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# Structure of the Core Oligosaccharide in the Serotype O8 Lipopolysaccharide from *Klebsiella pneumoniae*

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**Two classes of mutants with O-antigen-deficient lipopolysaccharides were isolated from the serotype O8 reference strain, belonging to *Klebsiella pneumoniae* subspecies *ozaenae*. These mutants were selected by resistance to bacteriophage KO1-2, which recognizes and lyses strains with lipopolysaccharide molecules containing the D-galactan II O antigen. Strain RFK-11 contains a defect in O-antigen synthesis and has a complete core, including the attachment site for O antigen. This mutation is complemented by a plasmid carrying the *rfb* (O-antigen biosynthesis) gene cluster from the related *K. pneumoniae* serotype O1. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the lipopolysaccharide from strain RFK-9 has a mobility typical of deep-rough lipopolysaccharide. RFK-9 lipopolysaccharide lacks the attachment site for O antigen. Lipopolysaccharides from strains RFK-9 and RFK-11 were isolated, and their structures were determined by methylation analyses, nuclear magnetic resonance spectroscopy, and mass spectroscopy. The deduced O8 core oligosaccharide includes the partial core structure reported for the *K. pneumoniae* subspecies *pneumoniae* serotype O1 lipopolysaccharide (M. Süsskind, S. Müller-Leonnies, W. Nimmich, H. Brade, and O. Holst, Carbohydr. Res. 269:C1–C7, 1995), consistent with the possibility of a conserved core structure within the species. The core oligosaccharide differs from those of the genera *Salmonella* and *Escherichia* by the absence of a hexose-containing outer core, the lack of phosphate residues in the inner core, and the presence of galacturonic acid residues.**

Lipopolysaccharides (LPSs; endotoxins) are biologically active components of gram-negative bacteria and represent important virulence factors in bacterial pathogens (42). The O-polysaccharide and core oligosaccharide moieties of LPS are immunogenic, giving rise to antibodies having specific serological properties that may be protective and that can also be of diagnostic importance.

In the genera *Salmonella* and *Escherichia*, the core oligosaccharide region of the LPS molecule serves to link the hydrophobic lipid A region to the distal O-specific polysaccharide. In these well-characterized LPSs, the core oligosaccharide may be divided into two regions, the inner and outer core, based on sugar composition. The inner core consists of L-*glycero*-D-*manno*-heptose (heptose), 3-deoxy-D-*manno*-2-octulosonic acid (Kdo), phosphate, and ethanolamine residues, and its carbohydrate structure is highly conserved in all enterobacterial LPSs so far studied (25). The outer core contains more common hexoses, such as glucose, galactose, and *N*-acetylglucosamine, and is known to vary slightly among and within bacterial species. Only one core oligosaccharide structure (designated Ra) has been characterized in the genus *Salmonella*. Closely related core structures are found in other genera of the *Enterobacteriaceae*, such as *Escherichia* and *Shigella* (25). However, five different core structures, designated R1, R2, R3, R4, and K-12, have been characterized in the LPSs from various *Escherichia coli* strains. While each of these core types is structurally related to the *Salmonella* Ra core, there are several differences in sugar composition and linkage details within the outer core region (25).

*Klebsiella pneumoniae* is an opportunistic pathogen causing bacteremia, pneumonia, and urinary tract infections in humans (36, 57). LPS is known to be an important virulence determinant in these infections, and injection of purified LPS significantly enhances the virulence of *K. pneumoniae* in a mouse model (19). Also, in strains of *K. pneumoniae* serotypes O1:K1 and O1:K2, a toxic complex is released from the cell surface during growth (48, 49). The complex consists of capsular polysaccharide, LPS, and proteins and is responsible for characteristic lung tissue damage caused by *K. pneumoniae* infections. It is known that LPS is the critical component of the complex, although the toxicity of purified LPS is lower than that of the complex (49). These toxic effects are abrogated by antibodies against LPS (48). However, LPS within the extracellular complex is also implicated in generating a transient reduction in the response of the reticuloendothelial system, allowing proliferation of the bacterium in the early stages of infection (19).

Consideration of conserved core structures has stimulated interest in their potential use in creation of a vaccine which might be cross-protective for pathogenic *Enterobacteriaceae* and perhaps other bacteria. This is not possible with O-specific polysaccharide-based vaccines because of the enormous structural variation in this portion of the LPS molecule. Passive immunization with antibodies directed against this conserved region of LPS might provide an alternative approach to chemotherapy for infections such as those resulting from *K. pneumoniae*. Such applications require a detailed knowledge of the molecular structure of the targeted LPS molecules and the diversity of structures in related bacteria. Early chemical analyses on the LPSs of *Klebsiella* spp. showed similar composition of cores from different serotypes (37). More recently, a cross-reactive monoclonal antibody (MAb) (V-9.5) was used to demonstrate a conserved epitope in the lipid A-core fraction of

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LPS from various *Klebsiella* O serotypes (51). While the work reported here was in progress, the lipid A-backbone and partial core structure was reported for LPS from a rough (R-LPS) mutant of *K. pneumoniae* serotype O1:K20 (50). This serotype is a representative of *K. pneumoniae* subsp. *pneumoniae* (33). The deduced structure contained several features which differ substantially from those of the cores of *E. coli* and *Salmonella enterica*. It has not yet been established whether this structure is found in all serotypes of *K. pneumoniae.*

Here we report the structure of the complete LPS core oligosaccharide from *K. pneumoniae* subsp. *ozaenae* serotype O8 and its relationship to the core from serotype O1.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *K. pneumoniae* subsp. *ozaenae* CWK47, serotype O8:K$^-$, was previously used to determine the structure of the O8 O polysaccharide (28). Strains RFK-9 and RFK-11 are bacteriophage KO1-2-resistant mutants derived from CWK47, with R-LPS. Bacteriophage KO1-2 recognizes and lyses *Klebsiella* strains with smooth LPS (S-LPS) belonging to serotypes O1 (56) and O8 (28). The O1 and O8 O antigens contain a common polysaccharide component, termed D-galactan II. Mutants which are resistant to phage KO1-2 either lack D-galactan II (28, 56) or contain rough LPS (R-LPS), suggesting a role for the D-galactan II O antigen as a receptor for the bacteriophage.

*S. enterica* serovar typhimurium strains SA1627, SL3748, and SL3789 provided LPS standards for the Ra, Rc, and $Rd_2$ chemotypes, respectively. These strains were obtained from K. E. Sanderson, Salmonella Genetic Stock Center, Calgary, Alberta, Canada.

Bacterial strains were routinely grown and maintained in Luria broth (LB) (35), and incubation was done at 37°C. For large-scale preparations of LPS, 28- and 75-liter bacterial cultures were grown for 18 h in a fermentor (Microfirm; New Brunswick Scientific) in a medium of brain heart infusion broth (3.7% [wt/vol]; Difco), operated at 37°C and 200 rpm, and with aeration at 25 liters/min. Cells were killed by the addition of phenol (0.75% [wt/vol] final concentration) and harvested with a Sharples continuous-flow centrifuge.

Plasmid pWQ5 contains the complete *rfb* gene cluster from *K. pneumoniae* subsp. *pneumoniae* serotype O1 cloned in the vector pBluescript (12, 16). The *rfb* cluster encodes products which synthesize the D-galactan I component of the O1 antigen. The plasmid was introduced into *K. pneumoniae* mutants by electroporation (9), and transformants were selected on LB containing ampicillin (100 μg/ml).

**SDS-PAGE.** LPS samples obtained by phenol extraction or by the sodium dodecyl sulfate (SDS)-proteinase K whole-cell lysate method (24) were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) with commercially prepared 1-mm-thick 10 to 20% gradient Tricine gels from Novex (San Diego, Calif.). The conditions for electrophoresis were those recommended by the manufacturer. Gels were silver stained by the method of Tsai and Frasch (52).

**LPS extraction.** Large-scale LPS preparations were made by the modified hot phenol extraction method of Johnson and Perry (27). Briefly, collected cells (approximately 500 g [wet weight]) were washed with 2.5% saline and digested with lysozyme, RNase, and DNase. The treated cells were then extracted with hot aqueous phenol as described in the original procedure (54). LPS was recovered as a pellet from the dialyzed and concentrated aqueous layer of the extract by ultracentrifugation (105,000 × *g*, 4°C, 16 h), treated with proteinase K (10 μg/ml, 2 h, 60°C), and purified by a further ultracentrifugation step. Normal yields were on the order of 2 g of pure LPS, reflecting approximately a 2% yield, based on the dry weight of the cells.

**Isolation and purification of core oligosaccharides.** For the preparation of core oligosaccharide, a suspension of the purified LPS (0.5 g) was first dephosphorylated by treatment with 48% aqueous hydrogen fluoride (48 h, 0°C). The resulting products were dried under a stream of nitrogen, resuspended in aqueous acetic acid (2% [vol/vol], 250 ml), and heated at 100°C for 2 h. Lipid A is cleaved from the LPS molecules and precipitates in this process, allowing its removal by low-speed centrifugation. The supernatant was concentrated by lyophilization. After reconstitution in distilled water, the residue was fractionated by gel filtration chromatography on a Sephadex G-50 column (Pharmacia; 3.5 by 100 cm) with pyridinium acetate (0.05 M, pH 4.7) as the elution buffer. The column eluant was continuously monitored for changes in refractive index with a Waters R403 differential refractometer, and fractions (10 ml) were collected and analyzed colorimetrically for neutral glycose (20). The core oligosaccharide, with a $K_{av}$ of 0.47, was collected and lyophilized.

