC557 and PAC605 were less stable to hydrolysis by 1 % acetic acid at 100 °C than those of PAC1, PAC608 and PAC7. Within 30 min, LPS solutions of the former group became cloudy and a white flocculent precipitate of lipid A was released, whereas even after 50 min hydrolysis the other LPS solutions merely appeared opalescent. Further hydrolysis for up to 2 h had no effect either on the lipid A released or on the amount or the composition of the polysaccharides formed.

The partially-degraded polysaccharides produced by acid hydrolysis of the LPSs were separated by elution from a column of Sephadex G75 and the fractions were analysed for total carbohydrate, 2-amino sugars and phosphorus (Fig. 1). The profile from PAC1R was like that of PAC1 (Fig. 1*a*), that from PAC7 resembled that of PAC608 (Fig. 1*b*) while PAC556

Fig. 1. Fractionation of polysaccharide obtained from LPSs from (a) PAC1, (b) PAC608, (c) PAC557 and (d) 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 (E_{490} , —), 2-amino sugars (E_{530} , ---) and total phosphorus (E_{630} , - · - · -).

was like PAC557 (Fig. 1c). All the strains gave the same low molecular weight solutes which contained ethanolamine, KDO and phosphate.

Strain PAC1 LPS gave two high molecular weight fractions of different amino sugar content (Fig. 1a). The major components of the H1 fraction were (molar ratios in parentheses) rhamnose (5), glucosamine (2) and 2-amino-2-deoxygalacturonic acid (0.6). The molar ratio given for the last compound is probably an underestimate since no attempt was made to allow for destruction during hydrolysis and, since no authentic compound was available for comparison, the estimate is based on the substance having the same colour yield to reaction with ninhydrin as norleucine. The H1 fraction also contained fucosamine, compound Y and post-arg with small amounts of the core components galactosamine and alanine. The H2 fraction contained mainly rhamnose (10) and glucose (1) with small amounts of the core components. These two fractions may therefore correspond to type-specific polysaccharide chains linked to the core polysaccharide. They would presumably have been released from the LPS by hydrolysis of the acid-labile KDO linkage between lipid A and the polysaccharide part of the molecule.

Very little high molecular weight material was detected in the elution profiles from the mutant LPSs (Fig. 1b, c, d). A small amount of material which reacted with the phenol-sulphuric acid reagent was detected in the high molecular weight fractions from PAC7 and

Fig. 2. Fractionation on Biogel P6 of the partially-degraded polysaccharides from (a) PAC608 and (b) PAC1R LPS (30 mg) after hydrolysis with 1 % (v/v) acetic acid. Fractions (4 ml) were analysed for total carbohydrate by the method of Dubois *et al.* (1956).

Table 3. *Composition of low molecular weight fractions eluted from Biogel P6*

The fractions (L1 and L2) analysed are those shown in Fig. 2. The samples analysed contained 100 to 200 nmol galactosamine. Results are expressed as molar ratios (galactosamine = 1). Compound Y and post-arg are unidentified amino compounds detected by the amino acid analyser.

			L1		L2	
	Source	...	PAC608	PAC1R	PAC608	PAC1R
Component	Wt (mg)	...	(4.6)	(3.2)	(6.7)	(5.9)
Glucose			3.7	3.3	4.4	5.5
Rhamnose			3.1	4.8	1.9	2.6
Heptose			1.9	2.1	1.7	2.1
Glucosamine			0.9	0.8	0	0
Galactosamine			1.0	1.0	1.0	1.0
Alanine			1.3	1.8	1.7	1.8
Aminogalacturonic acid			0.4	0.4	0	0
Fucosamine			+	+	0	0
Compound Y			+	+	0	0
Post-arg			+	+	0	0

+, Present, but not estimated; 0, not detected.

