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Chemical and Chromatographic Analysis of Lipopolysaccharide from an Antibiotic-Supersusceptible Mutant of *Pseudomonas aeruginosa*

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Lipopolysaccharides extracted from *Pseudomonas aeruginosa* strain K799 and its antibiotic-supersusceptible derivative Z61 were analyzed chemically and chromatographically. The side-chain polysaccharides purified by gel exclusion chromatography were compositionally identical, being composed of fucosamine (2-amino-2,6-dideoxygalactose), quinovosamine (2-amino-2,6-dideoxyglucose), and an unidentified amino sugar. In addition, low amounts of the core-specific components (glucose, rhamnose, alanine, and galactosamine) were found associated with the side chains from both strains. An average molecular weight of 38,000 to 50,000 was calculated for this fraction based on the glucose and rhamnose levels. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that the lipopolysaccharides from these two strains were microheterogeneous. Qualitative analysis of the lipopolysaccharide neutral sugars, using a series of single-step revertants of mutant Z61, demonstrated that full revertants showed patterns indistinguishable from those of the wild-type strain K799, whereas partial revertants had intermediate levels and mutant Z61 low levels of neutral sugars. Quantitative analysis revealed that the core oligosaccharide fraction from the wild-type strain had a glucose/rhamnose/galactosamine ratio of 4:1:1, whereas the core from Z61 exhibited major deficiencies in both glucose and rhamnose. The lipid A from both strains contained five fatty acids, namely, 3-hydroxydecanoate, dodecanoate, 2- and 3-hydroxydodecanoate, and hexadecanoate. Whereas the overall fatty acid content was equal, the mutant strain showed markedly lower levels of dodecanoate and hexadecanoate and increased levels of 2-hydroxydodecanoate. Results of whole-cell fatty acid analyses were consistent with this observation. Evidence for an additional alteration of the lipid A of strain Z61 was obtained from acid hydrolysis studies and infrared spectra of isolated lipid A, although the actual chemical basis could not be determined by a variety of techniques. It is suggested that the state of the lipopolysaccharide is able to influence the number of open functional protein F pores in the outer membrane of *P. aeruginosa*.

In the preceding paper (1), we provided evidence that the basis of the generally enhanced susceptibility to antibiotics of mutant Z61, when compared with its progenitor strain K799, was a more permeable outer membrane. In particular, the outer membrane of strain Z61 was more permeable to the hydrophilic antibiotic nitrocefin (1) and to other β-lactams (41), despite an apparently unaltered porin protein F, an outer membrane protein shown to form water-filled channels in model membrane systems (2a, 14). Indirect evidence was provided that mutant Z61 had an unspecified lipopolysaccharide (LPS) alteration (1). To gain further information regarding the mutation present in strain Z61, we characterized the LPS alteration in greater depth. Due to the rather complex method used to isolate mutant Z61 (41), we prepared a series of spontaneous revertants (1). Full revertants were isolated which were indistinguishable from the wild-type strain K799 in a wide variety of properties (1), including some LPS properties (see below). This suggests that Z61 and K799 differ by a single major mutation which is responsible for the antibiotic susceptibility and permeability differences between the strains as well as the

biochemical changes described in this paper. Thus, in the experiments reported below, K799 LPS was directly compared with Z61 LPS.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used, *P. aeruginosa* K799, Z61 and its revertants, and H103 of 0-5 serotype (4), were described in the preceding paper (1). Cultures were maintained at −70°C in broth containing 7.7% (vol/vol) dimethyl sulfoxide.

Medium and cell growth conditions. Cells were grown at 37°C with vigorous aeration in carboys containing 15 liters of TSY broth. This medium contained 30 g of tryptic soy broth (Difco Laboratories) and 5 g of yeast extract (Difco) per liter of distilled water. Cells were harvested by centrifugation when the cultures reached late log to early stationary phase, and the cell pellets were lyophilized to yield about 35 g (dry weight).

LPS extraction. LPS was extracted from the dried cells by the hot aqueous phenol procedure of Westphal and Jann (32). The aqueous layers were extracted twice with one-half volume of ethyl ether to remove residual phenol (27) and then centrifuged for 1 h at 20,000 × *g* to sediment peptidoglycan (24). The supernatants were centrifuged at 100,000 × *g* for 3.5 h, and the LPS pellets were suspended in distilled water, centrifuged as before, and then lyophilized to yield about 300 mg. The above methodology overcame the problem of low yields of LPS observed for mutant Z61 and partial revertants encountered by using a different procedure (15).

LPS extracted from the wild-type cells was found to contain high levels of material, presumably RNA, absorbing at 260 nm. K799 LPS (120 mg) was dissolved in 80 ml of 0.05 M Tris-hydrochloride, pH 7.5, and digested for 3 h at 37°C with 50 μg of RNase A per ml (Sigma Chemical Co., St. Louis, Mo.). The LPS was recovered by ultracentrifugation and washed with distilled water before lyophilizing to yield 110 mg.

