Pseudomonas aeruginosa Lipopolysaccharide: Evidence that the O Side Chains and Common Antigens Are on the Same Molecule

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We investigated whether *Pseudomonas aeruginosa* produces two distinct lipopolysaccharides (LPS) containing either serologically variable O side chains or a neutral polysaccharide common antigen, designated A bands, that reacts with monoclonal antibody (MAb) E87. Immunoprecipitation of LPS and free O side chains with O-side-chain-specific antibodies or MAb E87 resulted in coprecipitation of both polysaccharides when antibody of either specificity was employed. Chromatography of LPS and free O side chains in a disaggregating deoxycholate buffer indicated the two polysaccharide antigens cochromatograph when eluates were analyzed by sensitive and specific enzyme-linked immunosorbent assay inhibitions. The LPS from a mutant of strain PAO1 that lacks polymerized O side chains but retains the common antigen eluted in fractions containing smaller LPS molecules, indicating the necessity of polymerized O side chains for elution in early fractions containing large LPS monomers. A phosphomannomutase mutant of *P. aeruginosa* PAO1 makes a rough LPS lacking both O side chains and common antigen but still produces a small (<6-kDa) common antigen component detectable in cell lysates. Introduction of the cloned *pmm* gene into this strain restored production of a smooth LPS expressing large MAb E87-reactive common antigen. Destruction with NaOH of O side chains on recombinant LPS molecules eluting early from the molecular sieve column resulted in a shift of the MAb E87-reactive antigen to the late-eluting fractions. These results indicate that on most *P. aeruginosa* LPS molecules, O side chains and neutral polysaccharide common antigens are both present.

The natures of the polysaccharide substituents of the lipopolysaccharide (LPS) of *Pseudomonas aeruginosa* have recently come under close scrutiny. The classic picture of this LPS is of a standard gram-negative structure composed of lipid A-containing acyl- and ester-linked fatty acids and glycosidically and ester-linked phosphate (2, 5, 6); a core containing 2-keto-3-deoxyoctonate, heptose, glucose, rhamnose, *N*-acetylgalactosamine, and alanine (35); and a structurally variable O side chain, resulting in at least 20 different serotypes of *P. aeruginosa* (20, 21). Sawada et al. (36) described a monoclonal antibody (MAb), E87, that reacts with an antigenic component of LPS common to different serotypes. Yokota et al. (40, 41) showed that this common antigen was a D-rhamnan, as did Kocharova et al. (13). The D-rhamnan was composed of up to 30 repeat units and was part of a polysaccharide side chain that contained 3-*O*-methyl-6-deoxyhexose, glucose, xylose, alanine, galactosamine, and phosphorus. Arsenault et al. (1) have analyzed the polysaccharide portion of the LPS isolated from a LPS-rough mutant of *P. aeruginosa* PAO1, designated AK1401, and found that it contains a single trisaccharide O side chain repeat unit along with the D-rhamnan repeat structure as a major component. This LPS also contains additional minor sugars including 3-*O*-methylrhamnose, ribose, mannose, glucose, and a 3-*O*-methylhexose. Additional studies by Kocharova et al. (12, 14) have identified two other neutral polysaccharides in *P. aeruginosa* LPS preparations that cross-react among divergent serotypes. One of these structures (12), isolated from a Fisher immu-

notype 3 strain, also reacts with MAb E87, although it is not a D-rhamnan.

The relationship of the MAb E87-reactive, or common, antigen to the rest of the LPS structure is currently subject to debate. Yokota et al. (41) showed that the D-rhamnan was likely linked to the lipid A via core sugars; they could not separate out the D-rhamnan from O side chains by differential ultracentrifugation or chromatographic techniques and had to destroy the O side chains by alkali treatment in order to recover the D-rhamnan-rich fractions. In contrast, Rivera et al. (33) and Rivera and McGroarty (34) reported that *P. aeruginosa* LPS preparations could be fractionated by chromatography in deoxycholate buffers into larger polymers containing the traditional O side chains and smaller polymers reactive with MAb E87 by immunoblot; they designated the former fractions B bands and latter fractions A bands. However, in one report (34), the authors indicated that one fraction (peak 2) that reacted only with an O-side-chain-specific MAb in an immunoblot also reacted with the A band-specific MAb E87 in a dot blot, while the A-band-enriched fraction (peak X) reacted with both the O-side-chain-specific MAb and the MAb E87 in an immunoblot. Because the bands reacting with these two different MAbs were of different sizes after separation by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and visualization by immunoblot, Rivera and McGroarty (34) concluded that even though the peak X material was not pure, the A band and O-side-chain-bearing molecules (B bands) were distinct. They also described chemical and immunologic differences that differentiated the A-band-enriched peak X LPS from fractions enriched for O-side-chain-containing LPS, leading Rivera et al. (33) to suggest that *P. aeruginosa* can synthesize more than one type of LPS

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molecule. Lam and coworkers have extended these observations to characterization of genotypes (18, 19) and phenotypes (17) of strains of *P. aeruginosa* that differentially express A-band antigens. However, no group to date has reported on isolation of pure A-band LPS lacking detectable O antigen from strains that produce O-side-chain-bearing LPS, nor has a mutant strain that produces common-antigen-bearing LPS in the absence of at least a single O side chain repeat been described. This suggests that both the O side chains and neutral polysaccharide A bands are usually present on the same molecules. Although Rivera and colleagues (33, 34) observed differential migration in SDS-PAGE and differential reactivity in immunoblots of O-side-chain-bearing and A-band LPS, this could be readily explained by differences in the reactivity of subpopulations of LPS molecules due to differences in the quantity and/or size of the A band and O side chain antigens that might be present on subpopulations of LPS molecules. Because of the well-described variation in LPS structure ranging from small, rough molecules to larger ones with long O side chains, it seems possible that some molecules of *P. aeruginosa* LPS express more O side chain antigenic determinants and react preferentially with antibodies to these epitopes, while other, variant molecular species express more neutral polysaccharides (MAb E87-reactive common antigenic determinants) and react preferentially with antibodies to MAb E87 and similar A-band-specific MAbs (17). In this report we present data indicating that the common, neutral polysaccharide antigens reacting with MAb E87 are usually found on the same molecular structure as the O side chains in *P. aeruginosa* LPS.