PAC608. From its position and the lack of detectable amino compounds it may correspond to the H2 fraction of PAC1 but there was not enough material to analyse. The absence of high molecular weight material containing amino compounds in the mutant LPSs was checked by concentrating and hydrolysing the first 150 ml of eluate and applying it to the amino acid analyser. There were only traces of amino compounds in all except PAC556. The high molecular weight fractions from this mutant contained five amino compounds including material eluting in the position of aminogalacturonic acid or diaminopimelic acid. Paper chromatography of acid-hydrolysed LPS from PAC556 confirmed the presence of diaminopimelic acid. The molar ratios of the amino compounds detected were diaminopimelic acid 1.0, muramic acid 1.0, glutamic acid 1.2, alanine 2.0 and glucosamine 1.0, suggesting that this LPS contained mucopeptide though the amount present was small (158 nmol glutamic acid in 20 mg LPS). It is unlikely that this is a chance impurity since it

Table 4. *Composition of the core fraction of PACIR and the lipopolysaccharide-defective mutants*

The fractions analysed were the L peaks obtained after Sephadex G75 separation (see Fig. 1 *c, d*) and the L2 peak from Biogel P6 (Fig. 2 *b*). Results are expressed as molar ratios (galactosamine = 1). The fractions for PACIR, PAC557, PAC556 and PAC605 contained 1.4, 1.2, 1.3 and 2.2 mmol galactosamine respectively.

Component	Source ...	L2	L		
		PACIR	PAC557	PAC556	PAC605
Glucose		5.5	4.5	4.7	0
Rhamnose		2.6	1.9	0	0
Heptose		2.1	1.7	1.7	1.7
Galactosamine		1.0	1.0	1.0	1.0
Alanine		1.8	1.6	1.2	1.6

0, Not detected.

occurred in all LPS preparations from PAC556 and mutants derived from it, but not in those from any other PAC mutants we have analysed.

The largest polysaccharide fraction detected in eluates from all the mutants was the phosphorylated low molecular weight fraction labelled L. That from PAC7 and PAC608 and from PAC1 and PACIR consisted of two partially resolved components, the first of which had a higher proportion of 2-amino sugars than the second. This is particularly obvious in PAC7 and PAC608 which appeared to contain more of the leading fraction (Fig. 1 *b*). The two components L1 and L2 were separated by fractionation of the acid-hydrolysed LPS on Biogel P6 (Fig. 2). Analysis of the isolated fractions showed that they were similar in both mutant and parent strains (Table 3). The L2 fractions contained only those compounds thought to occur in the core (glucose, rhamnose, galactosamine, alanine and heptose) and are probably derived from LPS with unsubstituted core. The L1 fractions contained the same compounds as L2 but with additional rhamnose and amino sugars. These are the major components of the H1 polysaccharide suggesting that L1 may be derived from a molecule in which short side-chains are attached to the core polysaccharide. The L fractions from PAC557, PAC556 and PAC605 were eluted from Sephadex G75 as single peaks (Fig. 1 *c, d*) and contained core components only (Table 4). They were not further resolved by fractionation on Biogel P6. PAC557 appeared to have a complete core polysaccharide, while PAC556 and PAC605 lacked rhamnose and glucose plus rhamnose respectively. None of these fractions contained the additional amino sugars found in the L1 and H1 polysaccharides.

DISCUSSION

Although we detected two LPS-defective mutants by their increased sensitivity to carbenicillin, this did not provide a suitable selection method since some sensitive mutants had wild-type polysaccharide. We have since isolated revertants of PAC556 and PAC557 which have wild-type carbenicillin-sensitivity and found that they retained their mutant LPS (unpublished). Furthermore PAC605, the most defective mutant so far isolated, was no more sensitive than the parent strain to carbenicillin. Thus we have no evidence to suggest that increased sensitivity to carbenicillin might correlate with deficiency of LPS structure. However these experiments were limited to carbenicillin and other antibiotics may be different, a possibility which we are now studying.

Of the methods tried for isolating LPS-defective mutants, the only one which appeared

Fig. 3. Tentative partial structure of the core polysaccharide of *Pseudomonas aeruginosa* PAC1. Derived from the composition of mutant and parent LPSs and the analysis of the core polysaccharide of *P. aeruginosa* 1999 by Drewry *et al.* (1975). Dotted lines show possible sites of lesions of the mutants.

selective was that of resistance to aeruginocin P16. Suzuki (1974) suggested that only those strains containing quinovosamine were sensitive to this aeruginocin but there are some exceptions, PAC1 being one (Koval & Meadow, 1975*b*). The mutants we selected by their resistance to P16 aeruginocin all lacked the high molecular weight fractions H1 and H2 which could therefore contain the aeruginocin receptors. No single compound seemed to be responsible for sensitivity to P16 aeruginocin since the resistant mutant PAC608 contained all the compounds detected in the parent LPS. However we have so far only accounted for about 70 % of its dry weight and if the group or groups responsible for receptor activity were acid-labile we would not have detected them.