LPS hydrolysis and isolation of lipid A. LPS preparations (100 mg) were hydrolyzed in 1% (vol/vol) acetic acid for 90 min at 100°C. The hydrolysates were then chilled on ice and centrifuged at 10,000 × *g* for 10 min. The pellets containing crude lipid A were washed with 3.5 ml of 0.05 M pyridinium acetate buffer (pH 5.3) and lyophilized. Before further analysis, these preparations were suspended in 5 ml of distilled water, sonicated briefly, centrifuged, and then dried in vacuo. All preparations were stored in vacuo over phosphorus pentoxide.

Column chromatography of polysaccharide fractions. The degraded polysaccharide fractions derived from LPS by acetic acid hydrolysis (see above) were dissolved in 0.05 M pyridinium acetate buffer, pH 5.3, chilled on ice, and centrifuged for 5 min at 10,000 × *g* to remove insoluble material. The supernatant was lyophilized and, before column chromatography, redissolved in 1.5 ml of buffer. This material was applied to a column (2.5 by × 85 cm) containing Sephadex G-50 (medium), and 1.5-ml fractions were collected and monitored for hexose (9) and hexosamine (22) as described previously (25).

Fatty acid analysis. Samples (1 to 3 mg) were hydrolyzed in screw-capped tubes in the presence of 1 ml of 2 M methanolic HCl, prepared from acetyl chloride (Instant Methanolic HCl Kit; Applied Science Laboratories Inc., State College, Pa.). Hydrolysis was carried out under nitrogen for 16 h at 100°C. After this period, the samples were cooled to room temperature and neutralized with silver carbonate or Dowex AG1-X8 (HCO_3^-) (ca. 031 g). Internal standard (methyltetradecanoate; 829 μg) was added to each tube, and these were then centrifuged to remove the insoluble salts or Dowex. Portions (1 to 2 μl) of the supernatant were analyzed in a Perkin-Elmer Sigma 3 gas chromatograph equipped with a glass column (ca. 305 cm by 2 mm ID) containing 3% SP-2100 DOH on 100- to 200-mesh Supelcoport (Supelco Inc., Bellefonte, Pa.). The following program was used: initial temperature, 160°C; final temperature, 200°C; ramp rate, 2°C/min; final time, 3 min. Carrier gas (helium) was maintained at 18 ml/min. For whole-cell fatty acid analysis, 10 mg of lyophilized cells was premixed with internal standard (103 μg of pentadecanoic acid) before hydrolysis with methanolic HCl and analyzed as described above.

GLC analysis for neutral sugars. LPS (1 mg) was hydrolyzed, under nitrogen, in 0.6 ml of 1 M HCl for 4 h at 100°C. Internal standard (myoinositol; 100 μg) and 1.5 ml of distilled water were added to each sample before drying in vacuo. The residue was taken up in 0.2 ml of water and passed through a small column containing Dowex 1 (formate) and Dowex 50 (H^+). The resin was then washed sequentially with 0.6 ml of water and 0.8 ml of methanol-water (1:1, vol/vol). The eluants were pooled and lyophilized. The neutral sugar residue was dissolved in 0.2 ml of sodium borohydride (10 mg/ml in 0.1 M NH_4OH) and left at room temperature for 1 h, acidified with 0.05 ml of acetic acid, and dried in vacuo. This residue was extracted thrice with 0.25 ml of methanolic HCl (5:0.01, vol/vol) and three times with methanol to remove boric acid. The reduced sugars were then acetylated in pyridine-acetic anhydride (1:1, vol/vol) for 30 min at 100°C; these reagents were then removed in vacuo and the alditol acetates were dissolved in 0.05 ml of gas-liquid chromatography (GLC)-grade chloroform. Samples (1 to 2 μl) were analyzed with a nickel column (Ca. 366 by 0.3 cm) containing 10% Silar 10CP on 100 to 200-mesh Chromosorb W-HP (Chromatographic Specialties Ltd., Brockville, Ontario). Resolution of the alditol acetates was achieved by using the following program: injector/detector temperature, 250°C; initial temperature, 220°C; final temperature, 250°C; ramp rate, 1.5°C/min; helium flow rate, 40 ml/min.

Amino acid and amino sugar analyses. Samples (1 to 2 mg) of LPS, core oligosaccharide, and side chains were hydrolyzed, under nitrogen, in 6.1 M HCl (Pierce Chemical Co., Rockford, Ill.) for 4 h at 100°C. Samples of standards (glucosamine, galactosamine, glucosamine phosphate, *N*-acetyl-D-fucosamine, and *N*-acetylquinovosamine) were similarly hydrolyzed. The samples were dried in vacuo at room temperature and subjected to amino acid analysis, using a Beckman 120C modified single-column instrument.

Other chemical analyses. Heptose and hexose were determined by using the procedure of Wright and Rebers (38) with D-glycero-L-mannoheptose and D-glucose as standards. Wavelength scanning of the colored products of this assay provided a qualitative comparison of LPS neutral sugars.

3-Deoxy-D-mannooctulosonic acid (KDO) was determined by the colorimetric assay of Karkhanis et al.

