Structural Elucidation of the Lipopolysaccharide Core Region of the O-Chain-Deficient Mutant Strain A28 from *Pseudomonas aeruginosa* Serotype 06 (International Antigenic Typing Scheme)[†]

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The lipopolysaccharide (LPS) of the *Pseudomonas aeruginosa* serotype 06 rough-type mutant A28 was isolated by a modified phenol-chloroform-petroleum ether extraction method. Deoxycholate-polyacrylamide gel electrophoresis indicated a single band with mobility similar to that of the complete core region of the wild-type parent serotype 06 (International Antigenic Typing Scheme) strain. Compositional analysis of the LPS indicated that the core oligosaccharide was composed of D-glucose (three units), L-rhamnose (one unit), 2-amino-2-deoxy-D-galactose (one unit), L-glycero-D-manno-heptose (two units), 3-deoxy-D-manno-octulosonic acid (two units), L-alanine (one unit), and phosphate (two units). Under the mild conditions of hydrolysis with methanolic hydrogen chloride, a 7-O-carbamoyl substituent was observed on the second heptose residue. The glycan structure of the LPS was determined by employing one- and two-dimensional nuclear magnetic resonance spectroscopy and mass spectrometry-based methods with a backbone oligosaccharide that was obtained from the LPS by deacylation, dephosphorylation, and reduction of the terminal glucosamine. On the basis of the results of the present study and our earlier work with the *P. aeruginosa* 06-derived core-defective mutant R5 (H. Masoud, E. Altman, J. C. Richards, and J. S. Lam, *Biochemistry*, 33:10568–10578, 1994), a structural model for the complete core oligosaccharide is proposed.

Pseudomonas aeruginosa is an opportunistic pathogen that can cause fatal infections in immunocompromised or debilitated individuals, including those with severe burn wounds, cystic fibrosis, and cancer (1, 36). Lipopolysaccharide (LPS), an integral component of the outer membrane, is the most immunoreactive surface antigen of gram-negative bacteria. It is responsible for determining the O-antigen groups of bacteria and is implicated as a virulence factor involved in the pathogenesis of bacterial infections (13, 14, 23, 33). LPS of *P. aeruginosa* possesses a molecular architecture similar to that of members of the family *Enterobacteriaceae*, being composed of a hydrophilic polysaccharide region that is linked to hydrophobic lipid A via a core oligosaccharide (41). Twenty major serotypes of *P. aeruginosa* have been described on the basis of the structural diversity of their O antigens (30, 31). In order to understand the biochemistry, immunochemistry, and biosynthesis of LPS as well as to provide for future purposes a possible basis for developing specific reagents for intervention in *P. aeruginosa* infections, the complete and detailed structure of the LPS should be defined. Chemical structures of the O-antigen polysaccharides from 17 (International Antigenic Typing Scheme [IATS]) standard serotypes and of lipid A from *P. aeruginosa* LPS have been reported previously (22, 24). Until recently (34), only partial or tentative structures of the core regions of a few strains of *P. aeruginosa* had been reported (18, 27, 38). The complete and detailed structure is still unknown.

The core region of *P. aeruginosa* consists of an inner core

composed of 3-deoxy-D-manno-octulosonic acid (KDO), L-glycero-D-manno-heptose, and phosphate and an outer core composed of D-glucose, D-galactosamine, L-rhamnose, and L-alanine (15, 28). As expected, de Kievit and Lam (16), using monoclonal antibodies (MAbs) raised against core oligosaccharide (OS) of *P. aeruginosa*, found that the inner core region was more conserved among different serotypes than the outer core region.

Of the 20 *P. aeruginosa* serotypes (IATS), serotype 06 is the most prevalently encountered group of strains isolated from clinical sources. Interestingly, its LPS was also found to be the major protective component in the commercial polyvalent antipseudomonas vaccine PEV-01 (32). In order to facilitate the structural study of the core region, rough mutants, including strain R5 (core deficient), strain H4 (core deficient), and strain A28 (complete core), derived from *P. aeruginosa* serotype 06 were characterized and the constituent components of the LPS core OS were determined (15).

We have recently reported the partial elucidation of the core OS structure of *P. aeruginosa* 06 LPS based on LPS prepared from the core-deficient mutant strain R5 (34). In the present investigation core OS from the O-chain-deficient mutant strain A28 was used to elucidate the complete core structure of *P. aeruginosa* serotype 06 LPS.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. aeruginosa* IATS serotype 06 rough-type mutants were generated by transposon Tn5-751 mutagenesis (mutant A28) or by selecting mutant strains resistant to smooth-type LPS-specific phages E79 (mutant R5) and 2 Lindberg (mutant H4) (15). Bacterial strains were cultivated aerobically by using Trypticase soy broth-BBL medium in 25- and 75-liter fermentors (New Brunswick Scientific) at 37°C, and cultures were harvested in the stationary phase.