The R₃ aeruginocin of PAT1 binds to isolated LPS (Ito, Kageyama & Egami, 1970; Ikeda & Egami, 1973) and it is surprising that mutants of PAC1 which were resistant to this aeruginocin had apparently normal LPS. However Ito *et al.* (1970) and Kageyama (1975) have postulated that the receptor site for the R-type aeruginocins is a sequence of molecules projecting from the bacterial surface and that the receptor for R₃ is the most distal of those studied. Mutants resistant to it would thus differ depending on how much of the receptor was missing. Certainly all five of our defective mutants were resistant to the PAT1 aeruginocin (Table 1). Perhaps the mutants of PAC1 which were selected for resistance lacked only a small part of the aeruginocin receptor and this might not have been detected in our preliminary analyses of the LPS.

The analyses of the mutant and parent LPSs are consistent with our view that PAC1 may have four different types of LPS. One contains only the core polysaccharide and gives rise to the L2 fraction; this is the only one detected in PAC557, PAC556 and PAC605. A second contains core polysaccharide with short antigenic side-chains and yields the L1 fraction, also present in PAC7 and PAC608. In addition PAC1 contains LPSs in which the core is linked to long side-chains and which give H1 and H2 polysaccharides on hydrolysis. Since we have no mutants lacking only one of these H fractions, we do not know how they might be related or whether they might both be derived from the same molecule. Alkaline degradation suggested, however, that they were probably separate molecules (Chester & Meadow, 1975). LPSs in which short antigenic side-chains are linked to core polysaccharides have not previously been reported in the pseudomonads. The published Sephadex profiles of strains of Habs serotype 1, 2A, 2B, 3, 5D and 6 indicate that they all had core polysaccharides consisting of two fractions (Chester *et al.*, 1973; Wilkinson & Galbraith, 1975), suggesting that this type of heterogeneity may be common. The presence of the antigenic compounds in the L1 fractions is supported by the serological results (Table 1). Despite their lack of the high molecular weight polysaccharide fractions PAC7 and PAC608 reacted

with antisera to Habs type 3 and PAC1 whereas the mutants lacking both the H and LI fractions did not. Mutants producing short antigenic side-chains of this kind have been described in the Enterobacteriaceae and are termed semi-rough (SR) (Naide *et al.*, 1965)

Three of the mutants we isolated, PAC557, PAC556 and PAC605, appear to be core mutants and provided confirmatory evidence for the type of core structure proposed for *P. aeruginosa* 1999 by Drewry *et al.* (1975). They analysed the phosphorylated low molecular weight fraction isolated from the LPS after elution from Sephadex G50. The L2 fraction from PAC1 may contain an additional rhamnose and glucose molecule but its composition is otherwise identical. The progressive loss of sugars in our three core mutants is compatible with the structure proposed and their defects could be as shown in Fig. 3. The only mutant whose biochemical lesion has so far been partially identified is PAC556. Preliminary experiments have shown that membrane preparations from this mutant incorporate rhamnose from TDP[¹⁴C]rhamnose while comparable preparations from the parent strain and other mutants did not (Koval, Fensom & Meadow, unpublished). This mutant may therefore be unable to make TDP-rhamnose and hence to complete its core structure. PAC556 was, however, also unusual in that its LPS preparations contained mucopeptide and it grew more slowly than the parent strain, so there may be other lesions. The most defective mutant PAC605 apparently contained only the inner core components galactosamine, alanine and heptose linked through KDO to lipid A. It was a spontaneous mutant and may have only a single defect. It is somewhat surprising that this grossly deficient LPS did not appear to alter the growth rate, colonial morphology or sensitivity to carbenicillin of PAC605 but only to cause loss of O-antigenicity. Further work will be needed to relate LPS structure to phenotype in these mutants.