(23) on samples of LPS hydrolyzed in 0.2 N H_2SO_4 for 30 min at 100°C. The ammonium salt of KDO (Sigma) was used as the standard. Phosphorus was determined by the Analytical Services Unit, Department of Chemistry, Queen's University.

TLC of LPS hydrolysates. LPS was hydrolyzed in 1 M HCl at 100°C for 4 h (neutral sugars), 6.1 M HCl at 100°C for 4 h (amino sugars), or 0.1 M HCl at 100°C for 10 min (4-aminoarabinose). In the latter case, LPS purified from *Salmonella typhimurium* AK226 (Rc chemotype) was included as a positive control. The hydrolysates were lyophilized and suspended in distilled water to approximately 50 mg/ml, and samples (5 μl) were spotted on silica gel plates for neutral sugar separations and on cellulose thin-layer chromatography (TLC) plates for resolving amino compounds. The solvent systems used were: system I, isopropanol-formic acid-water (60:4:40, vol/vol); system II, 2,6-lutidine–water (65:35, vol/vol); system III, pyridine-acetic acid-ethyl acetate-water (25:5:25:15, vol/vol); and system IV, acetone-chloroform-water (85:10:5, vol/vol). The latter solvent system was used to separate the neutral sugars (40); systems I and II (31) and I and III were used to resolve amino compounds and 4-aminoarabinose, respectively. A chromogenic ninhydrin spray (31) was used to detect hexosamines and amino acids, and a diphenylamine-analine-phosphoric acid-containing reagent (17) was used for developing sugar chromatograms.

Infrared spectroscopy. Infrared spectra (KBr disk) were determined with a Perkin-Elmer model 598 spectrophotometer.

Polyacrylamide gel electrophoresis. LPS and side-chain polysaccharide fractions were analyzed by slab gel electrophoresis on 10% (wt/vol) or 10 to 20% (wt/vol) linear gradients of acrylamide in 0.4 M Tris (pH 8.8). The 10% (wt/vol) gel contained, per 30 ml: acrylamide, 3 g; *N,N'*-methylenebisacrylamide, 0.08 g; sodium dodecyl sulfate (SDS), 0.6 g; and EDTA, 10.5 mg. Polymerization was catalyzed by the addition of 10 mg of ammonium persulfate and 0.02 ml of *N,N,N',N'*-tetramethyl-ethylenediamine (TEMED). The stacking gel contained 5% (wt/vol) acrylamide-bisacrylamide (30:0.8, wt/wt) in 0.125 M Tris (pH 6.7) containing 0.002 M EDTA and 2% (wt/vol) SDS.

LPS (0.75 mg) and side-chain polysaccharide (0.5 mg) were dissolved in 0.1 ml of sample buffer (0.0625 M Tris-hydrochloride, pH 6.8; 0.002 M EDTA; 10% [wt/vol] glycerol; 4% [wt/vol] SDS; and 2% [wt/vol] 2-mercaptoethanol) and sonicated before gel electrophoresis. Pyronin Y (Bio-Rad Laboratories, Richmond, Calif.) was used as the tracking dye. Samples (0.02 ml) were applied to wells in a slab gel apparatus (1.2 mm by 16 cm; Aquebogue Machine Shop, Aquebogue, N.Y.) and overlayed with running buffer containing 0.025 M Tris-hydrochloride (pH 8.6), 0.192 M glycine, and 0.2% (wt/vol) SDS. Electrophoresis was carried out at 50 V until the tracking dye entered the resolving gel and then at 100 V until the dye was about 1 cm from the bottom.

Staining *Pseudomonas* LPS on gels. The gels were fixed, at room temperature for 1 h, in a fresh solution of 0.5% (wt/vol) phosphotungstic acid (Sigma) in 2 M HCl. They were then briefly rinsed in tap water and placed in a 0.2% (wt/vol) solution of Coomassie brilliant blue R250 in 50% (vol/vol) methanol–7% (vol/vol) glacial acetic acid. After staining overnight, the gels were briefly destained in 10% (vol/vol) methanol–14% (vol/vol) acetic acid.

RESULTS

Comparison of the LPS of Mutant Z61 and its revertants with the wild-type strain K799. We wished to establish a rapid, sensitive, and convenient qualitative method for studying the neutral sugar composition of the LPS of the various strains. The Dische cysteine-sulfuric acid method (2), and the related Wright and Rebers method (38), have been used for the qualitative and quantitative determination of hexoses, phosphorylated hexoses, methyl pentoses, pentoses, deoxypentoses, hexuronic acids, aldoheptoses, and ketoheptoses by measurement of adsorbance at different wavelengths. Therefore, we examined the LPS of our strains by this method (Fig. 1). The wild-type strains K799 and H103 and the full revertants H251, H252, and H253 gave almost identical products as revealed by wavelength scans between optical densities at 560 and 370 (see result for K799, Fig. 1). This strongly suggested that these strains had extremely similar neutral sugar compositions. In contrast, the profiles of these wavelength scans were substantially altered for the antibiotic-supersusceptible mutant Z61 and its partial revertants H254 and H257. Another clear difference we observed was that the amount of background color produced by H_2SO_4 hydrolysis of the LPS of mutant Z61 was considerably less than that produced from the wild-type strain K799 and the other strains noted above. Hydrolysis of the LPS of the partial revertants gave intermediate background adsorbances. Since we could find no qualitative or quantitative differences between K799 and the full revertants, by using a large range of criteria, we decided to use the LPS of the wild-type strain K799 as the basis for comparison with mutant Z61 LPS.