MATERIALS AND METHODS

Bacterial strains and plasmids. Fisher immunotype (IT) 1 through 7 strains were clinical isolates from bacteremic patients, and the Lányi serotype strain O6ac was kindly provided by B. Lányi, Budapest, Hungary. *P. aeruginosa* PAO1 (AK957) and the LPS-rough isogenic strains derived by selection for resistance to the LPS O-side-chain-specific phage E79, strains AK1012 and AK1401, were provided by A. Kropinski, Kingston, Ontario, Canada (15), and J. Lam, Guelph, Ontario, Canada, respectively. Strain AK1012 containing the recombinant plasmid designated pLPS1 was derived as previously described (7).

Antigens. LPS was initially extracted by the phenol-water procedure (39) from cell pellets of organisms grown at 37°C in Trypticase soy broth (TSB) for 24 h. To ensure recovery of representative smooth and rough fractions of LPS, we precipitated both the aqueous and phenol layers with 4 volumes of 95% ethanol to recover LPS and then combined these precipitates for further processing by ultracentrifugation, enzyme digestion, and chromatography on columns of Sepharose CL4B, as previously described (30). This procedure has been determined in our laboratory to give comparable yields of smooth and rough LPS fractions, as does the LPS extraction method of Darveau and Hancock (4). Intrinsically ¹⁴C-labelled LPS was extracted by the phenol-water method from cells grown in Trypticase soy broth containing 1 mCi of [¹⁴C]sodium acetate (29). The LPS component was purified as described above, and antigens had a specific activity of 2 to 5 cpm/ng. Free O side chains were obtained from LPS by mild acid hydrolysis (1% acetic acid, 3 h, 100°C) followed by removal of precipitated lipid A by centrifugation (5,000 × g) and dialysis against 0.1 M borate buffer, pH 8.6. For radiolabelled LPS, confirmation that the

lipid A portion was removed was obtained by comparative precipitation of intact LPS and O side chains in 50% ammonium sulfate. Intact LPS precipitates in this solution, while free O side chains do not. Confirmation of the serotype specificity of the radiolabelled O side chains was achieved by reacting each antigen with polyclonal antibodies raised to the intact LPS isolated from the seven Fisher IT strains (described below). All O side chains were precipitated only by homologous antibodies. Alkali-treated LPS was prepared by incubation in 0.1 N NaOH at 56°C for 4 h and neutralization with 0.1 N HCl. D-Rhamnan, which reacts with MAb E87, was kindly provided by Y. Knirel, Moscow, Russia, and purified neutral polysaccharide from a Fisher IT-3 strain was isolated as previously described (12). High-molecular-weight polysaccharides, which are chemically and immunologically similar to O side chains (27), were prepared as previously described (28).

Antisera and MAb. Polyclonal rabbit antibodies to purified *P. aeruginosa* LPS were prepared as previously described (31). The immunization dose and schedule were optimized to elicit antibodies to O side chains and minimize antibody production to common LPS antigens. Antisera raised to the IT-3 and IT-7 strains were adsorbed with 1 mg of lyophilized cells of the heterologous strain per ml to produce serotype-specific sera. The sera raised to purified LPS were thoroughly tested for reactivity to D-rhamnan and common LPS core antigens in an enzyme-linked immunosorbent assay (ELISA) and did not react at a level any different from that of preimmune rabbit sera despite the presence of the antigen in the LPS. In addition, these sera react in ELISA only with homologous LPS and not with heterologous LPS preparations bearing E87-reactive antigen and, most likely, other core antigens. MAb E87 was kindly provided by T. Kawamura, Teijin Limited, Tokyo, Japan, and MAb specific to the O side chains was kindly provided by J. Lam (16).

Immunoprecipitation with antibody-sensitized *Staphylococcus aureus*. The Cowan 1 strain of *S. aureus* (Sigma Chemical Co., St. Louis, Mo.) was sensitized with MAb E87 (12.5 µg of MAb per mg [dry weight] of bacteria) or polyclonal rabbit antibody to LPS (0.1 ml/mg [dry weight] of bacteria), and then 1 mg of *S. aureus* cells was added to 0.1-ml solutions containing 0.1 mg of LPS per ml. After 2 h at 37°C, the cells were recovered, washed three times in phosphate-buffered saline (PBS), resuspended in the original volume, and then boiled for 30 min. After cooling, the cells were removed, and the supernatant was diluted in ELISA inhibition assays (described below) to quantify the amount of MAb E87-reactive antigen (neutral polysaccharide) and O side chain antigen precipitated from the original solution by each antibody preparation. The amount of LPS antigen recovered is reported as a percentage of the original amount precipitated by the homologous antibody.

Immunoprecipitation of radiolabelled *P. aeruginosa* LPS was performed with 15 to 55 ng of labelled LPS and *S. aureus* sensitized with 250 ng of MAb E87 or 2 µl of O-side-chain-specific polyclonal antibodies. These amounts were derived from preliminary experiments that indicated that these amounts of antibodies were just below the level needed to precipitate 100% of labelled antigen. Thus, these experiments were not performed in antigen excess. For an individual LPS, an identical amount of labelled antigen was added to MAb E87-sensitized *S. aureus* and O-side-chain-antibody-sensitized *S. aureus*. After 2 h at 37°C, the bacterial cells were washed three times in PBS, and the bound ¹⁴C was counted in a Tri-Carb 4530 scintillation counter (Packard, Downers Grove, Ill.) with a window of energy of 10 to

156 keV to minimize background (20 to 30 cpm) and interference from chemiluminescence and other single-photon decay events. The counts per minute bound to *S. aureus* sensitized with normal rabbit serum served as a background control that was automatically subtracted. The counts per minute were determined by counting for a sufficient time to achieve a 2 σ level of accuracy, meaning that 2 standard deviations of the reported mean counts per minute were $\leq 5\%$ of these counts and that counts that differ by $>5\%$ are significantly different from each other at $P < 0.05$. The amount of LPS precipitated by the antibody-sensitized *S. aureus* was then determined from the specific activity, and the amount of an individual LPS precipitated by homologous O-side-chain-specific antibody was reported as a percentage of the same amount of LPS that could also be precipitated by MAb E87-sensitized *S. aureus*.