**Purification of LPS carbohydrate backbone oligosaccharides.** Carbohydrate backbone oligosaccharides consist of the LPS core oligosaccharide with the lipid A carbohydrate backbone still attached. For the preparation of LPS carbohydrate backbone oligosaccharides from strains RFK-9 and RFK-11, the ester-bound fatty acids of lipid A were first removed from LPS (400 mg) by treatment with anhydrous hydrazine under mild conditions (20 ml, 37°C, 30 min) by the procedure of Holst et al. (26). The reaction mixtures were cooled, and cold acetone (3 volumes, 70°C) was slowly added to destroy excess hydrazine and precipitate the product. The de-O-acylated LPS was then isolated by low-speed centrifugation (5,000 rpm, 10 min), and the pellet was washed three times with acetone, dissolved in water, reprecipitated by the addition of acetone, and lyophilized. The de-O-acylated sample (200 mg) was then de-N-acylated by heating in aqueous KOH (10 ml, 4 M, 100°C, 20 h). The solutions were cooled in an ice bath and neutralized with 4 M HCl, and the precipitated lipid was removed by centrifugation (12,000 × *g*, 30 min). The supernatants were dialyzed in an Amicon concentration cell with a 500-molecular-weight-cutoff membrane (Amicon; YCO5) and washed with distilled water until the eluant was free of chloride (determined with aqueous $AgNO_3$). The dialyzed material was then lyophilized to give 80 mg of crude deacylated LPS.

The deacylated oligosaccharides were purified by anion-exchange chromatography with a DEAE A-25 column (40 by 1 cm) equilibrated with aqueous 0.01 M NaCl. The column was washed with the same solution, and the products were eluted with a 0.01 to 0.25 M NaCl gradient. The collected fractions (2.5 ml) were assayed colorimetrically for neutral glycose (20), and the glycose-containing fractions were collected and lyophilized. The products were desalted on a Bio-Gel P2 column (BioRad; 2.6 by 140 cm) eluted with pyridinium acetate (0.05 M, pH 4.5). The column eluant was continuously monitored for changes in refractive index with a Waters R403 differential refractometer, and fractions (2.5 ml) were collected and analyzed as above. This yielded 43 mg of purified deacylated LPS (a 10.8% yield, based on LPS) suitable for carbohydrate sequence determination.

For carbohydrate structural analysis, the deacylated LPS was dephosphorylated by treatment with 48% aqueous hydrogen fluoride (48 h, 4°C). The resulting products were dried under a stream of nitrogen, resuspended in distilled water, and purified on a Bio-Gel P2 column.

**NMR spectroscopy.** Spectra were obtained on a Bruker AMX-500 spectrometer with standard Bruker software. Solutions of oligosaccharide samples in deuterium oxide (0.4 ml) were prepared at a concentration of 10 to 40 mg/ml subsequent to lyophilization from $D_2O$. All spectra were measured by using oligosaccharide samples in 5-mm tubes at 27°C. The pH of the deuterium oxide solution (pD) was approximately 2. $^1$H nuclear magnetic resonance ($^1$H-NMR) spectra were recorded at 500 MHz with a spectral width of 2.5 kHz, an acquisition time of 3.2 s, a 16K data block, and a 90° pulse. Broad-band proton-decoupled $^{13}$C-NMR spectra were recorded at 125 MHz with a spectral width of 25 kHz, a 32K data block, and a 90° pulse, employing WALTZ decoupling (46). Acetone was used as the internal standard, and chemical shifts were referenced to the methyl resonance ($\delta_H$, 2.225 ppm; $\delta_C$, 31.07 ppm). Heteronuclear $^1J_{C,H}$ couplings were measured by using gated decoupling.

Complete assignment of spectra was accomplished by two-dimensional homo- and heteronuclear chemical shift correlation techniques. Homonuclear two-dimensional (2D) chemical shift-correlated spectroscopy (COSY) (5) was measured in the phase-sensitive mode (41). Data were acquired over the full spectrum (2.5 kHz) or for the ring proton region (1.5 kHz) by using data sets of 512 by 2,048 points; 32 scans were acquired for each value of $t_1$.

Two-dimensional total correlation spectroscopy (TOCSY) (3) and nuclear Overhauser enhancement spectroscopy (NOESY) (32) experiments were performed in a phase-sensitive mode over a spectral width of 2.5 kHz, with a data set of 512 by 2,048 points and 32 scans. Mixing times of 76 and 400 ms were employed for each experiment.

Heteronuclear two-dimensional $^{13}$C- and $^1$H-chemical shift-correlated spectra were measured in the $^1$H-detected mode via multiple quantum coherence (HMQC) (6), with data sets of 512 by 2,048 points and spectral widths of 14 and 4.5 kHz for $^{13}$C and $^1$H domains, respectively. Thirty-two scans were acquired for each $t_1$ value, and $^{13}$C decoupling during $^1$H acquisition was achieved by using the GARP-1 composite pulse sequence (45). Heteronuclear multiple-bond-correlated (HMBC) experiments were carried out with the pulse sequence described by Bax and Summers (7) and employed a delay of 60 ms for the evolution of long-range couplings. The HMQC-TOCSY experiment (4) employed a mixing time of 76 ms.

$^{31}$P-NMR spectra were measured at 202 MHz by employing spectral widths of 10 to 12 kHz and a 90° pulse. Phosphoric acid (85%) was used as the external standard ($\delta_P$, 0.0 ppm). $^1$H-$^{31}$P correlations (HMQC) were made in the $^1$H-detected mode by using a data matrix of 16 by 1,024 points, sweep widths of 10 kHz for $^{31}$P and 13 kHz for $^1$H, and a mixing time of 60 ms.

**Quantitative determination of glycoses.** Glycoses were analyzed as their derived alditol acetates by capillary gas-liquid chromatography (GLC), as previously described for the characterization of the O antigens from *K. pneumoniae* serotype O1 (56). Briefly, samples (0.5 mg) were hydrolyzed by treatment in 1 ml of 2 M trifluoroacetic acid for 16 h at 100°C. The acid was then evaporated under a stream of nitrogen, and the resulting residues were reduced with sodium borodeuteride ($NaBD_4$; 6 h, 37°C). Following neutralization with glacial acetic acid, the reaction mixture was evaporated to dryness under nitrogen, and the borate esters were removed by distillation with methanol four times (2 ml each time). The liberated reduced glycoses were acetylated with acetic anhydride ($Ac_2O$; 2 h, 120°C) by standard protocols (58). Partially acetylated heptitol derivatives were found to have the L-*glycero*-D-*manno* (or D-*glycero*-L-*manno*) configuration by comparison of their GLC retention times with those of authentic standards. The L-*glycero*-D-*manno* absolute stereochemistry is assumed on biosynthetic grounds (17).

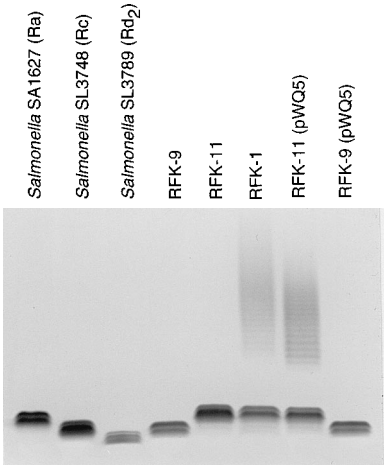


FIG. 1. Silver-stained SDS-PAGE gel showing the LPSs from *Klebsiella* mutants RFK-9 and RFK-11, derived from *K. pneumoniae* subsp. *ozaenae* serotype O8. Strain RFK-1 is an O8 derivative with S-LPS containing D-galactan I (28). RFK-11 contains an *rfb* defect, and the mutation is complemented to restore S-LPS expression by plasmid pWQ5, carrying the serotype O1 *rfb* gene cluster. Strain RFK-9 has a core defect and is not complemented by pWQ5. Standard LPS chemotypes (Ra, Rc, and $Rd_2$) from *S. enterica* serovar typhimurium are also shown. Samples were prepared from whole-cell lysates (24).

**Methylation analysis.** Methylation analysis was performed on dephosphorylated, deacylated oligosaccharide samples (2 to 5 mg) by the method of Hakomori (23). Briefly, samples were methylated with iodomethane in dimethyl sulfoxide containing an excess of sodium (methylsulfinyl)methanide. Excess iodomethane was evaporated under a stream of nitrogen, and the methylated products were recovered by partitioning the reaction mixture between water and methylene chloride, followed by concentration of the organic phase. The resulting carboxymethyl ester groups were reduced by treatment with 1 M lithium triethylborodeuteride in tetrahydrofuran (2 h, 2°C). Excess reagent was destroyed by the addition of glacial acetic acid, and the reaction mixture was evaporated under a stream of nitrogen, followed by evaporation from methanol to remove the borate esters. The methylated and reduced products were desalted on an ion-exchange column containing Dowex-50 ($H^+$ form), eluted with water-methanol (1:1), and lyophilized. Methylated and carboxyl-reduced products were solvolyzed with anhydrous HF (22°C, 3 h), and following removal of the HF, the products were hydrolyzed with 2% acetic acid (100°C, 2 h). The liberated glycoses were reduced with $NaBD_4$, and after neutralization with glacial acetic acid, the solutions were concentrated and distilled with methanol. Finally, the partially methylated alditols were acetylated by heating with $Ac_2O$ (120°C, 2 h), extracted into methylene chloride, and analyzed directly by GLC-mass spectrometry (MS).

**GLC-MS.** Analytical GLC was performed with a Hewlett Packard model P5890 GC fitted with a flame-ionization detector using a 3% DB-17 fused silica capillary column (0.3 mm by 25 m). The temperature employed for the separation of both alditol acetates and methylated alditol acetates was 180°C for 2 min and then increased at 4°C/min to 240°C. Partially methylated alditol acetates were separated by GLC as above and identified on a Varian Saturn II ion-trap GC-MS.

Samples for electrospray MS were analyzed on a Quatro mass spectrometer (VG Instruments, Manchester, United Kingdom) with an electrospray ion source. Oligosaccharide samples were first dissolved in distilled water and then diluted 1:1 with electrospray solvent (1 volume of $CH_3CN$ to 1 volume of $H_2O$, with 1% [vol/vol] acetic acid). Injection volumes were 10 μl, and the flow rate was set at 4 ml/min. The electrospray tip voltage was typically 2.7 kV. The mass spectrometer was scanned from *m/z* 50 to 2,500, with a scan time of 10 s. Data were collected in a multichannel analysis mode.