Isolation of LPS and core OS. LPS from *P. aeruginosa* IATS serotype 06

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mutant strains H4, R5, and A28 was isolated by a modified phenol-chloroform-petroleum ether method (9) and purified by repeated ultracentrifugation ($105,000 \times g$, 4°C , 4 h). Core OS was obtained from LPS by mild acid hydrolysis as described earlier for mutant R5 (34). Dephosphorylation of core OS was achieved with 48% aqueous HF (1 ml) at 4°C for 48 h, and this procedure was followed by evaporation under a stream of nitrogen.

Analytical methods. Glycoses were quantitated by gas-liquid chromatography (GLC) of their alditol acetates or *R*-2-butyl-glycoside derivatives as described earlier (34). Quantitative colorimetric methods used were that of Chen et al. (11) for phosphate and the periodate oxidation-thiobarbituric acid method for KDO (21). 7-*O*-Carbamoyl-*L*-glycero-*D*-manno-heptose was quantitated according to the method of Beckmann et al. (4). Briefly, LPS (5 mg) was dephosphorylated with aqueous 48% HF (1 ml) at 4°C for 48 h, dried under a stream of nitrogen, and treated with methanolic hydrogen chloride (2 M; 1 ml) at 85°C for 30 min. Following neutralization with Ag_2CO_3 and removal of excess methanol, the products were methylated with iodomethane in dimethyl sulfoxide containing NaOH (12). The mixture of permethylated methyl glycosides was analyzed by GLC-mass spectrometry (MS) directly or, following hydrolysis with 4 M trifluoroacetic acid (120°C , 1 h), as the reduced alditol acetate derivatives. The separated derivatives were identified by electron impact MS on a Hewlett-Packard 5958B or a Varian IonTrap GLC-MS system.

Methylation analysis. OS samples (2 mg) were methylated with methyl iodide in dimethyl sulfoxide containing an excess of potassium (methylsulfinyl) methanide, and the products were hydrolyzed and analyzed as their partially methylated reduced alditols by GLC-MS (34).

Preparation of LPS backbone OS. The backbone OS of the *P. aeruginosa* IATS serotype 06 mutant A28 was prepared according to the deacylation procedure of Holst et al. (20) as modified by Masoud et al. (34). For nuclear magnetic resonance (NMR) analysis the backbone OS was further purified by high-performance anion-exchange chromatography (HPAEC) on a Dionex Bio-LC system (35). Fractions were collected manually, neutralized directly with aqueous acetic acid (10%), and finally lyophilized. Salts were removed by application of the material to a Biogel P-2 (Bio-Rad) gel filtration system (2.6 by 140 cm) and eluted with pyridinium acetate (0.05 M; pH 4.5), and collected fractions (4.5 ml) were assayed colorimetrically for neutral glycoses by the phenol-sulfuric acid method (19).

DOC-PAGE. Polyacrylamide gel electrophoresis (PAGE) was performed by using the system of Laemmli and Favre (29) as modified by Komuro and Galanos (26) with deoxycholate (DOC) as the detergent. The separation gel contained final concentrations of 13% acrylamide, 0.5% DOC, and 375 mM Tris-HCl (pH 8.8), with the stacking gel containing 4% acrylamide, 0.5% DOC, and 125 mM Tris-HCl (pH 6.8). LPS samples were prepared at a concentration of 0.1% (wt/vol) in the sample buffer (0.25% DOC, 175 mM Tris-HCl [pH 6.8], 10% glycerol). Bromophenol blue (0.002% in sample buffer) was used as the tracking dye. The electrode buffer (pH 8.4) was composed of DOC (2.5 g/liter), glycine (14.4 g/liter), and Tris (3.0 g/liter). Electrophoresis was performed at a constant current of 30 mA. Immediately after the electrophoresis run, the gel was soaked in an aqueous solution containing 40% ethanol and 5% acetic acid with gentle shaking. LPS bands were stained and visualized by silver staining as described by Tsai and Frasch (39).

NMR spectroscopy. NMR spectra were obtained on a Bruker AMX 500 spectrometer using standard Bruker software. Measurements were made at 32°C with a solution containing 2.5 mg of material dissolved in 0.5 ml of D_2O (pH 8.8) subsequent to several lyophilizations with D_2O . Two-dimensional (2D) homonuclear proton correlation (correlated spectroscopy [COSY]), nuclear Overhauser effect (NOE) (2D NOE spectroscopy), and heteronuclear ^{13}C - ^1H chemical shift correlation (heteronuclear multiple quantum coherence [HMQC]) experiments were performed as previously described (35). Proton and ^{13}C chemical shifts were referenced to that of the methyl resonances of internal acetone (δ_{H} , 2.225 ppm; δ_{C} , 31.07 ppm).

