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Expression of Two Structurally Distinct D-Galactan O Antigens in the Lipopolysaccharide of *Klebsiella pneumoniae* Serotype O1

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The lipopolysaccharide (LPS) molecule is an important virulence determinant in *Klebsiella pneumoniae*. Studies on the serotype O1 LPS were initiated to determine the basis for antigenic heterogeneity previously observed in the O1 side chain polysaccharides and to resolve apparent ambiguities in the reported polysaccharide structure. Detailed chemical analysis, involving methylation and ^{1}H- and ^{13}C-nuclear magnetic resonance studies, demonstrated that the O- side chain polysaccharides of serotype O1 LPS contained a mixture of two structurally distinct D-galactan polymers. The repeating unit structures of these two polymers were identified as [→3)-β-D-Gal*f*-(1→3)-α-D-Gal*p*-(1→] (D-galactan I) and [→3)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→] (D-Galactan II). D-Galactan I polysaccharides were heterogeneous in size and were detected throughout the sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) profile of O1 LPS. In contrast, D-galactan II was confined to the higher-molecular-weight region. The structures of the two D-galactans were not influenced by simultaneous synthesis of a capsular K antigen. Apparently, neither of the D-galactans constitutes a common antigen widespread in *Klebsiella* spp. as determined by immunochemical analysis. Examination of the LPSs in mutants indicated that expression of D-galactan I can occur independently of D-galactan II. Transconjugants of *Escherichia coli* K-12 strains carrying the *his* region of *K. pneumoniae* were constructed by chromosome mobilization with RP4::mini-Mu. In these transconjugants, the O antigen encoded by the *his*-linked *rfb* locus was determined to be D-galactan I, suggesting that genes involved in the expression of D-galactan II are not closely linked to the *rfb* cluster.

The genus *Klebsiella* contains a number of opportunistic pathogens which cause several serious infections, including pneumonia, bacteremia, and urinary tract infections (36). Characteristically, *Klebsiella* spp. produce large mucoid colonies because of the synthesis of large amounts of capsular polysaccharide (CPS). There are 72 recognized K antigen serotypes of *Klebsiella* spp., which are based on the structures of the CPS (for a review, see reference 25). In contrast, the number of lipopolysaccharide (LPS) O antigen types is much smaller, reflecting a rather limited range of structures. Initially, 12 serotypes were reported, but chemical analysis suggests that there are actually only 8 unique structures (25).

The LPS molecule is a virulence determinant in *Klebsiella pneumoniae*. Virulent strains of *K. pneumoniae* serotypes O1:K1 and O1:K2 release an extracellular toxic complex from their cell surfaces during growth. This complex contains an aggregate of CPS, LPS, and protein and has been implicated in the tissue damage that typically results from active *K. pneumoniae* lobar pneumonia (14, 42, 43). Purified LPS components enhance virulence when coinjected into mice (14). The O1 LPS molecule is responsible for the resistance of *K. pneumoniae* to complement-mediated serum killing (33, 34, 44). Although serotype O1 LPS is common in clinical isolates, possession of this antigen is not sufficient in itself to confer virulence. The results of Mizuta et al. (35) clearly demonstrate that O1 strains with CPS types other than K1 or K2 are not virulent in a mouse model.

Previous work has indicated that the O side chain polysaccharide in serotype O1 LPS shows some heterogeneity in antigenicity (34). On the basis of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), two forms of smooth LPS molecules could be distinguished, and these were termed HMW (high-molecular-weight)- and LMW (low-molecular-weight)-LPS. HMW-LPS contained a unique epitope. Mutants deficient in HMW-LPS were used to demonstrate that HMW-LPS alone is responsible for the serum resistance of *Klebsiella* O1 strains. The structure of the O1 polysaccharide was first described as containing (1→3)-linked α-D-galactopyranose residues (7). In a more recent review, the repeating unit structure was modified as follows: →3)-α-D-Gal*p*-(1→3)-β-D-Gal*f*-(1→ (25). In order to resolve the contradiction in the reported structures and the possible relationships of these structures to the apparent antigenic heterogeneity, we have reevaluated the structure of the O-specific polysaccharide of *Klebsiella* serotype O1 LPS. We report here the presence of two structurally distinct galactans in the O1 LPS.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *K. pneumoniae* O1:K20 KD1 and its derivatives were reported previously (34). *K. pneumoniae* KD2 is a K-deficient mutant isolated from KD1. KD37 is a derivative of KD2 which produces smooth LPS but lacks HMW-LPS, the highest-molecular-weight O-substituted molecules. The following *Klebsiella* strains were obtained from I. Ørskov: NCTC 8172 (serotype O1 = O6 [originally identified as O6 but chemically identical to O1; 7]), 889 (serotype O8), 1205 (serotype O9), and the "O2" strains 2215/52, 2482, 7444, 5758, 6613, F5052, E5051, D5050, C5046, 5053, and 7380. Chemical analysis indicated that the O polysaccharides of serotype O2 LPSs were

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identical to either O8 or O9 (25). *Escherichia coli* PA360 *thi-1 leuB6 hisG1 serA1 argH1 thr-1 lacY1 gal-6 malA1 rpsL9 tonA2 supE44* (CGSC 228) was obtained from B. Bachmann. *E. coli* CWG100 is a His^+ transconjugant obtained by mobilizing the *his* region of the *K. pneumoniae* KD1 chromosome with RP4::mini-Mu (47). The resulting R-prime plasmids were transferred to *E. coli* PA360. Construction of these strains has been reported elsewhere (27). All strains were grown and maintained with Luria broth (LB) supplemented with antibiotics for the maintenance of R-prime plasmids; incubation was at 37°C. For large-scale preparation of LPS, bacteria were grown in a 28-liter Microfirm fermentor (New Brunswick Scientific) by using a medium of 3.7% (wt/vol) brain heart infusion (Difco) at 37°C, at 200 rpm, and with aeration at 25 liters/min for 18 h.

LPS extraction and purification of O polysaccharides. Cells (approximately 300 g[wet weight]) were washed in 2% (wt/vol) saline; digested with lysozyme, RNase, and DNase (23); and then extracted with hot, aqueous phenol (50). LPS was recovered from the dialyzed aqueous layer by repeated ultracentrifugation at 105,000 × *g* (12 h at 4°C), until judged to be pure by the carbocyanine dye assay (21). The LPS from *K. pneumoniae* KD1 and KD2 was obtained in yields of 10 and 12%, respectively. For the preparation of O polysaccharides, a solution of 0.6 g of LPS in 300 ml of 1.5% (vol/vol) aqueous acetic acid was heated at 100°C for 2 h and the precipitated lipid A (approximately 25%) was removed by low-speed centrifugation. The supernatant was lyophilized, and the concentrated residue was fractionated on a Sephadex G-50 column, with pyridinium acetate (0.05 M, pH 4.7) as an eluant. Fractions were monitored for hexoses, 2-keto-3-deoxyoctulosonic acid (KDO), and phosphate.