## RESULTS

**Isolation of R-LPS-containing mutants RFK-9 and RFK-11.** A collection of bacteriophage KO1-2-resistant mutants were initially screened for LPS defects by SDS-PAGE. Based on electrophoretic mobility relative to known standards, only two unique groups of R-LPS mutants were identified. However, subtle differences could have been overlooked in this initial screening.

One group of strains, represented by RFK-11, contained R-LPS which comigrated with *Salmonella* Ra-core standard in SDS-PAGE (Fig. 1). This is consistent with the possibility that RFK-11 contained a complete LPS core. Bacteria with R-LPS containing full-length cores can arise either by defects in the synthesis of O antigen (*rfb* defects) or by defects in *rfaL*, whose gene product is required for ligation of O antigen to lipid A-core (43). An *rfb* mutation could be investigated directly by using the cloned *rfb* cluster from *K. pneumoniae* serotype O1 (12, 16), which encodes the same enzymes as the *rfb* gene cluster from serotype O8, the original serotype of RFK-11 (29). RFK-11 transformants containing pWQ5 showed restoration of S-LPS (Fig. 1), indicating that RFK-11 indeed has an *rfb* defect and that the core of RFK-11 provides the O-antigen attachment site.

Mutants represented by RFK-9 contained LPS which comigrated with that from a *Salmonella rfaI* mutant, characteristic of R-LPS belonging to the deep-rough Rc core chemotype. The mutation in RFK-9 was not complemented by introduction of pWQ5 (Fig. 1), confirming that its truncated LPS core minimally lacks the terminal attachment site for O antigen.

These strains provided the starting points for structural analyses.

**Composition of the carbohydrate backbone oligosaccharides of RFK-9 and RFK-11 LPS.** Quantitative analysis of the sugar composition of the carbohydrate backbone oligosaccharides was performed by GLC-MS of the corresponding alditol acetate derivatives. These analyses revealed that the RFK-9 carbohydrate backbone oligosaccharide was composed of glucose (Glc), 2-amino-2-deoxyglucose (GlcN), and L-*glycero*-D-*manno*-heptose (L,D-Hep) residues in an approximate molar ratio of 1:2:2. The RFK-11 oligosaccharide contained the same sugar residues in the molar ratio of 1:2:3, respectively.

Methylation analysis of the RFK-9 carbohydrate backbone oligosaccharide (Table 1) employed a modified procedure involving carboxyl reduction of any methyl esters produced during methylation. Subsequent solvolysis with anhydrous HF and analysis by GLC-MS led to the identification of 4,5,7,8-tetra-*O*-methyl and 7,8-di-*O*-methyl derivatives of Kdo, which unambiguously established the presence of the terminal Kdo residue and identified the substitution sites at *O*-4 and *O*-5 on the branched Kdo residue. Additional methylated products were

TABLE 1. Methylation analysis of the oligosaccharides isolated from the LPS of *K. pneumoniae* serotype O8 mutants RFK-9 and RFK-11

| Methylated product[a] | Ratio[b] in: | | |
|---|---|---|---|
| | RFK-9[c] | RFK-11[c] | RFK-11 core[d] |
| 2,3-$Me_2$-hex-4-enuronopyranose-6,6-$d_2$ | | 0.3 | |
| 2,3,4,6-$Me_4$-Glc | 1.0 | | 1.0 |
| 2,3,4-$Me_3$-Glc | | 0.6 | 1.1 |
| 2,3,4-$Me_3$-Gal-6,6-$d_2$ | | 1.1 | 1.0 |
| 2,3-$Me_2$-Gal-6,6-$d_2$ | | | 1.0 |
| 2,3,4,6,7-$Me_5$-Hep | 1.2 | 1.0 | 0.9 |
| 2,4,6-$Me_3$-Hep | | 0.5 | 1.2 |
| 2,6,7-$Me_3$-Hep | 1.2 | 1.1 | 0.8 |
| 2-Deoxy-1,3,4,5-$Me_4$-2-(*N*-methylacetamido)-Glc | 0.3 | 0.4 | |
| 2-Deoxy-3,4-$Me_2$-2-(*N*-methylacetamido)-Glc | 0.3 | 0.4 | |
| 3-Deoxy-4,5,7,8-$Me_4$-Oct-1,1-$d_2$ | 0.1 | 0.2 | |
| 3-Deoxy-7,8-$Me_2$-Oct-1,1-$d_2$ | 0.1 | 0.2 | |

[a] As their peracetylated alditol derivatives.
[b] Relative to 2,3,4,6-tetramethylglucitol-1-d = 1.0.
[c] Carbohydrate backbone oligosaccharides from N- and O-deacylated and dephosphorylated LPS.
[d] Core oligosaccharide released by mild acid hydrolysis.

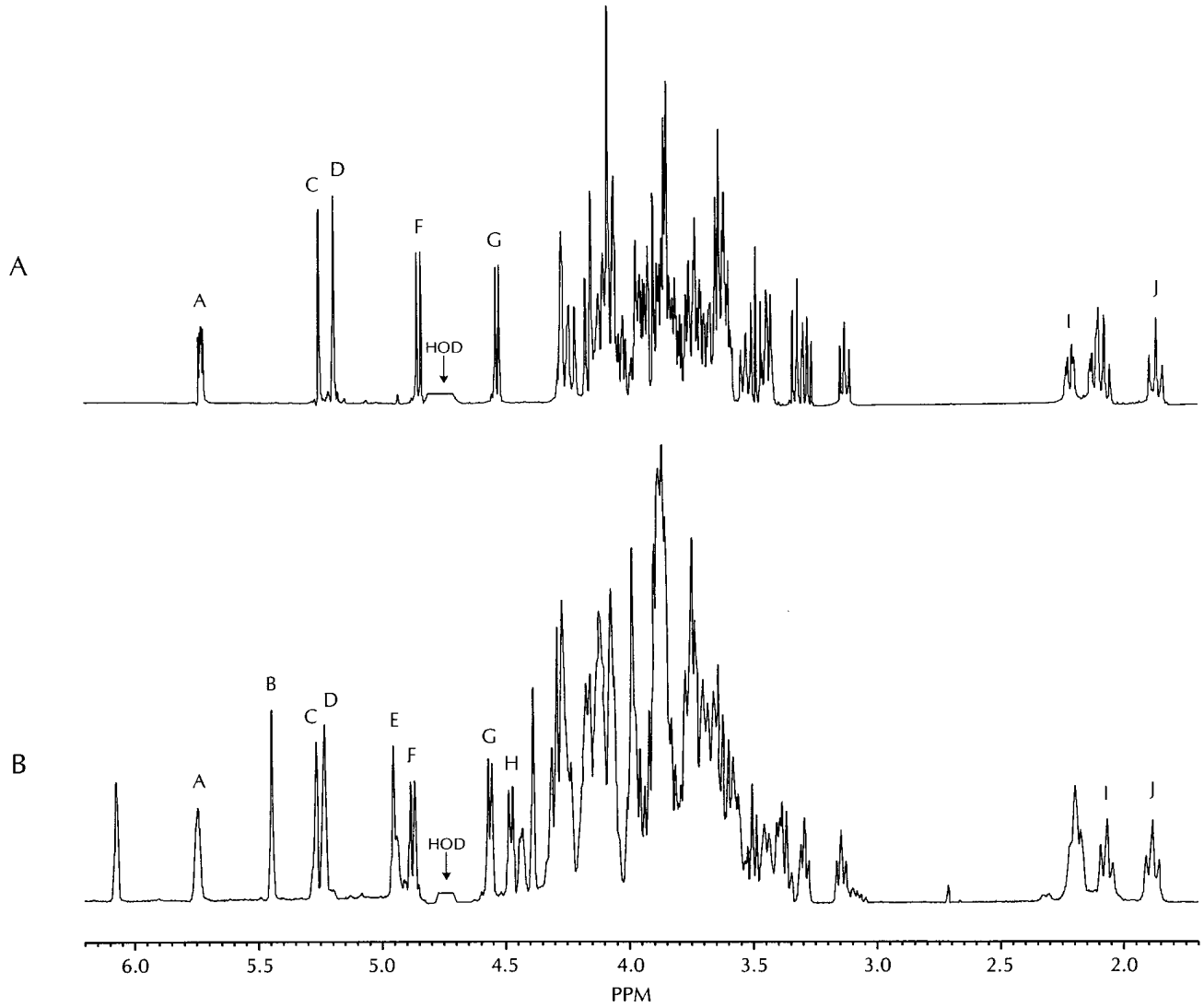


FIG. 2. $^1$H-NMR spectra of the deacylated carbohydrate backbone oligosaccharides from the LPSs of RFK-9 (A) and RKF-11 (B). The anomeric signals were labelled in order of decreasing $^1$H chemical shifts. The spectra were recorded at 27°C and pD $\simeq$ 2. The water peak (MOD) has been removed for clarity.

2,3,4,6-tetra-*O*-methyl-glucitol, 2,3,4,6,7-penta-*O*-methyl-D-*glycero*-L-*manno*-heptitol, 2,6,7-tri-*O*-methyl-D-*glycero*-L-*manno*-heptitol, 2-deoxy-1,3,4,5-tetra-*O*-methyl-2-(*N*-methylacetamido)-glucitol, and 2-deoxy-3,4-di-*O*-methyl-2-(*N*-methylacetamido)-glucitol (Table 1). These data indicate that the R-LPS core in strain RFK-9 contains two 2-aminoglycosyl residues, presumably comprising the disaccharide residue of the lipid A component, and a branched heptose residue substituted with terminal glucosyl and heptosyl residues.