Radioactive antigen binding assay with labelled O side chains. The radioactive antigen binding assay was a variation on the previously published assay utilized to measure antibodies in animals and humans vaccinated with high-molecular-weight polysaccharide antigens. The assay is highly sensitive and reproducible (23, 26, 29, 32), and it has been shown that an animal serum that binds as little as 25 cpm of radiolabelled antigen above background will indicate a level of immunity sufficient to protect against bacterial challenge (22). To measure binding of MAb E87 and O-side-chain-specific MAb to free O side chains, 100 to 200 cpm (80 to 200 ng) of these antigens was mixed with MAb for 2 h at 37°C and then overnight at 4°C. After this, saturated ammonium sulfate was added to achieve a final concentration of 50% of saturation, and the antigen-antibody precipitates were recovered by centrifugation (2,000 $\times$ g). Analysis of the supernatants indicated that when saturating levels of antibody are added to these antigens, 50% ammonium sulfate routinely brings down $>98\%$ of the counts per minute. The precipitates were washed twice with 50% saturated ammonium sulfate in 0.1 M borate–0.15 M NaCl, pH 8.6. The recovered radioactivity was counted as described above, and the absolute amount of O side chain antigen from the different immunotypes that was precipitated by MAb E87 was determined from the specific activity. We then added a corresponding amount of labelled O side chain to the homologous O-side-chain-specific MAb and determined the amount of O antigen bound to this MAb by ammonium sulfate precipitation as described above. Thus, an identical amount of O side chain antigen was added to the different antibody preparations. The percent of O side chains precipitated by MAb specific to O side chains is reported as a percentage of the amount of the same O side chains precipitated by 250 ng of MAb E87. In some cases the counts per minute of the precipitate obtained with O-side-chain-specific MAb was slightly higher (up to 5%) than the input counts per minute (equal to the amount of O side chains precipitated by MAb E87), resulting in values of $\geq 100\%$. This is most likely due to slight errors in counting and pipetting.

To determine whether the same population of molecules of radiolabelled O side chains was precipitated by MAb E87 and MAb to O side chains, about 6,000 cpm of free O side chains was mixed with 7.5 μ g of MAb E87 for 2 h at 37°C and then 2 h at 4°C, and antigen-antibody complexes were precipitated by addition of saturated ammonium sulfate to 50% saturation. After recovery and washing as described above, the precipitate was dissolved in water and heated at 95°C for 30 min to destroy MAb E87, and the sample was split into three pairs containing about 2,000 cpm each. One pair received 2.5 μ g of MAb E87, the second pair received 20

to 50 μ l of MAb to O side chains, and the third received 5 μ g of an irrelevant MAb to *P. aeruginosa* mucoid exopolysaccharide (37). After 2 h of incubation at 37°C and then overnight at 4°C, the immune complexes were precipitated, washed, and counted as described above.

Column chromatography. Fractionation of the LPS and O side chains on a Sephacryl S-200 column (0.9 by 100 cm) in 0.25% deoxycholate buffer was performed, as described by Rivera et al. (33), with the buffer system of Peterson and McGroarty (25). The material in the fractions (1 to 3 ml) was recovered by ethanol precipitation and redissolution in PBS.

SDS-PAGE and immunoblots. LPS fractions were subjected to SDS-PAGE analysis on 12.5% gels and visualized by silver staining as previously described (14). Immunoblots were obtained by electroblot transfer of fractions from SDS-PAGE to nitrocellulose membranes and subsequent incubation with either polyclonal rabbit antisera (1:25 dilution) and horseradish peroxidase-conjugated anti-rabbit immunoglobulin G or MAb E87 and alkaline phosphatase-conjugated anti-mouse immunoglobulin G (3).

ELISA and ELISA inhibition. ELISA wells were coated with purified LPS, alkali-treated LPS, or high-molecular-weight polysaccharide (10 μ g/ml in 0.04 M phosphate buffer, pH 7.0) or with D-rhamnan antigen (2.5 μ g/ml in 0.04 M phosphate buffer, pH 7.0) and blocked with 5% skim milk. For direct ELISA, serum samples were diluted twofold in PBS containing 0.5% Tween 20 and 5% skim milk and directly added to sensitized plates. For ELISA inhibitions, 100 μ l of either polyclonal rabbit antibodies (1:100 to 1:1,000 dilutions) or MAb E87 (12.2 ng/ml) was incubated with aliquots of column fractions or dilutions of purified antigens at 37°C for 2 h and then added to coated and blocked wells. After 2 h at 37°C, wells were washed, alkaline phosphatase-conjugated antibodies specific to the primary antibodies were added, plates were incubated for 1 h at 37°C and then washed again, and the O-para-nitrophenol phosphate substrate was added. Color development was measured at 405 nm in an ELISA reader after 30 to 60 min of incubation at 37°C. Percentage inhibition was calculated as follows: percent inhibition = $100 - \{[(\text{OD in test} - \text{background})/(\text{OD in uninhibited wells} - \text{background})] \times 100\}$, where OD is optical density.

Generation of elution profiles of antigens following column chromatography. To detect the various antigens in fractions collected following chromatography on the S-200 column in deoxycholate buffer, we used the ELISA inhibition assay. In each assay, a standard curve was generated by using duplicate dilutions of a known quantity of purified D-rhamnan, O side chain, or LPS. The mean percent inhibitions were calculated for each concentration of standard and plotted against the known concentration of antigen. The formula for the line was determined by best-fit analysis and was used to calculate quantities of antigen present in the aliquots of the various samples on the basis of their individual percent inhibitions. Occasionally dilutions of aliquots were used if $>80\%$ inhibition was obtained with undiluted samples. All standard curve formulas had r^2 values of ≥ 0.90 .

RESULTS

Immunoprecipitation of LPS with antibody-sensitized *S. aureus*. In order to determine whether the MAb E87-reactive antigen and the O side chain antigens are located on the same molecule, we performed immunoprecipitation experiments. We sensitized protein A-bearing *S. aureus* with MAb E87 and added it to LPS from *P. aeruginosa* strains IT-2, IT-3,

TABLE 1. Immunoprecipitation of radiolabelled LPS from *P. aeruginosa* by MAb E87 and O-side-chain-specific polyclonal antibodies

LPS Fisher immunotype	Amt (ng) of labelled LPS precipitated by 250 ng of MAb E87	% LPS precipitated by MAb E87 also precipitated by O-antigen-specific antibody ^a
1	0	NA
2	29.5	81
3	35	89
4	49.5	91
5	18	≥100
6	16.5	≥100
7	35	84

^a NA, not applicable. Values of ≥100% are due to slight variability in the quantitative inhibition ELISA.

IT-4, IT-5, and PAO1 (IT-7) and a LPS nonreactive with MAb E87 derived from strain O6ac. The amount of the LPS O side chains and common antigens precipitated by MAb E87 was then quantified by ELISA inhibitions specific for each antigen after releasing the precipitated LPS from MAb E87-sensitized *S. aureus* by heat treatment. Of the total starting amount of MAb E87-reactive antigen from the different serogroups, 44 to 94% could be immunoprecipitated by *S. aureus* sensitized with MAb E87. When we then quantified the amount of O-side-chain-reactive material in these precipitates, 41 to 59% of the initial amount of the available O side chain antigens was detected, except when LPS from the MAb E87-nonreactive strain, O6ac, was used. No O side chain reactivity was found in this latter immunoprecipitate.