Fractionation of whole LPS. Fractionation of LPS was achieved by using a modification of the method of MacIntyre et al. (29). Briefly, 30 mg of purified LPS was resuspended in 2 ml of 0.25% (wt/vol) sodium deoxycholate, 0.2 M NaCl, 0.001 M EDTA, 0.01 M Tris hydrochloride (pH 8.0) and subjected to gel filtration chromatography on a column (68 by 2.5 cm) of Sephadex G-200. Elution was performed in the deoxycholate-containing buffer. Fractions of 2.25 ml were collected at a flow rate of 2.5 ml/h. The fractions were assayed for KDO and neutral sugar content, and those containing LPS were analyzed by SDS-PAGE and Western immunoblotting by using monoclonal antibodies (MAbs).

SDS-PAGE and Western immunoblotting. LPS obtained by phenol extraction or by the SDS-proteinase K method (20) was analyzed by SDS-PAGE. The conditions for electrophoresis (12) and silver staining (46) have been reported previously. Western blotting was performed by using modifications (10) of the original method of Towbin et al. (45). Immunoblots were developed with alkaline phosphatase-conjugated goat anti-mouse $F(ab')_2$ (Jackson Laboratories). The color reagent was Nitro Blue Tetrazolium with 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

MAbs. The procedures used for the production of MAbs are described elsewhere (34). MAb O1-52.6 was obtained by fusion of spleen cells from a mouse immunized with Formalin-killed *K. pneumoniae* KD2 cells. This MAb recognized O-substituted LPS in *K. pneumoniae* KD1 and KD2 and in several independent O1 isolates but did not react with LPS from strain KD37 in either Western blots or enzyme-linked immunosorbent assays (ELISAs) (34). MAb O1-2.6 was obtained after immunization of mice with Formalin-killed *K. pneumoniae* KD37. Both MAbs were class M immunoglobulins.

Competition ELISA. The basic ELISA procedure of Voller et al. (48) was adapted. The coating antigen was *K. pneumonia* KD2 LPS resuspended at a final concentration of 5 μg/ml in carbonate buffer, pH 9.8. Wells were blocked by using 3% (wt/vol) bovine serum albumin. The competition step was carried out in the well by first adding 100 μl of the tested competing antigen and then adding 100 μl of antibody. The concentration of MAb was adjusted to give a control (no competing antigen) A_{405} of approximately 0.5. After incubation at 37°C for 2 h and then at 4°C overnight, the plates were washed and developed by using a detection system comprising alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G and immunoglobulin M (BioCan, Mississauga, Ontario, Canada) and *p*-nitrophenyl phosphate (Sigma). All determinations were made in triplicate.

NMR spectroscopy. Spectra were obtained at 30°C with solutions in deuterium oxide by using a Bruker AM-500 spectrometer equipped with an Aspect 3000 computer and by using standard Bruker software. Proton spectra, recorded at 500 MHz, were obtained by using a spectral width of 2.5 KHz, a 16 K data block, and a 90° pulse. Chemical shifts are expressed relative to internal acetone (δ 2.225 ppm). Broadband proton-decoupled ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded at 125 MHz with a spectral width of 31 KHz, a 32 K data block, and a 90° pulse employing WALTZ decoupling (41). The internal reference was acetone (δ 31.07 ppm). Heteronuclear $^1J_{C,H}$ couplings were measured by using gated decoupling.

Homonuclear two-dimensional chemical shift-correlated experiments (COSY; one- and two-step relay COSY) using the conventional pulse sequences (3, 4, 49) were carried out. The experiments were acquired over the full spectral width (960 Hz) by using data sets ($t_1 \times t_2$) of 256 by 2,048 points which were processed to give magnitude spectra. Nuclear Overhauser enhancement spectroscopy (NOESY) experiments were carried out in the phase-sensitive mode (26, 32) by using 256 by 2,048 datum point sets over the full spectral width by employing a 200-ms mixing time. Resolution enhancement was achieved by means of sine-bell window functions, and the processed data is presented with symmetrization.

Heteronuclear two-dimensional ^{13}C and ^1H chemical shift-correlated spectra (5) were obtained by using an initial data set of 128 by 4,096 points that was zero filled and Fourier transformed to give 1,024 by 4,096 points. Spectral widths of 3,125 and 400 Hz for the respective ^{13}C (F_2) and ^1H (F_1) chemical shift domains and 1,060 transients were acquired for each t_1 value. In order to select for all multiplicities, the fixed delays t_1 and t_2 were set at 3.3 and 1.7 ms, respectively. Resolution enhancement was applied in both dimensions by means of Lorentz-to-Gauss transformation, and the data were processed to give a power spectrum.

Methylation analysis. Samples (1 to 2 mg) were methylated by using the Hakamori procedure (19). Briefly, the methylated products were recovered from the reaction mixtures by chloroform extraction (5 ml) against water washing (five times, 5 ml [each]). Methylated products were hydrolyzed in sealed glass tubes with 90% (vol/vol) formic acid at 100°C for 2 h and, following removal of the formic acid, with 1 ml of 1 M H_2SO_4 for 16 h at 100°C. The neutralized ($BaCO_3$) and filtered hydrolysates were reduced with sodium borodeuteride (30 mg), and after acidification with 2 N acetic acid, the solutions were concentrated, distilled with methanol (four times, 10 ml [each]) to remove borate, acetylated by heating with acetic anhydride (1 ml) at 116°C for 2 h, and analyzed directly by gas-liquid chromatography (GLC)-mass spectrometry.

Periodate oxidation. Polysaccharide (60 mg) was treated in the dark with 0.05 M sodium metaperiodate (50 ml) for 2 days at 4°C. Excess periodate was consumed by using ethylene glycol (0.2 ml), and the oxidized polymer was reduced with sodium borohydride (200 mg) for 15 h at 4°C (17). The solution was neutralized with dilute acetic acid, dialyzed against water, lyophilized, and purified by Sephadex G-50 gel filtration chromatography.

Analytical methods. Neutral sugars were estimated by the phenol-sulfuric acid method (15), and KDO was estimated by the method of Aminoff (2). Phosphate was determined by the method of Chen et al. (11). Quantitative analyses and the absolute configurations of neutral sugars were determined by capillary GLC of their trimethylsilylated 2-(*R*)-butyl glycoside derivatives (16). GLC was performed by using a Hewlett-Packard model 5958B gas chromatograph fitted with a fused silica capillary column (0.3 by 25 m) containing OV17 (Quadrex Corp.) and by using a flame ionization detector. The operational temperature programs were (i) 200°C → 2 min delay → 220°C, at 1°C/min and (ii) isothermal at 180°C. Development was made with dry nitrogen.

RESULTS

Purification of the O polysaccharides of *K. pneumoniae* O1 LPS. Structural studies were initiated with *K. pneumoniae* KD2 (O1:K$^-$), to prevent any complications arising from the contamination of LPS with capsular polysaccharide. However, the O polysaccharide of *K. pneumoniae* KD1 (O1:K20) was also examined to confirm the results from strain KD2 and to verify that those results were not influenced by the use of a mutant derivative.