Results obtained from the methylation analysis of the RFK-11 carbohydrate backbone oligosaccharide (Table 1) indicated that it was more complex than that of RFK-9 and contained additional single residues of heptose, galacturonic acid (GalA), and hex-4-enuronose. The latter two substituents were identified as their carboxyl-reduced derivatives. Furthermore, the identification of 2,3,4-tri-*O*-methyl-glucitol and 2,4,6-tri-*O*-methyl-D-*glycero*-L-*manno*-heptitol indicated that additional residues in the RFK-11 carbohydrate backbone oligosaccharide were located at the 6 position of the glucose residue and the 3 and 7 positions of the heptose residue of RFK-9 LPS. This is consistent with the finding that glucose and heptose were terminal glycoses in the RFK-9 oligosaccharide.

**Glycose sequence determinations for the carbohydrate backbone oligosaccharide in RFK-9 and RFK-11 LPS.** The sequence of the glycosyl residues within the carbohydrate backbone oligosaccharides was established by high-resolution NMR techniques. The $^1$H-NMR spectrum of the RFK-9 carbohydrate backbone oligosaccharide showed discrete resonances of equal signal areas in the low-field region of the spectrum (Fig. 2A), corresponding to the anomeric protons from five aldose residues (Table 2). Four high-field resonances were observed in the 1.5- to 2.5-ppm region of the spectrum, and these were attributed to the H-3 methylene protons of two Kdo residues. The $^1$H-NMR spectrum of RFK-11 carbohydrate backbone oligosaccharide (Fig. 2B) contained the anomeric resonances found for RFK-9 together with four additional peaks. Three of these resonances were attributed to 2-α- and 1-β-anomeric signals from three glycose residues, and the peak at 6.05 ppm was representative of the H-4 of a hex-4-enurono*p* residue (11) (Table 2). The $^1$H-NMR resonances of the oligosaccharides

TABLE 2. Proton chemical shifts and coupling constants for the deacylated carbohydrate backbone oligosaccharides from the LPS of *K. pneumoniae* serotype O8 mutants RFK-9 and RFK-11[a]

| Residue | Monosaccharide unit | Oligosaccharide | Proton chemical shift (coupling constant, Hz) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | H-1 ($J_{1,2}$) | H-2 ($J_{2,3}$) | H-3 ($J_{3,4}$) | H-3′ ($J_{3,3'}$, $J_{3',4}$) | H-4 | H-5 | H-6 ($J_{5,6}$) | H-6′ ($J_{5,6'}$, $J_{6,6'}$) | H-7 ($J_{6,7}$) | H-7′ ($J_{6,7'}$, $J_{7,7'}$) | H-8 ($J_{7,8}$) | H-8′ ($J_{7,8'}$, $J_{8,8'}$) |
| A | →6)-α-GlcN*p*-(1→P | RFK-11 | 5.74 (3.6) | 3.44 (10.5) | 3.94 (9.8) | | 3.62 (9.8) | 4.13 | 4.11 (2.0) | 3.83 (8.0, 12.1) | | | | |
| | | RFK-9 | 5.74 (3.7) | 3.43 (10.5) | 3.92 (9.6) | | 3.61 (9.6) | 4.12 | 4.10 (2.2) | 3.80 (4.8, 12.0) | | | | |
| B | α-*threo*-Hex-4-enurono*p*-(1→ | RFK-11 | 5.46 (2.8) | 3.89 (7.6) | 4.45 (3.0) | | 6.05 | | | | | | | |
| C | →3)-L-α-D-Hep*p*-(1→ | RFK-11 | 5.27 (1.0) | 4.30 (4.0) | 4.12 (9.8) | | 3.99 (9.8) | 3.70 | 4.16 (2.0) | | 3.70 (6.0) | 3.75 (8.0, 9.8) | | |
| | 7 ↑ | | | | | | | | | | | | | |
| | L-α-D-Hep*p*-(1→ | RFK-9 | 5.25 (1.0) | 4.15 | 3.84 | | 3.64 | 3.91 | 4.09 | | 3.79 | | | |
| D | →3)-L-α-D-Hep*p*-(1→ | RFK-11 | 5.25 (1.0) | 4.06 (3.8) | 4.14 (9.8) | | 4.26 (9.8) | 4.17 | 4.12 (2.8) | | 3.83 (5.8) | 3.89 (8.3, 10.0) | | |
| | 4 | RFK-9 | 5.20 (1.1) | 4.08 | 4.10 | | 4.26 | 4.14 | 4.10 | | 3.80 | 3.91 | | |
| | ↑ | | | | | | | | | | | | | |
| E | L-α-D-Hep*p*-(1→ | RFK-11 | 4.96 (0.6) | 3.98 (3.8) | 3.83 (9.5) | | 3.87 (9.5) | 3.66 | 3.98 (3.0) | | 3.68 (6.0) | 3.72 (8.3, 10.0) | | |
| F | →6)-β-GlcN*p*-(1→ | RFK-11 | 4.88 (8.5) | 3.15 (10.5) | 3.88 (9.2) | | 3.92 (10.3) | 3.76 | 3.69 (2.0) | 3.81 (5.8, 10.0) | | | | |
| | 4 | RFK-9 | 4.85 (8.6) | 3.13 (10.6) | 3.89 (9.2) | | 3.90 (10.4) | 3.74 | 3.65 (2.4) | 3.80 (6.5, 10.5) | | | | |
| | ↑ | | | | | | | | | | | | | |
| | P | | | | | | | | | | | | | |
| G | →6)-β-Glc*p*-(1→ | RFK-11 | 4.56 (8.1) | 3.30 (9.3) | 3.52 (9.3) | | 3.37 (9.4) | 3.41 | 3.77 (2.0) | 3.88 (6.2, 12.3) | | | | |
| | β-Glc*p*-(1→ | RFK-9 | 4.55 (8.1) | 3.28 (9.4) | 3.49 (9.4) | | 3.32 (9.4) | 3.46 | 3.70 (1.8) | 3.93 (6.0, 12.0) | | | | |
| H | β-Gal*p*A-(1→ | RFK-11 | 4.48 (8.0) | 3.59 (10.0) | 3.76 (3.8) | | 4.26 (1.0) | 4.39 | | | | | | |
| I | →5)-α-Kdo*p*-(2→ | RFK-11 | | | 2.07[b] (4.0, 12.3) | 2.21[c] (12.0) | 4.17 (3.2) | 4.29 | 4.12 (1.0) | | 4.10 (9.0) | | 3.87 (2.6) | 3.82 (7.0, 12.0) |
| | 4 | RFK-9 | | | 1.88 (4.0, 12.0) | 2.19 (12.0) | 4.08 (3.0) | 4.27 | 4.16 (1.2) | | 4.10 (9.2) | | 3.84 (2.6) | 3.81 (7.0, 12.3) |
| | ↑ | | | | | | | | | | | | | |
| J | α-Kdo*p*-(2→ | RFK-11 | | | 1.89[b] (4.9, 13.0) | 2.18[c] (12.2) | 4.09 (3.1) | 4.26 | 3.83 (1.0) | | 4.12 (9.0) | | 3.99 (3.5) | 3.79 (7.0, 12.5) |
| | | RFK-9 | | | 2.08 (5.0, 12.8) | 2.22 (12.4) | 4.12 (2.8) | 4.12 | 3.86 (1.0) | | 4.15 (9.0) | | 3.96 (3.6) | 3.87 (7.1, 12.6) |

[a] Observed first-order chemical shifts and coupling constants (hertz) (in parentheses), measured at 27°C in $D_2O$, pD ≃ 2.
[b] H-3 axial.
[c] H-3 equitorial.

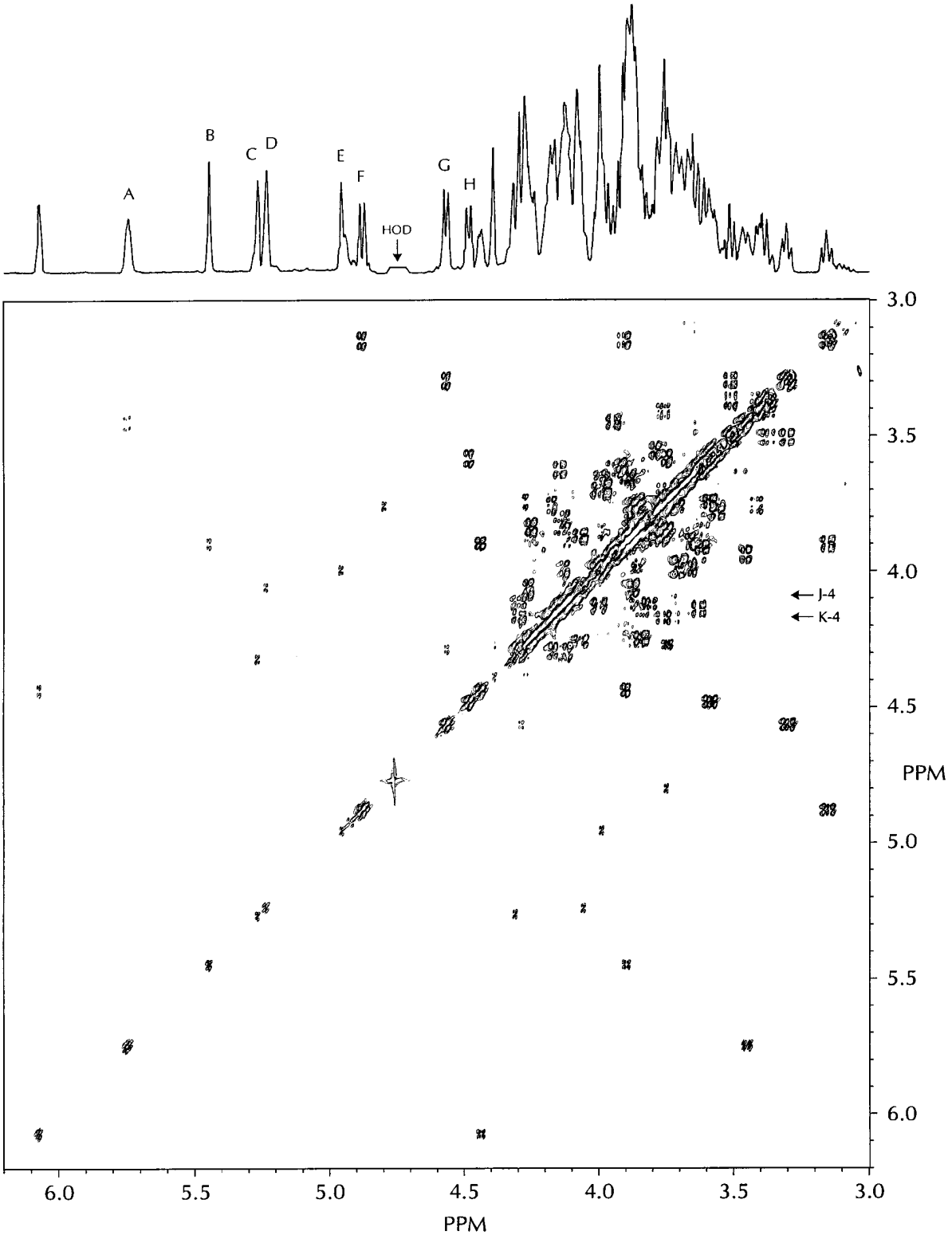


FIG. 3. Partial phase-sensitive COSY contour map of the *K. pneumoniae* mutant RFK-11 deacylated LPS carbohydrate backbone oligosaccharide, showing correlations of the ring protons from 3.0 to 6.2 ppm. The spectra were recorded at 27°C and pD ≃ 2.

