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Characterization of Lipopolysaccharide-Deficient Mutants of *Pseudomonas aeruginosa* Derived from Serotypes O3, O5, and O6

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Well-characterized rough mutants are important for the understanding of structures, functions, and biosynthesis of lipopolysaccharide (LPS) in gram-negative organisms. In this study, three series of *Pseudomonas aeruginosa* LPS-deficient mutants, namely PAC strains derived from serotype O3, AK strains derived from strain PAO1 (serotype O5), and serotype O6-derived mutants were subjected to biochemical analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining as well as immunochemical characterization using LPS-specific monoclonal antibodies. The O-side-chain deficiency among the O6-derived mutants was also examined, and three mutants, A28, R5, and H4, were subsequently chosen for the elucidation of component sugars of the core structure of serotype O6 LPS. LPS of strain A28 has L-rhamnose and proportionally higher amounts of D-glucose, a feature shared by the O5-derived mutant, strain AK1401 (previously demonstrated as a mutant with a core-plus-one O repeat). In contrast, strains R5 and H4 were shown to be devoid of L-rhamnose and have low and undetectable amounts of D-glucose, respectively, which indicated their core deficiency. The LPS-deficient or -sufficient characteristics of the *P. aeruginosa* strains examined correlated well with serum sensitivity data. This report represents a comprehensive analysis of rough mutants derived from O3 and O5 strains that have been used by others in many studies and a first look at the core oligosaccharide region of serotype O6 LPS obtained with the O6-derived mutants generated in this study.

Pseudomonas aeruginosa continues to be an important pathogen among debilitated, burn-wounded, and immunocompromised individuals (5, 6). Lipopolysaccharide (LPS) is one of the major virulence factors of *P. aeruginosa*, especially manifested in wild-type strains as adjuvant activities, resistance to the bactericidal activities of normal human serum (25), antibiotic susceptibility (3, 20), and lower lethal dose in the burned mouse sepsis model (12).

As in most gram-negative bacteria, the LPS of *P. aeruginosa* forms an integral part of the outer membrane, and despite its distinctive features (68), it shares the general architecture found in members of the family *Enterobacteriaceae*, viz., an outermost domain of O-specific heteropolysaccharide covalently linked to the outer membrane-embedded lipid A via a core oligosaccharide. The core oligosaccharide is further divided into the inner core and the more distal outer core region. The majority of *P. aeruginosa* strains can coexpress two chemically and antigenically distinct forms of LPS, namely, a serotype-specific (or O polysaccharide-containing) B-band LPS and a common antigen A-band LPS (41, 55). The differences in the structures of the O polysaccharide of B-band LPS form the basis of the 17 serotypes in the International Antigenic Typing Scheme (IATS) (44). Recently, three new major somatic antigens have been described which extended the existing scheme to 20 (45). The O-antigen structures of the 17 IATS serotypes have been determined (35), but details of the LPS core structure are still inadequate. Like *Escherichia coli*, which is reported to have five unique core types (46), it is believed that the core region of *P. aeruginosa* contains some degree of heterogeneity (57, 71). Recent structural studies have demonstrated that there is more than one core type among *P. aeruginosa* strains since serotype O5 and O6 strains differ in the outer core region (2, 48).

The composition of the *P. aeruginosa* core has been investigated by several groups (14, 31, 37, 50, 56, 68). Drewry et al. (14) isolated the core material from the LPS of *P. aeruginosa* NCTC 1999 and found the major components to be glucose, rhamnose, heptose, 3-deoxy-2-octulosonate (KDO), galactosamine, alanine, and phosphate. More recently, Rowe and Meadow (56) isolated LPS from *P. aeruginosa* PAC1R (serotype O3) and its LPS-defective mutants, some of which lacked rhamnose and/or glucose units. A series of O-antigen-deficient or core-deficient mutants with the "AK" designation derived from strain PAO1 (serotype O5) that were generated by Kropinski and coworkers (4, 28, 30, 38) were also used for structural elucidation of the LPS core. On the basis of these studies, partial structures were proposed for the LPS core oligosaccharide of NCTC 1999, PAC1R, and O5. To date, a complete and accurate elucidation of the core structure from either the O5 or O3 LPS has not been achieved. Series of both O3- and O5-derived rough mutants have been isolated previously and were used by others for the purpose of structural and immunochemical studies of the LPS core of *P. aeruginosa*; however, these mutants have not been characterized with regard to the presence of either A-band or B-band LPS, particularly since these mutants were derived before the first report of A-band LPS. In addition, it is not known whether the structure of the heptose-KDO region of the core varies from strain to strain in *P. aeruginosa* (34). Earlier reports indicated

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TABLE 1. *P. aeruginosa* strains used in this study