The Sephadex G-50 elution profiles of the two samples of O polysaccharide were qualitatively similar (Fig. 1). In each case, four peaks, corresponding to high-molecular-weight O polysaccharides (approximately 24%, in two poorly resolved peaks), core oligosaccharides (11%), and a fraction containing KDO (14%), were observed. In both cases, the high-molecular-weight O polysaccharides eluted in two poorly separated peaks. Since previous results indicated some antigenic heterogeneity in O1 LPS (34), the two peaks of O polysaccharide were collected separately. The O polysaccharide of *K. pneumoniae* KD2 was retained as the high-molecular-weight fraction A (K_{av}, 0.02 to 0.2; 14%) and the lower-molecular-weight fraction B (K_{av}, 0.25 to 0.6; 17%). Similarly, the O polysaccharide of *K. pneumoniae* KD1 was also collected in two parts as fraction C (K_{av}, 0.01 to 0.18; 18%) and fraction D (K_{av}, 0.22 to 0.50; 15%). Fraction C was initially contaminated with a small amount of nucleic acid which was removed by DEAE-Sephacel chromatography. The structures of the polysaccharide fractions A to D were determined separately.

Structure of the O polysaccharide fraction of *K. pneumoniae* KD2 LPS. Fractions A and B had an $[\alpha]_D$ of +138° (c 7, water) and an $[\alpha]_D$ of +99° (c 6, water), respectively. Both fractions were composed of D-galactose (>95%) which was characterized by capillary GLC of its trimethysilylated 2-(*R*)-butyl glycoside derivatives (program B).

Fraction B O polysaccharide gave a ^{1}H-NMR spectrum (Fig. 2B) and a ^{13}C-NMR (Fig. 3B) spectrum which both showed two major (>75%) anomeric signals, suggesting that the product could be a polysaccharide composed of a repeating disaccharide unit. Methylation of fraction B gave 2,5,6-tri-*O*-methyl-D-galactose and 2,4,6-tri-*O*-methyl-D-galactose (1:1.4) (Table 1), indicating that all the D-galactose units were 1,3 linked and that the D-galactose was present in

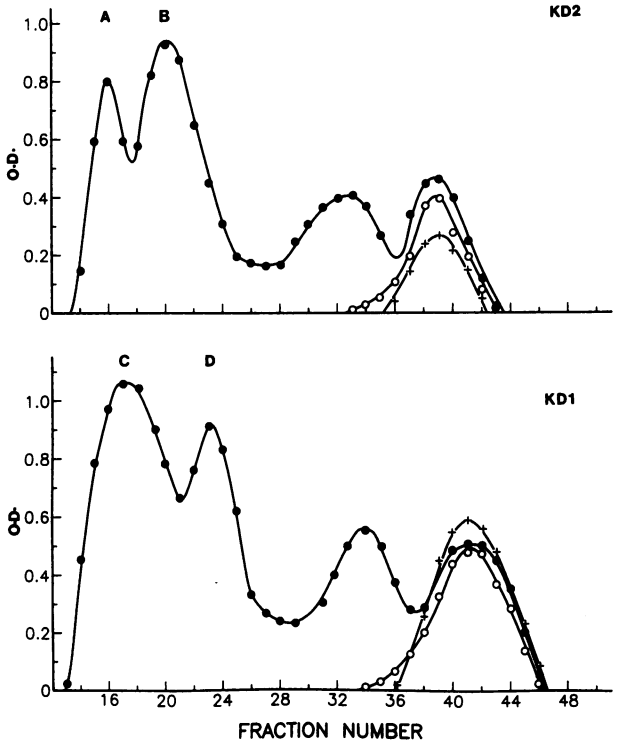

FIG. 1. Sephadex G-50 gel filtration chromatography of the water-soluble products (approximately 450 mg) from the acetic acid-hydrolyzed LPS. The upper panel shows LPS from *K. pneumoniae* KD2 and the lower panel shows LPS from strain KD1. Aliquots were analyzed for neutral glycose (●), phosphate (+), and KDO (○). The fractions collected for structural analysis are indicated by the letters A to D. O.D., Optical density.

furanoside and pyranoside forms in the ratio 1:1.4. This conclusion was confirmed by the fact that periodate oxidation and $NaBH_4$ reduction of fraction B afforded a new product which on hydrolysis gave L-arabinose and D-galactose (1:1.4). L-Arabinose is the predicted product of periodate oxidation of the D-galactofuranose units. The low-field signal at 110.3 ppm in the ^{13}C-NMR spectrum was characteristic of a D-galactofuranose residue having the β configuration, while the second major anomeric signal at 100.4 ppm was characteristic of an α-D-galactopyranose residue. Therefore, it was concluded that the major O polysaccharide component of fraction B was a polymer of a repeating disaccharide residue having the structure →3)-β-D-Gal*f*-(1→3)-α-D-Gal*p*-(1→ (D-galactan I). Polymers having this structure have been described in the LPS O polysaccharides produced by *Pasteurella haemolytica* serotypes 4 and 10 (38, 39). The major signals seen in the ^{1}H- and ^{13}C-NMR spectra of fraction B have chemical shifts identical to those of the fully identified *P. haemolytica* LPS O chains. The presence of minor signals in the NMR spectra of fraction B suggested that D-galactan I is contaminated with a second polysaccharide (D-galactan II).

Methylation of the O polysaccharide in fraction A gave 2,5,6-tri-*O*-methyl-D-galactose and 2,4,6-tri-*O*-methyl-D-galactose (1:3) (Table 1), indicating that all the D-galactose residues were 1,3 linked and that the ratio of furanoside to pyranoside residues present was 1:3. The latter conclusion was supported by the yields of L-arabinose and D-galactose

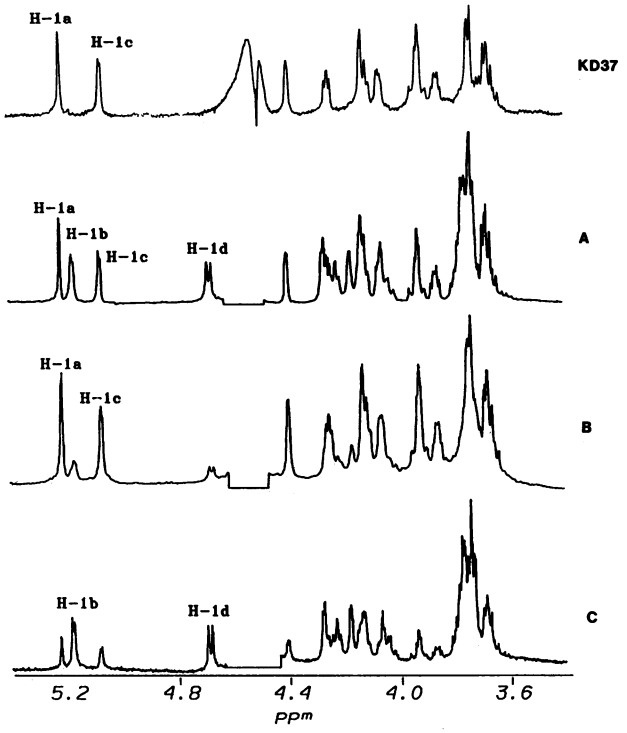

FIG. 2. ^{1}H-NMR spectra of LPS O polysaccharides from *K. pneumoniae* strains. Fractions A and B from strain KD2 and fraction C from strain KD1 are shown. Fraction B is enriched in D-galactan I, whereas fraction C contains mainly D-galactan II. Fraction A is an approximately equimolar mixture of the two D-galactans. The spectrum of the O polysaccharide of strain KD37, which synthesizes only D-galactan I is shown for reference. The four D-galactosyl residues were labeled (a to d) according to decreasing anomeric ^{1}H shifts.