were fully assigned by COSY (5) (Fig. 3) and TOCSY (3) experiments. From the $^{1}H$ chemical shifts (10) and vicinal proton coupling constants (1), subspectra corresponding to all of the glycosyl residues were identified (Table 2).

Seven anomeric $^{13}C$ resonances were observed within the 90- to 110-ppm region of the one-dimensional $^{13}C$-NMR spectrum of the RFK-9 carbohydrate backbone oligosaccharide (Table 3). The $^{13}C$-NMR spectrum of the RFK-11 carbohy-

TABLE 3. Carbon 13 chemical shifts and $^1J_{C,H}$ values for the deacylated carbohydrate backbone oligosaccharides from the LPS of *K. pneumoniae* serotype O8 mutants RFK-9 and RFK-11[a]

| Residue | Monosaccharide unit | Oligosaccharide | $^{13}$C chemical shift ($^1J_{C,H}$, Hz) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | C-1 ($J_{C-1,H-1}$) | C-2 | C-3 | C-4 | C-5 | C-6 | C-7 | C-8 |
| A | →6)-α-Glc*p*N-(1→P | RFK-11 | 92.0 (177) | 54.2 | 69.8 | 69.7 | 72.9 | 69.2 | | |
| | | RFK-9 | 92.0 | 54.3 | 69.7 | 69.6 | 72.6 | 69.0 | | |
| B | →α-*threo*-Hex-4-enurono*p*-(1→ | RFK-11 | 100.8 (177) | 70.2 | 65.8 | 111.6 | 142.3 | 167.0 | | |
| C | →3)-L-α-D-Hep*p*-(1→ | RFK-11 | 101.9 (175) | 69.5 | 72.5 | 65.6 | 72.2 | 69.2 | 70.5 | |
| | 7 ↑ L-α-D-Hep*p*-(1→ | RFK-9 | 102.0 | 70.7 | 69.3 | 72.9 | 72.0 | 69.1 | 64.2 | |
| D | →3)-L-α-D-Hep*p*-(1→ | RFK-11 | 99.6 (177) | 70.7 | 75.8 | 73.4 | 68.8 | 69.2 | 64.1 | |
| | 4 ↑ | RFK-9 | 99.2 | 70.6 | 74.5 | 73.2 | 69.5 | 69.4 | 64.1 | |
| E | L-α-D-Hep*p*-(1→ | RFK-11 | 101.5 (171) | 70.2 | 71.0 | 66.8 | 72.8 | 70.2 | 63.3 | |
| F | →6)-β-Glc*p*N-(1→ | RFK-11 | 99.2 (162) | 55.6 | 74.3[b] | 74.4[b] | 72.8 | 63.5 | | |
| | 4 ↑ P | RFK-9 | 99.2 | 55.8 | 74.4[b] | 74.5[b] | 72.6 | 63.4 | | |
| G | →6)-β-Glc*p*-(1→ | RFK-11 | 102.6 (161) | 73.9 | 75.8 | 70.2 | 76.5 | 70.2 | | |
| | β-Glc*p*-(1→ | RFK-9 | 102.6 | 73.8 | 75.8 | 70.3 | 76.5 | 61.8 | | |
| H | β-Gal*p*A-(1→ | RFK-11 | 102.8 (164) | 70.5 | 72.5 | 69.7 | 74.5 | 172.5 | | |
| I | →5)-α-Kdo*p*-(2→ | RFK-11 | 175.5[c] | 100.5[c] | 34.0 | 70.6 | 73.4 | 72.8 | 69.2 | 64.2 |
| | 4 ↑ | RFK-9 | ND[d] | ND | 34.0 | 70.5 | 73.2 | 72.2 | 69.1 | 64.2 |
| J | α-Kdo*p*-(2→ | RFK-11 | 175.8[c] | 100.8[c] | 34.4 | 66.1 | 67.5 | 72.2 | 72.6 | 63.5 |
| | | RFK-9 | ND | ND | 34.4 | 66.2 | 66.4 | 71.8 | 72.1 | 63.5 |

[a] Assignments were determined by $^{13}$C-$^1$H chemical shift correlation (HMQC) unless otherwise indicated, measured at 27°C in $D_2O$, pD ≈ 2.
[b] Assigned by HMQC-TOCSY experiment.
[c] Identified by long-range $^{13}$C-$^1$H chemical shift correlation (HMBC).
[d] ND, not determined.

drate backbone oligosaccharide exhibited three additional anomeric signals representing the addition of three glycoses to form the RFK-11 oligosaccharide. Unambiguous assignment of the $^{13}$C resonances was done by correlation with the $^1$H resonances of the directly attached protons in a heteronuclear $^{13}$C-$^1$H chemical shift correlation experiment (HMQC) (38), and the data are given in Table 3.

In consideration of the results of 2D subspectral and compositional analyses, the carbohydrate backbone oligosaccharide in RFK-9 LPS was found to contain the following components: α-D-Glc*p*N (A), β-D-Glc*p*N (F), two L-α-D-Hep*p* (C and D), β-D-Glc*p* (G), and two Kdo*p* (I and J) residues. The same components were present in the RFK-11 carbohydrate backbone oligosaccharide; however, additional L-α-D-Hep*p* (E), β-D-Gal*p*A (H), and hex-4-enurono*p* (B) residues were also identified.

The sequence of the glycosyl residues within these oligosaccharides was established by interresidue $^1$H-$^1$H nuclear Overhauser enhancement (NOE) measurements (8, 39) and long-range $^1$H-$^{13}$C couplings (Table 4). NOE measurements were made in the 2D mode and were employed qualitatively to establish short through-space connectivities between the anomeric and aglyconic protons of the adjacent glycosidically linked residues. Long-range $^1$H-$^{13}$C couplings across the glycosidic linkages also gave accurate sequence information and were obtained by $^1$H-detected HMBC.

The accumulated evidence from NMR and methylation analyses permits the carbohydrate backbone oligosaccharides from RFK-9 and RFK-11 to be assigned the structures shown in Fig. 4B and C. The structure of the carbohydrate backbone oligosaccharide in the LPS of RFK-11 is identical to that reported for the LPS from *K. pneumoniae* O1 (50). Differences in the chemical shifts for similar residues in the O1 and O8 structures can probably be attributed to the use of NMR at pH(pD) values of approximately 2 in this work and 9.4 for the O1 analysis (50).

**Structure of the complete core oligosaccharide in RFK-11 LPS.** The terminal α-L-*threo*-hex-4-enurono*p* residue in the RFK-11 carbohydrate backbone oligosaccharide structure described above was predicted to arise from a (1→4)-linked α-Gal*p*A moiety which had undergone β-elimination under the alkaline conditions used to de-N-acylate the LPS during isolation of the carbohydrate backbone oligosaccharide. This would also result in loss of any distal outer core sugars in RFK-11 LPS. The structure in Fig. 4C can therefore be regarded as only a partial structure. To test this hypothesis, the core oligosaccharide from the dephosphorylated RFK-11 lipopolysaccharide was isolated by selective cleavage with dilute acetic acid of the acid-labile 3-deoxyaldulosonic acid glycosidic linkages.

Methylation analysis of the RFK-11 core oligosaccharide (Table 1) gave all of the expected methylated derivatives already described for the carbohydrate backbone oligosaccharide of RFK-11 with the exception of 2,3-di-*O*-methyl-hex-4-enuronopyranose-6,6-$d_2$ derivative. This was replaced by 2,3-di-*O*-methyl-galactopyranose-6,6-$d_2$, resulting from an intact Gal*p*A residue. An additional 2,3,4,6,-tetra-*O*-methyl-glucopyranose residue was detected. This confirms the presence of the →4)-α-Gal*p*A-(1→ residue and indicates that the main chain of the core oligosaccharide terminates in a glucopyranose residue. Furthermore, the $^1$H-NMR spectrum of the core oligosaccharide shows the expected anomeric proton from the glucopyranose residue (5.14 ppm) (44). The Glc*p* residue has a vicinal coupling ($J_{1,2}$ = 3.4 Hz) indicative of its possessing the α-configuration.