Strain	Phenotype and relevant properties	Reference and/or source
Serotype O3	Wild type, A^+ B^+	ATCC 33350
Serotype O5 (PAO1)	Wild type, A^+ B^+	24
IATS O6	Wild type, A^+ B^+	ATCC 33354
PAC556, -557, and -608	Serotype O3-derived mutants, A^- B^-	55
PAC605, -609, and -611	Serotype O3-derived mutant, A^+ B^+	55
AK43 and AK1012	Serotype O5-derived mutant, core deficient, A^- B^-	28
AK44	Serotype O5-derived mutant, complete core, A^+ B^-	38
AK1401	Serotype O5-derived mutant, core-plus-one O-antigen unit (no B-band polymer), A^+ B^-	4
A18, A19, A28, and A34	IATS O6-derived, transposon Tn*5-751*-induced mutants, A^+ B^-	This study
R1–R5	IATS O6-derived mutant, phage E79 resistant, A^+ B^-	This study
H4, H5, H23, and H29	IATS O6-derived mutant, phage 2 Lindberg resistant, A^+ B^-	This study
Gt520, Gt580, Gt620, Gt680, and Gt700	IATS O6-derived; resistant to gentamicin concentrations (μg/ml) of 520, 580, 620, 680, and 700, respectively; A^- B^-	This study
P1	Serum-sensitive control strain, isolated from a cystic fibrosis patient, polyagglutinable, rough LPS	61
M2	Serum-resistant control, mouse-virulent strain, LPS wild type	63

that the highly phosphorylated inner core is composed of heptose and KDO; phosphates are presumably present as triphosphates, pyrophosphate monoester, pyrophosphate diester, and monophosphate (50).

Structural, biosynthetic, and molecular details of the LPS core have been well studied in members of the *Enterobacteriaceae*, particularly *Salmonella typhimurium* (51). These studies were facilitated by the isolation of a set of well-defined rough mutants. In order to more clearly define the biochemistry and the structure of the core region of *P. aeruginosa* LPS, we report the characterization of rough mutants derived from serotype O6 in our laboratory as well as rough mutants of O3 and O5. Serotype O6 strains are the most commonly encountered *P. aeruginosa* strains from clinical sources; thus, it is important to define the core region of this serotype for future purposes of developing therapeutic agents or new antibiotics against *P. aeruginosa*. O6-derived mutants were generated by means of transposon insertion, phage infection, and resistance to high concentrations of the antibiotic gentamicin. The determination of structural components of the LPS core region in *P. aeruginosa* serotypes O5 and O6 will also be discussed.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. All strains were cultured on tryptic soy broth or agar plates (Difco Laboratories, Detroit, Mich.) except where indicated.

Isolation of mutants. The A series of mutants were generated by Tn*5-751* mutagenesis as described by Rella et al. (54). The R and H series of mutants were selected by screening for mutants resistant to the smooth-LPS-specific phages E79 (29) and 2 Lindberg (1), respectively, according to the method used by Jarrell and Kropinski (27). The Gt series of mutants were isolated by growing serotype O6 in the presence of progressively higher concentrations of gentamicin in Mueller-Hinton broth (Difco Laboratories) as described by Galbraith et al. (18). These mutants were maintained on Mueller-Hinton agar containing the appropriate concentration of gentamicin. All the above-mentioned mutants were screened for the absence of B-band LPS by their lack of reactivity with the serotype O6-specific monoclonal antibody (MAb) MF23-2 (40) in colony immunoblots (described below). In the colony immunoblots, mutants that did not react with the MAb appeared faint purple, as opposed to the wild types, which were distinctly dark purple.

Isolation and dephosphorylation of the LPS. Small-scale LPS preparations were made from proteinase K digests of whole-cell lysates as described by Hitchcock and Brown (26). LPS from rough-type mutants was isolated by a modified phenol-chloroform-petroleum ether extraction method (7). LPS from the wild-type strain was isolated by 95% hot phenol–water extraction and purified by ultracentrifugation (80,000 × *g*, 4 h, 4°C) (67). The LPS was obtained in a yield of about 5% (dry weight) bacteria. For the dephosphorylation, LPS samples (150 mg) were treated with 48% aqueous hydrofluoric acid (HF, 15 ml) at 4°C for 48 h; the excess of HF was removed under a stream of nitrogen, and the residue was redissolved in distilled water and lyophilized.

Preparation of the core oligosaccharide. A solution of LPS or dephosphorylated LPS (150 mg) in aqueous 1.5% acetic acid (50 ml) was heated for 2 h at 100°C, and the precipitated lipid A was removed by low-speed centrifugation. The soluble products were fractionated by gel filtration on a column (1.6 by 100 cm) of Bio-Gel P-2 (Bio-Rad, Richmond, Calif.), using 0.05 M pyridinium acetate buffer (pH 5.4). Fractions of the eluate (6 ml) were analyzed colorimetrically for neutral aldose (15).

SDS-PAGE. Isolated LPS was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Hancock et al. (25) and visualized by the silver staining method of Dubray and Bezard (16). Protein molecular weight standards are not presented in the LPS gels for two reasons: first, the relative mobilities of the LPS do not correlate with those of the protein standard markers; second, many of the standard protein bands do not stain well by the silver staining method designed for LPS.

Tricine SDS-PAGE gels. In the Tricine gel procedure, glycine is replaced with Tricine in the cathode buffer; this modification provides a higher resolution of proteins smaller than 20 kDa (59). We used the modification of Lesse et al. (42) for improved resolution of the core oligosaccharide bands. Gels were silver stained according to the method of Dubray and Bezard (16).

Western and colony immunoblots. Western immunoblots of SDS-PAGE gels were done as described by Burnette (8), and Towbin et al. (66). The enzyme-substrate system used in developing the blots was alkaline phosphatase conjugated to

goat anti-mouse immunoglobulin G F(ab')$_2$ fragment (Jackson Immunoresearch Laboratories, West Grove, Pa.) and a solution consisting of nitroblue tetrazolium (30 mg) and 5-bromo-4-chloro-3-indolyl phosphate toluidine (15 mg) in 100 ml of 100 mM sodium bicarbonate buffer (pH 9.8) (41). Colony immunoblotting was done with hydrophobic grid membrane filters or nitrocellulose membranes as described by Lightfoot and Lam (43).