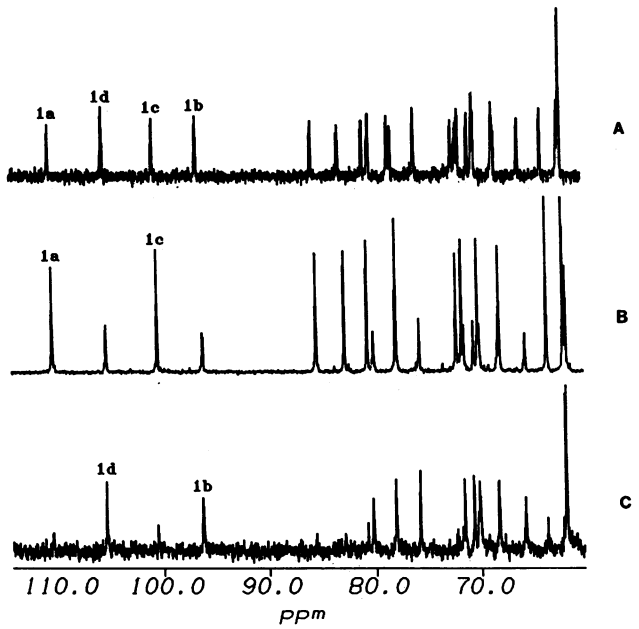

FIG. 3. ^{13}C-NMR spectra of the LPS O polysaccharides from *K. pneumoniae* strains. Fractions A, B, and C are shown. Fraction B is enriched in D-galactan I, whereas fraction C contains mainly D-galactan II. Fraction A is an approximately equimolar mixture of the two D-galactans. The four D-galactosyl residues were labeled (a to d) according to decreasing anomeric ^{1}H shifts, as shown in Fig. 2.

(1:3) resulting from the hydrolysis of the glycan obtained from the periodate oxidized and reduced fraction A.

Fraction A gave ^{1}H-NMR (Fig. 2A) and ^{13}C-NMR (Fig. 3A) spectra showing four anomeric atom signals of approximately equal intensity, initially suggesting that this fraction was possibly a polymer of a repeating tetrasaccharide unit. However, the presence of signals in both the ^{1}H- and ^{13}C-NMR spectra corresponding to those already described for D-galactan I suggested that fraction A could also be a mixture of D-galactan I and a second D-galactan composed of a structurally different repeating disaccharide unit. The latter possibility was confirmed by two-dimensional NMR analysis of fraction A.

Assignment of the ^{1}H-NMR spectrum of fraction A (Fig. 2A) was achieved by application of a series of homonuclear chemical shift correlation experiments. The ^{13}C-NMR resonances were then assigned by heteronuclear ^{13}C-^{1}H chemical shift correlation. The four D-galactosyl residues were labeled a, b, c, and d according to the order of decreasing anomeric ^{1}H chemical shifts.

Examination of the connectivities defined by cross-peaks in the COSY (Fig. 4) and the one- and two-step relayed COSY (data not shown) spectra led to the identification of subspectra corresponding to each of the D-galactosyl ring systems. The resonances arising from the β-D-galactofuranosyl residue (residue a) were well resolved, and the corresponding subspectrum was readily identified from the ^{1}H chemical shift and vicinal-coupling constant (^{3}J) values (Table 2) (1). That this residue was a β-furanose configuration was evident from ^{13}C chemical shifts (18), assigned (Table 3) from the ^{13}C-^{1}H chemical shift correlation map (Fig. 5). The magnitude of the observed ring proton coupling constants from the D-galactosyl residues b and d indicated that these residues were present as α- and β-pyranosyl units, respectively (8). Strong coupling between overlapping ^{1}H signals (H-2/H-3 and H-4/H-5) from the D-galactosyl residue c precluded measurement of the associated ^{3}J values. However, the subspectrum from this residue could be identified

TABLE 1. Methylation analysis of *K. pneumoniae* KD1 and KD2 O chain fractions

Methylated glycose[a]	T_{GM}[b]	Molar ratio[c]			
		Fraction A	Fraction B	Fraction C	KD37
2,5,6-Tri-*O*-methyl-D-galactose	1.33	0.31	0.67	0.09	1.00
2,4,6-Tri-*O*-methyl-D-galactose	1.44	1.00	1.00	1.00	1.00

[a] As alditol acetates.
[b] GLC program A. Retention time is quoted relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol value (1.00).
[c] Molar ratios relative to 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol value (1.00).