Isolation of LPS from strains K799 and Z61. LPS was extracted from both strains by using hot aqueous phenol (32) in approximately 1% yields by weight. Upon ultracentrifugation, all preparations of mutant Z61 LPS formed firm pellets which were considerably more difficult to resuspend in water than those obtained with wild-type K799 LPS. As estimated by absorbance at 260 nm, Z61 LPS contained 6.1% (by weight) nucleic acid, whereas K799 LPS contained over 40%. This high level of nucleic acid contamination was reduced to 1.6% by digestion with RNase. It should be noted that these values obtained by UV absorbance are an overestimate since they were not supported by the expected levels of ribose in the neutral sugar analyses by GLC.

Isolation of lipid A and polysaccharide fractions. Hydrolysis of the LPS preparations in

FIG. 1. Wavelength scans of colored products produced by a Wright and Rebers cysteine-H_2SO_4 treatment (38) of *P. aeruginosa* LPS isolated as described previously (15) from the wild-type strain K799, partial revertants H254 and H257, and the antibiotic-supersusceptible mutant Z61. The scans were performed with water as a reference. Such derivatizations have been used to estimate, for example, heptoses (difference between absorbance at 505 and 545 nm), hexoses (difference between absorbance at 415 and 380 nm), and methyl pentoses such as rhamnose (difference between absorbance at 396 and 427 nm).

dilute acetic acid resulted in the cleavage of the ketosidic linkages between KDO residues and the liberation of insoluble lipid A. This material was obtained from the LPS of both strains in approximately 35% yield. Hydrolysis of mutant Z61, but not K799, LPS was accompanied by a darkening of the hydrolysis medium. This phenomenon was also noted when 0.1 to 6.1 M HCl or 2 M trifluoroacetic acid was used with Z61 LPS. In addition, lipid A of K799 purified as described in Materials and Methods was white, whereas Z61 preparations were light brown.

Chromatography of polysaccharide fractions. Strain K799 polysaccharide (61 mg) and 54 mg of Z61 polysaccharide were applied to Sephadex G-50 columns and eluted with 0.05 M pyridinium acetate (pH 5.3). The column fractions were monitored for hexosamine by the Elson-Morgan procedure (22) and for total hexose by the phenol-sulfuric acid procedure of Dubois et al. (9). The results for the two polysaccharide preparations were superficially similar in that both gave rise to three subfractions (Fig. 2). Fraction I eluted just after blue dextran (i.e., V_0) and was particularly high in hexosamine. This high-molecular-weight fraction, by analogy with other studies on *Pseudomonas* LPS (14, 21, 29, 30) represented the O-antigenic side-chain polysaccharide with the attached core. The second peak (fraction II), which had higher levels of hexose than hexosamine, corresponded to the core oligosaccharide (21, 25, 34, 35). It should be noted that both preparations showed some heterogeneity in this region, with two peaks of Elson-Morgan-reactive material being noted (fractions IIA and IIB). Only fraction IIB was analyzed further (see below). Fraction III material had been previously shown to contain hydrolysis products such as KDO, ethanolamine, and phosphorylated derivatives of the latter (7) and was not studied further. Although we noted no differences in the chromatographic properties of fraction I material from the two strains, fraction IIB oligosaccharide from the mutant strain showed two changes. The peak material was shifted by approximately 20 fractions to a lower molecular weight, and there was a change in the hexose/hexosamine ratio. In addition, there was an increased amount of fraction III materials in the degraded polysaccharide of strain Z61. These three observations suggested the existence of an altered rough core in a portion of the LPS of the mutant strain. Since fraction III material represented degraded LPS (7, 34), it was not studied in detail.

Fatty acid analysis of whole cells, LPS and lipid A. LPS and lipid A fraction were assayed for fatty acid methyl esters after hydrolysis in 2 M methanolic HCl. The LPS from these two organisms contained the five fatty acids commonly found in the LPS of *P. aeruginosa* strains (34): dodecanoic acid, 3-hydroxydecanoic acid, 2- and 3-hydroxydodecanoic acid, and hexadecanoic acid (Table 1). These were identified on the basis of their retention times and quantitated by using response factors calculated for each, using commercial standards. The fatty acid content of K799 and Z61 lipid A represented approximately 50% of the weight of the lipid A preparations, but the percentage of each fatty acid showed marked variation. We observed (Table 1) a 40 to 50% decrease in the levels of dodecanoate and hexadecanoate and a 20% increase in the level of 2-hydroxydodecanoate. Only the level of 3-hydroxydodecanoate remained approximately constant.