When we switched the antibody sensitizing *S. aureus* to O-side-chain-specific antibody, we immunoprecipitated 40 to 96% of the starting amount of O-side-chain-reactive material. These precipitates also contained 89 to 100% of the initial amount of MAb E87-reactive common antigen. In control tubes, the polyclonal antibodies to O side chains did not precipitate (≤2%) either O side chains or common antigens from nonhomologous LPS, a result confirming that these antibody preparations do not cross-react with nonhomologous LPS preparations. Thus, these initial experiments showed that we could precipitate both O side chains and common antigens on *P. aeruginosa* LPS when antibody specific to either antigen was employed in immunoprecipitation experiments.

Although the above experiments suggested that the common antigen and O side chain epitopes could often be coimmunoprecipitated, we did not always precipitate close to 100% of the starting amounts of LPS antigens by using *S. aureus* sensitized with the antibodies, most likely because the experiments were performed with an excess of antigen. Therefore, we devised a method to avoid antigen excess by determining the amount of labelled LPS antigen that was just sufficient to saturate *S. aureus* sensitized with 250 ng of MAb E87 and then added an equivalent amount of labelled antigen to O-side-chain-specific antibodies attached to *S. aureus*. We reasoned that if the antibodies specific for the different epitopes were directed at structures present on the same molecule, then both MAb E87 and O-side-chain-specific antibodies would precipitate the majority of LPS added. As shown in Table 1, MAb E87 precipitated 16.5 to 49.5 ng of LPS from Fisher IT-2 to IT-7. The IT-1 LPS from the clinical isolate used here does not express MAb E87-reactive antigens. When 16.5- to 49.5-ng amounts of radiolabelled LPS

TABLE 2. Immunoprecipitation of radiolabelled O side chains from *P. aeruginosa* by MAb E87 and O-side-chain-specific MABs

O side chain Fisher immunotype ^a	Amt (ng) of labelled O side chain precipitated by 250 ng of MAb E87	% O side chains precipitated by MAb E87 also precipitated by O-antigen-specific MAB ^b
1	<0.1	NA
2	62.4	79.8
3	78.9	89.1
4	165	95.8
5	76.7	84.6
6	102	4.1
7	96.3	≥100

^a Fisher IT-1 LPS does not express MAb E87 common antigen. Fisher IT-6 O antigens are acid labile and are destroyed by the procedure used to obtain O side chains.

^b NA, not applicable. A value of ≥100% is due to slight variability in counting.

(equivalent to the amount precipitated by MAb E87) were added to suspensions of *S. aureus* sensitized with homologous O-side-chain-specific antibodies, ≥81% of the total LPS was precipitated. This result indicates that only 0 to 19% of the LPS molecules express MAb E87-reactive antigens and do not express O side chains.

The above conclusions were potentially confounded by two issues: the presence of low levels of antibodies to D-rhamnan in the polyclonal sera raised to LPS and the possibility of micellar mixing of different LPS-like antigens, thus accounting for coprecipitation. We reasoned that if O side chains and MAb E87-reactive antigens were on the same molecule, then mild acid hydrolysis of LPS should release this complex as well as eliminate micelle formation. Therefore, we prepared ¹⁴C-labelled O side chains via mild acid hydrolysis of labelled LPS and then employed O-side-chain-specific MAB in immunoprecipitation experiments to determine if O side chains and common antigens were linked together on the same molecule. Table 2 shows that 250 ng of soluble MAb E87 precipitated 62.4 to 165 ng of available O side chain antigen. As before, no radioactivity was precipitated from the IT-1 LPS lacking common antigen by MAb E87. When we then added the same quantity of labelled O side chain precipitated by 250 ng of MAb E87 to homologous O-side-chain-specific MAB, we found that 79.8 to ≥100% of the O side chain antigen was precipitated by their respective MAB, except for that of IT-6. This O antigen is not precipitated by its specific MAB, since it contains an internal ketosidic linkage that is destroyed by mild acid hydrolysis (10, 11). However, the acid-stable common antigen was preserved, and this could be precipitated by MAb E87. These results show that when comparable amounts of *P. aeruginosa* O side chain antigens are reacted with either MAb E87 or O-side-chain-specific MAB, the majority (>80%) of the antigen is precipitated by both MAB, indicating the epitopes that they bind are mostly found on the same molecules.

A final study was performed to confirm these results. MAB E87 was incubated with labelled O side chains from the IT-2, IT-4, and IT-7 strains, and immune complexes were precipitated with ammonium sulfate, redissolved in water, heated, and split into portions containing about 2,000 cpm for reaction with MAB specific to O side chains, MAB E87, or an irrelevant MAB. Less than 2% of the added counts were recovered in the initial supernatants following precipitation with MAB E87. As shown in Table 3, O-side-chain-specific MAB reprecipitated between 95 and 100% of the antigen

TABLE 3. Precipitation of radiolabelled O side chains by homologous MAb after an initial precipitation by MAb E87

O side chain Fisher immunotype	% MAb E87-precipitated O side chains reprecipitated by the following:		
	Homologous MAb	MAb E87	Irrelevant MAb ^a
2	≥100	92	1
4	95	100	4
7	97	100	8

^a MAb specific to *P. aeruginosa* mucoid exopolysaccharide antigen.

originally precipitated by MAb E87. Fresh MAb E87 reprecipitated 92 to 100% of the antigens. Only small amounts (1 to 8%) of labelled O side chains were bound to the irrelevant MAb after the MAb E87 that brought down the antigen initially was destroyed. This set of results further indicates that most of the molecules of LPS made by different *P. aeruginosa* strains contain both O side chains and MAb E87-reactive common antigens that are linked together.