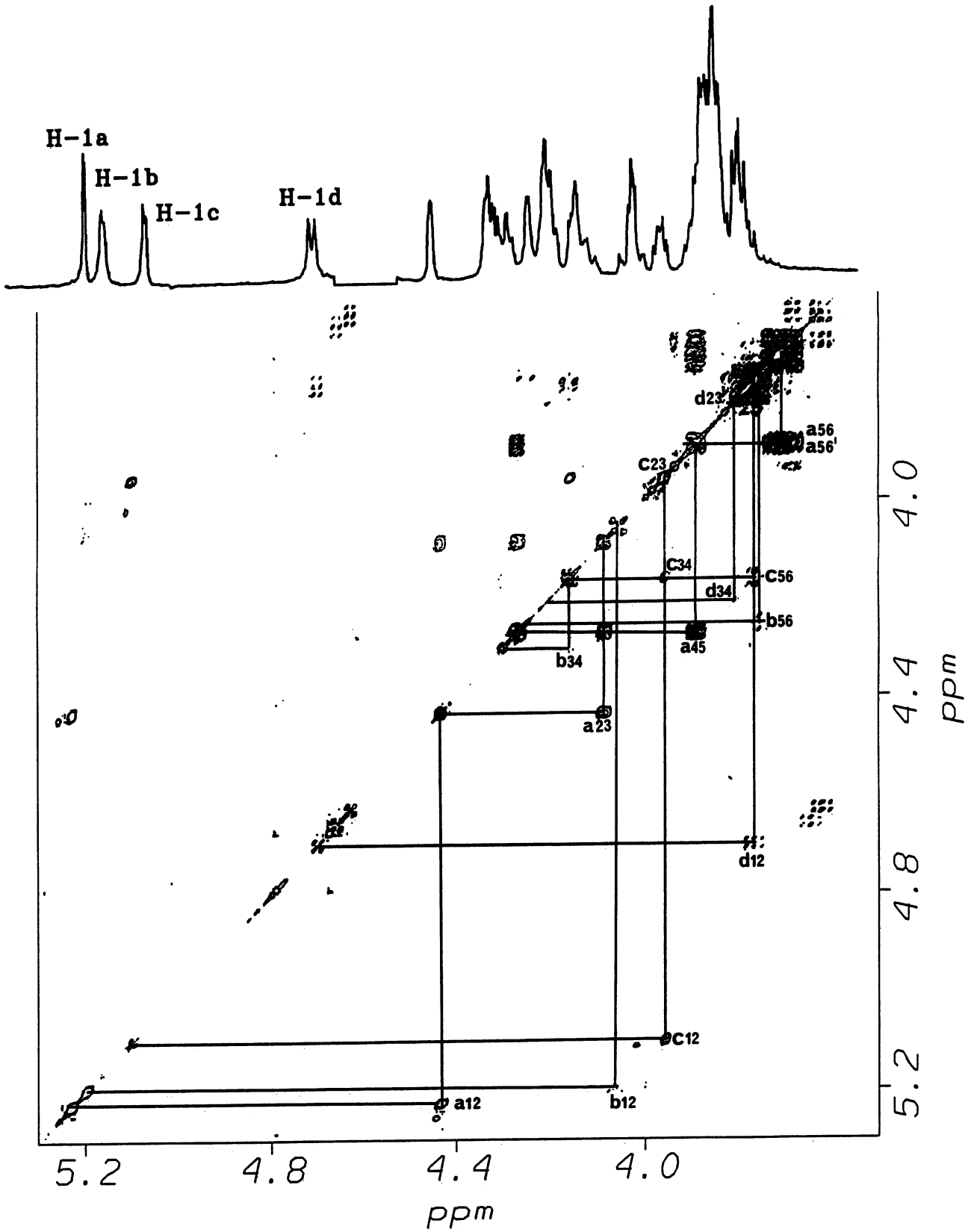

FIG. 4. Contour plot of the ring proton region (5.3 to 3.5 ppm) of the COSY spectrum of fraction A from *K. pneumoniae* KD2 LPS, containing a mixture of D-galactans I and II. Resonance assignments are shown for H-1 to H-5 for each of the component monosaccharide residues (a, b, c, and d).

TABLE 2. ^{1}H-NMR chemical shifts and ring proton coupling constants of D-galactose units present in the D-galactans I and II produced by *K. pneumoniae* KD1 and KD2

D-Galactose unit	Chemical shift[a] (coupling constant[b]) in:						
	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5}$)	H-5	H-6	H-6^1
a, -3)-β-D-Gal*f*-(1-	5.18 (≤1)	4.43 (5.1)	4.11 (8.0)	4.29 (4.0)	3.86	3.73	3.77
b, -3)-α-D-Gal*p*-(1-	5.15 (3.6)	4.07 (10.0)	4.17 (~3)	4.30 (≤1)	4.28	3.77	3.77
c, -3)-α-D-Gal*p*-(1-	5.06 (3.1)	4.97 (−)[c]	4.97 (−)[c]	4.16 (−)[c]	4.16	3.78	3.78
d, -3)-β-D-Gal*p*-(1-	4.66 (7.3)	3.80 (~9)	3.81 (≤3)	4.21 (≤1)	3.71	3.76	3.76

[a] Measured at 30°C in deuterium oxide, in parts per million.
[b] Observed first-order coupling constants, in hertz.
[c] Unresolved because of second-order effects.

since the connectivity pathway observed in the COSY contour plot (Fig. 4) was identical to that previously observed for the →3)-α-D-Gal*p*-(1→ residue of the *P. haemolytica* serotype 10 O polysaccharide (39). Indeed, the ^{1}H and ^{13}C data (Tables 2 and 3) for both the residue c and the residue a corresponded to the reported values for the repeating disaccharide →3)-β-D-Gal*f*-(1→3)-α-D-Gal*p*-(1→, representing D-galactan I.

The structure of D-galactan I was confirmed by ^{1}H-^{1}H NOE measurements. The NOE data obtained from a two-dimensional phase-sensitive NOESY experiment identified connectivities between the anomeric and aglyconic protons of contiguous residues. The occurrence of interresidue NOE between H-1a and the H-2c/H-3c proton pair and between H-1c and H-3a established the linear sequence of a repeating disaccharide, →a→c→, corresponding to the D-galactan I structure. Interresidue NOE relating the H-1b/H-3d and the H-1d/H-3b proton resonance pairs led to the identification of a second repeating disaccharide sequence, →b→d→, corresponding to the structural unit, →3)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→ (D-galactan II). No NOE connectivities were detectable between the two repeating disaccharide units →a→c→ and →b→d→, confirming the conclusion that fraction A is a mixture of two polymeric D-galactans.

Structure of the O polysaccharide fraction of *K. pneumoniae* KD1 LPS. Fraction C was a D-galactan having an $[\alpha]_D$ of +170° (c 5.2, water). The ^{1}H-NMR (Fig. 2C) and ^{13}C-NMR (Fig. 3C) spectra of fraction C showed major signals (including two major anomeric signals) corresponding in chemical shifts with those associated with the D-galactan II. In this fraction the minor signals (approximately 10%) were those associated with D-galactan I. Methylation of fraction C gave 2,4,6-tri-*O*-methyl-D-galactose and 2,5,6,-tri-*O*-methyl-D-galactose in the ratio 1:0.1 (Table 1). It can be inferred that the 2,5,6-tri-*O*-methyl-D-galactose arose from the D-galactofuranose residues in the minor D-galactan I component present in this fraction.

From the application of NMR, methylation, and periodate oxidation analyses as previously described it was found that fraction D contained an approximately equal proportion mixture of D-galactan I and D-galactan II (data not shown). In all respects, this fraction was similar in composition to fraction A (described above) from *K. pneumoniae* KD2. This result was unexpected since it was predicted that fraction D would be identical to fraction B.

Structure of the O polysaccharide fraction of *K. pneumoniae* KD37 LPS. Previous work (34) demonstrated that the mutant *K. pneumoniae* KD37 produced LPS lacking the highest-molecular-weight O-substituted LPS molecules. Methylation analysis (Table 1) indicated that KD37 LPS was a homopolymer comprising only D-galactan I. This conclusion was confirmed by examination of spectra from ^{1}H-NMR (Fig. 2) and ^{13}C-NMR (data not shown).

MAb probes specific for D-galactans I and II. In a previous study, MAb O1-52.6 was developed as a probe specific for O1 HMW-LPS (34). This MAb reacted with LPS from *K. pneumoniae* KD2 but not from the mutant KD37 (34) (Fig. 6). The precise D-galactan recognized by O1-52.6 was determined by competition ELISA using purified O polysaccharides. MAb O1-52.6 reactivity was not affected by the addition of D-galactan I obtained in purified form as the O side chain polysaccharide from *K. pneumoniae* KD37 (Fig. 7). In contrast, polysaccharide C, which contains D-galactan II with approximately 10% contamination by D-galactan I, inhibited the reactivity of MAb 01-52.6 by 80% at concentrations as low as 2 μg/ml. MAb O1-52.6 is therefore specific for epitopes associated with D-galactan II.