We thank the Medical Research Council for a project grant, Mr P. D. Laverack for the amino compound analyses, Mr T. L. Pitt for the serotyping and Mr P. Wells for excellent technical assistance.

REFERENCES

- BARTLETT, G. R. (1959). Phosphorus assay in column chromatography. *Journal of Biological Chemistry* **234**, 466–468.
- BRAMMAR, W. J., CLARKE, P. H. & SKINNER, A. J. (1967). Biochemical and genetic studies with regulator mutants of the *Pseudomonas aeruginosa* 8602 amidase system. *Journal of General Microbiology* **47**, 87–102.
- BROWN, P. R. & CLARKE, P. H. (1972). Amino acid substitution in an amidase produced by an acetanilide mutant of *Pseudomonas aeruginosa*. *Journal of General Microbiology* **70**, 287–298.
- CHESTER, I. R. & MEADOW, P. M. (1975). Heterogeneity of the lipopolysaccharide from *Pseudomonas aeruginosa*. *European Journal of Biochemistry* **58**, 273–282.
- CHESTER, I. R., MEADOW, P. M. & PITT, T. L. (1973). The relationship between the O-antigenic lipopolysaccharides and serological specificity in strains of *Pseudomonas aeruginosa* of different O-serotypes. *Journal of General Microbiology* **78**, 305–318.
- CLARKSON, C. E. & MEADOW, P. M. (1971). Diaminopimelic acid and lysine auxotrophs of *Pseudomonas aeruginosa* 8602. *Journal of General Microbiology* **66**, 161–169.
- DREWRY, D. T., SYMES, K. C., GRAY, G. W. & WILKINSON, S. G. (1975). Studies of polysaccharide fractions from the lipopolysaccharide of *Pseudomonas aeruginosa* NCTC1999. *Biochemical Journal* **149**, 93–106.
- DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**, 350–356.
- FENSOM, A. H. & MEADOW, P. M. (1970). Evidence for two regions in the polysaccharide moiety of the lipopolysaccharide of *Pseudomonas aeruginosa* 8602. *FEBS Letters* **9**, 81–84.
- GOVAN, J. R. W. (1974). Studies on the pyocins of *Pseudomonas aeruginosa*: morphology and mode of action of contractile pyocins. *Journal of General Microbiology* **80**, 1–15.
- GUSTAFSSON, P., NORDSTROM, K. & NORMARK, S. (1973). Outer penetration barrier of *Escherichia coli* K12: kinetics of the uptake of gentian violet by wild type and envelope mutants. *Journal of Bacteriology* **116**, 893–900.