Column chromatography of LPS into different-size fractions. The above conclusions were in contrast to those of Rivera and McGroarty (34), so we went on to repeat their experimental protocol that reportedly resolved *P. aeruginosa* LPS into distinct populations to ascertain the basis for our differing conclusions. When 30 mg of LPS from strain PAO1 or IT-4 *P. aeruginosa* was applied to a column of Sephacryl S-200 (0.9 by 100 cm) in deoxycholate buffer (25), different-size O side chains were resolved, as seen by application of the material in the fractions to SDS-polyacrylamide gels that were silver stained after electrophoresis (Fig. 1). A similar pattern was seen when LPS from the Lányi O6ac strain was used (not shown). When samples from the individual fractions were run on SDS-PAGE, electroblotted to nitrocellulose membranes, and reacted with polyclonal rabbit antibody to the LPS O side chains, antibody reactivity was present in the same fractions as those visualized in the silver-stained gel (Fig. 2). The reactivity of the O-side-chain-

specific antibody with the fast-migrating fraction reportedly containing mostly rough LPS has recently been determined by us to be due to the presence of small O side chains in molecules migrating with this fraction (7). When the column fractions run on SDS-PAGE and electroblotted to nitrocellulose were reacted with MAb E87, the common antigen was visualized among the middle- to late-eluting fractions of PAO1 LPS and among the late-eluting fractions of IT-4 LPS (Fig. 3). Some slight reactivity of the earliest eluting fractions of PAO1 LPS (fractions 81 to 93 [Fig. 2]) was seen with MAb E87, and the reactive material migrated more quickly through the gel than did the antigen reactive with the antibody to the O side chain (Fig. 2, fractions 81 to 90). This is similar to the reactivity of the peak X material described by Rivera and McGroarty (34). This result may be caused by incomplete disaggregation of the A-band material in the deoxycholate buffer or by the presence of differential amounts of O side chain or MAb E87-reactive antigens substituting the LPS that eluted in these fractions. Application of smaller amounts of LPS to the sizing column resulted in no visualization of material reacting with MAb E87 in these early-eluting fractions and clear visualization in late-eluting fractions (not shown). Application of 50 mg of LPS increased the material reactive with MAb E87 in the early fractions (not shown). Rechromatography of LPS eluting early from the molecular sieve column showed that it eluted in the same volume during the second chromatographic step, indicating that the LPS had been properly disaggregated (not shown). LPS from strain Lányi O6ac does not react with MAb E87, and fractions from this preparation could not be visualized in immunoblots with MAb E87. These results basically replicate the findings of Rivera and coworkers (33, 34), wherein LPS eluting early from the molecular sieve column in deoxycholate buffer reacted with antibody to O side chains, while a ladder-like pattern could be visualized in material eluting later from the column after SDS-PAGE and immunoblotting with MAb E87.

FIG. 1. Separation of LPS from strains PAO1 and Fisher IT-4 on a S-200 column in deoxycholate buffer. Fractions (3 ml) were collected, alcohol precipitated, redissolved, and applied to polyacrylamide gels. Numbers above lanes indicate elution volume in milliliters. Material in fractions of IT-4 LPS eluting prior to 108 ml were not visualized by silver staining.

FIG. 2. Immunoblot of PAO1 and Fisher IT-4 LPS after separation of LPS from strains PAO1 and Fisher IT-4 on a S-200 column in deoxycholate buffer and visualization by staining with serotype-specific antibody to O side chains. Numbers above lanes indicate elution volume in milliliters.

ELISA inhibition for presence of MAb E87-reactive antigen. To resolve the apparent discrepancy between the immunoprecipitation and column chromatography results, we hypothesized that the immunoblotting technique was insufficiently sensitive to detect common antigen in fractions containing higher-molecular-weight O side chains. We therefore set out to determine if an ELISA inhibition would be considerably more sensitive than immunoblotting for detecting common antigen and O side chains. We initially investigated the inhibitory activity on 100 μ l of MAb E87 (122 ng/ml) by various concentrations of the following antigens, all previously reported to react with this antibody: LPS from strain PAO1 (34), alkali-treated LPS from this strain (41), the neutral polysaccharide isolated from an IT-3 strain of *P. aeruginosa* (12), and D-rhamnan (13). The negative control was LPS from strain O6ac. After inhibition, the MAb was added to ELISA plates sensitized with PAO1 LPS. We found that all of the antigens, except LPS from strain O6ac

(not shown), had a high inhibitory activity (Fig. 4), with 50% inhibition occurring at concentrations of <25 ng/ml. The neutral polysaccharide from the IT-3 strain was about 10 times less inhibitory than the other three antigens but still expressed a good level of inhibition. Both D-rhamnan and the neutral polysaccharide from IT-3 *P. aeruginosa* share an α 1-3-linked rhamnose disaccharide structure (12, 41), although the rhamnose is in the L configuration in the neutral polysaccharide. This structural similarity may be a basis for the reactivity of both D-rhamnan and the IT-3 neutral polysaccharide with MAb E87.

When MAb E87 was inhibited with the four antigens listed above and then added to ELISA plates sensitized with alkali-treated LPS, D-rhamnan, or IT-3 neutral polysaccharide, a similar pattern of inhibition was observed (not shown). In contrast, a minimum of 5 μ g of LPS from strain PAO1 had to be applied to an SDS-polyacrylamide gel and electroblotted to nitrocellulose in order for it to be visualized

FIG. 3. Immunoblot of PAO1 and Fisher IT-4 LPS after separation of LPS from strains PAO1 and Fisher IT-4 on a S-200 column in deoxycholate buffer and visualization by staining with MAb E87. Numbers above lanes indicate elution volume in milliliters.

FIG. 4. Inhibition of the binding of MAb E87 to intact PAO1 LPS coated onto an ELISA plate by the antigens indicated in the legend. Each point represents the mean of duplicate determinations.

by reaction with MAb E87. Thus, the ELISA inhibition was >1,000-fold-more sensitive than immunoblotting for detecting MAb E87-reactive antigen. In a similar vein, the ELISA inhibition assay for O side chain antigens with high-molecular-weight polysaccharide antigens to sensitize ELISA plates was found to be specific to a single serotype, and 1 μ g of antigen per ml inhibited the reaction by >20%.

