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Heterogeneity of Lipopolysaccharides from *Pseudomonas aeruginosa*: Analysis of Lipopolysaccharide Chain Length

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Lipopolysaccharide (LPS) from smooth strains of *Pseudomonas aeruginosa* 503, PAZ1, PAO1715, PAO1716, and Z61 was fractionated by gel filtration chromatography. LPS samples from the first four strains, all PAO1 derivatives, separated into three major size populations, whereas LPS from strain Z61, a Pac K799/WT mutant strain, separated into two size populations. When column fractions were applied to sodium dodecyl sulfate-polyacrylamide gels in their order of elution, molecules of decreasing size were resolved, and the ladder of molecules with different-length O antigens formed a diagonal across the gel. The LPS from the PAO1 derivatives contained two distinct sets of bands, distinguished on the gels as two sets of diagonals. The set of bands with the faster mobility, the B bands, was found in column fractions comprising the three major amino sugar-containing peaks. In the sample from strain 503, a fourth minor peak which contained B bands was resolved. The slower-moving set of bands, the A bands, were recovered in a minor peak. LPS from strain Z61 contained only one set of bands, with the higher-molecular-weight molecules eluting from the column in a volume similar to that of the B bands of the PAO1 strains. Analysis of the fractions of LPS from all strains indicated that less than 8% of the LPS molecules had a long, attached O antigen. Analysis of the peak that contained mainly A bands indicated a lack of reactive amino sugar and phosphate, although heptose and 2-keto-3-deoxyoctulosonic acid were detected. Reaction of isolated fractions with monoclonal antibody specific for the PAO1 O-antigen side chain indicated that only the B bands from the PAO1 strains were antigenically reactive. The bands from strain Z61 showed no reactivity. The data suggest that the A and B bands from the PAO1 strains are antigenically distinct. We propose that PAO1 strains synthesize two types of molecules that are antigenically different.

Lipopolysaccharide (LPS), a major component of the outer membranes of gram-negative bacteria, is important in the structure (33, 37) and function (33, 36) of this membrane. Structural microheterogeneity in several regions of LPS molecules from members of the family *Enterobacteriaceae* (2, 14, 22, 37, 38, 48, 51) and *Pseudomonas aeruginosa* strains (33, 57) has been demonstrated. Of the several methods used to separate the subclasses of LPS from individual strains, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20, 24, 45, 48) and gel filtration (9, 26, 30, 32, 34, 48) are the best. Either of these two methods by themselves, however, may be insufficient to completely characterize the high- and low-molecular-weight fractions of LPS. Peterson and McGroarty (48) demonstrated that the SDS-PAGE of the column fractions of samples from *Salmonella typhimurium*, *Salmonella minnesota*, and *Escherichia coli* was instrumental in characterizing the various-sized fractions. Analysis of the isolated fractions allowed for the estimation of the average number of O-antigen repeat units per LPS from each of the size fractions.

Compositional analysis of LPS from *P. aeruginosa* has indicated that the LPS molecules are structurally similar to enterobacterial LPS molecules, but possess several distinctive features (33, 57). The most outstanding differences include the unusually high phosphate content (33, 58), the presence of L-alanine in the core (33, 57), and the high levels of amino sugars and uronic acids in the O side chain (8, 33). The characterization of *P. aeruginosa* O-specific polysaccharides has been complicated in some cases by chemical heterogeneity of the polysaccharide chains (8, 9, 30, 58). In some instances, the polymeric material has been resolved into amino sugar-rich and neutral sugar-rich fractions (30, 58). The biological significance of such fractions is unclear, but a possible explanation is that *P. aeruginosa* strains produce multiple types of molecules with chemically distinct polysaccharide chains.

In the work described in this paper, we have analyzed the size heterogeneity of LPS isolates from several *P. aeruginosa* strains by both gel filtration and SDS-PAGE. These studies have revealed that LPS isolates from PAO1 strains contain two distinct sets of bands, suggesting that PAO1 strains are capable of synthesizing more than one type of chemically and antigenically distinct molecule. We also present evidence that the fraction of core oligosaccharides carrying the O-specific polymer is less than 8%.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* Z61 is a mutant derived from strain Pae K799/wt selected for antibiotic supersusceptibility, and PAZ1 (*met-28 trp-6 lysA12 his-4 ile-226 absA*) is a PAO222 derivative into which the *absA* mutant gene from Z61 encoding antibiotic sensitivity has been conjugated (1a). Strains PAO1716 (*ade-136 leu-8 rif-1*) (revertant) and PAO1715 (*ade-136 leu-8 rif-1 tolA12*) (an aminoglycoside-supersensitive mutant) were described previously (40). Strain PAO503 (*met-9011*) is a methionine auxotroph of *P. aeruginosa* PAO1. Strains PAZ1, 1715, 1716, and 503 are serotype O5. *Escherichia coli* D21 and D21f2 are derived

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from strain K-12 and were characterized as Ra and Re chemotypes, respectively (3).

Growth media. Strains PAO1716, PAO1715, Z61, and PAZ1 were grown at 37°C to mid-logarithmic phase in a 100-liter fermentor containing 80 liters of proteose peptone no. 2 (Difco) medium from a 1-liter overnight culture grown in the same medium. Strain 503 was grown as previously described (4).

E. coli D21 and D21f2, grown as described by Coughlin et al. (10), were harvested in late log phase.

Isolation of LPS. LPS from *P. aeruginosa* Z61 and PAO1 derivatives 1715, 1716, PAZ1, and 503 were isolated by the method of Darveau and Hancock (12) and then subjected to two extractions in chloroform-methanol (1:1, vol/vol), resulting in recovery of approximately 80% of the total LPS. The LPS from *E. coli* D21 and D21f2 was isolated by the hot aqueous phenol (56) and the chloroform-petroleum ether (16) extraction procedures, respectively.