The differences in LPS fatty acids were reflected in analyses of whole-cell fatty acids (Table 1). These analyses demonstrated no differences in five of the seven major fatty acids,

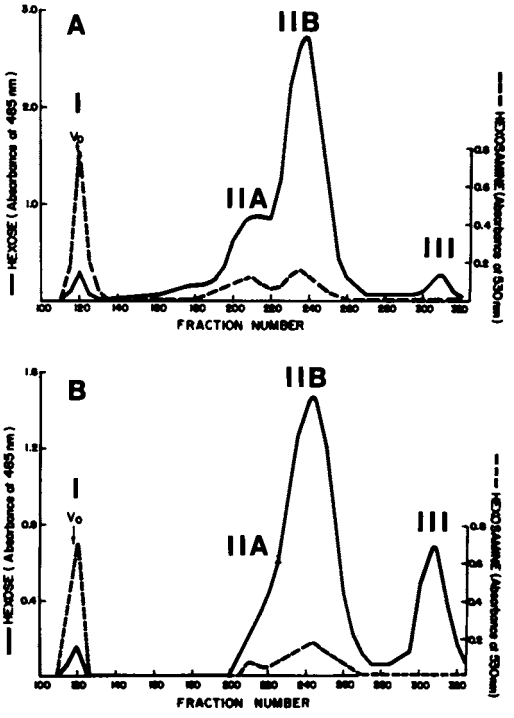

FIG. 2. Chromatographic profile of the degraded polysaccharide fractions derived from the hydrolysis of K799 (61 mg; A) and Z61 (54 mg; B) LPS in 1% (vol/vol) acetic acid (100°C, 1.5 h). The column (Sephadex G-50; 2.5 by 85 cm) was developed with 0.05 M pyridinium acetate buffer, pH 5.3, and the eluant was analyzed for total carbohydrates (absorbancy at 485 nm; _____) and hexosamine (absorbancy at 530 nm; - - -). The void volume (V_0) was determined by using blue dextran.

but significant ($P < 0.1$ by the Student t test) reductions in the amounts of dodecanoic acid and hexadecanoic acid in whole cells of mutant Z61 compared with the wild type, K799. The magnitudes of these reductions were similar to those observed for LPS and lipid A (when one takes into account that hexadecanoic acid is a major fatty acid of phospholipids [12]). The levels of each of the seven major fatty acid peaks in the full revertants were not significantly different ($P > 0.5$) from the amounts in K799 (Table 1), whereas partial revertants showed intermediate phenotypes.

Infrared spectra of lipid A samples from the two organisms were superimposable with the exception that the absorbance at 805 cm^{-1} and 1,260 cm^{-1} was much reduced in the mutant Z61 spectrum.

TLC. No differences were observed in the number of UV-adsorbing, ninhydrin-positive or diphenylamine-reactive components on TLC plates when we used the techniques described in Materials and Methods. LPS from the mutant strain showed qualitatively less glucose and rhamnose, which was confirmed subsequently by GLC (see below). In addition, we found no evidence for 4-aminoarabinose in these strains.

Analysis of core oligosaccharide fractions. The core oligosaccharide fractions (IIB) contained glucose, rhamnose, heptose, galactosamine, and alanine as indicated by GLC and automated amino acid analyses (Table 2). In addition, there was evidence for trace amounts of ethanolamine phosphate along with high levels of phosphorus (Table 2). Mutant strain Z61, but not wild-type strain K799, LPS contained low levels of ribose. Core oligosaccharide from the mutant strain had 4-fold less glucose and 11-fold less rhamnose than the corresponding fraction from K799 LPS. On the other hand, it had higher levels of heptose, phosphorus, alanine, and galactosamine. This would be expected for a rough core. The core from the wild-type strain had glucose, rhamnose, heptose, and galactosamine in a molar ratio of 4:1:2:1; the ratio for the mutant strain was approximately 1:0:2:1.

Fraction IIA and B material from strain Z61 also possessed the brownish coloration of the lipid A preparations, whereas the side-chain polysaccharide material from this strain was, like that of K799, white.

Infrared spectra of both preparations showed no additional bands, but Z61 fraction IIB exhibited a decrease in the 1,050/920 cm^{-1} absorbance ratio. This change closely mimicked the analogous changes observed with the rough mutants of strain PAO (A. M. Kropinski, unpublished data).

Chemical analysis of side chain. Hydrolysis of both LPS preparations and analysis of the amino compounds with an amino acid analyzer indicated that both Z61 and K799 LPS contained glucosamine, glucosamine phosphate, fucosamine, quinovosamine, and galactosamine (Table 2). In addition, an unknown material was observed which eluted just before the ammonia peak. The side-chain oligosaccharide fractions contained this material (unknown A) along with fucosamine and quinovosamine. In addition, lesser amounts of galactosamine and alanine, together with glucose and rhamnose, were found. The presence of these neutral sugars could have been due to contamination of the side-chain polysaccharides with undegraded LPS or could indicate the presence of core units at the reducing end of the polysaccharide chains. This latter explanation appears the more reasonable, since there was no evidence for the lipid A component glucosamine in these preparations. Of additional interest was the fact that the glucose/rhamnose ratio was 4:1 in both preparations—a value identical to that in the core oligosaccharide from