MAb O1-2.6 recognized *K. pneumoniae* KD2 and KD37 (Fig. 6), suggesting that it reacts with epitopes found in D-galactan I. MAb O1-2.6 also recognized epitopes in the O-substituted LPS of *P. haemolytica* serotype 4 and *Serratia marcescens* O20 (results not shown), which is consistent with the above suggestion. However, since we have been unable to obtain a pure sample of D-galactan II, we have been unable to directly confirm this result by competition

TABLE 3. ^{13}C-NMR chemical shifts and $^1J_{C,H}$ coupling constants of the D-galactose units present in the D-galactans I and II produced by *K. pneumoniae* KD1 and KD2

D-Galactose unit	Chemical shift[a] (coupling constant[b]) in:					
	C-1	C-2	C-3	C-4	C-5	C-6
a, -3)-β-D-Gal*f*-(1-	110.29 (174)	80.62	85.40	82.76	71.68	63.64
b, -3)-α-D-Gal*p*-(1-	96.09 (159)	68.23	80.07	70.04	71.47	62.09
c, -3)-α-D-Gal*p*-(1-	100.39 (171)	68.11	77.96	70.15	72.21	62.09
d, -3)-β-D-Gal*p*-(1-	105.21 (169)	70.56	77.96	65.67	75.73	62.09

[a] In parts per million.
[b] In hertz.

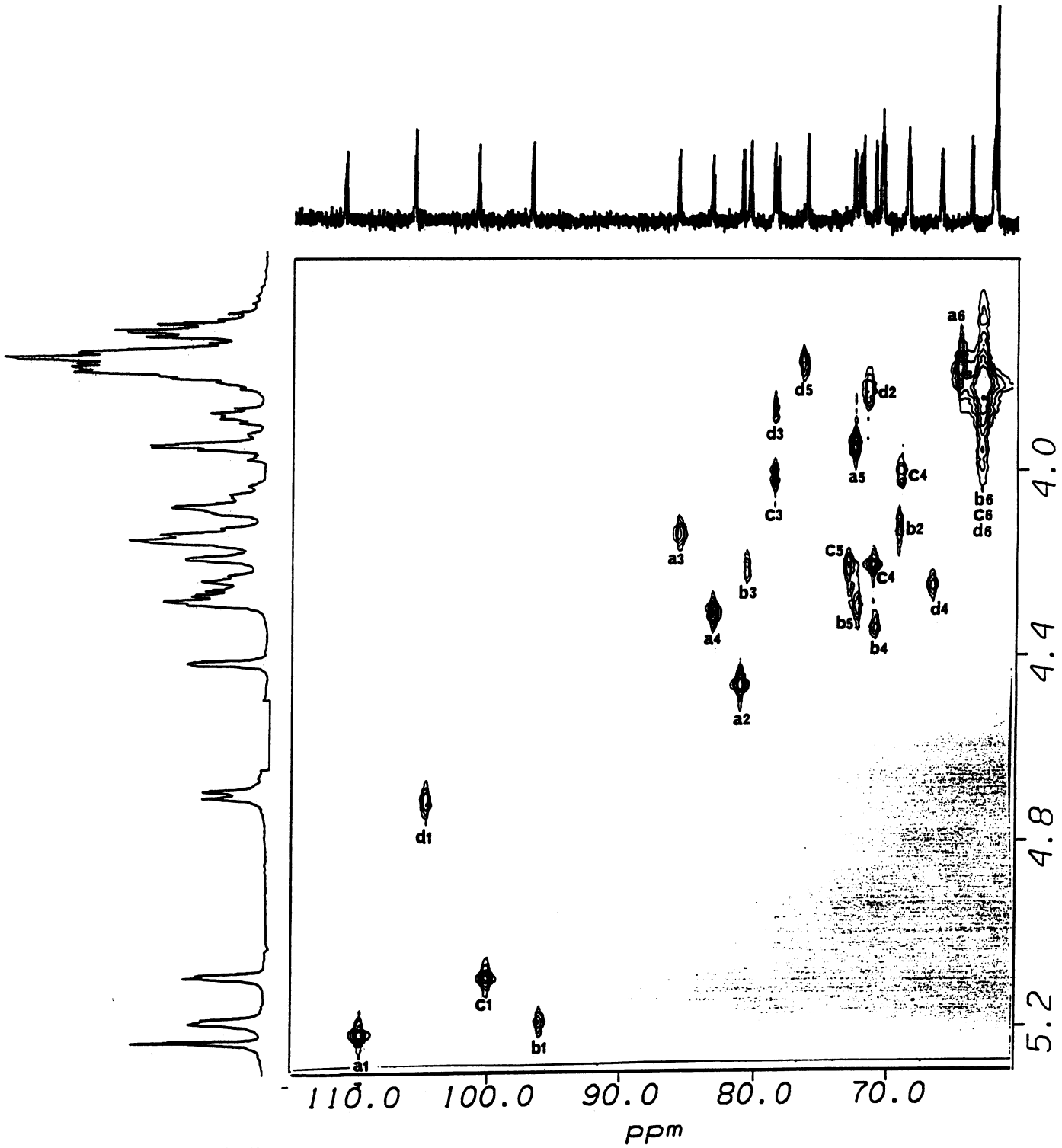

FIG. 5. Heteronuclear ^{1}H-^{13}C chemical shift correlation map of fraction A from *K. pneumoniae* KD2 LPS, a mixture of D-galactans I and II. The respective one-dimensional ^{1}H and ^{13}C spectra are displayed along the F_1 (5.3 to 3.5 ppm) and F_2 (115 to 60 ppm) axes. The correlated resonances are labeled (a, b, c, and d).

ELISA. Immunoprecipitation experiments using this MAb have not given unequivocal results.

The distribution of epitopes recognized by both MAbs within the heterogeneous O-substituted LPS molecules was examined in more detail, by using LPS fractionated on a Sephadex G-200 column. The size-fractionated LPS was examined by SDS-PAGE and Western immunoblotting (Fig. 8). Only the results obtained with *K. pneumoniae* KD2 LPS

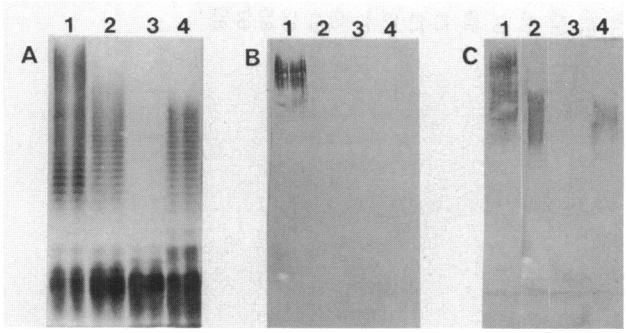

FIG. 6. Identification of D-galactans I and II in LPS samples from whole-cell lysates. The strains analyzed are *K. pneumoniae* KD2 (lanes 1), *K. pneumoniae* KD37 (lanes 2), *E. coli* K-12 PA360 (lanes 3), and an *E. coli* transconjugant, strain CWG100 (His^+ O^+ $K20^+$) (lanes 4). The SDS-PAGE profile is shown in panel A. The samples were tested by Western blotting against MAb O1-52.6 (B) and MAb O1-2.6 (C).

are shown, but the distribution of epitopes for the two MAbs was the same when LPS extracted from the encapsulated strain, *K. pneumoniae* KD1, was examined.