Electrospray MS. Samples were analyzed on a VG Quattro triple quadrupole mass spectrometer (Fisons Instruments) with an electrospray ion source. OS samples were first dissolved in H_2O and then diluted 1:1 with a solvent composed of acetonitrile, H_2O , MeOH, and 10% ammonia (4:4:1:1). The sample was injected by direct infusion at 4 $\mu\text{l}/\text{min}$ with a Harvard syringe pump 22. The electrospray tip voltage was 3.5 kV, and the mass spectrometer was scanned from m/z 50 to m/z 2,500 with a scan time of 10 s. Data were collected in multichannel analysis mode, and data processing was handled by the VG data system (Masslynx).

RESULTS

Isolation and characterization of rough-type mutants. Rough-type mutants of *P. aeruginosa* IATS serotype 06 were generated by transposon mutagenesis (mutant A28) or by means of phage infection (mutants H4 and R5) (15). LPS was isolated by a modified phenol-chloroform-petroleum ether extraction method (9) in a yield of ca. 5% from the dried bacterial cells. DOC-PAGE analysis of LPS from these serotype 06-derived mutants indicated that all were completely devoid

FIG. 1. DOC-PAGE pattern of LPS from *Salmonella* strains and *P. aeruginosa* IATS 06. Lane 1, *S. milwaukee* (S-type LPS; 10 μg); lane 2, *S. minnesota* (Re mutant; 2 μg); lane 3, *P. aeruginosa* mutant A28 (2 μg); lane 4, *P. aeruginosa* mutant R5 (2 μg); lane 5, *P. aeruginosa* mutant H4 (2 μg); lane 6, *P. aeruginosa* wild-type strain (10 μg). The vertical arrow indicates the direction of migration.

of O-antigen polysaccharide; each showed bands corresponding to rough LPS composed of a lipid A and low-molecular-weight core OS (Fig. 1, lanes 3 to 5). This gel system was used instead of the standard sodium dodecyl sulfate-PAGE because it provided improved resolution of the low-molecular-weight (rough) LPS and, in contrast to a Tricine PAGE system (16), provided good resolution between bands from both smooth and rough LPSs. LPS from mutant A28 showed a single band that exhibited electrophoretic mobility similar to that of the complete core region present in the wild-type parent strain (Fig. 1, lanes 3 and 6), suggesting that LPS from this mutant contains a complete core. LPSs from mutant strains R5 and H4 each showed single-band patterns with relative mobilities consecutively faster than that of the complete core, indicating several sugar deletions in the core regions. The structure of the core region of the R5 LPS was recently determined in detail (34).

Composition analysis. Cleavage of the KDO ketosidic linkages by treatment of intact LPS with dilute aqueous acetic acid afforded a water-insoluble lipid A and soluble products. The concentrated water-soluble products were fractionated and purified by size exclusion chromatography on a Biogel P-2 gel filtration system which gave fractions containing monomeric KDO and core OS. In a recent study (15), the core OS of the A28 LPS was found to be composed of *L*-rhamnose (*L*-Rha), *D*-glucose (*D*-Glc), 2-amino-2-deoxy-*D*-galactose (*D*-GalN), and *L*-glycero-*D*-manno-heptose (*LD*-Hep), which were identified by GLC-MS analysis of the corresponding alditol acetate and *R*-2-butyl glycoside derivatives. In addition, KDO, *L*-alanine (*L*-Ala), and phosphate were identified by colorimetric analyses. Compositional analysis indicated the absence of *L*-Rha and a diminished amount of *D*-Glc in mutant R5 LPS and the complete absence of both *L*-Rha and *D*-Glc residues in mutant H-4 (15). In the present study, it was found that permethylation of the methanolysis product of dephosphorylated LPS (from mutants H4, R5, and A28) afforded the expected mixture of methylated methyl glycosides together with the corresponding derivative of 7-*O*-carbamoyl-*L*-glycero-*D*-manno-heptopyranoside, which was identified by GLC-MS analysis (Fig. 2A). Carbamoyl substitution at the *O*-7 position of heptose was verified from the MS fragmentation pattern of the derived

FIG. 2. Electron impact mass spectra of methyl 7-*O*-carbamoyl-L-glycero-D-manno-heptopyranoside (A) and the derived alditol acetate (B), measured following GLC separation. The MS fragmentation patterns are shown in the inserts.

alditol acetate derivative of the permethylated methyl glycoside (Fig. 2B).