SDS-PAGE. SDS-PAGE gels were prepared and run by using the buffer system of Laemmli (35). Unless otherwise noted, separating gels were formed with 15% acrylamide–0.1% SDS, with a 7.5% acrylamide stacking gel. Samples were mixed 1:1 with sample buffer (containing 4% SDS) and applied to the gel. Electrophoresis was performed with a constant current of 15 mA per gel until the tracking dye entered the separating gel and then at 30 mA per gel until the tracking dye reached the bottom of the gel. LPS bands were detected by the silver staining method of Dubray and Bezard (15).

Column chromatography. Samples were fractionated on a Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) column (64 cm by 25 mm) at room temperature by using the column buffer system of Peterson and McGroarty (48). Approximately 30 mg of LPS was applied to the column, and 5-ml fractions were collected at a flow rate of 8 ml/h. To remove detergent and buffer, pooled fractions were extensively dialyzed (12,000- to 14,000-molecular-weight cutoff membranes) against column buffer without deoxycholate at 37°C and then against distilled water at 4°C. The dialyzed fractions were lyophilized and suspended to a concentration of 10 mg/ml in water. All fractionations were done at least twice.

Western blots. Western immunoblots of SDS-polyacrylamide gels were prepared as previously described (5, 54). The gels were electrotransferred with a model TE Transphor Electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) at a constant current of 150 mA for 18 h by using the electrode buffer described by Otten et al. (44) unless otherwise noted. The nitrocellulose blots were visualized as described previously (44) with monoclonal anti-503 antibody (titer, ca. 1:100,000 [19]) diluted 1:10,000 in blocking solution. In addition, dot blots were performed by applying known quantities of LPS isolates directly on nitrocellulose. The blots were washed and visualized by using horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G antibody (Sigma Chemical Co., St. Louis, Mo.) as described above or a silver stain protocol identical to that described by Dubray and Bezard (15).

Assays. Assays for amino sugars (17), heptose (59), and 2-keto-3-deoxyoctulosonic acid (KDO) (28) were performed as described previously except when noted. Phosphate analyses were performed by using either a colorimetric assay (1) or inductively coupled plasma emission spectroscopy (10). Protein concentrations were estimated by the Pierce BCA protein assay (Pierce Chemical Co., Rockford, Ill.) with bovine serum albumin as the standard.

RESULTS

Silver staining of LPS from strains of *P. aeruginosa* separated by SDS-PAGE revealed a progressive ladderlike pattern of bands up the gel (Fig. 1A). For members of the family *Enterobacteriaceae* and *P. aeruginosa* strains, these bands have been reported to represent LPS molecules containing increasing lengths of O antigen (4, 20, 45). The intensity of staining indicated three to four regions of bands representing as many as four populations of molecules (Fig. 1A, band sets 1, 2, 2a, and 3) differing in O-antigen length. The electrophoretic pattern of LPS from strain 503 showed a set of bands (set 2a) which were closely spaced and slower-migrating bands immediately above the 2a set which had wider spacing (Fig. 1A, lane 2). In the banding pattern of LPS from PAO1 derivatives 1715, 1716, and PAZ1 (lanes 1, 3, and 5, respectively), we observed irregularities in the spacing and intensities of bands up the gel. In contrast, LPS from strain Z61 appeared to have a regular spacing and intensity in the banding pattern (lane 4). The average length of the highest-molecular-weight LPS of strains Z61 and PAZ1 seemed shorter than that of the other PAO1 derivatives: a phenomenon observed previously and ascribed to the *absA* (antibiotic supersusceptibility) mutant locus (1a). When small amounts of LPS were applied to the gel, only the fastest-migrating bands were stained and there was no difference in migration patterns of LPS of strains 1715, 503, 1716, and PAZ1 (lanes 8, 9, 10, and 12, respectively). On the other hand, the low-molecular-weight bands from strain Z61 migrated faster than those of the other *P. aeruginosa* strains (lane 11) as previously observed (34), owing to an apparent truncation in the rough core of the short-chain LPS molecules in this strain. LPS from *E. coli* D21 (Ra chemotype) and D21f2 (Re chemotype) was used to compare and characterize the electrophoretic mobilities of the short-chain populations.

Using antibodies specific to the PAO1 O antigen, we analyzed Western blots of the LPS separated by SDS-PAGE

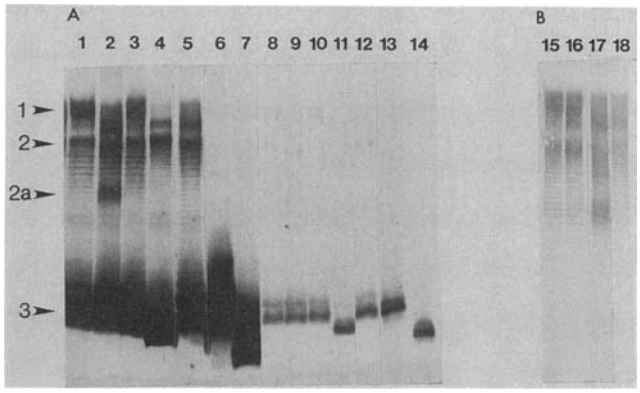

FIG. 1. (A) Silver-stained SDS-polyacrylamide gel of LPS from *P. aeruginosa* 1715 (lanes 1 and 8), 503 (lanes 2 and 9), 1716 (lanes 3 and 10), Z61 (lanes 4 and 11), and PAZ1 (lanes 5 and 12) and from *E. coli* D21 (Ra, lanes 6 and 13) and D21f2 (Re, lanes 7 and 14). Samples of either 5 μg (lanes 1 to 7) or 0.1 μg (lanes 8 to 14) were applied to a 15% acrylamide gel which had been polymerized overnight with a butanol overlay. Arrows indicate the four intensively stained regions of the *P. aeruginosa* samples: band sets 1, 2, 2a, and 3. (B) Western blots of LPS from *P. aeruginosa* 1715 (lane 15), 1716 (lane 16), 503 (lane 17), and PAZ1 (lane 18) reacted with monoclonal anti-503 antibody. Samples of 2.5 μg were applied to a 12% acrylamide gel which had been polymerized overnight with a butanol overlay. The gel was blotted as described in Materials and Methods.

to help clarify the irregularities in the banding pattern. The blots of LPS isolated from *P. aeruginosa* 1715, 1716, 503, and PAZ1 revealed a ladder pattern of molecules that consisted of doublet bands (Fig. 1B). Furthermore, the level of one of the bands in the doublet was lower in the isolates from strains 1715, 1716, and PAZ1 than in the isolate from strain 503. Presumably this reflects a difference in substoichiometric modification within the core-lipid A region of the molecules. Interestingly, the spacing and intensity of the ladder pattern seen in the Western blot were much more regular than those in the silver-stained gel. This suggested that the stained sample may contain bands superimposed on the ladder pattern of the main antigen.