As expected, D-galactan II was confined to fractions 59 to 73, in the higher-molecular-weight range. Epitopes recognized by MAb O1-2.6 were detected throughout the profile. These results confirmed the chemical data, indicating that traces of D-galactan I are found even in the highest-molecular-weight LPS molecules. Interestingly, the spectrum of LPS recognized by MAb O1-2.6 was identical to that observed if rabbit antiserum raised against *P. haemolytica* was used as the immune probe (data not shown).

In the mutant KD37, the maximum chain length of D-galactan I was altered; in addition, D-galactan II expression was lost. This phenomenon was clearly evident when Western blots of KD37 and KD2 LPS with MAb O1-2.6 were compared (Fig. 6).

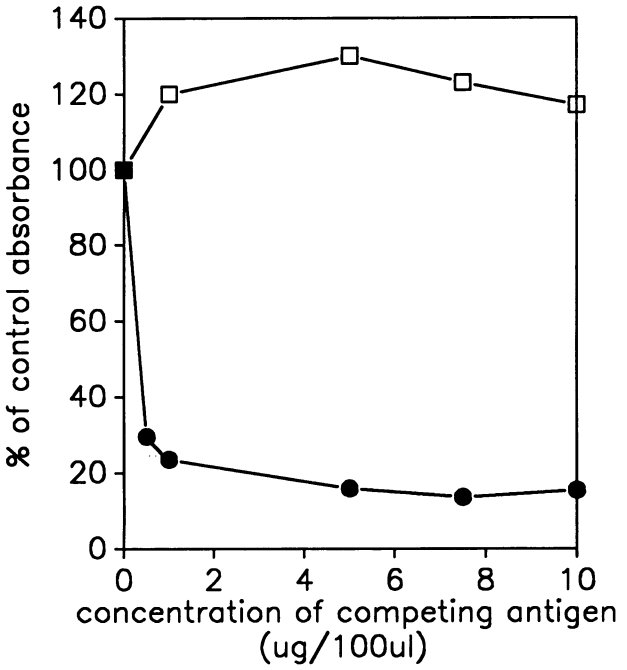

FIG. 7. Specificity of MAb O1-52.6 determined by competition ELISA. The control A_{405} (100%) was 0.5. The antigens tested for inhibition were D-galactan I from *K. pneumoniae* KD37 (□) and D-galactan II obtained at 90% purity in polysaccharide C (●).

Determination of the nature of the O polysaccharide determined by the *K. pneumoniae* O1 *rfb* gene locus. In a previous report (27), RP4::mini-Mu was utilized to mobilize the *K. pneumoniae* chromosome and to mediate transfer of the *his* region from *K. pneumoniae* KD1 to an *E. coli* K-12 recipient. In enteric bacteria, the *rfb* gene cluster is linked to *his* (31), and *E. coli* K-12 His^+ transconjugants inherited the ability to synthesize O antigen. MAbs O1-52.6 and O1-2.6 were used to determine the nature of the O polysaccharides expressed in these transconjugants. LPS synthesized in a representative strain (*E. coli* CWG100) displayed a SDS-PAGE profile similar to that of *K. pneumoniae* KD37 (Fig. 6). This LPS reacted with MAb O1-2.6 but not O1-52.6, indicating that only D-galactan I was expressed. Approximately 50 independent transconjugants have been examined; some (e.g., CWG100) simultaneously express the K20 antigen (27). No expression of D-galactan II was detected in any of the transconjugants.

Distribution of D-galactans I and II in LPS from *Klebsiella* spp. To determine whether D-galactan I or II was unique to serotype O1 or whether either formed a widespread common antigen in *Klebsiella* spp., several different O serotype LPS samples were probed with MAbs O1-52.6 and O1-2.6. The LPSs chosen were representatives of serotypes O6, O8, O9, and the O2 group, all of which have been reported to be galactans (Fig. 9). The primary structures of O1 and O6 (strain 8172) have been reported to be identical (7), and this was confirmed by similar SDS-PAGE profiles and by reaction with both MAbs. Serotype O8 contains an acetylated galactan with a trisaccharide repeating unit (Fig. 10). Surprisingly, the SDS-PAGE profile of strain 889 was identical to the O1 LPS, and strain 889 LPS also reacted with both MAbs (Fig. 10). The reason for this result is unclear, but since the "backbones" of O1 D-galactan I and O8 differ only in the *O* acetylation of the latter, they may contain a cross-reactive epitope recognized by MAb O1-2.6. However, strain 889 apparently also contains D-galactan II. In a previous report, Mizuta et al. (35) speculated that strain 889 may in fact belong to serotype O1; the observations presented here support that possibility. Anomalies in serotyping could easily result from the difficulties associated with determining the O type of strains which are characteristically heavily encapsulated. Serotype O9 comprises an acetylated galactan pentasaccharide repeating unit (Fig. 9), and a representative strain, 1205, showed no reactivity with either MAb (Fig. 10). Strains originally placed in serotype O2 were subsequently reported to belong to either serotype O8 or serotype O9 on the basis of chemical analysis (25). Several distinct SDS-PAGE profiles were visible in the 11 O2 group isolates examined, and one strain (5052) produced very little O-substituted LPS (Fig. 10). No O2 group strain reacted with MAb O1-52.6. Strains 2482 and 5758 did react with MAb O1-2.6 and produced an SDS-PAGE profile similar to the mutant *K. pneumoniae* KD37 (Fig. 10).

DISCUSSION

From the chemical evidence presented, it can be concluded that the *K. pneumoniae* O1 polysaccharide consists of a mixture of two structurally distinct D-galactans with the repeating disaccharide structures shown in Fig. 9. D-Galactan I has also been reported in the LPS of *P. haemolytica*