The negative electrospray mass spectrum of the RFK-11

TABLE 4. Long-range $^{1}H$-$^{13}C$ and $^{1}H$-$^{1}H$ NOE intra- and interresidue correlations obtained for the deacylated carbohydrate backbone oligosaccharides from the LPS of *K. pneumoniae* serotype O8 mutants RFK-9 and RFK-11

| Residue | Monosaccharide unit | Oligosaccharide | Observed atom | NOE contacts | | Long-range $^{1}H$-$^{13}C$ correlations | |
|---|---|---|---|---|---|---|---|
| | | | | Intraresidue | Interresidue | Intraresidue | Interresidue |
| A | →6)-α-Glc*p*N-(1→P | RFK-11 | H-1 | 3.44, **A**2 | | 72.8, **A**5 | |
| | | RFK-9 | H-1 | 3.43, **A**2 | | | |
| B | α-*threo*-Hex-4-enurono*p*-(1→ | RFK-11 | H-1 | 3.89, **B**2 | 4.30, **C**2; 4.12, **C**3 | 65.8, **B**3 | |
| | | | C-1 | | | 4.17, **B**6 | |
| C | →3)-L-α-D-Hep*p*-(1→ 7 ↑ L-α-D-Hep*p*-(1→ | RFK-11 | H-1 | 4.30, **C**2; 4.12, **C**3 | 4.14, **D**3; 4.06, **D**2 | 72.5, **C**3 | |
| | | | C-1 | | | 4.11, **C**3 | |
| | | RFK-9 | H-1 | 4.15, **C**2 | 4.10, **D**3; 4.08, **D**2 | | |
| D | →3)-L-α-D-Hep*p*-(1→ 4 ↑ | RFK-11 | H-1 | 4.06, **D**2 | 4.29, **I**5 | | |
| | | RFK-9 | H-1 | 4.06, **D**2 | 4.27, **I**5 | | |
| E | L-α-D-Hep*p*-(1→ | RFK-11 | H-1 | 3.98, **E**2 | 3.70, **C**7; 3.75, **C**7′; 4.16, **C**6 | 72.8, **E**2 | 70.5, **C**7 |
| | | | C-1 | | | 4.13, **E**3 | 3.70, **C**7; 3.75, **C**7′ |
| F | →6)-β-Glc*p*N-(1→ 4 ↑ P | RFK-11 | H-1 | 3.88, **F**3; 3.76, **F**5 | 3.83, **A**6′ | | |
| | | | C-1 | | | 3.15, **F**2 | 3.83, **A**6′ |
| | | RFK 9 | H-1 | 3.88, **F**3; 3.76, **F**5 | 3.80, **A**6′ | | |
| G | β-Glc*p*-(1→ | RFK-11 | H-1 | 3.52, **G**3; 3.41, **G**5 | 4.26, **D**4 | 73.9, **G**2 | |
| | | | C-1 | | | 3.30, **G**2 | 4.26, **D**4 |
| | | RFK-9 | H-1 | 3.49, **G**3; 3.28, **G**2 | 4.27, **D**4 | | |
| H | β-Gal*p*A-(1→ | RFK-11 | H-1 | 4.39, **H**5 | 3.88, **G**6′ | 72.5, **H**3 | 70.2, **G**6 |
| | | | C-1 | | | 4.39, **H**5; 3.59, **H**2 | |
| I | →5)-α-Kdo*p*-(2→ 4 ↑ | RFK-11 | H-3 | 4.29, **I**5; 4.17, **I**4 | 3.69, **F**6 | 100.5, **I**2; 70.6, **I**4 | |
| | | | C-2 | | | 4.17, **I**4 | |
| | | RFK-9 | H-3 | 4.08, **I**4 | 3.65, **F**6 | | |
| J | α-Kdo*p*-(2→ | RFK 11 | H-3 | 4.09, **J**4 | 4.17, **I**4 | 100.8, **J**2 | |
| | | RFK 9 | H-3 | 4.12, **J**4 | 4.08, **I**4 | | |

core oligosaccharide showed a strong $(M-2)^{2-}$ ion at 735 Da (44). This corresponds to the (predicted) molecular mass of 1,472 Da and is consistent with the presence of 2 uronic acids, 3 heptoses, 2 hexoses, and an anhydro-Kdo residue.

Compilation of these data and the partial structure defined above resulted in the carbohydrate structure of the RFK-11 core oligosaccharide shown in Fig. 4A.

**Sites of phosphorylation on the carbohydrate backbone of the RFK-11 LPS.** The $^{31}P$-NMR spectrum of the phosphorylated RFK-11 carbohydrate backbone oligosaccharide showed only two major phosphate monoester peaks, at 0.20 and −1.88 ppm. As expected, strong connectivities were observed in the 2D $^{31}P$-$^{1}H$ HMBC correlation map (44) between the $^{31}P$ signals and the $^{1}H$ resonances for H-1 of residue A (5.74 ppm) and H-4 of residue F (3.92 ppm), respectively, indicating that the phosphate groups are confined to the lipid A region of the molecule at the O-1 position of the α-Glc*p*N residue and at the O-4 position of the β-Glc*p*N residue.

**MS analysis of the LPS of RFK-9 and RFK-11.** MS was used to confirm the predicted LPS structures. The negative-ion electrospray mass spectra of the RFK-9 and RFK-11 phosphorylated carbohydrate backbone oligosaccharides (44) gave triply and doubly charged ions corresponding to molecular ions at 1,487 and 2,013 Da, respectively, in excellent agreement with the calculated masses for $C_{48}H_{85}O_{46}N_2P_2$ (1,488 Da) and $C_{67}H_{111}O_{63}N_2P_2$ (2,014 Da). The complete structure of the RFK-11 LPS is therefore that shown in Fig. 4A.

## DISCUSSION

In contrast to the highly variable structure of the O-antigen portion of the LPSs of the *Enterobacteriaceae*, the relatively few LPS core oligosaccharide structures described to date indicate much higher levels of conservation. This is particularly true for the inner regions of the LPS core oligosaccharide. Overall, the architecture of the *K. pneumoniae* inner core oligosaccharide reported here shows general similarities to the enterobacterial core structures so far elucidated. The internal heptose-Kdo backbone region, which constitutes the linkage between lipid A and the outer core oligosaccharide, is common. An inner core-specific MAb (WN1) has been isolated and shown to recognize a conserved epitope widespread among the *Enterobacteriaceae* (18). MAb WN1 is protective against LPS-induced pyrogenicity in rabbits. However, most *Klebsiella* isolates did not react with MAb WN1, and this is likely related to interesting and unique features in the *K. pneumoniae* core. Substitution of the Hep-I residue at the O-4 position is a feature seen only in the core of strains of *Proteus mirabilis* (40), *Yersinia enterocolitica* (25), and *K. pneumoniae*. In *K. pneumoniae* serotypes O1 (50) and O8 (this study), the Hep-I substitution consists of a β-D-Gal*p*A-(1→6)-β-D-Glc disaccharide. The truncated RFK-9 core lacks the terminal β-D-Gal*p*A residue.

One of the more striking features of the *K. pneumoniae* core oligosaccharide is the absence of phosphate residues. Kdo and GalA residues provide the only negative charges in the core oligosaccharides from serotypes O1 and O8. Divalent cations stabilize adjacent LPS molecules by interacting with negatively charged groups on LPS (53). In *E. coli*, the most probable binding sites for the cations are the phosphate residues (21, 22, 47), and it has been shown that free carboxyl groups (on Kdo residues) do not play a major role in cation binding (22). Consequently, the absence of phosphate outside of the *K. pneumoniae* lipid A region may adversely affect outer mem-

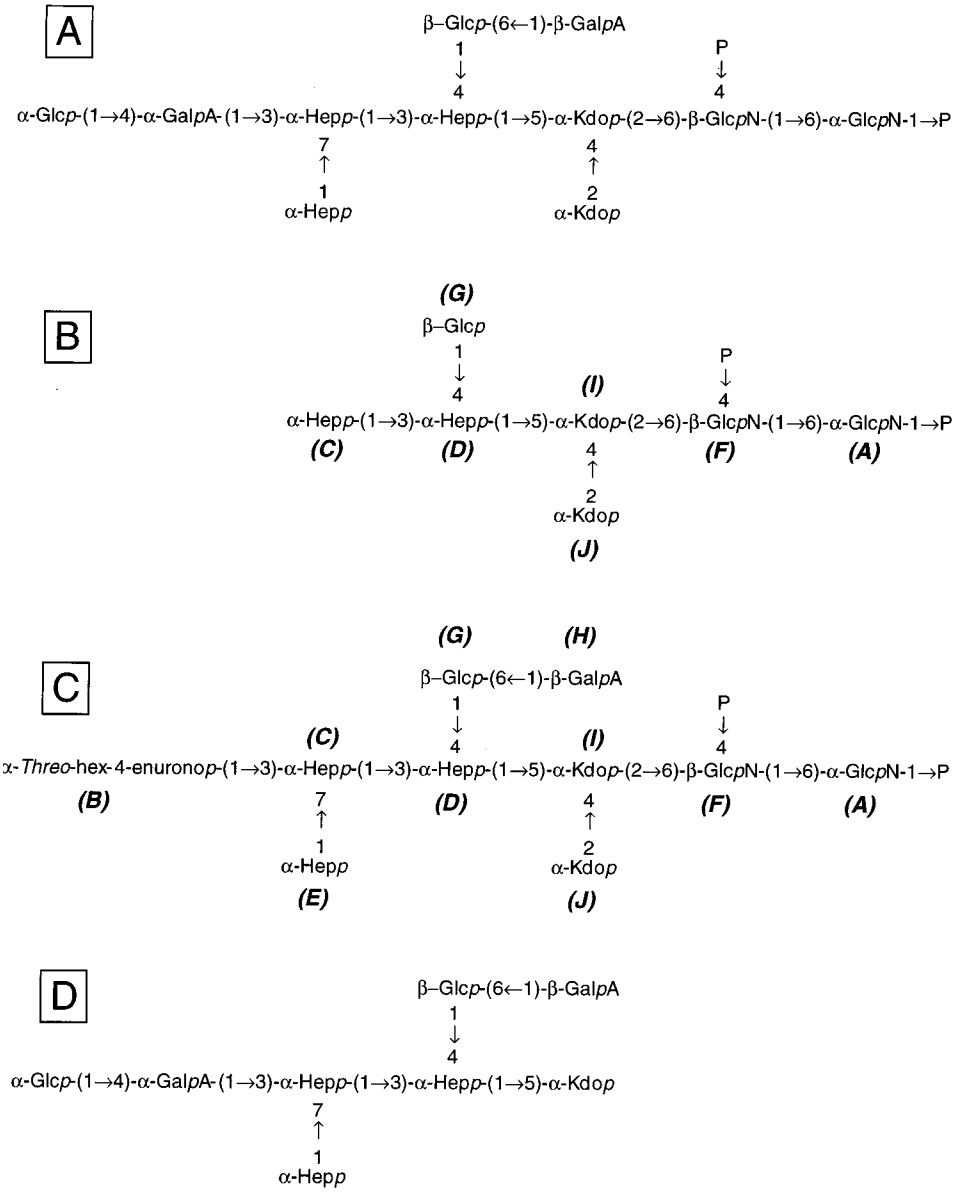


FIG. 4. Deduced structures of the oligosaccharides from the LPSs of *K. pneumoniae* mutants RFK-9 and RFK-11. (A) Complete carbohydrate backbone of the LPS of RFK-11 and the locations of phosphate residues. (B and C) Carbohydrate backbone oligosaccharides of the deacylated LPSs from RFK-9 and RFK-11, respectively. (D) Structure of the RFK-11 core oligosaccharide prepared by mild acid hydrolysis. Letters in parentheses are the designations given each residue in NMR analyses (see Fig. 2).