TABLE 1. Fatty acid composition of whole cells, LPS, and lipid A from strain K799, mutant Z61, and revertant derivatives

Fatty acid source	Strain	Fatty acid composition (% of total fatty acids)[a]							Total fatty acid content	
		β-OH 10:0	12:0	α-OH 12:0	β-OH 12:0	16:1	16:0	18:1	nmol/mg of cell dry wt	% (wt/wt) of LPS or lipid A
Whole cells	K799	5.5	4.3	5.9	6.9	15.1	33.1	29.7	315.8	
	Full revertants	6.5	4.0	5.6	7.3	13.6	33.1	29.8	300.6	
	Partial revertants	7.7	3.3	6.7	7.7	13.7	30.4	29.5	290.4	
	Z61	5.9	2.2	6.3	7.1	14.7	29.9	32.5	285.7	
LPS	K799	20.1	12.2	32.9	28.0	0	6.7	0		16.4
	Z61	19.0	6.0	40.8	29.3	0	4.9	0		18.4
Lipid A	K799	20.5	11.9	33.0	30.2	0	4.5	0		53.7
	Z61	18.9	5.8	40.8	31.2	0	3.4	0		50.3

[a] For fatty acid nomenclature, the number to the left of the colon represents the number of carbon atoms, and the number to the right represents the number of double bands. OH refers to hydroxy fatty acids. The results presented represent the mean of three to six separate determinations. The results for strains H257, H258, and H259 (full revertants) and for strains H254, H255, H256, and H257 (partial revertants) have been pooled.

TABLE 2. Chemical composition of the LPS, core oligosaccharides, and side-chain polysaccharides from *P. aeruginosa* strains K799 and Z61

Component	Composition (% by weight)					
	Wild-type K799			Mutant Z61		
	LPS	Side chain	Core	LPS	Side chain	Core
Glucose	9.3	1.8	29.3	5.2	1.4	8.8
Rhamnose	2.0	0.4	6.7	0.6	0.3	0.6
Heptose	7.4		13.0	7.9		14.0
KDO	4.3			4.8		
Ribose	ND[a]	ND	ND	0.9	ND	ND
Phosphorus	5.5		6.6	7.8		7.3
Alanine	1.5	0.2	3.6	1.7	0.3	4.6
Galactosamine	4.3	1.1	7.8	4.8	0.9	9.7
Glucosamine	4.3	ND	ND	5.1	ND	ND
Glucosamine-phosphate	3.5	ND	ND	3.9	ND	ND
Ethanolamine-phosphate			tr			tr[b]
Fucosamine	1.9	6.9	ND	1.4	7.1	ND
Quinovosamine	2.6	16.1	ND	2.7	17.2	ND
Unknown A[c]	2.1	12.5	ND	1.9	11.6	ND
Lipid A	34.2			38.2		

[a] ND, Not detected.

[b] tr, trace amount present.

[c] Unknown A was not 2-amino-2-deoxygalacturonic acid, since the latter compound elutes before glucosamine on an amino acid analyzer (37), whereas unknown A eluted after the hexosamines. In addition, it is unlikely that it was a 4-amino sugar, such as 4-aminoarabinose or 2,4-diamino-2,4,6-trideoxy-D-glucose (33), since these compounds are highly acid labile. Furthermore the latter compound has a 570/440 absorbance ratio of 2.1:1, whereas unknown A exhibited a 570/440 ratio of 4.8:1. A possible candidate could be 2,3-diamino-2,3-dideoxy-D-glucose (36), which is more acid stable and is found in the lipid A of certain *Pseudomonas* strains. In calculations of the concentrations of unknown A, we have assumed a molecular weight of 178 and a response factor equivalent to that of glucosamine.

the wild-type strain. Assuming that the side chains are terminated with normal core regions, then the molecular weight of the side chain can be calculated on the basis of the weight percentage of glucose or rhamnose, assuming four and one residues, respectively. Values of 38 and 50 kilodaltons (Kdal), respectively, were obtained for the side chains of the K799 and Z61 LPS.

The low quantitative recovery of the side-chain material after mild acetic acid hydrolysis suggested either incomplete release of side chain constituents during hydrolysis or the presence of acid-labile compounds. In addition, this material

could have contained other residues, such as acetyl groups, which were not assayed for. The fact that the actual response factor of unknown A in ninhydrin was not known may also have introduced a sizable error in quantitation.