If the irregular silver-stained banding pattern was a result of heterogeneity in the LPS samples, this heterogeneity could have been due to contamination of the culture or to true heterogeneity of the sample. The possibility that the cultures were contaminated is very low, since after growth, all cultures were streaked out onto protease peptone no. 2 agar plates for observation of characteristic colony morphologies and pigmentation, and Z61, PAZ1, and PAO1715 cultures were tested for characteristic antibiotic supersusceptibilities. Furthermore, the irregular banding patterns were seen in samples from PAO1 derivatives (Fig. 1A) isolated in two different laboratories, as well as in several independently isolated LPS samples.

To further characterize the heterogeneity of the LPS isolates from the PAO1 strains, we separated the samples on a Sephadex G-200 column. The elution profile showed three major amino sugar-containing peaks for strains 503 (Fig. 2), 1715 (Fig. 3), 1716, and PAZ1 (results not shown). In contrast, the elution profile of the LPS sample from strain Z61 showed only two major peaks (Fig. 4). Both gel permeation chromatography and SDS-PAGE separate molecules on the basis of size; therefore, a diagonal banding pattern should be expected across SDS-PAGE gels of column fractions when applied in the order of elution. SDS-PAGE of the column fractions of samples from each of the PAO1 derivatives studied revealed two distinct ladder patterns of apparently different sizes, the A bands (later-eluting ladder) and the B bands (earlier-eluting ladder) (see Fig. 2 and 3 for elution profiles of strains 503 and 1715, respectively). In contrast, the LPS isolate from strain Z61 showed only one ladder set (Fig. 4). Peterson and McGroarty (48) reported that the LPS from *Salmonella* species separated in this type of column as molecules of decreasing size. The presence of two distinct ladder patterns suggests that either stable aggregates were present (48) or there were two types of molecules

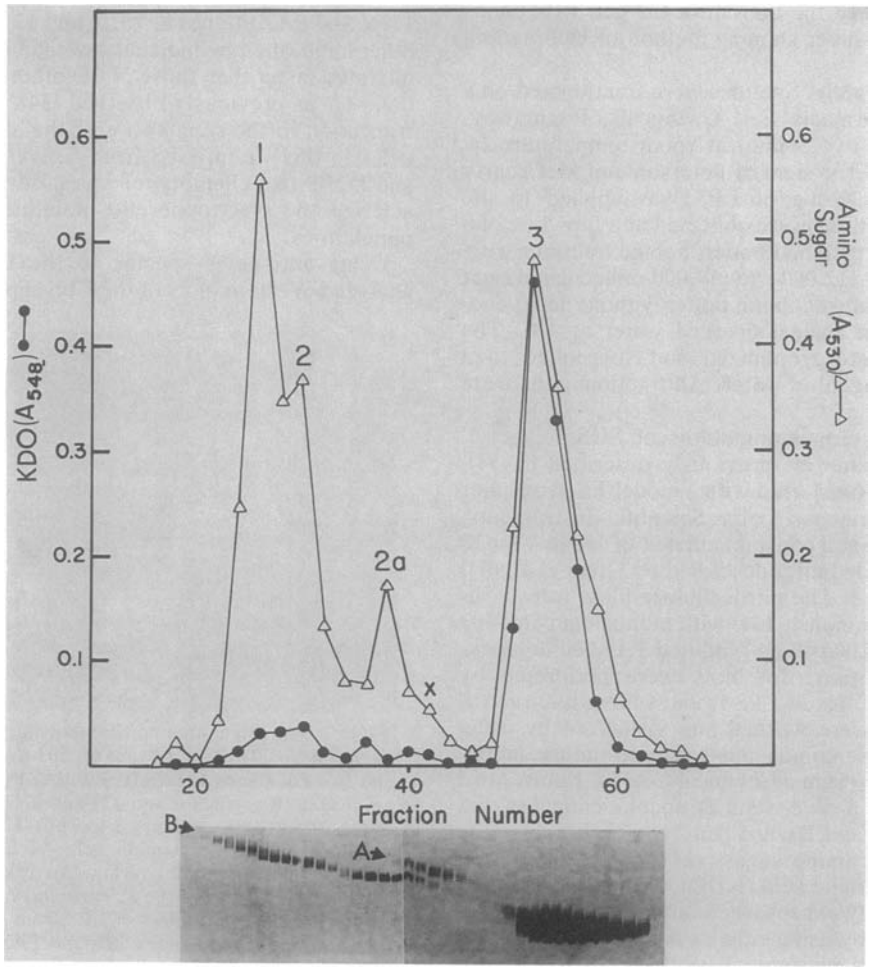

FIG. 2. Fractionation of LPS from *P. aeruginosa* 503 on Sephadex G-200. Fractions were analyzed for KDO (●) and amino sugar (△). Silver-stained SDS-polyacrylamide gels of column fractions are aligned under their appropriate fraction number. A represents the slow-moving set of bands, and B represents the faster-moving set.