Glycosyl linkage analysis of mutant A28. The core OS and its dephosphorylated analog were subjected to methylation analysis (Table 1), which indicated that the OS component contained 3,6-di-*O*-substituted Glcp, 3,4-di-*O*-substituted GalpN, 3-*O*-substituted Hepp, and terminal Glcp and Rha residues. Results from analysis of the native core OS revealed significant

TABLE 1. Methylation analysis of core OS and the dephosphorylated analog of *P. aeruginosa* serotype 06 mutant A28 LPS

Methylated product	Relative detector response ^a	
	Core OS	Dephosphorylated core OS
2,3,4-Me ₃ -Rha	0.52	0.22
2,3,4,6-Me ₄ -Glc ^b	1.00	1.00
2,4-Me ₂ -Glc	0.63	0.31
2,4,6,7-Me ₄ -Hep	1.01	1.35
4,6,7-Me ₃ -Hep	0.70	0.00
6,7-Me ₂ -Hep	0.48	0.00
2,6-Me ₂ -GalN	0.78	0.20

^a Relative to the response with 2,3,4,6-Me₄-Glc, which is set at 1.00.

^b 2,3,4,6-Me₄-Glc corresponds to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol-d₁, etc.

FIG. 3. ¹H NMR spectrum of A28 backbone OS after HPAEC purification recorded in D₂O at pH 8.8 and 32°C. The H-3e doublet of KDOII overlaps the methyl resonance of an internal acetone reference, but it was fully resolved in the full-scale COSY experiment. The sharp resonance at 1.90 ppm is from the methyl group of an acetate impurity.

amounts of 6,7-di-*O*-methyl- and 4,6,7-tri-*O*-methyl-heptose among the hydrolysis products. The di- and tri-*O*-methyl-heptose derivatives were absent from the hydrolysis products of the methylated dephosphorylated core OS, while the relative proportion of 2,4,6,7-tetra-*O*-methyl-heptose was increased. This indicated that one of the 3-*O*-substituted heptoses is phosphorylated at the O-2 and O-4 positions.

NMR analysis of the A28 backbone OS. The backbone OS, obtained from mutant A28 LPS by deacylation, dephosphorylation, and reduction of the terminal glucosamine, was examined in detail by NMR spectroscopy. Several minor signals were observed in the anomeric region of the ¹H NMR spectrum (5.5 to 4.4 ppm) of this sample (data not shown), indicating considerable structural heterogeneity. These minor signals were absent from the spectrum of the OS following further purification of the sample by HPAEC. All subsequent NMR experiments were performed with the HPAEC-purified sample.

The one-dimensional (1D) ¹H NMR spectrum of HPAEC-purified backbone OS (Fig. 3) showed nine resonances of approximately equal signal areas in the low-field region (5.5 to 4.4 ppm), of which eight were attributed to resonances from anomeric protons by direct correlation with attached ¹³C resonances in a 2D heteronuclear ¹³C-¹H correlation (HMQC) spectrum (Fig. 4).

A pair of overlapping resonances at 1.30 to 1.33 ppm in the high-field region of the 1D ¹H NMR spectrum (Fig. 3) could be attributed to the methyl protons of L-alanine and L-rhamnose. This was confirmed by a COSY experiment which showed cross-peaks to proton resonances attached to the α-carbon of L-alanine (3.55 ppm) and to H-5 (3.74 ppm) of L-rhamnose, respectively. Equatorial (2.10 and 2.22 ppm) and axial (1.98 and 1.78 ppm) methylene protons (H-3e and H-3a, respectively) from two α-linked pyranosyl KDO residues (10, 34, 35, 40, 42) were also observed in the high-field region of the spectrum (Fig. 3).

The ¹H NMR spectrum of the backbone OS was fully assigned (Fig. 5; Table 2) by 2D homonuclear chemical shift correlation techniques (COSY), and the relative stereochemistries and ring sizes of the component monosaccharides were established from the ¹H chemical shifts (8) and the magnitudes of the proton coupling constants (3). Assignment of the ¹³C resonances (Table 3) was carried out by direct correlation of the ¹H resonances with the ¹³C resonances in an HMQC experiment and by comparison of ¹³C resonances with similar chemical shift data (6, 7, 34).

Large vicinal proton coupling constants for *J*_{2,3}, *J*_{3,4}, and *J*_{4,5} (8 to 10 Hz) indicated the presence of four hexopyranosyl residues having the *gluco* configuration (labelled GI to GIII and GNII). From the magnitude of the *J*_{1,2} couplings, two of these residues (GI and GII) were assigned the α-D configuration (*J*_{1,2} ≈ 3.5 Hz), while the other two residues (GIII and

FIG. 4. Heteronuclear 2D ^{13}C - ^1H chemical shift correlation map of the anomeric region of the A28 backbone OS. Assignments are indicated by the following abbreviations: GI to GIII, Glc; HI and HII, Hep; GaN, GalN; R, Rha; and GNII, GlcN.