Polyacrylamide gel electrophoresis. LPS from *P. aeruginosa* PAO1 has been shown to be heterogeneous by SDS-polyacrylamide gel electrophoresis (D. Ho, unpublished data). LPS from this bacterium can be resolved into three periodate-Schiff-reactive fractions, one of which corresponds to lipid A-core oligosaccharide (R-form) LPS (3, 4). Unfortunately, the periodate-Schiff procedure is particularly insensitive as a stain for *Pseudomonas* LPS (5), perhaps due to the low concentrations of vicinal hydroxyls in the side-chain polysaccharides. The recent observation (Kuzio et al., submitted for publication) that PAO1 LPS can be stained on gels with Coomassie brilliant blue R250 after appropriate pretreatment prompted us to apply this technique to K799 and Z61 LPS. The results (Fig. 3) indicated clearly that LPS from these strains, like that of strain PAO1, was microheterogeneous. For both K799 and Z61 LPS, we observed a series of discrete bands with an interband distance of 1.6 Kdal. In both LPS samples, certain size fractions, namely, those at approximately 55 and 82 Kdal, showed increased staining intensity with minor bands at 66 and 72 Kdal (we have used the molecular weights of standard proteins as our reference points for identifying bands in Fig. 3; this is not meant to imply that proteins can be used to estimate the molecular weights of saccharides). We have observed that side-chain polysaccharides from *Pseudomonas*, unlike that of *Alteromonas* (5), entered the gels. Again, no major differences were observed between side chains of strains K799 and Z61. The average molecular size of the side-chain polysaccharide bands appeared to be approximately 5 Kdal less than that of the whole LPS.

In addition to the LPS bands and corresponding side-chain polysaccharide bands noted above, K799 and Z61 LPS exhibited bands at 13.5 Kdal, although the intensity of the band from the mutant was reduced. Immediately below this was a turbid area (hatched area in Fig. 3) with estimated average molecular sizes of 11.5 (K799) and 10.5 (Z61) Kdal. Based on the mobility of these two turbid areas, we feel that it was these bands which were stained by the Schiff staining procedure used in the preceding paper (1). The turbid band probably represented the R-form LPS, since the different mobilities of this band with K799 and Z61 LPS reflected both the chemical (Table 2) and chromatographic (Fig. 2) properties of the rough cores. Gels of Z61 LPS also had a pink-staining band running just behind the tracking dye. The nature of this material is unknown, but it migrated faster than lipid A-core oligosaccharide from AK1012 (21a; Kuzio et al., submitted for publication); a strain with a more defective core than Z61. The only other major difference was the appearance of an intense band in the wild-type strain running ahead of the putative R-form LPS.

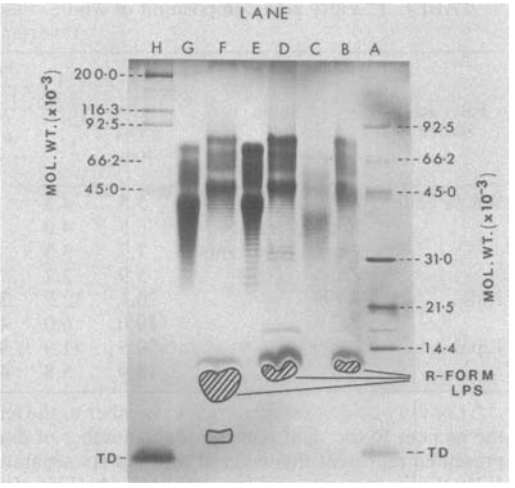

FIG. 3. A 10 to 20% (wt/vol) linear polyacrylamide gradient gel with PAO1 (lane B), K799 (lane F), and Z61 (lane D) LPS. The O-antigenic side chains for these strains are in lanes C, G, and E, respectively. Molecular weight markers (Bio-Rad Laboratories; lanes A and H) are lysozyme (14,400), soybean trypsin inhibitor (21,000), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200), phosphorylase B (92,500), β-galactosidase (116,250), and myosin (200,000). Pronin Y was used as the tracking dye (TD).

DISCUSSION

In the preceding paper (1), we obtained indications that the major biochemical alterations in the antibiotic-supersusceptible mutant strain Z61 compared with its parent strain K799 were probably associated with the LPS. In this study, we have confirmed by chemical analysis that the LPS of strain Z61 has at least one and possibly two alterations. Furthermore, our data, taken overall, favor the hypothesis that it is the different LPS composition of the mutant strain Z61 which gives rise to its antibiotic-supersusceptible phenotype and higher outer membrane permeability (1).

Both the parental strain K799 and the mutant Z61 appeared smooth on the basis of lack of agglutination in acriflavine (25), extraction of the LPS by the phenol-water procedure of Westphal and Jann (32), susceptibility to smooth-specific and resistance to rough-specific phages (1), and

the presence of side-chain polysaccharides. The O-antigenic side chain was unusually rich in amino sugars, two of which were identified by TLC and amino acid analysis as fucosamine (2-amino-2,6-dideoxygalactose) and quinovosamine (2-amino-2,6-dideoxyglucose). In addition, an unknown basic amino compound was also detected in these samples. The occurrence of such compounds in the side-chain region of *P. aeruginosa* LPS has been noted by several workers (3, 8, 10, 35).

In addition to the high levels of the above-mentioned hexosamines, we have also observed in both K799 and Z61 side chains low levels of core components in the same molar ratios as in the core oligosaccharide of K799. These core components include glucose, rhamnose, galactosamine, alanine, heptose, and phosphorus (Table 2), all of which have been found in the analysis of core regions from other *P. aeruginosa* strains (8, 33, 35; Kropinski, unpublished data). The glucose/rhamnose/galactosamine ratio (4:1:1) was also consistent with most previous studies, with some exceptions (23; D. Horton, personal communication). In the side-chain fraction, the absence of glucosamine and glucosamine-phosphate strongly suggested that the presence of core components was not due to undegraded LPS (lipid A) in our preparations. We therefore suggest that this is the first clear-cut evidence that the side chains of *Pseudomonas* strains are terminated with core oligosaccharide units.