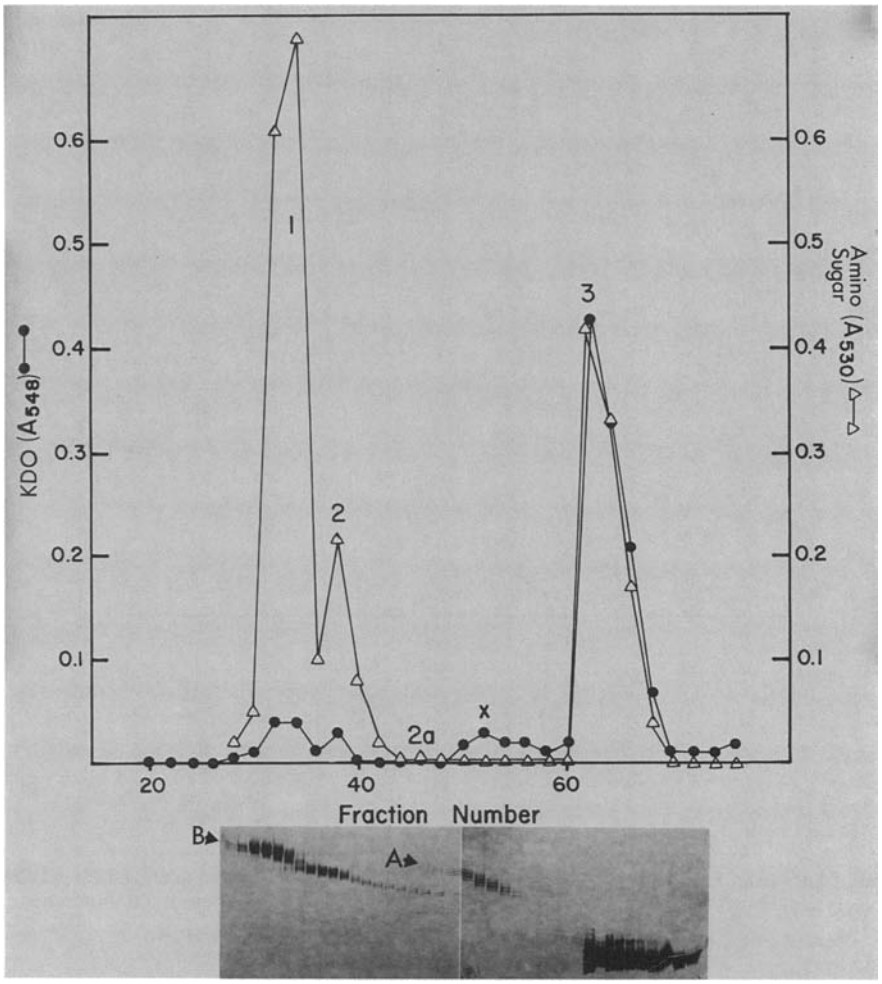

FIG. 3. Fractionation of LPS from *P. aeruginosa* 1715 on Sephadex G-200. Fractions were analyzed for KDO (●) and amino sugar (△). Silver-stained SDS-polyacrylamide gels of column fractions are aligned under their appropriate fraction number. A represents the slow-moving set of bands, and B represents the faster-moving set.

with different charges; electrophoresis separates molecules on the basis of both size and charge. To eliminate the possibility that the A bands were aggregates of the B bands stabilized by hydrogen bonding between O polymers, SDS-PAGE of the column fractions of samples from strain 503 was run in the presence of 4 M urea (final concentration). The same two sets of bands were observed in 4 M urea–SDS gels (results not shown). In addition, two-dimensional electrophoresis of column fractions of LPS from strains 503 and 1716, which contained approximately equal amounts of A and B bands, showed that both the A and B bands ran with the same mobilities in the second dimension. Since the gels in both dimensions consisted of standard SDS-gels and the gel strip from the first dimension was heated before the second dimension was run, the results indicate that the bands do not interconvert (results not shown). This again indicates that one set of bands is not an aggregate or different conformational state of the other. Amino sugar analysis of our column fractions indicated that the fractions containing mainly A bands (Fig. 3, peak X) lacked reactive amino sugars, whereas the other fractions (Fig. 2 and 3, peaks 1, 2, 2a, and 3) showed reactivity.

The individual fractions from the elution profile of each of the LPS samples separated in a Sephadex G-200 column were also analyzed for KDO and phosphate content, and the relative molar ratios of amino sugar to KDO and amino sugar to phosphate were determined for the three or four major peaks. Also, the fractions corresponding to each of the major peaks in the elution profile of strains 1715 and PAZ1 were pooled, dialyzed, lyophilized, and suspended in water to a final concentration of 10 mg/ml and analyzed as described above. The data shown in Table 1 for the pooled fractions and data from the individual fractions (not shown) indicated that with phosphate as an indicator of molar amounts of the LPS, the short-chain peak 3 sample represented 92 to 97% of the total LPS molecules, peak 2a represented 0.4 to 2%, peak 2 represented 1 to 3%, and the very-long-chain fraction, peak 1, represented 3 to 4%. The fifth population of molecules, the A bands, were resolved as a separate population on the column (peak X) in the samples from strains 1715 (Fig. 3), 1716, and PAZ1, whereas A bands overlapped with peak 2a of the main ladder set in strain 503 fractionation (Fig. 2). As stated above, strain Z61 showed only two different LPS size populations: the short-chain fraction (peak 3 [97% of total]) and the long-chain fraction (peak 2 [2.8% of total]) (Fig. 4).

The O antigen of *P. aeruginosa* is reportedly rich in amino sugars (33, 34, 39, 57), although the reported sequence of the