GNII) were assigned the β -D configuration ($J_{1,2} \approx 8$ Hz). The H-2 resonance of GNII (2.72 ppm) was directly correlated with a ^{13}C resonance at 57.6 ppm in the HMQC experiment, indicating that it was a glucosamine residue (6). Three other ^{13}C resonances in the region (50 to 60 ppm) characteristic of amino-substituted carbons were observed in the HMQC spectrum. A ^{13}C resonance at 50.5 ppm correlated with the H-2 resonance (4.52 ppm) from the α -D-galactosamine (GalN), of which the ^1H spin system was identified on the basis of the characteristic vicinal ring coupling constant pattern, which was

FIG. 5. Contour plot of the 2D COSY of the ring proton region (4.6 to 3.1 ppm) of the A28 backbone OS. Cross-peaks relating ring protons are indicated.

as follows: $J_{1,2}$, 4.4 Hz; $J_{2,3}$, 8.5 Hz; $J_{3,4}$, 3.2 Hz; and $J_{4,5}$, ≈ 1.0 Hz. The ^{13}C resonance at 55.8 ppm showed a correlation with H-2 (3.27 ppm) of the D-glucosaminitol end group (GNI') (34, 35), while the fourth amino-substituted ^{13}C resonance (51.6 ppm) correlated with the α -proton resonance (3.55 ppm) of the L-alanine unit.

Three subspectra (labelled HI, HII, and Rha) having ^1H spin systems typical of *manno* pyranose ring systems were identified on the basis of the observed small $J_{1,2}$ and $J_{2,3}$ values (< 3.0 Hz) and the large $J_{3,4}$ value (≈ 10.0 Hz) (Table 2). Each was found to have the α -configuration on the basis of the $^1J_{\text{C-1,H-1}}$ values (~ 170 Hz) (Table 3) (5). The ^1H and ^{13}C subspectra for the HI and HII residues were associated with eight proton resonances and seven ^{13}C resonances, respectively (Tables 2 and 3), indicating that they were the L-glycero-D-manno-heptopyranose residues. The other residue (Rha) was identified as the L-rhamnopyranose residue on the basis of high-field values for the H-6 (1.33 ppm) and C-6 (18 ppm) resonances.

The anomeric configurations of the glycopyranosyl residues were confirmed from intrasidue NOEs relating the anomeric proton resonances to protons within the same pyranose ring systems (Fig. 6). The β -linked glycopyranoses showed NOEs between H-1, H-3, and H-5 resonances within the same ring systems (GIII and GNII), while the α -linked glycopyranoses showed intrasidue NOEs between the anomeric proton and H-2 resonances only (HI, HII, GalN, GI, GII, and Rha).

Structure of the A28 backbone OS. The sequence of the glucose residues within the inner core region of the A28 backbone OS was established from NOE measurements. Transglycosidic NOE connectivities relating anomeric and aglyconic protons on adjacent glycosyl residues were determined in a 2D NOE spectroscopy experiment (Fig. 6). The occurrence of transglycosidic NOEs between H-1 of GI and H-4 and H-6 of GalN, between H-1 of GalN and H-3 of HII, between H-1 of HII and H-3 of HI, and between H-1 of HI and H-5 of KDOI (Fig. 6) established the partial sequence GI $\rightarrow$ GalN $\rightarrow$ HII $\rightarrow$ HI $\rightarrow$ KDOI $\rightarrow$ in the inner core region of the molecule. In conjunction with the methylation results (Table 1) and chemical shift data (Tables 2 and 3), the NOE connectivities indicated that the backbone structure of the inner core region of the A28 LPS was identical to that of the inner core region of the R5 LPS. Correspondingly, the ^1H chemical shift values of the inner core KDO and Hep residues closely corresponded (≤ 0.1 ppm) to those previously reported for the truncated backbone OS obtained from the R5 LPS (34). (It is noteworthy that the proton chemical shift values of H-1 to H-3 of the glucosamine and glucosaminitol residues are observed to be less than those previously reported for the truncated backbone OSs [by 0.11 to 0.29 ppm] because of the difference in the pH values at which the two spectra were recorded [pHs 8.8 and 7.4, respectively].) Glycoses within this region of the OS form a linear chain having the structure α -D-Glcp-(1-4)- α -D-GalpN-(1-3)-L- α -D-Hep-(1-3)-L- α -D-Hep-(1-5)- α -D-KDOp.