Detection of O side chain and neutral polysaccharide antigens by ELISA inhibition. When we tested the fractions from the molecular sieve column for their ability to inhibit binding of MAb E87 to ELISA wells sensitized with PAO1 LPS, we found inhibitory activity in most of the fractions, including those with low reactivity to this antibody in immunoblots (Fig. 5A). This phenomenon was particularly noticeable for LPS from IT-4 *P. aeruginosa*, in which good inhibitory activity was detected in elution volumes of 60 to 140 ml, even though no MAb E87-reactive antigen was visualized in these fractions by immunoblot. Indeed, the ELISA inhibition was sufficiently sensitive to detect LPS in elution volumes of 60 to 108 ml even though IT-4 LPS was not visualized in these fractions by using either silver-stained gels or immunoblots. Also, most fractions contained between 1 and 3% of the applied LPS, indicating that the sensitive ELISA inhibition assay was not detecting a low-level contaminant in the fractions. On the basis of the standard curve run in the ELISA inhibition, we estimated recovery of 99.3% of the common antigen present on PAO1 LPS and 73.7% of this antigen on the IT-4 LPS. Calculation of the amount of MAb E87-reactive antigen in IT-4 LPS eluting between 60 and 141 ml indicated that 81% of the recovered antigen was accounted for in this elution volume. Similarly, 73% of the recovered MAb E87 antigen in PAO1 LPS eluted with ≤ 141 ml of buffer. Fractions obtained after chromatography of the LPS from the Lányi O6ac strain, which is negative for the MAb E87 antigen, did not inhibit this antibody's reactivity (Fig. 5A), indicating that nonspecific inhibition from buffers or other column effluents did not account for the observed inhibition. When the fractions eluting from the molecular sieve column were used to inhibit binding of polyclonal antibodies raised to LPS to the high-molecular-weight polysaccharide version of O side chains, these antigens were detected in the fractions eluting early

FIG. 5. Elution profile of intact LPS obtained from *P. aeruginosa* strains PAO1, Fisher IT-4, and Lányi O6ac (which fails to react with MAb E87) on the S-200 column in deoxycholate buffer by using ELISA inhibition to detect antigen in the fractions obtained following chromatography. (A) Inhibition of binding of MAb E87 to wells sensitized with PAO1 LPS. (B) Inhibition of binding of polyclonal rabbit antibodies to IT-4 and IT-7 LPS by fractions of homologous antigen prior to addition to wells sensitized with the high-molecular-weight polysaccharide version of homologous O antigen. The milligrams of LPS per fraction was determined as indicated in Materials and Methods, and the percent applied LPS per fraction was calculated on the basis of the initial amount of LPS applied to the S-200 column. Symbols for the different LPS antigens applied to the S-200 column are identified in the legend on the upper graph.

from the column (Fig. 5B), as well as at the end of the column run when MAb E87-reactive antigens were detected by immunoblot (shown in Fig. 3) and ELISA inhibition (Fig. 5A).

To determine further that the above results were not due to incomplete disaggregation of the common antigen fractions during the column chromatography, we prepared free O side chains (<0.001% lipid present) from 30 mg of LPS from strains PAO1, IT-4, and O6ac and applied them to the deoxycholate column. The resulting fractions were then used in the ELISA inhibition assay to detect MAb E87-reactive antigens (Fig. 6A) and O side chains (Fig. 6B). Again we found strong inhibition of the reaction of MAb E87 with its target antigen in the earlier-eluting fractions containing the largest polymers of O side chains. ELISA inhibition for O side chain antigens also showed the presence of these antigens in most of the fractions. Interestingly, after mild acid hydrolysis of LPS, some of the MAb E87-reactive antigen was detected in the very late eluting fractions in which O side chain reactivity was minimal. This may represent a fraction of LPS wherein the common antigen has been attached to the LPS core but polymerized O side chains have

FIG. 6. Elution profile of free O side chains obtained from hydrolyzed LPS isolated from *P. aeruginosa* strains PAO1, Fisher IT-4, or Lányi O6ac (which fails to react with MAb E87) on the S-200 column in deoxycholate buffer by using ELISA inhibition to detect antigen in the fractions obtained following chromatography. (A) Inhibition of binding of MAb E87 to wells sensitized with PAO1 LPS. (B) Inhibition of binding of polyclonal rabbit antibodies to IT-4 and IT-7 LPS by fractions of homologous antigen prior to addition to wells sensitized with the high-molecular-weight polysaccharide version of homologous O antigen. The milligrams of LPS O side chains per fraction was determined as indicated in Materials and Methods, and the percent applied LPS O side chains per fraction was calculated on the basis of the initial amount of LPS applied to the S-200 column. The different O side chain antigens are identified in the legend on the upper graph.

not. Free O side chains did not migrate into SDS-polyacrylamide gels and could not be visualized by this method.

Finally we analyzed the elution profile of LPS isolated from strain AK1401, a phage E79-resistant, LPS-rough mutant of strain PAO1. This strain's LPS contains a single O side chain trisaccharide repeat unit, the D-rhamnan polymer, and associated core sugars (1) and likely lacks the enzymes needed for O side chain polymerization. The chromatography results (Fig. 7) showed that only 20% of the applied antigen eluted in early fractions (between 80 and 140 ml) and most of the LPS from strain AK1401 eluted in the late fractions containing small material. The early-eluting material likely represents incompletely disaggregated LPS. The ELISA inhibition data indicated 96% of the applied antigen was detected in the fractions. The presence of E87-reactive antigen mostly in the latter fractions contrasts with the presence of this material in earlier-eluting fractions (60 to 140 ml) of LPS obtained from parental strain PAO1 chromatographed under identical conditions (Fig. 5A).

Immunologic analysis of LPS from strain AK1012. Further efforts were directed towards using a genetic approach to

address the issue of the incorporation of common antigens on LPS molecules bearing O side chains. We have recently shown that the LPS-rough derivative of strain PAO1, strain AK1012, is a *pmm* mutant (7). We utilized this strain, plus a derivative wherein the *pmm* defect was complemented by cloned DNA contained on plasmid pLPS1, to characterize the LPS of strain AK1012 and AK1012(pLPS1). LPS isolated from *P. aeruginosa* strains AK1012 and AK1012(pLPS1) was analyzed by ELISA inhibition (Fig. 8) to quantify the amount of O side chain and common antigens present. LPS from strain AK1012 contained <0.05% of the amount of O side chains expressed by the parental PAO1 LPS and the control IT-7 LPS (Fig. 8A) and barely detectable MAb E87-reactive antigen. The recombinant LPS from strain AK1012(pLPS1) had a somewhat reduced expression of O side chains (Fig. 8A) compared with O side chains expressed on LPS from strain PAO1 and the IT-7 strain (approximately a fourfold increase in the amount of recombinant LPS needed for 50% inhibition compared with LPS from strains PAO1 and IT-7). LPS prepared from strain AK1012(pLPS1) contained an amount of common antigen comparable to that in the PAO1 LPS and the control LPS from an IT-7 LPS strain (Fig. 8B). LPS from strain AK1012(pLAFR1) bearing only the cloning vector behaved identically to LPS from the parental rough strain AK1012 (not shown). LPS from strain AK1012(pLPS1) has been shown to have detectable ladders by SDS-PAGE that reacted with both MAb E87- and O-side-chain-specific antibodies by immunoblot, whereas LPS from strain AK1012 lacks ladders and does not react with these antibodies in immunoblots (7).