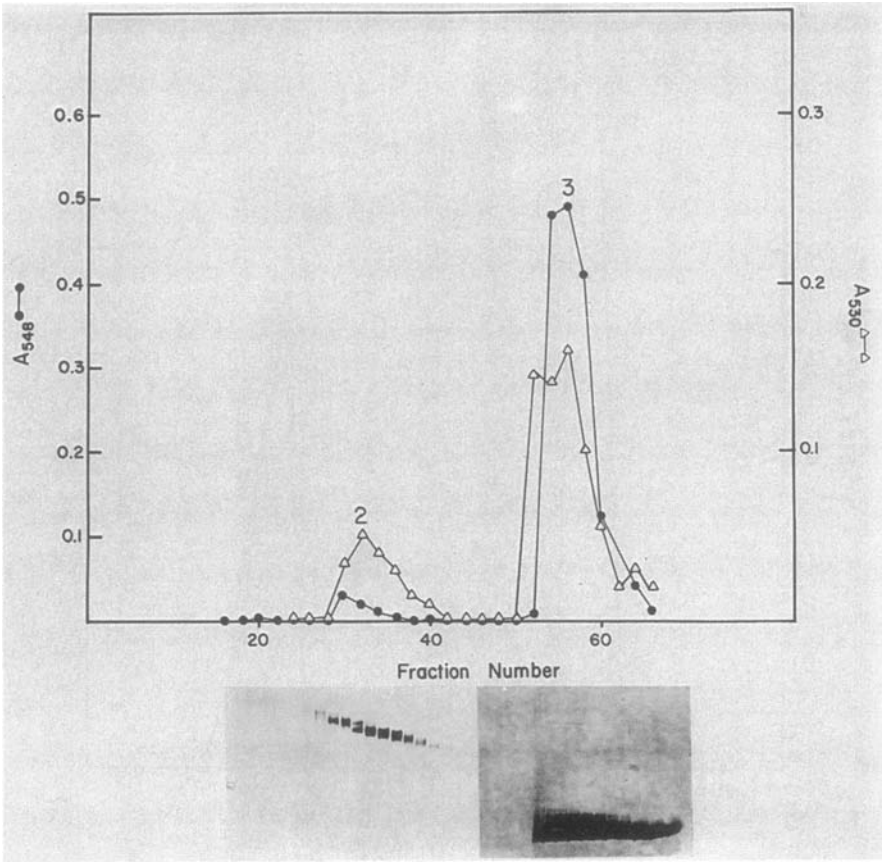

FIG. 4. Fractionation of LPS from *P. aeruginosa* Z61 on Sephadex G-200. Fractions were analyzed for KDO (●) and amino sugar (△). Silver-stained SDS-polyacrylamide gels of column fractions are aligned under their appropriate fraction number.

O5 serogroup (equivalent to Lanyi type 3a,d) indicates that most of the amino groups are acetylated (29). The phosphate and KDO assays measure residues in the core region of LPS (34), whereas the assay for amino sugars detects residues both in the core-lipid A region and O-antigen repeat region. The data reported in Table 1 show that the molar ratios of

TABLE 1. Chemical analysis of *P. aeruginosa* B-band LPS fractions

Peak no.	% P recovered[a]	Amino sugar/KDO[b]	Amino sugar/P[c]	P/KDO (mole/mole)
Strain Z61[d]				
2	2.8	5.5	9.3	ND[e]
3	97.0	1.0	1.0	5.5
Strain PAZ1[f]				
1	2.3	24.8	30.8	4.8
2	3.2	19.5	18.8	6.1
3	94.3	1.0	1.0	5.9
Strain 1715[f]				
1	3.4	29.3	31.4	5.4
2	1.4	17.0	18.2	5.4
3	95.1	1.0	1.0	6.0

[a] Percentage of total amounts of phosphate in each of the peaks.
[b] Relative molar ratio; KDO and amino sugar levels were normalized to a value of 1.0 for peak 3 samples.
[c] Relative molar ratios; phosphate levels were determined for the individual fractions of the Z61 sample by using the colorimetric assay and for the pooled fractions from samples of strains 1715 and PAZ1 by inductively coupled plasma emission spectroscopy, as described in Materials and Methods. Levels were normalized to a value of 1.0 for peak 3 samples.
[d] Individual fractions from the elution profile of the LPS samples separated on a Sephadex G-200 column, as described in Material and Methods, were analyzed and the amounts in all the fractions in each peak were added together.
[e] ND, Not determined.
[f] Pooled fractions corresponding to each of the peaks in the elution profile of LPS samples separated on a Sephadex G-200 column were dialyzed, lyophilized, and suspended in distilled water for analysis.

TABLE 2. Composition of the pooled column fractions of LPS from *P. aeruginosa* 1715 and PAZ1

Peak no.[a]	Wt (% total recovered)		KDO[b] (nmol/mg of LPS)		Phosphate[c] (nmol/mg of LPS)		Amino sugars[d] (A_{530}/mg per ml of sample)		Heptose[e] (nmol/mg of LPS)
	1715	PAZ1	1715	PAZ1	1715	PAZ1	1715	PAZ1	1715
1	23	14	28	31	150	150	4.2	3.4	39
2	10	15	26	31	140	190	2.3	2.6	34
3	56	55	292	272	1,740	1,600	1.5	1.2	272
X	11	15	2	3	7	9	0.6	0.2	83
Unfractionated LPS sample	NA[f]	NA	162	ND[g]	1,200	ND	2.7	ND	ND

[a] Column fractions of peaks 1, 2, 3, and X, along with an unfractionated sample of LPS, were pooled, dialyzed against column buffer without deoxycholate and then against distilled water, and then lyophilized and weighed. The samples were suspended in distilled water to a concentration of 10 mg/ml for analysis.
[b] KDO was assayed by using the standard procedure.
[c] Phosphate levels were determined by inductively coupled plasma emission spectroscopy, as described in Materials and Methods.
[d] A_{530} was corrected to that of a sample at 1 mg/ml (final concentration).
[e] D-Glycero-D-guloheptose was used for the standard curve.
[f] NA, Not applicable.
[g] ND, Not determined.

phosphate to KDO for the three major B-band-containing peaks of the pooled fractions are similar for strains PAZ1 and 1716. Also, this ratio for peak 3, calculated from the individual fractions for all strains, appeared to be relatively constant from strain to strain and was determined to be between 5.5 and 6.6 (mole per mole, data not shown). Since the level of phosphate or KDO can be used as a measure of the relative molar amount of LPS in each peak, comparison of the ratios of amino sugar to KDO and/or amino sugar to phosphate in the three major peaks of the pooled fractions should reflect the O-antigen length. For the individual fractions a discrepancy existed in these ratios, such that the values are lower than for the pooled fractions. This may be due to the elution buffer present in the fractions before dialysis. Since the pooled fractions were dialyzed to remove the detergent and salts and were suspended in H_2O to a known concentration, the pooled-fraction data were more reliable. Furthermore, the small amount of sample in the individual fractions decreased the sensitivity of the assays.