brane stability. This in turn could explain the propensity of *K. pneumoniae* to release the LPS-containing extracellular toxic complex (48, 49) from its cell surface and therefore would have a significant impact on the pathogenesis of this organism.

The outer core from *K. pneumoniae* is quite different from the equivalent region from *E. coli*, *Shigella* spp., and *S. enterica* (25). Both the presence of GalA at O-3 of Hep II and the absence of the typical hexose-containing region are unusual. However, studies have shown that enterobacterial species that are genetically more distantly related to *E. coli* and *Salmonella* spp., such as *P. mirabilis* (40), *Providencia rettgeri* (2), and *Morganella morganii* (31), express characteristically deviating core structures, with terminal D-GalA as a characteristic core constituent. It has been suggested that comparison of core oligosaccharide structures is of taxonomic and phylogenetic significance and is in accord with rRNA sequence similarity analyses (40). Galacturonic acid residues have also been detected as components of LPS core oligosaccharides in *Vibrio cholerae* H11 (11), *Rhizobium trifolii* ANU843 (14), *Rhizobium leguminosarum* (13), and *Rhodocyclus gelatinosa* (34).

Analysis of core oligosaccharides is complicated by heterogeneity in isolated material. To overcome this, mutants with core or O-antigen synthesis defects have typically been used for structural analyses. Truncated cores can provide a useful starting point, as is the case in this study. However, for bacteria whose LPS structure and genetics are not well established, it is often impossible to determine whether the final deduced core structure represents that of a full-length molecule. In this study, an *rfb* mutant, RFK-11, was examined, and the presence of an O-antigen attachment site was confirmed. These data

strongly support the proposal that the structure described here for RFK-11 LPS reflects a full-length core oligosaccharide. It is considered unlikely that RFK-11 has an additional core biosynthesis defect which does not compromise the O-antigen attachment site. The absence of O antigen in the LPS preparations means that the precise attachment site for O antigen remains unknown in *K. pneumoniae*, but based on the organization of other core oligosaccharides, the most likely attachment site is the terminal α-Glc residue. In some bacteria, semirough LPS (SR-LPS), containing a single repeat unit of O antigen attached to the core, provides a means to characterize the attachment site. Unfortunately, the synthesis pathway for the O1 and O8 antigens precludes isolation of *K. pneumoniae* mutants with SR-LPS (12, 15, 55), and as a consequence, resolution of this question is impossible at this time.

There are several potential explanations for the mutation resulting in defects in two regions of the RFK-9 core oligosaccharide. The simplest possibility is a defect in the formation of the precursor for Gal*p*A residues. The absence of the α-Hep*p* side branch can be explained by the requirement for a main-chain Gal*p*A residue in the receptor which is recognized by the heptosyl transferase. In a similar way, an *rfaB* mutant of *S. enterica* contains LPS lacking a side branch galactose and cannot efficiently complete the core (43). Alternatively, the enzymes involved in core synthesis may operate in a complex, and mutations in one *rfa* gene might influence protein-protein interactions in the complex, affecting additional steps. These possibilities have been discussed elsewhere by Schnaitman and Klena (43). Finally, the phenotype of RFK-9 may result from a double mutation affecting different steps of core biosynthesis.

In earlier analyses by others (51), Western immunoblotting and enzyme-linked immunosorbent assays were used to demonstrate the reactivity of MAb V/9.5 with LPS core fractions from seven *Klebsiella* serotypes (1 to 6 and 8), suggesting that these serotypes possess structurally related core oligosaccharides. The O7 serotype reference strain did not react, but there is some question whether this strain is in fact a member of the genus *Klebsiella* (51). The majority (>90%) of isolates of *K. pneumoniae* subsp. *pneumoniae* and *Klebsiella oxytoca* were reactive with MAb V/9.5, whereas two isolates of *K. pneumoniae* subsp. *rhinoscleromatis* were not. Since LPSs from other enteric bacteria did not react with MAb V-9.5, the epitope is considered genus specific. The chemical basis for the cross-reactivity between the core oligosaccharides of serotypes O1 and O8 is now evident.

The complete molecules of O1 and O8 LPS show considerable similarity. The LPSs of both serotypes contain a D-galactan I polysaccharide domain attached to lipid A-core (28, 30, 56), and the O-acetylation of D-galactan I in serotype O8 provides the only distinction between the serotypes (28). Long O-antigen chains in both serotypes contain identical domains of a second O-polysaccharide structure (D-galactan II). As a result, the O1 and O8 antigens show extensive O-serological cross-reactions (28). Despite the similarity in the O antigens, there are significant differences in the reference strains which express the O1 and O8 antigens. First, the O1 strain and the O8 isolate represent different subspecies (*K. pneumoniae* subsp. *pneumoniae* and *K. pneumoniae* subsp. *ozaenae*, respectively). Second, although the *rfb* clusters from both serotypes encode highly conserved proteins involved in synthesis of D-galactan I (29), the nucleotide sequences are sufficiently divergent that these isolates contain *rfb* genes from quite distinct clonal groups (28). The similarities between the cores of the O1 and O8 type strains are therefore significant, and although the complete structure of the O1 core is not yet available, it is reasonable to speculate that the core described here in strain RFK-11 may represent a common core oligosaccharide structure in *Klebsiella* LPS. If further studies confirm a common LPS core structure within *Klebsiella* spp. or, minimally, a unique genus-specific inner core region, an alternative immunotherapy-based approach to the treatment of *Klebsiella* infections may prove viable. From existing chemical and immunochemical data, antibodies against the core oligosaccharide structure might serve as useful diagnostic reagents.

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## REFERENCES

1. **Altona, C., and C. A. G. Haasnoot.** 1980. Prediction of *anti* and *gauche* vicinal proton-proton coupling constants in carbohydrates: a simple additivity rule for pyranose rings. Org. Magn. Reson. **13:**417–429.
2. **Basu, S., J. Radziejewska-Lebrecht, and H. Mayer.** 1986. Lipopolysaccharide of *Providencia rettgeri*: chemical studies and taxonomical implications. Arch. Microbiol. **144:**213–218.
3. **Bax, A., and D. G. Davis.** 1985. MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. J. Magn. Reson. **65:**335–360.
4. **Bax, A., D. G. Davis, and S. K. Sarkar.** 1985. An improved method for two-dimensional heteronuclear relayed-coherence-transfer NMR spectroscopy. J. Magn. Reson. **63:**230–234.
5. **Bax, A., R. Freeman, and G. Morris.** 1981. Correlation of chemical shifts by two-dimensional Fourier transfer NMR. J. Magn. Reson. **42:**164–168.
6. **Bax, A., R. H. Griffey, and B. L. Hawkins.** 1983. Correlation of proton and nitrogen-15 chemical shifts by multiple quantum NMR. J. Magn. Reson. **55:** 301–315.
7. **Bax, A., and M. F. Summers.** 1986. Proton and $^{13}C$ assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. J. Am. Chem. Soc. **108:**2093–2094.
8. **Bernstein, M. A., and L. D. Hall.** 1982. De novo sequencing of oligosaccharides by proton NMR spectroscopy. J. Am. Chem. Soc. **104:**5553–5555.
9. **Binotto, J., P. R. MacLachlan, and P. R. Sanderson.** 1991. Electrotransformation of *Salmonella typhimurium* LT2. Can. J. Microbiol. **37:**474–477.
10. **Bock, K., and H. Thögersen.** 1982. Nuclear magnetic resonance spectroscopy in the study of mono- and oligosaccharides. Annu. Rep. NMR Spectrosc. **13:** 1–57.
11. **Bock, K., E. V. Vinogradov, O. Holst, and H. Brade.** 1994. Isolation and structural analysis of oligosaccharide phosphates containing the complete carbohydrate chain of the lipopolysaccharide from *Vibrio cholerae* strain H11 (non-O1). Eur. J. Biochem. **225:**1029–1039.
12. **Bronner, D., B. R. Clarke, and C. Whitfield.** 1994. Identification of an ATP-binding cassette transport system required for translocation of lipopolysaccharide O-antigen side chains across the cytoplasmic membrane of *Klebsiella pneumoniae* serotype O1. Mol. Microbiol. **14:**505–519.
13. **Carlson, R. W., F. Garci, D. Noel, and R. Hollingsworth.** 1989. The structures of the lipopolysaccharide core components from *Rhizobium leguminosarum* biovar *phaseoli* CE3 and two of its symbiotic mutants, CE109 and CE309. Carbohydr. Res. **195:**101–110.
14. **Carlson, R. W., R. L. Hollingsworth, and F. B. Dazzo.** 1988. A core oligosaccharide component from the lipopolysaccharide of *Rhizobium trifolii* ANU843. Carbohydr. Res. **176:**127–135.
15. **Clarke, B. R., D. Bronner, W. J. Keenleyside, W. B. Severn, J. C. Richards, and C. Whitfield.** 1995. Role of Rfe and RfbF in the initiation of biosynthesis of D-galactan I, the lipopolysaccharide O antigen from *Klebsiella pneumoniae* serotype O1. J. Bacteriol. **177:**5411–5418.
16. **Clarke, B. R., and C. Whitfield.** 1992. Molecular cloning of the *rfb* region of *Klebsiella pneumoniae* serotype O1:K20: the *rfb* gene cluster is responsible for synthesis of the D-galactan I O polysaccharide. J. Bacteriol. **174:**4614–4621.
17. **Coleman, W. G. J.** 1983. The *rfaD* gene codes for ADP-L-glycero-D-manno-heptose-6-epimerase. J. Biol. Chem. **258:**1985–1990.
18. **Di Padova, F. E., H. Brade, G. R. Barclay, I. R. Poxton, E. Liehl, E. Schuetze, H. P. Kochner, G. Ramsay, M. H. Schreier, D. B. L. McClelland, and E. Rietschel.** 1993. A broadly cross-protective monoclonal antibody binding to *Escherichia coli* and *Salmonella* lipopolysaccharides. Infect. Immun. **61:** 3863–3872.
19. **Domenico, P., D. L. Diedrich, and D. C. Straus.** 1985. Extracellular polysac-