Antibiotic sensitivity tests. The test of the cultures for sensitivity to antibiotics was performed using antibiotic-incorporated discs (Becton Dickinson Microbiology Systems, Cockeysville, Md.) by the Kirby-Bauer method as described by Reeves et al. (53). Sensitivity or resistance to an antibiotic was judged by comparison of the size of the zone of inhibition to a standard sensitivity chart (Becton Dickinson).

Outer membrane protein preparations. Outer membranes were isolated from French press lysates of *P. aeruginosa* cells by the one-step sucrose gradient method of Hancock and Carey (24).

Serum sensitivity test. The susceptibility of different *P. aeruginosa* strains to the bactericidal effect of normal human serum was assessed as described previously (61). Strain identity was not revealed until after all tests were performed. Bacteria were grown overnight, washed in Hanks' balanced salt solution (Gibco/BRL, Gaithersburg, Md.) with 0.1% gelatin (gHBSS), and adjusted spectrophotometrically to 10^8/ml in gHBSS. Serum was obtained from five healthy adults, pooled, and frozen at −70°C until use. Approximately 10^6 bacteria were incubated in gHBSS with 10% serum, tumbling, at 37°C for 120 min. All strains were evaluated on three separate days and the means ± standard errors for CFU at 0 and 120 min were calculated. A serum-sensitive strain was defined as one in which ≥90% of the initial inoculum was killed in 120 min. *P. aeruginosa* P1 (serum sensitive) and M2 (serum resistant) were included as controls in all experiments.

Analytical methods. Quantitative colorimetric methods used were that of Chen et al. (10) for phosphate and the periodate oxidation-thiobarbituric acid method for KDO (33). Glycoses were determined by their alditol acetate derivatives (23), using inositol as an internal standard. For analysis of constituent glycoses, core oligosaccharide samples (1 mg) were hydrolyzed with 2 M trifluoroacetic acid (0.5 ml) for 1.5 h at 125°C followed by concentration to dryness. Analytical gas-liquid chromatography–mass spectrometry was done with a Hewlett-Packard 5710A (Series II) system (Mississauga, Ontario, Canada) fitted with a flame ionization detector and an DB-17 column (Chromatographic Specialities Inc., Brockville, Ontario, Canada). The following program was employed: 180°C for 2 min and then 2°/min to 240°C.

For amino acid analysis, core oligosaccharide and LPS samples were hydrolyzed under vapor hydrolysis conditions in 6 N hydrochloric acid–1% phenol for 1 h at 155°C and analyzed on an Applied Biosystems (Mississauga, Ontario, Canada) 420 A/H amino acid analyzer.

RESULTS

Analysis of O3 and O5 mutants. LPS-defective mutants of *P. aeruginosa* serotype O3 (56) and serotype O5 (4, 28, 30, 38) have been reported previously, but the extent of the deficiency in LPS is not clear. In the present study, LPS isolated from serotype O3 and O5 mutants (Table 1) was characterized by SDS-PAGE and Western immunoblots. Strains PAC556, -557, -605, and -608 did not appear to express high-molecular-weight LPS bands in silver-stained gels. The core regions in strains PAC557 and -608 had similar relative mobilities, while the same regions in strains PAC556 and -605 showed higher relative mobilities compared with that of the wild-type core region. Strain PAC605 seemed to be the most core deficient among those analyzed (Fig. 1). PAC609 and -611 closely resembled the wild type in the expression of both the complete core and high-molecular-weight LPS bands (Fig. 1). Among the serotype O5-derived mutants, strain AK1401 (4), which is reported to possess the core plus one O-antigen repeat unit and A-band LPS, showed the presence of some ladder-like LPS bands and a core region with a relative mobility similar to that of the parent strain. The core region of strain AK44 exhibited a slight increase in relative mobility compared with that of the wild-type strain; in addition, some high-molecular-weight LPS bands were also expressed. Strains AK43 and -1012 showed a marked increase in relative mobility of the core region and did not demonstrate the presence of any high-molecular-weight LPS bands in silver-stained LPS gels.

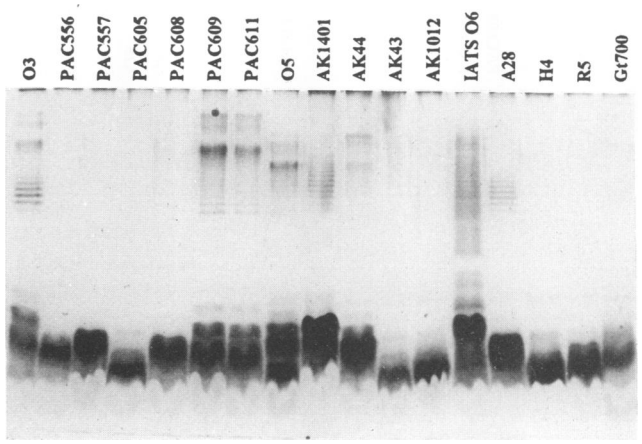

FIG. 1. Silver-stained SDS-PAGE gel of LPS from *P. aeruginosa* serotypes O3, O5, and O6 and LPS-defective mutants. High-molecular-weight LPS bands can be seen in strains O3, PAC609, PAC611, O5, AK1401, AK44, O6, and A28. These bands could be identified as either A-band or B-band LPS by their reactions with specific MAbs.