Although O side chain and common antigens were not detected on the LPS from strain AK1012, this did not preclude the possibility that it synthesized the antigen but failed to incorporate it onto the core-lipid A. To determine whether strain AK1012 produced O-side-chain- or MAb E87-reactive antigens, we prepared both autoclaved extracts of 10^9 CFU of strain AK1012 and lysates of the same number of whole cells by the method of Hitchcock and Brown (9). These extracts were found to inhibit the binding of MAb E87 to D-rhamnan, as did autoclaved extracts of the parent strain PAO1 (Fig. 9A). The extract and lysate of strain AK1012 contained minimal amounts of O side chain antigens, since

FIG. 7. Elution profile of LPS from *P. aeruginosa* LPS-rough strain AK1401, deficient in polymerization of O side chains, in fractions obtained following chromatography on the S-200 column in deoxycholate buffer by using ELISA inhibition to detect antigen in the fractions. The amount (in milligrams) of LPS per fraction was determined as indicated in Materials and Methods, and the cumulative percentage of applied LPS antigen eluted was calculated by dividing the amount of LPS in an individual fraction by the total amount initially applied and multiplying by 100.

FIG. 8. Analysis by ELISA inhibition of the amount of O side chains and MAb E87-reactive antigen in LPS purified from the strains indicated in the legend on the upper graph. (A) Inhibition of binding to the high-molecular-weight polysaccharide antigen of polyclonal rabbit antibody raised to LPS by the purified LPS. (B) Inhibition of binding to D-rhamnan antigen by MAb E87.

they only slightly inhibited antibodies specific to the O side chain from binding to intact PAO1 LPS (Fig. 9B). These data indicate that this LPS-rough strain derived by selection for resistance to the LPS-specific smooth phage E79 produces only minimal levels of O side chains and does not synthesize the MAb E87-reactive antigen but fails to incorporate it into the rough LPS structure.

To determine the relative size of the MAb E87-reactive antigen in extracts of strain AK1012, we applied this material to the S-200 column in deoxycholate buffer (25, 33, 34). The MAb E87-reactive antigen was found exclusively in late-eluting fractions, indicating a relatively small molecular size for this antigen (Fig. 10). As described above for use of LPS from strain AK1401, this result contrasts with the presence of the MAb E87-reactive antigen in earlier-eluting (i.e., larger) fractions of LPS obtained from strain PAO1 chromatographed under identical conditions (Fig. 5A).

Chromatographic analysis of the LPS from AK1012 (pLPS1). LPS isolated from strain AK1012(pLPS1) was fractionated on the S-200 column in deoxycholate buffer. An elution profile similar to that observed in Fig. 1 for the parental PAO1 strain was obtained after SDS-PAGE analysis of material in individual fractions. The pattern observed in immunoblots with antibody to the O side chain and MAb E87 was also very similar to that of LPS from strain PAO1 (Fig. 2 and 3). As we did for analysis of LPS from strain PAO1 (Fig. 5), we used the ELISA inhibition assay employing D-rhamnan as a coating antigen and MAb E87 for detection to determine whether the MAb E87-reactive antigen in the LPS from strain AK1012(pLPS1) was present in

FIG. 9. (A) Inhibition of binding to D-rhamnan of MAb E87 by autoclave extracts and lysates of *P. aeruginosa* strains PAO1 and AK1012. (B) Inhibition of binding of IT-7 high-molecular-weight polysaccharide of polyclonal rabbit antibody raised to purified IT-7 LPS by autoclaved extracts and lysates of *P. aeruginosa* strains PAO1 and AK1012.

the fractions of the larger LPS O side chains. Similarly, we used an ELISA inhibition specific for the O side chains to detect these antigens in the fractions eluting from the deoxycholate column. As seen in Fig. 11A, over 60% of the total common antigen eluted with 120 ml of buffer. MAb E87-reactive antigen was found in the same fractions as was O side chain antigen. Thus, after reconstituting strain AK1012's ability to make O side chains by the cloned *pmm* gene in pLPS1, we also reconstituted this strain's ability to incorporate the small-molecular-size MAb E87-reactive antigen into the LPS where it is associated with O side chains

FIG. 10. Elution profile of an autoclaved extract of *P. aeruginosa* AK1012 following application onto the S-200 deoxycholate column. The micrograms of antigen per milliliter was determined as indicated in Materials and Methods.

FIG. 11. (A) Elution profile of LPS obtained from recombinant *P. aeruginosa* AK1012(pLPS1) after application to the S-200 deoxycholate column. (B) Elution profile of LPS from the recombinant strain AK1012(pLPS1) eluting between 60 and 75 ml in panel A after treatment with 0.1 N NaOH for 4 h at 56°C and reapplication to the S-200 deoxycholate column. The milligrams of LPS per fraction was determined as indicated in Materials and Methods, and symbols for the results of assays specific for O side chains or MAB E87-reactive antigen are indicated in the legend. The cumulative percentage of applied LPS antigen eluted was calculated by dividing the amount of LPS determined to be present in an individual fraction (by using inhibition of MAB E87 binding to D-rhamnan) by the total amount initially applied and multiplying by 100.

of all lengths. Quantitative recovery of the applied LPS was 87%.

We sought additional confirmation that the material eluting early from the deoxycholate column that reacted with MAB E87 was linked to the larger molecules containing O side chains. Material eluting with 75 ml of buffer (15 ml after the start of the void volume at 60 ml) of the deoxycholate column (18% of the total MAB E87-reactive antigen applied) was recovered by ethanol precipitation and treated with 0.1 N NaOH at 56°C for 4 h. This treatment has been shown by Yokota et al. (41) to destroy the O side chain and preserve activity of the MAB E87 antigen (Fig. 4). As shown in Fig.

11B, 83% of the recovered MAB E87-reactive antigen that originally eluted with ≤ 75 ml of buffer now required >125 ml of buffer to elute from the S-200 column after alkali treatment. Only 17% of the antigen eluted with ≤ 125 ml of buffer. Minimal inhibition of O-side-chain-specific antibody was seen ($<10\%$ of the original amount), indicating that most of this antigen had been destroyed. Presumably this remaining activity, along with the 17% of the MAB E87 reactivity that eluted early from the column, represents residual intact molecules not hydrolyzed by the alkali treatment. In this series of experiments, we documented the presence of small common antigen in lysates of *P. aeruginosa* AK1012, showed that the antigen converted to an apparent larger size when this strain's defect in O side chain production was genetically complemented, and finally showed the common antigen reverted to a small size when the O side chains were chemically destroyed. Thus, the apparent larger size of the MAB E87-reactive antigen in the intact LPS must be due to linkage of both O side chains and this common antigen to the same structure.