The chemical composition of the dialyzed A-band samples from strains 1715 and PAZ1 was analyzed, and the results were compared with those of the three major amino sugar-containing peaks (Table 2). The results indicated that peak X (A bands) made up 10 to 15% of the total LPS sample by weight. Furthermore, under the normal conditions of the assay, it appeared to have low levels of reactive KDO, amino sugars, and phosphate. When the pooled samples were hydrolyzed for a longer period (20 min) and at a higher acid concentration (0.5 N H_2SO_4), the KDO levels detected in the B-band fractions were similar to those in previous assays, but the KDO levels in the A-band fraction increased 10-fold. Thus, the A-band material appeared to contain KDO residues which are much less reactive than those in the B-band isolates. Analysis of the isolates from strain 1715 indicated that the A-band material had levels of heptose intermediate between those found in peaks 2 and 3 (Table 2). Analysis for protein in the pooled fractions indicated less than 1.5% (wt/wt) in all fractions. From the SDS-PAGE of the separated fractions (e.g., Fig. 3) and from the chemical characterization of the pooled samples, it was observed that the A bands of strains 1715 and PAZ1 were contaminated with only minor amounts of B-band material. The A-band material is probably a glycolipid, since the isolate, suspended in the absence of detergent at 10 mg/ml, was cloudy and not completely water soluble and since it contained heptose and KDO.

To better compare the migration patterns of equivalent LPS populations from different strains, comparable column fractions of LPS containing both A and B bands from the different strains were run together on SDS-PAGE (Fig. 5). The A bands from all four PAO1 strains had very similar spacings (lanes 2 to 5), and the spacings of the B bands of strains PAZ1, 1716, and 1715 (lanes 2, 4, and 5, respectively) appeared very similar. In contrast, the B bands of strain 503 (lane 3) appeared much more closely spaced than the same bands of the other strains owing to an increased amount of the second band in the B-band set of doublets (see also Fig. 1B, lane 17). Fractions from peak 3 of LPS from strains Z61, 503, PAZ1, 1715, and 1716 were run on an SDS–18% acrylamide gel. No differences were seen in band mobilities of the short-chain isolates except for that from strain Z61, which migrated faster (results not shown), corroborating the results with unfractionated LPS (Fig. 1A, lanes 8 to 12).

To determine the antigenic reactivity of the A and B bands, the LPS fractions containing approximately equal amounts of A and B bands were subjected to Western

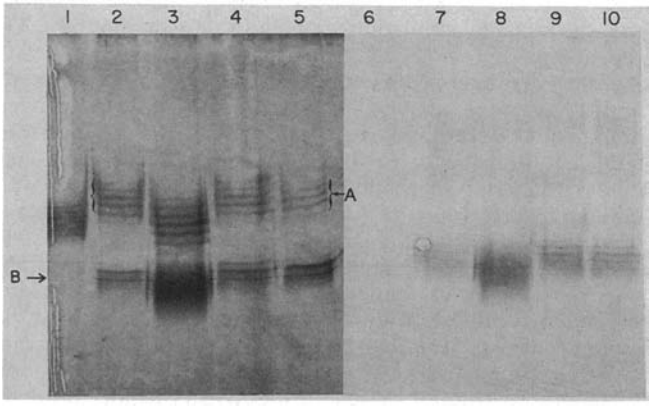

FIG. 5. Silver-stained SDS-PAGE (11% acrylamide) (lanes 1 to 5) and Western blots (lanes 6 to 10) of LPS fractions from *P. aeruginosa* reacted with monoclonal anti-503 LPS antibody. Lanes: 1 and 6, LPS from strain Z61 (fraction 36); 2 and 7, strain PAZ1 (fraction 44); 3 and 8, strain 503 (fraction 40); 4 and 9, strain 1716 (fraction 42); 5 and 10, strain 1715 (fraction 48). Samples of 100 μl were applied to the SDS-polyacrylamide gel, which had been polymerized overnight with a butanol overlay. The gel was blotted as described in Materials and Methods. Arrows indicate the A and B bands of the respective fractions.

immunoblotting. Figure 5 (lanes 6 to 10) shows a Western blot of these LPS fractions from strains PAZ1, 503, 1716, and 1715 and a fraction of Z61 whose LPS had an electrophoretic mobility similar to that of the A bands of the other four samples. Reaction with monoclonal anti-503 LPS antibody (specific for the O-antigen side chain) indicated that only the B bands, and not the A bands, were antigenically reactive. There was a weak antigenic reactivity of closely spaced bands above the region corresponding to A bands, which presumably was due to reaction with aggregated B band-type LPS (Fig. 5, lanes 7 to 10). Peterson and McGroarty (48) have shown that the high-molecular-weight fractions of LPS from *E. coli* can migrate as multimers. As expected, the bands from strain Z61 also showed no reactivity with the antibody, since it is known that the LPS from strain Z61 is antigenically distinct from that of PAO1 derivatives (1a).

To show that the lack of immunoreactivity of the A bands was not due to lack of transfer or recovery of samples on the nitrocellulose, Western blots were performed under different conditions. We used 150 and 400 mA and also transferred for 24 h. The gels were stained for LPS with and without transblotting to determine the level of electrotransfer of the different bands. The results indicated that, at the lower voltage, A bands did not transfer as well as B bands, consistent with their mobility on the gel. However, at the higher transblotting voltage, at which the A-band type of molecules were removed from the gel, no immunoreactivity was detected on the nitrocellulose (data not shown).