Western blots of LPS from the O3- and O5-derived mutants were reacted with both O-polysaccharide-specific MAbs and a MAb specific for the A-band common antigen LPS. Western blots of LPS from the O3 mutants reacted with the O3-specific MAb MF57-9 (40) revealed that PAC605, -609, and -611 are leaky, producing O antigen. In addition, PAC556 and -557 were found to produce minute quantities of O3 LPS (Fig. 2). Similarly, blots of LPS from the O5 mutants reacted with the O5-specific MAb MF15-4 (40) demonstrated that AK1012, AK44, and AK43 all expressed O antigen while AK1401 was only weakly positive for O5 LPS expression (Fig. 2). Western immunoblots of LPS from these mutants reacted with the A-band-specific MAb N1F10 (41) revealed that three of the O3-derived mutants, PAC605, -609, and -611, and two of the O5-derived mutants, AK1401 and AK44, expressed A-band LPS (Fig. 3). Interestingly, AK1401 appeared to produce significantly increased amounts of the common antigen compared with that expressed by the O5 wild type. Two of these mutants, namely, AK1012 and AK1401, were chosen for structural elucidation of the complete core of IATS serotype O5 LPS. Comparison of the phosphate and KDO contents in the purified LPS from strain PAO1 and mutants AK1012 and AK1401 indicated a truncation in the core structure in mutant AK1012 (Table 2). Compositional analysis of the LPS core from mutant AK1401 showed that it was composed of D-

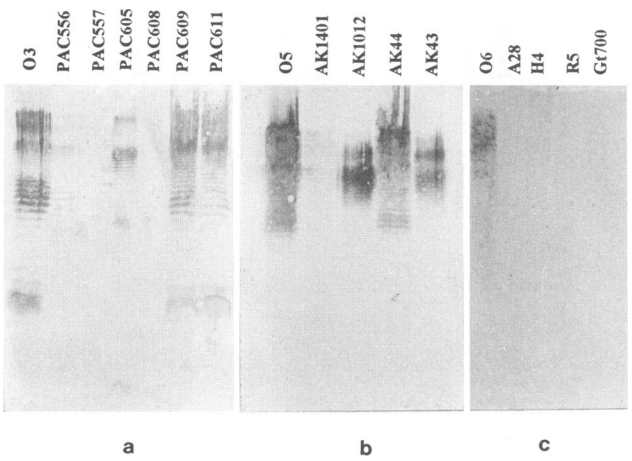

FIG. 2. Western immunoblots of the LPS from the wild-type and mutant strains of *P. aeruginosa* reacted with serotype-specific MAbs. (a) LPS from wild-type serotype O3 and mutants reacted with MAb MF57-9. (b) LPS from wild-type serotype O5 and mutants reacted with MAb MF15-4. (c) LPS from IATS O6 and mutants reacted with MAb MF23-2.

glucose, L-rhamnose, 2-amino-2-deoxy-D-galactose, L-glycero-D-mannoheptose, L-alanine, and KDO (Tables 2 and 3). In contrast, mutant AK1012 was devoid of L-rhamnose and D-glucose, indicating a deficiency in the outer core region of the LPS. Aqueous hydrofluoric acid treatment of the core oligosaccharide gave an increase in the amount of heptose detected; this indicated phosphate substitution on a heptose residue (Table 3).

Isolation of IATS serotype O6 LPS-defective mutants. Several mutagenesis methods were employed in order to generate as broad a panel of rough mutants as possible, with various degrees of LPS deficiency. The transposon Tn*5-751*-induced mutants were designated as the A series of mutants; the H and the R series of mutants were mutants that were found to be resistant to the LPS-specific phages 2 Lindberg and E79, respectively. The Gt series consisted of the gentamicin-resistant mutants. The A and the Gt series were stably maintained with the inclusion of trimethoprim-kanamycin and gentamicin, respectively, in the growth medium. Nineteen mutants were isolated by these three methods and characterized in Western immunoblots (Table 1). Three of these mutants, namely, A28,

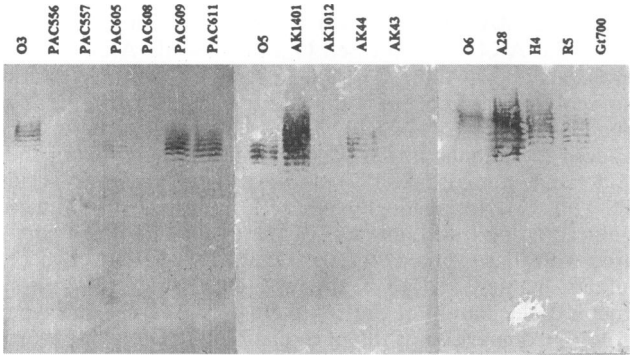

FIG. 3. Western immunoblots of the LPS from wild-type and mutant strains of *P. aeruginosa* reacted with A-band-specific MAb N1F10.