The NOE data indicated that this structural unit is further substituted by three terminal residues in the outer core region as follows:

The occurrence of transglycosidic NOEs between H-1 of GII and H-3 of GI and between H-1 of Rha and H-6 of GI (Fig. 6) established that the α -D-Glcp residue, GI, is substituted at the O-3 and O-6 positions by the α -D-Glcp and α -L-Rhap end groups, respectively. In addition, a transglycosidic NOE be-

TABLE 2. Proton chemical shifts and coupling constants of backbone OS from *P. aeruginosa* 06 mutant A28 LPS^a

Residue	Glycose unit	Proton chemical shift (ppm) and coupling constant(s) (Hz)											
		H-1 (<i>U</i> _{1,2})	H-2 (<i>U</i> _{2,3})	H-3a (<i>U</i> _{3a,4}) ^b	H-3c (<i>U</i> _{3c,4c} , <i>J</i> _{3c,3b}) ^b	H-4 (<i>U</i> _{4,5})	H-5	H-6 (<i>U</i> _{5,6})	H-6' (<i>U</i> _{5,6'} , <i>J</i> _{6,6'})	H-7 (<i>U</i> _{6,7})	H-7' (<i>U</i> _{6,7'} , <i>J</i> _{7,7'})	H-8 (<i>U</i> _{7,8})	H-8' (<i>U</i> _{7,8'} , <i>J</i> _{8,8'})
GI	→3)-α-D-Glcp-(1→ 6 ↑	4.95 (3.7)	3.52 (9.5)	3.85 (9.5)		3.59 (8.8)	4.35	3.79 (2.5)	3.86 (4.5,10.0)				
GII	α-D-Glcp-(1→ β-D-Glcp-(1→	4.98 (3.0) 4.56 (8.7)	3.58 (9.5) 3.14 (8.7)	3.69 (8.8) 3.47 (9.3)		3.47 (9.5) 3.30 (9.3)	3.69 3.65	3.85 (≈2.0) 3.88 (≈2.0)	3.81 (4.3,12.5) 3.83 (3.7,10.0)				
GalN	→4)-α-D-GalpN-(1→ 2 3 ↓	5.26 (4.4)	4.52 (10.5)	4.32 (≈3.2)		4.26 (≈1.0)	4.24	3.90 (≈2.0)					
Rha	α-L-Rhap-(1→	4.80 (≈1.0)	4.02 (3.8)	3.82 (9.5)		3.46 (10.0)	3.74	1.33 (6.5)					
HI	→3)-L-α-D-Hepp-(1→	5.29 (≈1.0)	4.13 (4.4)	4.04 (9.3)		3.95 (8.7)	3.88	4.04 (<2.9)	3.76				
III	→3)-L-α-D-Hepp-(1→	5.16 (≈1.0)	4.28 (≈4.4)	4.05 (≈9.3)		4.07 (8.7)	3.80	4.07 (<2.9)	3.75 (<2.0)	3.71 (3.5,9.4)			
KDOI	→5)-α-KDOp-(2→ 4 ↑			1.98 (12.2)	2.10 (≈4.0,12.2)	4.26	4.25	3.66 (≈1.5)	3.83 (8.7)			3.64 (≈2.0)	3.92 (≈2.5,10.5)
KDOI	α-KDOp-(2→												
GNII'	→6)-D-GlcNol	3.78 ^c (≈2.9)	3.27	1.78 (12.8)	2.22 (4.8,12.2)	4.11 (3.8)	4.04	3.67 (≈1.0)		3.98 (7.5)		3.99 (≈2.5)	3.78 (3.8,10.5)
GNII	→6)-β-D-GlcpN-(1→	4.42 (7.8)	2.72 (8.8)	3.36 (9.5)		3.74							
Ala	L-Ala		3.55	1.30		3.42 (8.2)	3.60	3.55	3.58				

^a Measured at 32°C in D₂O (pH ≈8.8).
^b Letters c and a indicate equatorial and axial H-3s of KDOs, respectively.
^c Proton chemical shifts for H-1' equal 3.64, and coupling constants *J*_{1,1'} and *J*_{1,2} equal 10.8 and 4.7 Hz, respectively.
^d —, chemical shift unresolved.

TABLE 3. ^{13}C chemical shifts and coupling constants of backbone OS from *P. aeruginosa* 06 mutant A28 LPS^a