We also performed a dot blot immunoassay with the pooled column fractions to characterize differences in the antigenic reactivity of the two types of molecules. The four pooled fractions of LPS from strain 1715 (peaks 1, 2, 3, and X) were dialyzed, lyophilized, and suspended in water. A 10-μg portion of each of the fractions was spotted along the top of a nitrocellulose strip. Subsequent rows were spotted with the same volume of sample serially diluted 10-fold in each row. The spots were dried and visualized with the anti-503 monoclonal antibody as described for the Western blots. The results (Fig. 6A) indicated that the very-long-chain and long-chain populations of B bands had similar reactivity, and as little as 1 ng could be detected. In contrast, the short-chain population, containing no or only one O-repeat unit per molecule, showed no reactivity. Interestingly, the A-band sample showed weak reactivity when 10 and 1 μg were applied, but no reactivity with smaller amounts. The reactivity seen with 10 μg of the A bands was similar to that seen with 10 ng of long-chain and very-long-chain fractions, suggesting that the A-band fraction was contaminated with <1% (wt/wt) of B-band-type molecules. That the A-band fraction remained bound to the nitrocellulose was shown by staining the dot blot with the silver stain used for the polyacrylamide gels. We found that the intensity of staining indicated the approximate molar amount of material applied (Fig. 6B). The levels detected were not changed if the blots were washed extensively with buffer or the blocking solutions used in the Western blots prior to staining (data not shown).

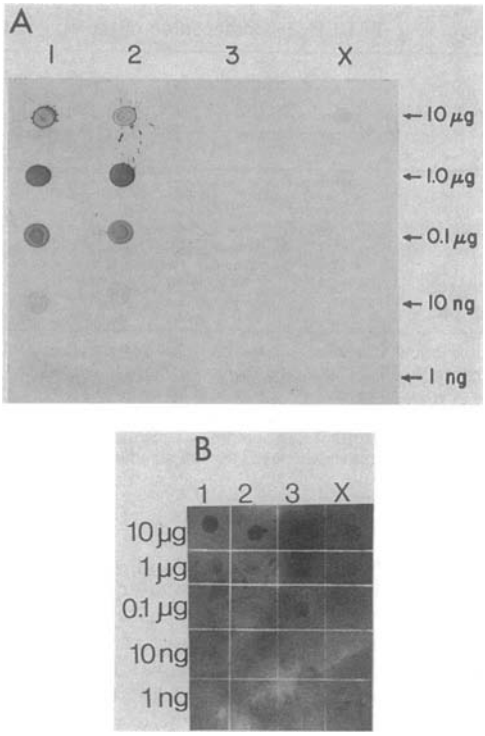

FIG. 6. Dot blots of pooled column fractions from *P. aeruginosa* 1715 LPS. Peaks 1, 2, 3, and X were separated on Sephadex G-200, dialyzed, and lyophilized as described in Materials and Methods and suspended in distilled water to a final concentration of 10 mg/ml. In the top row, 10 μg of each sample was applied on the nitrocellulose strip, and subsequent rows were spotted with an equal volume of 10-fold serially diluted samples. The sample spots were dried, and the nitrocellulose was either reacted with monoclonal anti-503 antibody and developed as described in Materials and Methods (A) or washed with 10 mM Tris–150 mM NaCl (pH 7.0) and reacted with the silver stain as indicated in Materials and Methods for polyacrylamide gels (B).

DISCUSSION

The LPS isolates of *P. aeruginosa* 503, PAZ1, 1715, 1716, and Z61 were separated by SDS-PAGE into as many as four major size populations. The ladderlike banding patterns represent molecules with increasing numbers of O-antigen repeat units (20, 24, 45). Other investigators have reported similar heterogeneity in the LPS from strains 503 and Z61, as well as other *P. aeruginosa* smooth strains (4, 24, 33, 34). An O-antigen ladder pattern has recently been described for several smooth strains of *P. aeruginosa* as a result of using silver staining and immunoblotting techniques (53). Our Western blot of unfractionated LPS (Fig. 1B) revealed a ladderlike banding pattern with regular spacing. The bands were resolved as doublets (Fig. 1B, lanes 15 to 18), suggesting substoichiometric modification in the core-lipid A similar to that seen with *Salmonella* LPS (42, 55). The irregular spacing that we observed in the SDS-PAGE silver-stained ladder pattern of LPS from strains 503, PAZ1, 1715, and 1716 (Fig. 1A) suggested the possibility that PAO1 derivatives may be producing LPS with more than one type of O polymer.

Peterson and McGroarty (48), using strains of the family *Enterobacteriaceae*, have shown that LPS molecules of different sizes can be partially separated on Sephadex G-200 in the presence of deoxycholate and EDTA. Two or three major populations of LPS could be resolved as detected by sugar analysis. These populations represent sets of molecules with O antigens of different lengths which are made in large amounts. Other investigators have also demonstrated, using gel permeation chromatography in combination with other methods, that the LPS from members of the family *Enterobacteriaceae* and other gram-negative bacteria could

be resolved into at least two main populations of LPS, differing in the length of their O-polysaccharide chain (9, 26, 30, 32, 34). In the results presented here, we found that the LPS from strains 503 and 1715 (Fig. 2 and 3, respectively), as well as strains 1716 and PAZ1, separated into three major populations (peaks 1, 2, and 3) and two minor populations (peaks 2a and X). Interestingly, the short-chain population made up more than 90% of the total sample on a molar basis (Table 1). In addition, there were larger amounts of the very-long-chain population from the PAO1 strains than of the long- and intermediate-chain populations. These results agree with those of Wilkinson (57) and Hancock et al. (23), who estimated the mole percent of S-form LPS to be between 0.2 and 14%. On the other hand, members of the family *Enterobacteriaceae* show a distribution of 44 to 60% of the LPS molecules in the low-molecular-weight population and 30 to 50% in the high-molecular-weight fractions (36, 48). It has been demonstrated that *S. typhimurium* synthesizes LPS molecules with over 80 O-antigen repeating units and that this population constitutes about 6% of the total LPS sample (48). Since the hydrophilic O-polysaccharides extend from the bacterial surface into the aqueous environment, the observed heterogeneity of O-chain lengths suggests that the surface topography of the gram-negative bacteria is irregular and that accessibility of the lipid A head group of the LPS could vary in different regions on the surface and on different bacterial species. It has been shown that the presence of O-antigen-containing LPS influences various cell surface phenomena, including antibiotic binding to LPS (47), antibiotic susceptibility (1a, 4, 18), LPS aggregate structure (47), bacteriophage recognition (25, 27), immunochemical characterization (8, 9, 49), virulence (11, 50), protection against the bactericidal action of serum (21, 41), polyclonal B cell activation, and macrophage cytotoxicity (43). The low level of LPS on *P. aeruginosa* that contains a long O polymer, however, may be sufficient to form a uniform cover over the cell, since the surface is inaccessible to rough-core-specific monoclonal antibodies (52).