TABLE 2. Phosphorus and KDO content in LPS samples of *P. aeruginosa* serotypes O5 and O6

LPS	%P (wt/wt)	μmol of P/mg of LPS	% KDO
H4	8.00	2.50	4.60
R5	7.70	2.40	5.00
A28	5.00	1.60	4.20
IATS O6	4.90	1.50	2.00
AK1012	6.90	2.10	4.20
AK1401	5.20	1.60	3.80
PAO1	4.30	1.40	3.50

R5, and H4, were selected for structural analysis of the core and for further characterization and comparison with serotype O3- and O5-derived mutants. This selection was based on the mobility of the fastest-migrating bands (i.e., predominantly core LPS bands) observed in Tricine gels (Fig. 4) and the stability of these mutants. Silver-stained gels of the LPS from mutant A28 revealed core region bands with relative mobilities similar to that of the complete core region present in the wild-type parent IATS O6 and some high-molecular-weight LPS bands (Fig. 1). Mutants H4, R5, and Gt700 had core regions migrating faster than that of the wild-type parent, indicating a loss in certain core components (Fig. 1). Western immunoblots of LPS from these mutants reacted with O6-specific MAb MF23-2 were negative (Fig. 2), thus validating the results obtained in the colony immunoblots done earlier (data not shown); blots reacted with A-band-specific MAb N1F10 illustrated the presence of A-band LPS in mutants A28, H4, and R5, while Gt700 remained negative (Fig. 3). Western immunoblots reacted with MAbs are sensitive means of detecting small amounts of LPS which remain undetected in silver-stained SDS-PAGE gels. Hence, even though we could not detect any high-molecular-weight LPS bands in silver-stained gels of LPS isolated from mutants H4 and R5 (Fig. 1), A-band LPS could be detected in immunoblots reacted with MAb N1F10 (Fig. 3).

Characterization of the IATS serotype O6 LPS core. Tricine SDS-PAGE gels were used in addition to SDS-PAGE gels for a better resolution of the core and lipid A regions. The relative electrophoretic mobilities of the fast-migrating bands from the LPS of mutant A28 were similar to those of the core LPS bands of IATS serotype O6 (Fig. 4, arrow), indicating that A28 likely has a complete core. Mutant R5 showed a faster-migrating band, indicating sugar deletions in the core; mutant H4 presumably had the highest extent of deficiency in the core region as reflected by the highest mobility of the fast-moving band in the Tricine gel when compared with those of the LPS bands of the other strains. Purified LPS from the wild type and mutant strains H4, R5, and A28 was analyzed for phosphate and KDO contents. The percentage of phosphate and KDO in the LPS from the wild-type O6 strain and the mutants indicated progressive truncation of the core structure (Table 2). Partial acid hydrolysis of the LPS of mutants with dilute aqueous acetic acid gave insoluble lipid A and core oligosaccharide fractions which were subsequently purified by gel permeation chromatography. Compositional analysis of the LPS core from mutant A28 showed that it was composed of D-glucose, L-rhamnose, 2-amino-2-deoxy-D-galactose, L-glycero-D-manno-heptose, L-alanine, and KDO (Tables 2 and 3). In comparison, mutant R5 contained significantly less D-glucose and no L-rhamnose, while mutant H4 was completely devoid of D-glucose and L-rhamnose (Table 3). All of the mutants contained 2-amino-2-deoxy-D-galactose, KDO, and

TABLE 3. Chemical analysis of the core oligosaccharides of *P. aeruginosa* O5 and O6 mutants

Component	Concn (μmol/mg of PS[a]) in strain:									
	A28		R5		H4		AK1401		AK1012	
	Untreated	HF treated[b]	Untreated	HF treated	Untreated	HF treated	Untreated	HF treated	Untreated	HF treated
L-Rha	0.32	0.37	0.00	0.00	0.00	0.00	0.28	0.45	0.00	0.00
D-Glc	1.04	1.46	0.69	0.83	0.00	0.00	1.26	1.76	0.00	0.00
D-GalN	0.06	0.12	0.20	0.27	0.22	0.45	0.05	0.08	0.06	0.13
L-D-Hep	0.09	0.59	0.06	0.97	0.12	0.78	0.18	0.83	0.13	1.52

[a] PS represents the dry weight of polysaccharide.
[b] HF treated denotes polysaccharides that were hydrolyzed with hydrofluoric acid for dephosphorylation.

L-alanine. Treatment of the core oligosaccharide samples with aqueous HF prior to sugar analysis gave an increase in the amount of heptose detected. This suggested phosphate substitution on a heptose residue (Table 3).

The structures of deacylated LPS obtained from mutants H4, R5, and A28 were investigated in detail using high-field nuclear magnetic resonance, fast atom bombardment-mass spectrometry, and methylation analysis. A common basal structure consisting of an α-D-Gal*p*N-(1-3)-L-α-D-Hep*p*-(1-3)-L-α-D-Hep*p* trisaccharide unit linked to a lipid A via a branched bis-KDO moiety was found to be representative of the mutant H4 LPS core component. The terminal 2-amino-2-deoxy-D-galactose residue was N acylated by L-alanine, and it served as the point for further substitution in the LPS from mutants R5 and A28. In addition to the L-glycero-D-manno-heptose and 2-amino-2-deoxy-D-galactose residues present in the inner core region, the LPS core of mutant A28 contained D-glucose (three residues) and L-rhamnose (one residue). The detailed structural elucidation of the IATS serotype O6 LPS is reported elsewhere (48).