DISCUSSION

The results of our fractionation of *P. aeruginosa* LPS in deoxycholate buffers and subsequent analysis by SDS-PAGE are comparable to those reported previously by Rivera and coworkers (33, 34). The LPS could be resolved into fractions containing different lengths of O side chain, and material reacting with MAB E87 in immunoblots was found mostly in the late-eluting fractions. Also, the MAB E87-reactive antigens in the late-eluting fractions migrated in the SDS-polyacrylamide gel more slowly than the O antigen containing semirough LPS fractions as visualized by immunoblot. However, we also found that we could coimmunoprecipitate both O side chain antigens and the MAB E87-reactive antigens by using polyclonal and monoclonal antibodies specific to either determinant, indicating linkage of these determinants on the same molecule. The apparent discrepancy between the immunoprecipitation and the chromatography and immunoblot data in regards to the linkage of common antigens and O side chains on *P. aeruginosa* LPS was resolved by use of a sensitive ELISA inhibition assay documenting that common antigens cochromatograph with high-molecular-size O side chains on intact LPS as well as on free O side chains. Additional support for our conclusion was derived from the observation that LPS from an O-side-chain-defective mutant of strain PAO1, strain AK1401, elutes from the deoxycholate column late, indicating that loss of O side chains rendered this strain's LPS small. Thus, we do not believe that the MAB E87-reactive antigen is usually on a separate LPS-like molecule from that bearing the serotype-specific O side chains, but on most molecules of *P. aeruginosa* LPS, the O-side-chain- and MAB E87-reactive antigens are both present. In addition, since other common antigens with different polysaccharide structures associated with *P. aeruginosa* LPS have been described (12, 14), it is possible that this molecule is a multiantennary structure wherein the O side chain repeat units predominate, while small amounts of additional polysaccharides are also present.

The results of the genetic experiments, in which the defect in LPS O side chain production in strain AK1012 was complemented with a cloned *pmm* gene, provide further support for our conclusion that the MAB E87-reactive antigen is part of the same LPS structure that contains the O side chain. When pLPS1 containing the *pmm* gene was intro-

duced into strain AK1012, both O side chains and MAb E87-reactive antigen were incorporated onto LPS, and the small common antigen present in extracts of strain AK1012 increased in size as determined by molecular sieve chromatography of the purified LPS from the recombinant strain. When O side chains were destroyed by alkali treatment, the apparent size of the MAb E87-reactive antigen decreased. The increased size of the common antigen in strain AK1012(pLPS1) is likely because of the linkage of this antigen either directly to the O side chain or the same core structure that the O side chain is linked to. Taken together, the data derived from the immunoprecipitation studies, column chromatography, and genetic reconstitution experiments indicate that the MAb E87-reactive common antigen is usually present on the same molecule as the O side chains. However, given the continuum of molecular species that compose an LPS preparation, it is reasonable to acknowledge that there could be a minority of molecules wherein the common antigen was attached to LPS cores without any polymerized O side chains added. Indeed, this appears to be the case for LPS isolated from strain AK1401, wherein the LPS contains a single O side chain trisaccharide repeat unit. However, our results indicate that LPS molecules bearing common antigen and lacking O side chains would compose at most 20% of the *P. aeruginosa* LPS molecules. These findings are consistent with the data of Yokota et al. (41), who, using a variety of methods, could not separate the D-rhamnan (i.e., MAb E87-reactive antigen) component from the O side chains of LPS from *P. aeruginosa* IID 1008.

We did not evaluate the findings of Rivera and McGroarty (34) that their A-band LPS preparation had low levels of phosphate, contained stoichiometric amounts of sulfate, and did not react with a MAb against *Escherichia coli* lipid A that could recognize *P. aeruginosa* LPS bearing long O side chains. Given that the A-band fraction characterized (peak X; Table 1 of reference 34) was at least minimally contaminated with O-side-chain-expressing molecules, no conclusions associating a chemical species identified in this material as part of a particular component identified by immunoblot can be drawn. The lack of reactivity of the peak X fraction with a MAb against lipid A from *E. coli* could be explained by slight chemical or conformational differences between LPS containing long O side chains and those expressing short O side chains that were contained in peak X. For example, Bhat et al. (2) showed that an ester-bound phosphate at C-4' of the nonreducing glucosamine in the lipid A of *P. aeruginosa* was nonstoichiometrically substituted with 4-amino-4-deoxy arabinose, and this was affected by culture conditions. The failure of Rivera and McGroarty (34) to detect a chemical difference in the lipid composition of the A-band and B-band LPS is consistent with our interpretation that there is variation in the molecular species of LPS made by *P. aeruginosa*, with the variation due to the amount of O side chains and neutral polysaccharides present on a single molecule.

One consideration in regards to the interpretation of our data is that MAb E87 might cross-react with a core epitope in *P. aeruginosa* LPS distinct from the common antigen, and this could account for the apparent detection of common antigen in fractions with large O side chains by using the ELISA inhibition assay. MAb E87 binds to many different strains of *P. aeruginosa*, including those of different LPS serotypes (17). However, it is unlikely that MAb E87 binds to the common core structure of *P. aeruginosa* LPS, since a human MAb reactive with this structure and dependent upon the presence of the core rhamnose residue (38) has been

shown to react with a different set of strains than MAb E87 (40). The human core-specific MAb does not react with strain PAO1 (38). Most importantly, we recently have found by chemical analysis that the high-molecular-weight polysaccharide versions of the O side chains contain core sugars, except 2-keto-3-deoxyoctonate. These results also showed that all of the rhamnose is present in the L configuration, and we have determined that high-molecular-weight polysaccharide versions of the O side chain fail to bind MAb E87 (8). Furthermore, any interpretations of our results potentially confounded by a cross-reactivity of MAb E87 with core antigens would similarly confound the results of Rivera and colleagues (33, 34), who used the same MAb and *P. aeruginosa* strain (PAO1) to suggest that two distinct LPS species were made by this organism.

In conclusion, we have used an immunochemical and a genetic approach to define the structure of the *P. aeruginosa* LPS. These approaches indicate that both serologically variable O side chains and serologically conserved MAb E87-reactive antigens are usually found on the same molecule. While O-side-chain-deficient mutants (18) and variable growth conditions (24) can produce LPS molecules enriched for or devoid of polymerized O side chains, our results indicate that under conditions when both antigens are made, they most often associate together on the same lipid A-core structure.

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