- HABS, I. (1957). Untersuchungen über die O-antigene von *Pseudomonas aeruginosa*. *Zeitschrift für Hygiene und Infektionskrankheiten* **144**, 218–228.
- HOARE, D. S. & WORK, E. (1955). The stereoisomers of α,ϵ -diaminopimelic acid: their distribution in nature and behaviour towards certain enzyme preparations. *Biochemical Journal* **61**, 562–568.
- HOLLOWAY, B. W. & KRISHNAPILLAI, V. (1974). Bacteriophages and bacteriocins. In *Genetics and Biochemistry of Pseudomonas*, pp. 99–132. Edited by P. H. Clarke and M. H. Richmond. London: John Wiley.
- IKEDA, K. & EGAMI, F. (1973). Lipopolysaccharides of *Pseudomonas aeruginosa* with special reference to pyocin R receptor activity. *Journal of General and Applied Microbiology* **19**, 115–128.
- ITO, S., KAGEYAMA, M. & EGAMI, F. (1970). Isolation and characterisation of pyocins from several strains of *Pseudomonas aeruginosa*. *Journal of General and Applied Microbiology* **16**, 205–214.
- KAGEYAMA, M. (1975). Bacteriocins and bacteriophages in *Pseudomonas aeruginosa*. In *Microbial Drug Resistance*, pp. 291–305. Edited by S. Mitsuhashi and H. Hashimoto. Tokyo: University of Tokyo Press.
- KOVAL, S. F. & MEADOW, P. M. (1975*a*). Lipopolysaccharide-defective mutants of *Pseudomonas aeruginosa* PAC1. *Proceedings of the Society for General Microbiology* **3**, 53.
- KOVAL, S. F. & MEADOW, P. M. (1975*b*). The relationship between aminosugars in the lipopolysaccharide, serotype and aeruginocin sensitivity in strains of *Pseudomonas aeruginosa*. *Journal of General Microbiology* **91**, 437–440.
- LÜDERITZ, O., WESTPHAL, O., STAUB, A. M. & NIKAIIDO, H. (1971). Isolation and immunological characterisation of bacterial lipopolysaccharides. In *Microbial Toxins*, vol. IV, pp. 145–233. Edited by G. Weinbaum, S. Kadis and S. J. Ajl. London and New York: Academic Press.
- MARTIN, D. (1971). *Variation in Pseudomonas aeruginosa*. Ph.D. thesis, University of London.
- MEADOW, P. M. (1975). Wall and membrane structure in the genus *Pseudomonas*. In *Genetics and Biochemistry of Pseudomonas*, pp. 67–98. Edited by P. H. Clarke and M. H. Richmond. London: John Wiley.
- NAIDE, Y., NIKAIIDO, H., MÄKELÄ, P. H., WILKINSON, R. G. & STOCKER, B. A. D. (1965). Semi-rough strains of *Salmonella*. *Proceedings of the National Academy of Sciences of the United States of America* **53**, 147–153.
- NOWOTNY, A. (1971). Chemical and biological heterogeneity of endotoxins. In *Microbial Toxins*, vol. IV, pp. 309–329. Edited by G. Weinbaum, S. Kadis and S. J. Ajl. London and New York: Academic Press.
- RONDLE, C. G. M. & MORGAN, W. T. J. (1955). Determination of glucosamine and galactosamine. *Biochemical Journal* **61**, 586–589.
- SANDERSON, K. E., MACALISTER, T. & COSTERTON, J. W. (1974). Permeability of lipopolysaccharide-deficient (rough) mutants of *Salmonella typhimurium* to antibiotics, lysozyme and other agents. *Canadian Journal of Microbiology* **20**, 1135–1145.
- SANDERSON, K. E., VAN WYNGAARDEN, J., LÜDERITZ, O. & STOCKER, B. A. D. (1974). Rough mutants of *Salmonella typhimurium* with defects in the heptose region of the lipopolysaccharide core. *Canadian Journal of Microbiology* **20**, 1127–1134.
- SCHLECT, S. & WESTPHAL, O. (1968). Antibiotica-Empfindlichkeit bei S- und R-Formen von *Salmonella minnesota*. *Naturwissenschaften* **10**, 494–495.
- SUZUKI, N. (1974). Correlation between pyocin sensitivity and 2-aminosugar composition of *Pseudomonas aeruginosa*. *FEBS Letters* **48**, 301–305.
- TAMAKI, S. & MATSUHASHI, M. (1973). Increase in sensitivity to antibiotics and lysozyme on deletion of lipopolysaccharides in *Escherichia coli* strains. *Journal of Bacteriology* **114**, 453–454.
- TAMAKI, S., SATO, T. & MATSUHASHI, M. (1971). Role of lipopolysaccharides in antibiotic resistance and bacteriophage absorption of *Escherichia coli* K12. *Journal of Bacteriology* **105**, 968–975.
- WAHBA, A. H. & DARRELL, J. H. (1965). The identification of atypical strains of *Pseudomonas aeruginosa*. *Journal of General Microbiology* **38**, 329–342.
- WILKINSON, S. G. & GALBRAITH, L. (1975). Studies of lipopolysaccharide from *Pseudomonas aeruginosa*. *European Journal of Biochemistry* **52**, 331–343.
- WILKINSON, R. G., GEMSKI, P. & STOCKER, B. A. D. (1972). Non-smooth mutants of *Salmonella typhimurium*: differentiation by phage sensitivity and genetic mapping. *Journal of General Microbiology* **70**, 527–554.