Serum sensitivity. Rough mutants of the O3, O5, and O6 series were subjected to serum sensitivity tests, which should reveal the effect of O-antigen deficiency on the susceptibility of these strains to the bactericidal effect of serum components. The wild-type O3 strain grew in the presence of 10% human serum from 1.08×10^5 to 2.97×10^5, showing an increase of approximately threefold in CFU, while rough mutants PAC556, -557, -605, and -608 (all B^-) were all killed after 120 min of exposure to human serum. As expected, mutants PAC609 and -611, which express B-band LPS, were resistant to serum after 120 min of exposure (Fig. 5a). The wild-type O5 strain grew in the presence of 10% human serum from 2.3×10^5 to 3.5×10^5, while strain AK1401 ($A^+ B^-$) was completely killed. Not surprisingly, strain AK44 and strain AK1012, which were shown earlier to be leaky mutants expressing B-band LPS, were found to be resistant and partially resistant, respectively, to serum after 120 min of exposure (Fig. 5b). Among the O6 strain and its rough mutants, the parent O6 strain was partially resistant to serum; the viability of O6 cells decreased from 3.1×10^5 to 2.1×10^5. All the O6-derived mutants were serum sensitive, with little to no growth after 120 min of exposure to serum (Fig. 5c). Strains P1 and M2 were controls included in all three sets of serum sensitivity determinations, and they were serum sensitive and serum resistant, respectively, as expected (Fig. 5).

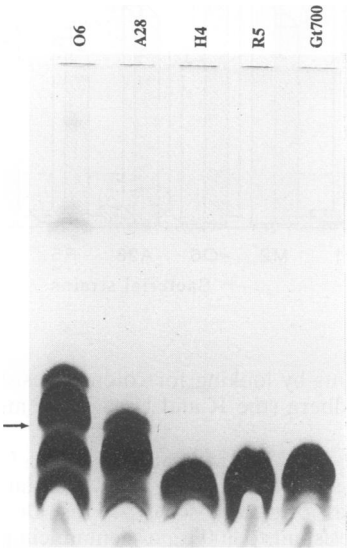

FIG. 4. Silver-stained Tricine SDS-PAGE gel of the LPS from IATS O6 and derived mutants for analysis of the LPS core lipid A region. The high-molecular-weight LPS bands do not resolve in this gel system and hence are not visible as a distinct ladder-like profile. The arrow indicates the LPS band of mutant A28, corresponding to a complete core.

DISCUSSION

The core region of *P. aeruginosa* LPS has been much investigated in attempts to devise an immunoassay for detection of this organism, especially in pathological samples, and in mouse protection studies (19, 49, 57, 63). The structure of the LPS core oligosaccharide and/or lipid A is presumed to play a role in both cross-protection against other serotypes and the conformation of the LPS molecule (65). All of the studies with MAbs directed towards the LPS core oligosaccharide (13, 25, 49, 71) have shed some light on the possible location of common epitopes and the composition of the LPS core in a limited number of strains, namely, of serotypes O3 and O5; however, an accurate model of the core structure has not been clearly defined. Stable LPS-defective mutants are an absolute necessity in such studies. LPS-defective (R) mutants of the family *Enterobacteriaceae* synthesize incomplete LPS and are recognized by their rough colony morphology (47). LPS-specific bacteriophages are generally used in the isolation of such mutants, which can be further characterized by using R-specific phages (70). A similar approach has not been possible for *P. aeruginosa* because of a lack of R-specific phages.

Rowe and Meadow (56), investigated the structure of the core oligosaccharide of strain PAC1R and its LPS-defective mutants. They reported that mutants PAC556, -557, and -605 all lacked the O-antigenic side chains; in addition, PAC605 was