The mutant strain, Z61, contained a side chain-linked polysaccharide which was chemically identical to that of the parent. Of particular interest was the existence of glucose and rhamnose in the molar ratio of 4:1. The core oligosaccharide (fraction IIB) from strain Z61, however, possessed a glucose/rhamnose ratio of approximately 10:1. Also noted with both strains was the existence of core heterogeneity, and it is possible that the presence of low amounts of rhamnose in Z61 fraction IIB was due to cross-contamination of the latter with material from fraction IIA. This fraction may represent a complete core containing one side-chain subunit or an alternate core structure. The simplest interpretation based on these analyses is that the antibiotic-supersusceptible mutant Z61 is a leaky rough mutant. It may be that when a complete R-form LPS molecule is synthesized as a result of the leakiness of the mutation in strain Z61, it is capped at a high frequency with side-chain oligosaccharide units. These results are consistent with the qualitative results obtained for strain K799, mutant Z61, and its revertants (Fig. 1).

The lipid A contained the five fatty acids which have been shown to be commonly found in the LPS from *P. aeruginosa* strains: 3-hydroxydecanoate, dodecanoate, 2- and 3-hydroxydodecanoate, and hexadecanoate. One of the largest differences between these two strains was the change in the fatty acid content. Although no alterations had occurred in the overall fatty acid concentration, the levels of several of the fatty acids were modified. Most noticeable were the decreased levels of dodecanoate and hexadecanoate, which were also noted in whole-cell fatty acid analyses of mutant Z61 (Table 1), and the increased amounts of 2-hydroxydodecanoic acid. The presence of a further difference in the lipid A was also observed; that is, the appearance of a brownish coloration when Z61 LPS was hydrolyzed under a variety of conditions, including dilute acetic acid, but not after hydrolysis of K799 LPS. Unfortunately, we could obtain no other evidence for the presence of a compound unique to Z61 LPS by TLC, GLC, amino acid analyses, or infrared spectra. Indeed, the comparison of the infrared spectra of the lipid A molecules of mutant strain Z61 and wild-type K799 suggested that the Z61 LPS was in fact missing a component present in K799 LPS. Possibly the lack of a chemical modification of a component of the lipid A of strain Z61 may make this component acid labile, resulting in the observed brown color on acid treatment. It is tempting to speculate that such an alteration or the fatty acid changes are primarily responsible for the observed changes in phenotype, in particular the changes in susceptibility to antibiotics, outer membrane permeability, and leaky rough character of the Z61 LPS.

In the accompanying paper (1), evidence is provided that the antibiotic supersusceptibility of mutant Z61 is caused by an enhancement in outer membrane permeability which is proposed to be due to an increase in the number of open functional porin pores in Z61 compared with K799. Furthermore, evidence is provided that argues against a porin protein F alteration. In this paper, we have shown by qualitative sugar and whole-cell fatty acid analyses that the mutant Z61 and partial revertants have LPS alterations, the magnitude of which correlates with the changes in susceptibility to 12 antibiotics (1) and outer membrane permeability (1). In contrast, spontaneous full revertants have LPS neutral sugars and fatty acids, antibiotic susceptibilities (1), and outer membrane permeabilities (1) indistinguishable from those of the wild-type K799. Therefore, we postulate that the state of the LPS in *P. aeruginosa* can directly influence the number of open functional pores (and hence antibiotic susceptibility and outer membrane permeability). Other studies suggest that purified porin protein F is usually associated with LPS (13) and that the removal of LPS from

protein F renders this porin inactive in black lipid bilayer permeability assays (Hancock, unpublished data).

We have provided two pieces of evidence in the accompanying paper (1) which support the idea of the ability of LPS to interact with outer membrane proteins and thus alter the surface properties of the cell. First, changes in phage susceptibility occurred in the LPS-altered mutant Z61 for phages which had surface receptors other than LPS. The LPS may therefore interact with porin and other proteins in such a way as to determine their orientation or surface exposure in the outer membrane. A similar conclusion was made from an extensive study of phage resistant, LPS-altered mutants of *E. coli* (16). Second, the LPS of the mutant Z61 reacted differently with the lipoprotein I as seen on polyacrylamide gels. An interaction of LPS with porin and a modulation of the conformation of porin by LPS has been demonstrated in *E. coli* by Yamada and Mizushima, using X-ray diffraction as a tool (39). Both the lipid and polysaccharide regions of LPS were thought to interact with the protein to determine the lattice conformation. From the results presented here, we suggest that LPS influences the conformation and hence the function of porin in the outer membrane of *P. aeruginosa*, perhaps ionically through charged phosphate groups or via hydrophobic interactions with acyl chains on lipid A residues, or both.

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