A striking feature of PAO1 strains from *P. aeruginosa* is the presence of the A bands, which constitute a significant amount of the isolated LPS (Table 2). This set of bands was observed as a slow-moving diagonal banding pattern across the SDS-PAGE of the column fractions (Fig. 2 and 3, peak X). LPS from strain Z61 contained only a single diagonal banding pattern, corresponding to the bands in peaks 2 and 3 (Fig. 4). Although SDS-PAGE separates LPS molecules according to size (26, 45, 48), we propose that the anomalous migration in SDS-PAGE of the A bands is due to a difference in the charge in the core-lipid A region of the molecules. The lack of phosphate substituents in the A-band sample would make the molecules much less negatively charged than the B-band fractions, which are high in phosphate groups. To further explore this possibility, we compared the mobility of the A and B bands of the PAO1 strains by SDS-PAGE (11% acrylamide) (Fig. 5). We observed that there was a difference in the spacing between the A and B bands and that the B bands consisted of sets of doublet bands in which the members of the doublets differed in their staining intensities (Fig. 1B and 5), whereas the A bands seemed to lack this doublet pattern. This doublet probably represents substoichiometric modification in the core or lipid A of the B-band components. It has been reported for different gram-negative bacteria that there is microheterogeneity in the structure of lipid A (phosphate levels and types and numbers of fatty acids) (2, 51, 57), in the substituents in the core (14, 22, 36–38, 57), and in modifications of the O-antigen side chain (36, 37, 57). The heterogeneity of LPS molecules presumably depends in part on the strain and on growth conditions (13, 36).

We have presented several pieces of evidence which indicate that the molecules represented by the A and B bands are chemically distinct. Only the B bands from the PAO1 strains reacted with anti-O-antigen antibodies in Western blots (Fig. 5). Also, the A bands lacked reactive amino sugars detected in the B band fractions. Since the pooled fractions corresponding to A bands (peak X) contained very low levels of reactive KDO unless hydrolyzed with high concentrations of acid, and since no phosphate or amino sugar was detected (Table 2), it is possible that these A bands represent another type of molecule different from LPS, that is, an O repeat attached to a molecule which is not lipid A. In the past 10 years the chemical structure of the lipid A's from gram-negative bacteria other than members of the family *Enterobacteriaceae* have been studied, and the existence of unusual lipid A's has been noted (39). For instance, the lipid A from *Pseudomonas paucimobilis* contains a number of sugars, in addition to glucosamine, in the bound-lipid fraction, and phosphate as well as KDO appears to be lacking (39). Also, *Thermus* species have been reported to make LPS that lacks detectable heptose, KDO, glucosamine, and phosphorus (39). However, negative reactivity in the thiobarbiturate assay may not reflect the lack of KDO residues. Recently, Parr and Bryan (46) demonstrated that more rigorous hydrolysis conditions were required to release KDO from *Haemophilus influenza* LPS than from LPS of other species. Subsequently, Carof et al. (7) demonstrated that after treatment with aqueous hydrofluoric acid, the presence of KDO could readily be demonstrated in LPS of *Bortedella*, *Bacteroides*, *Aeromonas*, and *Vibrio* species which had been reported to be KDO deficient. We therefore hydrolyzed our A-band sample with a higher acid concentration for longer times and observed a 10-fold increase in the KDO level. This suggests that the A bands are resistant to hydrolysis owing to substitutions of the KDO units in positions 4 and 5 or 5 and 7 (6). Other evidence that suggests that the A bands represent LPS molecules is the ability to silver stain the A-band molecules; Kropinski et al. (31) and Lam (personal communication) reported that the silver-staining reaction for *P. aeruginosa* LPS occurs in the lipid A rather than the core sugars.

Analysis of the Western and dot blots (Fig. 5 and 6) yielded several additional interesting observations. The pooled fractions containing the higher-molecular-weight LPS (peaks 1 and 2) from the PAO1 derivatives reacted with the antibody (Fig. 6), indicating that the B bands make up the serotype-specific LPS. However, *P. aeruginosa* synthesizes a significant amount of A-band-type molecules (Table 2) with presumably a different antigenicity. It has been shown that *S. paratyphi* B and *S. typhimurium* can synthesize a T1 polysaccharide and an O polysaccharide attached to the same core (37, 41) and that the synthesis of the two molecules is independent (37). Other *P. aeruginosa* strains may also be able to synthesize more than one type of LPS with different O-antigenic side chains (8, 30, 57, 58). The high-molecular-weight polysaccharide released from LPS of these strains has been resolved into amino sugar-rich and neutral sugar-rich fractions when separated by gel permeation chromatography (30, 57, 58). Finally, Carof et al. (6) have shown that *Bortedella pertussis* produces two LPS, one of which does not give a positive reaction for KDO under normal thiobarbituric assay conditions. This is very similar to our findings for the A- and B-band-type LPS of the PAO1 derivatives.

The effect of the presence of a unique, A-band-type LPS on the physical interactions within the outer membrane, as well as the immunological reaction with the cell surface, may be important. It has been proposed that bacteria synthesizing O side chains of an unusual structure might escape the immune system of the host, which might have difficulties in producing effective antibody molecules against these O side chains (41, 50).

In summary, our data suggest that PAO1 strains from *P. aeruginosa* are capable of synthesizing more than one type of LPS-like molecule differing in their antigenic reactivities. Although the LPS isolated from the different strains used in this study was shown to be heterogeneous on SDS-PAGE, this method by itself does not have the power to predict the presence of more than one type of LPS-like molecule with a different O polymer. However, by combining SDS-PAGE with gel permeation chromatography, Western blots, and sugar analysis, we have been able to distinguish chemically distinct subclasses of molecules from individual strains. We have also demonstrated that the fraction of core oligosaccharides carrying the O-specific polymer is less than 8%.

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