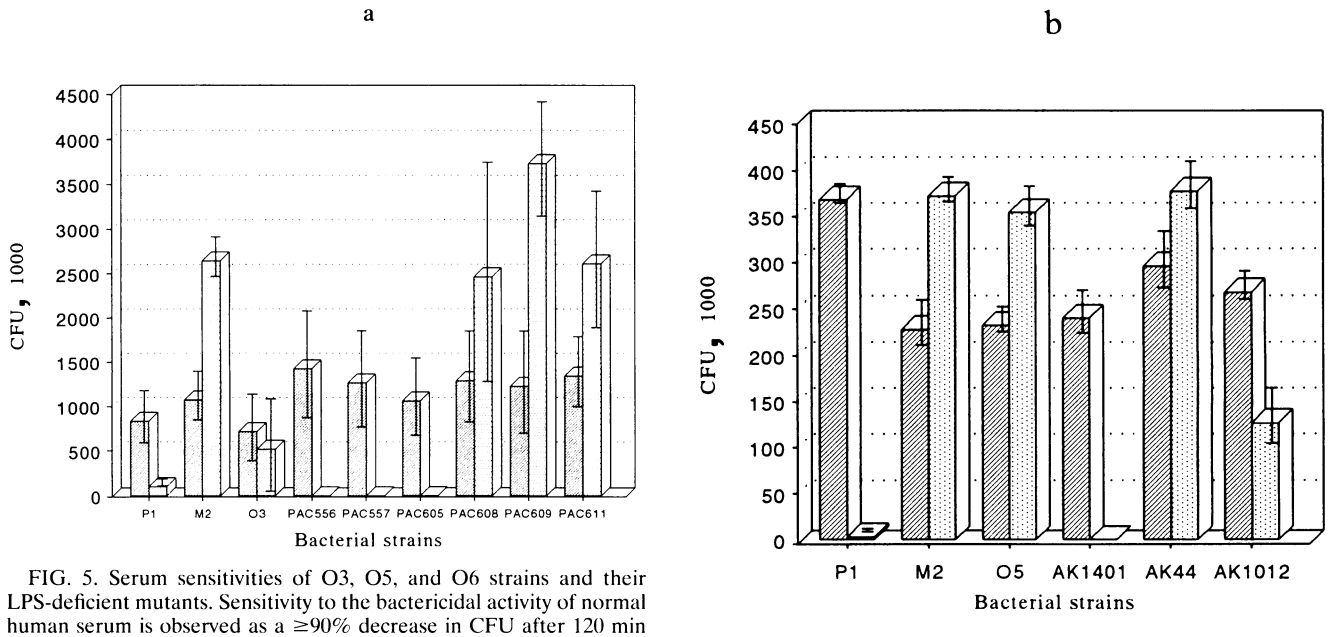

FIG. 5. Serum sensitivities of O3, O5, and O6 strains and their LPS-deficient mutants. Sensitivity to the bactericidal activity of normal human serum is observed as a ≥90% decrease in CFU after 120 min of exposure to serum. The data presented are mean values of three experiments, and the calculated standard errors are shown as error bars. Strains P1 and M2 are included as serum-sensitive and serum-resistant controls, respectively. (a) O3 wild-type strain and rough mutants; (b) O5 wild-type strain and rough mutants; (c) O6 wild-type strain and rough mutants. ▨, 0 min; ▩, 120 min.

reported to be the most defective mutant, containing heptose as its sole neutral sugar in the core fraction. In our present studies we could not visualize any high-molecular-weight LPS in silver-stained gels of LPS preparations isolated from these three mutants (Fig. 1); however, in Western immunoblots reacted with O3-specific MAb MF57-9, we could detect traces of O-antigenic bands in PAC556 and, surprisingly, in PAC605 (Fig. 2), even though the core region in PAC605 appeared to be migrating faster than those of the other serotype O3-derived mutants analyzed in SDS-PAGE gels (Fig. 1). The production of B-band LPS by both strains PAC605 and -556 suggests that these strains are leaky, unlike the earlier study in which the production of O-antigenic sugars was not detected (56). In Western blots, mutants PAC609 and PAC611 reacted with the B-band-specific MAb. These results are consistent with those observed by Rowe and Meadow (56), who also found that mutants PAC609 and PAC611 were leaky and produced O antigen.

Rough colonial morphology could not always be related to defective LPS in *P. aeruginosa* (36), unlike observations for members of the *Enterobacteriaceae*. One of the LPS-defective mutants reported earlier, PAC556, was isolated by selection for defective glucose metabolism (17); attempts to isolate more mutants by this method were unsuccessful. Sensitivity to deoxycholate is associated with defective LPS in some mutants of members of the *Enterobacteriaceae* (58). In our preliminary experiments we could not detect any such change in sensitivity to deoxycholate; similar observations were also made by Koval and Meadow (36) in their studies with LPS-defective mutant PAC556. Resistance to smooth-LPS-specific phages is a commonly used method for the isolation of LPS-defective mutants (38). In this study, two different phages were used to determine whether we could isolate rough mutants with different degrees of deficiencies in their LPS. We were successful in isolating defective mutants by looking for colonies resistant to phages E79 and 2 Lindberg (the R and H series of mutants, respectively).

Galbraith et al. (18) reported loss of the O antigen in a gentamicin-resistant mutant of a clinical isolate of *P. aeruginosa* which was originally serotyped as O6. This variant, P28-800, was resistant to 800 μg of gentamicin per ml and had a core oligosaccharide similar to that of the parent strain. In our studies, the Gt series of mutants were found to be devoid of the O-specific polymer and part of the core sugars. Kadurugamuwa et al. (32) investigated the interaction of gentamicin with the A- and B-band LPS of *P. aeruginosa* PAO1 (serotype O5). Their data indicated that the affinity of gentamicin

binding to bacteria was higher in strains possessing B-band than in strains with A-band LPS. The strains with the B-band (or smooth) LPS were more prone to the lethal effects of gentamicin. The difference in affinity explains the increased resistance to gentamicin in our Gt series of mutants, which also lacked the B-band LPS. This observation is significant especially when one considers antibiotic therapy in cystic fibrosis cases, in which a progressive loss of O antigen is observed in *P. aeruginosa* isolates (41). In a study by Shearer and Legakis (60), an altered LPS structure was observed in *P. aeruginosa* isolates that were sensitive to carbenicillin and gentamicin; their results supported the hypothesis that a primary mutation involving LPS, in combination with some undefined secondary mutation, determined the permeability of the outer membrane to carbenicillin and gentamicin. In the present investigation, the characteristics of the A series of mutants appeared to be similar to those of the group B mutants isolated by Shearer and Legakis (60); these mutants lacked long-chain LPS and became sensitive to 100 μg of carbenicillin as well as 10 μg of gentamicin (data not shown).

We could not observe any significant changes in the growth rate and colony morphology in the A, R, and H series of mutants. However, the gentamicin-resistant mutants showed smaller colonies and had a slower growth rate compared with the wild-type parent (data not shown). Rough colony morphology was not observed in any of our mutants. Comparisons of the outer membrane protein profile of the mutants and the wild-type O6 strain did not reveal any qualitative difference in the major outer membrane protein banding profile in SDS-PAGE gels (data not shown); this probably indicates that the altered characteristics in the mutants are due to a change in their LPS profiles.