Residue	Glycose unit	^{13}C chemical shift (ppm) and coupling constant (Hz)							
		C-1 ($J_{\text{C,H}}$)	C-2	C-3	C-4	C-5	C-6	C-7	C-8
GI	$\rightarrow 3\text{)-}\alpha\text{-D-Glcp-(1}\rightarrow$ 6 ↑	100.3 (167)	73.0	≈ 73.9	70.1 ^b	71.3	67.5		
GII	$\alpha\text{-D-Glcp-(1}\rightarrow$	99.2 (170)	72.5 ^b	≈ 74.6	70.5	72.3	62.0		
GIII	$\beta\text{-D-Glcp-(1}\rightarrow$ 2 ↓ 3 ↑	105.0 (159)	74.3	77.0	71.8	≈ 75.5	61.5		
GalN	$\rightarrow 4\text{)-}\alpha\text{-D-GalpN-(1}\rightarrow$	100.8 (174)	50.5	76.8	77.0	73.9	61.9		
Rha	$\alpha\text{-L-Rhap-(1}\rightarrow$	102.2 (170)	≈ 71.0	71.3	≈ 73.1	69.9 ^b	18.0		
HI	$\rightarrow 3\text{)-L-}\alpha\text{-D-Hepp-(1}\rightarrow$	102.0 (170)	71.3	80.0	66.9	≈ 73.9	≈ 70.2	65.0	
HII	$\rightarrow 3\text{)-L-}\alpha\text{-D-Hepp-(1}\rightarrow$	103.5 (171)	71.1	80.0	≈ 67.1	73.4	69.8	64.1	
KDOI	$\rightarrow 5\text{)-}\alpha\text{-KDOp-(2}\rightarrow$ 4 ↑	— ^c	— ^c	35.5	70.5	73.9	≈ 72.5	≈ 70.7	64.5
KDOII	$\alpha\text{-KDOp-(2}\rightarrow$	— ^c	— ^c	35.5	67.4	67.5	≈ 72.7	71.2	64.3
GNI'	$\rightarrow 6\text{)-D-GlcNol}$	62.3	55.8	69.8	73.1 ^b	71.0	73.1		
GNII	$\rightarrow 6\text{)-}\beta\text{-D-GlcpN-(1}\rightarrow$	105.5 (161)	57.6	77.3	71.2	75.7	63.0		
Ala	L-Ala		51.6	20.8					

^a Measured at 32°C in D₂O (pH $\approx$ 8.8).
^b Assignments may be reversed.
^c —, not determined.

tween H-1 of GIII and H-3 of GalN suggested that the α -D-GalpN residue also forms a branch point in which the O-3 position carries the β -D-Glcp residue (GIII). The NOE connectivities and the linkage positions of the glycosyl residues of the backbone OS deduced from the NOE connectivities are illustrated in Fig. 7. In accord with this structure, the backbone OS showed an abundant doubly charged ion, [M-2]²⁻, at m/z 1,014 in the negative-ion electrospray MS experiment, corresponding to an M_r of 2,029. **Structure of the A28 LPS.** On the basis of the combined NMR spectroscopic and methylation data in conjunction with the inner core data deduced from structural analysis of mutant

R5 (34), the LPS structure from the *P. aeruginosa* serotype 06 IATS mutant strain A28 was established (Fig. 8). In agreement with this structure, an O-deacylated sample of the A28 LPS produced major ions in the negative-ion electrospray mass spectrum that corresponded to the species containing four phosphates and the *O*-carbamoyl substituent (M_r , 2,788), as indicated in Fig. 8.

DISCUSSION

In previous studies, only partial and tentative structures for the core OSs from LPSs of serologically distinct strains of *P.*

FIG. 6. 2D NOE spectrum of the A28 backbone OS showing NOE connectivities involving the anomeric proton resonances of the eight glycopyranosyl residues.

FIG. 7. Structure of the A28 backbone OS from *P. aeruginosa* serotype 06 illustrating the network of observed interresidue NOE connectivities used to establish the sequence of the glycosyl residues.

FIG. 8. LPS structure for *P. aeruginosa* serotype 06 IATS mutant strain A28.

aeruginosa belonging to serotypes 02, 03, and 05 have been proposed (Fig. 9) (18, 28, 38); an accurate chemical structure for this region has not yet been reported. Determination of detailed structures of the core LPSs from different serotypes is necessary to better understand their immunochemical reactions and thereby provide a possible basis for the development of specific diagnostic antibody reagents and agents protective against *P. aeruginosa*. In this study we have achieved the elucidation of the complete chemical structure of the core OS from the *P. aeruginosa* IATS serotype 06 mutant A28 by analytical and NMR techniques. The structural model determined for the core OS region (Fig. 9) of the LPS is representative of the structure of the core OS region in the parent wild-type strain. The core OS component of mutant A28 LPS consists of a nonasaccharide moiety attached through a ketosidic linkage from one of the KDO residues to the β -D-GlcpN component of the lipid A moiety (22). The structure of LPS backbone OS from mutant A28 indicated that the inner core region was identical to those of core-deficient mutants H4 and R5 (34).

Indeed, the *P. aeruginosa* mutant H4 expresses LPS which contains only the inner core pentasaccharide unit terminating in α -D-galactosamine. This terminal α -D-GalpN is capped in the LPS of mutant R5 by an α -D-Glcp residue, while the complete core OS expressed by mutant A28 contains three additional glucose end groups (Fig. 9) which provide unique structural epitopes.