charide production by *Klebsiella pneumoniae* and its relationship to virulence. Can. J. Microbiol. **31:**472–478.
20. **Dubois, M., K. A. Gillies, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for the determination of sugars and related substances. Anal. Chem. **28:**167–171.
21. **Ferris, F. G., and T. J. Beveridge.** 1984. Binding of a paramagnetic metal cation to *Escherichia coli* K-12 outer-membrane vesicles. FEMS Microbiol. Lett. **24:**43–46.
22. **Ferris, F. G., and T. J. Beveridge.** 1986. Site specificity of metallic ion binding in *Escherichia coli* K-12 lipopolysaccharide. Can. J. Microbiol. **32:**52–55.
23. **Hakomori, S.** 1964. Rapid permethylation of glycolipids and polysaccharides catalysed by methylsulfinyl carbanion in dimethyl sulfoxide. J. Biochem. (Tokyo) **55:**205–208.
24. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154:**269–277.
25. **Holst, O., and H. Brade.** 1992. Chemical structure of the core region of lipopolysaccharides, p. 171–205. *In* D. C. Morrison and J. L. Ryan (ed.), Bacterial endotoxic lipopolysaccharides, vol. I. CRC Press, Boca Raton, Fla.
26. **Holst, O., U. Zähringer, H. Brade, and A. Zamojski.** 1991. Structural analysis of the heptose/hexose region of the lipopolysaccharide from *Escherichia coli* K-12 strain W3100. Carbohydr. Res. **215:**323–335.
27. **Johnson, K. J., and M. B. Perry.** 1976. Improved techniques for the preparation of bacterial lipopolysaccharide. Can. J. Microbiol. **22:**29–34.
28. **Kelly, R. F., W. B. Severn, J. C. Richards, M. B. Perry, L. L. MacLean, J. M. Tomás, S. Merino, and C. Whitfield.** 1993. Structural variation in the O-specific polysaccharides of *Klebsiella pneumoniae* serotype O1 and O8 lipopolysaccharide: evidence for clonal diversity in *rfb* genes. Mol. Microbiol. **10:**615–625.
29. **Kelly, R. F., and C. Whitfield.** Unpublished results.
30. **Kol, O., J.-M. Wieruszeski, G. Strecker, B. Fournet, R. Zalisz, and P. Smets.** 1992. Structure of the O-specific polysaccharide chain of *Klebsiella pneumoniae* O1:K2 (NCTC 5055) lipopolysaccharide: a complementary elucidation. Carbohydr. Res. **236:**339–344.
31. **Kotelko, K., M. Deka, W. Gromska, W. Kaca, J. Radziejewska-Lebrecht, and A. Rozalski.** 1983. Galacturonic acid as the terminal constituent in the R-core polysaccharide of *Proteus* R110 (Ra) mutant. Arch. Immunol. Ther. Exp. **31:**619–624.
32. **Kumar, A., R. R. Ernst, and K. Wüthrich.** 1980. A two-dimensional nuclear overhauser enhancement (2D NOE) experiment for the elucidation of complete proton-proton cross-relaxation networks in biological macromolecules. Biochem. Biophys. Res. Commun. **95:**1–6.
33. **Laakso, D. H., M. K. Homonylo, S. J. Wilmot, and C. Whitfield.** 1988. Transfer and expression of the genetic determinants for O and K antigen synthesis in *Escherichia coli* O9:K(A)30:H12 and *Klebsiella* sp. O1:K20, in *Escherichia coli* K-12. Can. J. Microbiol. **34:**987–992.
34. **Masoud, H., H. Mayer, T. Kontrohr, O. Holst, and J. Weckesser.** 1991. The structure of the core region of the lipopolysaccharide from *Rhodocyclus gelatinosus* Dr2. Syst. Appl. Microbiol. **14:**222.
35. **Miller, J. H.** 1992. A short course in bacteria genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
36. **Montgomerie, J. Z.** 1979. Epidemiology of *Klebsiella* and hospital-associated infections. Rev. Infect. Dis. **1:**736–753.
37. **Nimmich, W., and G. Korten.** 1970. Die chemische zusammensetzung der Klebsiella-lipopolysaccharide (O-antigene). Pathol. Microbiol. **36:**179–190.
38. **Pearson, G. A.** 1985. High-accuracy proton-carbon chemical-shift correlations from one-dimensional polarization-transfer $^{13}C$ NMR spectra. J. Magn. Reson. **64:**487–500.
39. **Prestegard, J. H., T. A. W. Koerner, Jr., P. C. Demou, and R. K. Yu.** 1982. Complete analysis of oligosaccharide primary structure using two-dimensional high-field proton NMR. J. Am. Chem. Soc. **104:**4993–4995.
40. **Radziejewska-Lebrecht, J., and H. Mayer.** 1989. The core region of *Proteus mirabilis* R110/1959 lipopolysaccharide. Eur. J. Biochem. **183:**573–581.
41. **Rance, M., O. W. Sörensen, G. Bodenhausen, G. Wagner, R. R. Ernst, and K. Wüthrich.** 1983. Improved spectral resolution in COSY $^{1}H$ NMR spectra of proteins via double quantum filtering. Biochem. Biophys. Res. Commun. **117:**479–485.
42. **Rietschel, E. T., L. Brade, B. Lindner, and U. Zähringer.** 1992. Molecular biochemistry of lipopolysaccharides, p. 3–42. *In* D. C. Morrison and J. L. Ryan (ed.), Bacterial endotoxic lipopolysaccharides, vol. I. CRC Press, Boca Raton, Fla.
43. **Schnaitman, C. A., and J. D. Klena.** 1993. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiol. Rev. **57:**655–682.
44. **Severn, W., R. F. Kelly, J. C. Richards, and C. Whitfield.** Unpublished results.
45. **Shaka, A. J., P. B. Barker, and R. Freeman.** 1985. Computer-optimized decoupling scheme for wideband applications and low-level operation. J. Magn. Reson. **64:**547–552.
46. **Shaka, A. J., J. Keeler, T. Frenkiel, and R. Freeman.** 1983. An improved sequence for broadband decoupling: WALTZ-16. J. Magn. Reson. **52:**335–338.
47. **Strain, S. M., S. W. Fesiik, and I. M. Armitage.** 1983. Structure and metal-binding properties of lipopolysaccharide from heptoseless mutants of *Escherichia coli*: studies by $^{13}C$ and $^{31}P$ nuclear magnetic resonance. J. Biol. Chem. **258:**13466–13477.
48. **Straus, D. C.** 1987. Production of an extracellular toxic complex by various strains of *Klebsiella pneumoniae*. Infect. Immun. **55:**44–48.
49. **Straus, D. C., D. L. Atkisson, and C. W. Garner.** 1985. Importance of a lipopolysaccharide-containing extracellular toxic complex in infections produced by *Klebsiella pneumoniae*. Infect. Immun. **50:**787–795.
50. **Süsskind, M., S. Müller-Leonnies, W. Nimmich, H. Brade, and O. Holst.** 1995. Structural investigation on the carbohydrate backbone of the lipopolysaccharide from *Klebsiella pneumoniae* rough mutant R20/O1$^-$. Carbohydr. Res. **269:**C1–C7.
51. **Trautmann, M., K. Vogt, C. Hammack, and A. S. Cross.** 1994. A murine monoclonal antibody defines a unique epitope shared by *Klebsiella* lipopolysaccharides. Infect. Immun. **62:**1282–1288.
52. **Tsai, G. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
53. **Vaara, M.** 1992. Agents that increase the permeability of the outer membrane. Microbiol. Rev. **56:**395–411.
54. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharide extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. **5:**83–91.
55. **Whitfield, C.** 1995. Biosynthesis of lipopolysaccharide O-antigens. Trends Microbiol. **3:**178–185.
56. **Whitfield, C., J. C. Richards, M. B. Perry, B. R. Clarke, and L. L. MacLean.** 1991. Expression of two structurally distinct D-galactan O-antigens in the lipopolysaccharide of *Klebsiella pneumoniae* serotype O1. J. Bacteriol. **173:**1420–1431.
57. **Williams, P., and J. M. Tomás.** 1990. The pathogenicity of *Klebsiella pneumoniae*. Rev. Med. Microbiol. **1:**196–204.
58. **York, W. S., A. G. Darvill, M. McNeil, T. T. Stevenson, and P. Albersheim.** 1985. Isolation and characterization of plant cell walls and cell wall components. Methods Enzymol. **118:**3–40.