Results from the serum sensitivity tests correlated well with the SDS-PAGE and Western immunoblot analysis. Strains PAC609 and -611, which produce B-band LPS (Fig. 2), were serum resistant (Fig. 5a), while strains PAC556, -557, and -605, which do not express B-band LPS (Fig. 2), were serum sensitive (Fig. 5a). Both strains AK44 and AK1012, which were shown to be leaky and to produce B-band LPS (Fig. 2), were serum resistant and only partially sensitive, respectively (Fig. 5b). Mutant AK1401 produces A-band LPS but remains serum sensitive. The group of O6-derived mutants, which all lacked B-band LPS (Fig. 1 and 2), were sensitive to serum; A-band-producing mutant A28 remained sensitive to serum. Thus, it appears that the A-band common LPS antigen does not play a role in protecting the organisms against serum-mediated killing. Another interesting observation was the difference in serum sensitivity between O5 and O6 parent strains. The O5 strain was serum resistant, while the O6 strain was partially sensitive. This observation is consistent with our previous observations in an ultrastructural study (39) that the B-band LPS molecules of the O5 strain form an extensive fibrous coat on the outer leaflet of the outer membrane while the B-band LPS of the O6 strain could be discerned only as patches of fibrous material on the bacterial surface which apparently does not provide sufficient protection for the cells against serum components. A similar correlation between serum sensitivity and the presence of the O antigen was observed by Grossman et al. (22) in their investigation of *Salmonella montevideo* SL5222 and by Porat et al. (52) in a study with *E. coli*. Their results indicated that the O-antigen polysaccharide protected the cells from serum-mediated killing by sterically preventing access of the complement to the outer membrane. In our studies, comparison of the serum sensitivities of strains O5 and O6 indicates that the distribution of the LPS molecules bearing the long O-Antigen side chains also influences the serum sensitivity.

The structural component determinations for the O5- and O6-derived mutants were in agreement with the observed relative mobilities of the LPS bands in the gels. Strains H4, R5, and AK1012, which all exhibited a faster-migrating LPS band in silver-stained gels (Fig. 1), were clearly shown to be lacking L-rhamnose and have diminished amounts of D-glucose (Table 3). The absence of L-rhamnose in mutants R5, H4, and AK1012 indicates a deficiency in the core region, while the presence of this sugar in A28 and AK1401 indicates a complete LPS core in these strains. The absence of D-glucose in mutants H4 and AK1012 confirms a defective outer core region in the LPS of these mutants. These observations have been further verified by structural studies using high-field nuclear magnetic resonance techniques (2, 48). As one would expect, because of the lack of O-polysaccharide sugars in the LPS of the rough mutants, the relative amounts of phosphorous and KDO in these core-deficient mutants were proportionally higher than in their wild-type parent strains (Table 2). The phosphorus content in the LPS from PAO1 and IATS O6 at 4.3 and 4.9% (wt/wt), respectively, indicated a high level of phosphorylation in the LPS of these two serotypes. However, these observed values are within the ranges of 3.6 to 5.6% (wt/wt) found in the LPS of several Habs serotypes of *P. aeruginosa* reported by Chester et al. (11). Relative to that of the corresponding wild-type parent strains, the LPS of AK1401 and AK1012 (O5 mutants) and A28, H4, and R5 (O6 mutants) had, on a weight basis, increased amounts of phosphorus. These results are consistent with the fact that strains AK1012, H4, and R5 are deficient in core components and hence show proportionally elevated levels of phosphorus. SDS-PAGE analysis of the LPS from these mutants supports the data obtained by chemical analysis of the corresponding LPS. The KDO content in the LPS of PAO1 is higher than that in IATS O6; the 2% KDO content in IATS O6 is lower than the reported average range of 2.2 to 4.8% observed in the Habs serotypes and PAC strains analyzed by other workers (11, 56, 69). The differential deficiency of sugars in the LPS among the mutants A28, R5, and H4 has been effectively exploited for the elucidation of the O6 core structure (48).

The importance of LPS as a virulence factor has led to significant research in the pharmaceutical industry directed towards the design of inhibitors of CMP-KDO synthase (9). Goldman et al. (21) targeted the KDO pathway by using 8-amino (compound 3) and 8-aminomethyl-2-deoxy-KDO (compound 6) analogs; though the effects of these compounds on enteric bacteria and *P. aeruginosa* were essentially the same, there were species-specific differences between the two organisms when the pathways of fatty acid and KDO addition were inhibited. Thus, information on the core structure would be significant in the study of LPS core synthesis, which could ultimately be used in drug design studies directed at different organisms. Structural information would also be necessary for the preparation of antigens and vaccines, which could be used for immunoprophylaxis, diagnosis, and therapy of *P. aeruginosa* infections.

In conclusion, this study reports a comprehensive comparison of some of the better known (i.e., serotypes O3- and O5-derived) LPS-defective mutants of *P. aeruginosa* with respect to their ability to express the common A-band and serotype-specific B-band antigens. We have been able to generate a group of stable IATS O6-derived mutants defective in LPS synthesis, which have been used in the analysis of the O6 core structure.

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