The characterization of the core region from mutant R5 facilitated the localization of the phosphate groups in the inner core region of the mutant A28 LPS. Phosphate residues are substituted at positions O-2 and O-4, positions of the first heptose residue adjacent to KDO in the backbone OS structure. One of the heptose residues was also found to carry a carbamoyl group at position O-7. Recent studies of the *P. aeruginosa* deep rough mutant strain PAC 65 (4) suggest that carbamoyl substitution occurs on the second heptose. While no data are available to indicate a biological role of this novel carbamoyl substituent in the inner core of *P. aeruginosa*, it is of interest that O-carbamoyl and other functional substituents

FIG. 9. Proposed or tentative core OS structures of *P. aeruginosa*. Core OS structures of three serotypes, IATS 02 (strain Habs 2) (18), IATS 03 (strain PAC1R) (38), and IATS 05 (strain PAO1) (28), are presented as they were reported earlier, and the core OS structures of the O-chain-deficient mutants H4, R5, and A28 of an IATS 06 strain are also shown.

have been found to be components of a unique class of lipooligosaccharides expressed by rhizobial bacteria which serve as nodulation signals. It was suggested (17, 37) that these novel substituents may mediate the specificity in the interaction of rhizobia with specific receptors of legumes.

When the complete core OS structure of mutant A28 was compared with the aforementioned proposed and tentative core structures, significant differences were apparent. The partial core structure proposed by Drewry et al. (18) for NCTC 1999 contains an α -1,6-linked Glc disaccharide and a Glc-Rha-Glc trisaccharide glycosidically linked to a GalN residue (Fig. 9).

Comparison of the structure of strain A28 core OS with that proposed by Rowe and Meadow (38) for strain PAC1R core OS revealed that both contain L-Ala and a diglucoside moiety glycosidically linked to D-GalN (Fig. 9). In the core structure of mutant A28, an α -L-Rhap end group is linked to the -3)- α -D-Glcp-(1- residue at the 6 position, whereas in the PAC1R core this residue is reported to be linked to the α -GalpN residue. Moreover, an α -Glc residue, which is absent in A28, is proposed to be substituted at position O-7 of the Hep residue in the PAC1R core. The presence of only a single Hep residue in the inner core region of PAC1R also differs from the two Hep residues observed in A28. The tentative core structure proposed by Kropinski and colleagues (27) for PAO1 and the mutant strain AK44, which contains a complete core, is similar to that proposed by Drewry and coworkers for NCTC 1999 in that it contains a Glc-Rha-Glc trisaccharide and a Glc-Glc disaccharide linked to GalN (Fig. 9). Similarly, both PAO1 and NCTC 1999 are reported to contain an L-Ala residue linked to the GalN residue which is also observed in the mutant A28 core structure. The proposed structure of the PAO1 core OS, however, contains three Hep residues instead of the two found in A28. Examination of the reported linkages between the individual sugars in these proposed core structures also reveals substantial differences between them.

Variations in the previously reported core OS structures may reflect structural differences that exist in the core regions of the different serotypes, particularly within the outer core. In a recent study, de Kievit and Lam (16) observed that an inner core-specific MAb, MAb 7-4, raised against the core LPS of *P. aeruginosa* serotype O5 reacted with LPS from the twenty serotypes as well as other *Pseudomonas* species, suggesting that they all have a common inner core structure. In contrast, an outer core-specific MAb, MAb 101, reacted with LPS from nine serotypes, namely, 02, 05, 07, 08, 010, 016, 018, 019, and 020, but not with LPS from any of the other serotypes. This suggested that structural differences between the different serotypes exist within the outer core region, and in agreement with these findings, recent structural studies by our group (2) have indicated that the serotype O5 core OS carries an additional α -D-Glcp residue substituting for the terminal L-Rhap in the serotype O6 core. In contrast to the structures proposed previously (Fig. 9), our analytical and immunochemical data indicate that the inner core region is conserved among *P. aeruginosa* serotypes; this likely reflects limitations of the earlier methods employed. Most of the chemical studies reported to date have relied on the acid lability of the KDO ketosidic linkage and subsequent dissociation of the lipid A moiety for LPS structural determinations. While this strategy has proven successful for studying the structure of O polysaccharides, it has revealed only limited information about the structure of the core OS region. Furthermore, because the KDO residues are cleaved off during the mild acid hydrolysis treatment, very little structural information regarding the inner core region can be obtained. In this study an alternative approach, which

we had previously reported (34), in which some or all of the fatty acyl groups were removed from the lipid A moiety of the molecule to obtain OSs that are representative of the complete core OS backbone structure was used. Using this approach has enabled us to report for the first time the detailed chemical structure of the complete core OS region from IATS serotype O6.

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