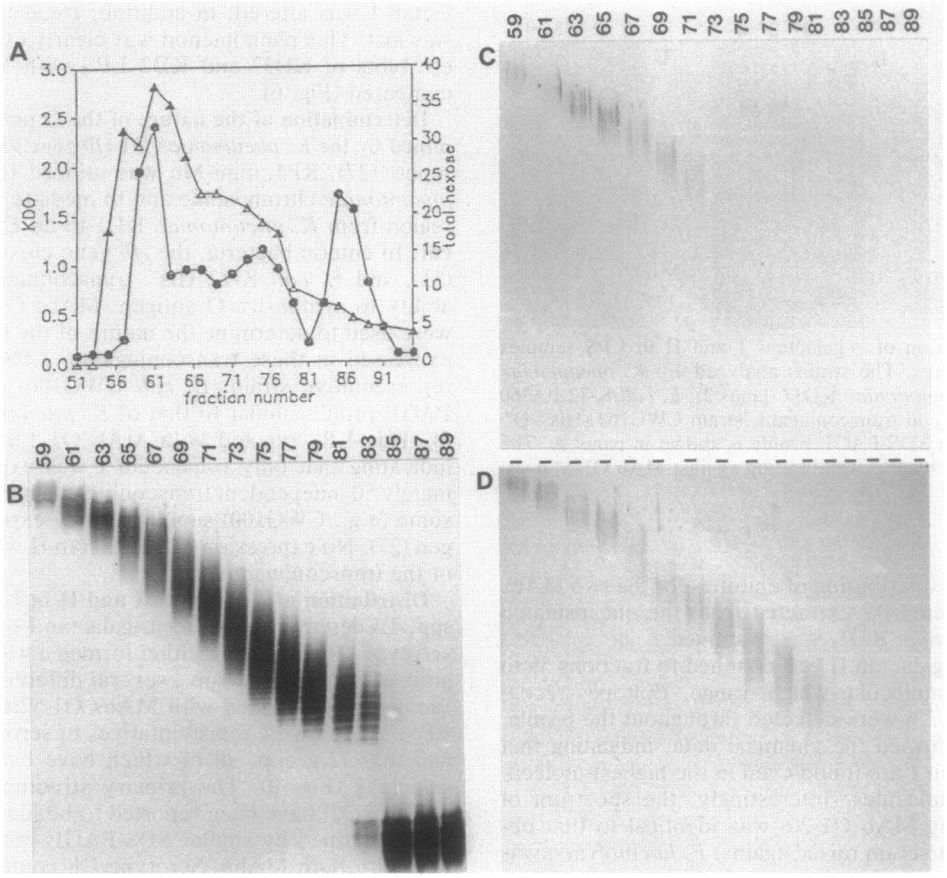

FIG. 8. Distribution of D-galactan I and D-galactan II epitopes in fractionated *K. pneumoniae* O1 LPS. LPS from *K. pneumoniae* KD2 was separated by column chromatography on Sephadex G-200 in deoxycholate buffer. Elution was followed (A) by monitoring both total hexose (△) and KDO (●). Alternate samples were analyzed by SDS-PAGE (B). The presence of D-galactans I and II was determined by Western blotting using MAb O1-2.6 (D) and MAb O1-52.6 (C), respectively.

serotypes 4 (38) and 10 (39) and in *S. marcescens* serotypes O16 and O20 (37). D-Galactan II consists of a unique polymer and, to our knowledge, this structure has not been reported in other bacteria.

SDS-PAGE and Western immunoblotting indicated that the size range of the two D-galactans was not influenced by the simultaneous synthesis of CPS. However, chemical analysis and the G-50 chromatography profiles (Fig. 1) of fractionated O polysaccharide suggest that the relative amount of D-galactan I is higher in *K. pneumoniae* KD2 compared with strain KD1. Since both O polysaccharides and the K20 CPS (25) contain D-Gal*p* residues, it is possible that the increased demand for available UDP-D-Gal*p* precursor in the encapsulated strain KD1 may have detrimental effects on the expression of D-galactan I. On the basis of the results presented, the anomalies in previous reports of the O1 polysaccharide structure (7, 25) could be explained simply by the arbitrary selection of O polysaccharides of different molecular weights for chemical analysis.

In *K. pneumoniae* KD37, synthesis of the two polymers was uncoupled and only D-galactan I was synthesized. On the basis of the SDS-PAGE and Western blots in Fig. 6 it appears that the O chain length of D-galactan I in *K. pneumoniae* KD37 differs from that in strain KD2. The reason(s) for the reduced chain length in strain KD37 remains unclear. Since the assembly of LPS requires the coordinated activity of enzyme complexes which separately

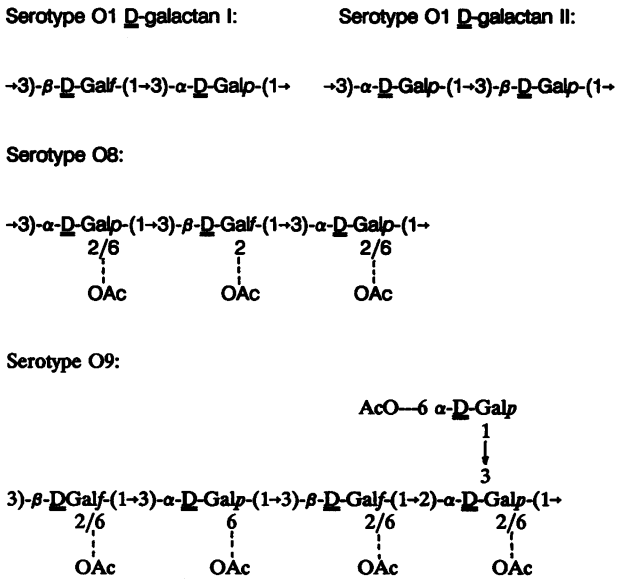

FIG. 9. Structures of D-galactan containing O polysaccharides in *Klebsiella* spp. LPS. The structures for serotypes O8 and O9 were previously described by Kenne and Lindberg (25).

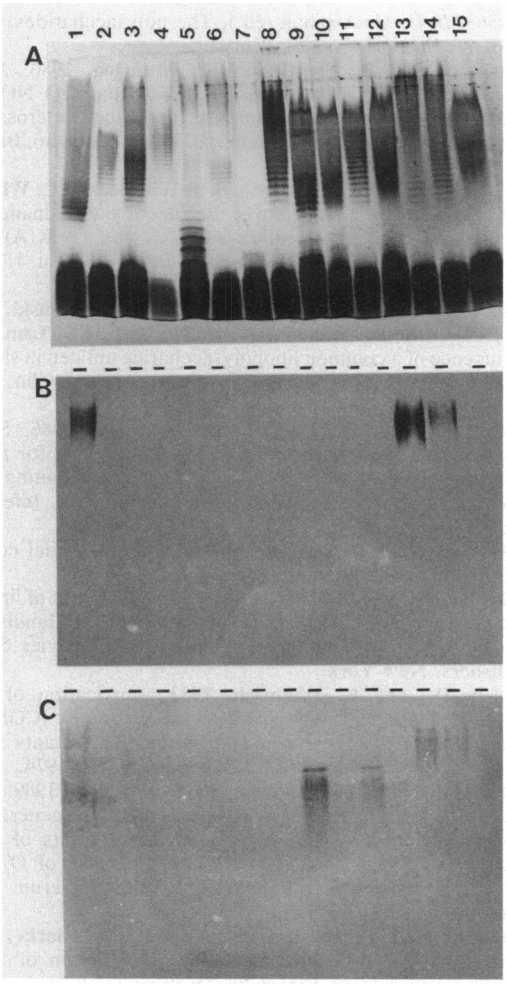

FIG. 10. Distribution of D-galactan I and D-galactan II in different *Klebsiella* spp. O serotypes. LPS samples were analyzed by SDS-PAGE (A) and by Western blotting against MAb O1-52.6 (B) and MAb O1-2.6 (C). The strains (serotypes) examined were as follows: lane 1, KD2 (O1); lane 2, 7380 (group O2); lane 3, 5053 (O2); lane 4, C5046 (O2); lane 5, D5050 (O2); lane 6, E5051 (O2); lane 7, F5052 (O2); lane 8, 6613 (O2); lane 9, 5758 (O2); lane 10, 7444 (O2); lane 11, 2482 (O2); lane 12, 2212/52 (O2); lane 13, NCTC 8172 (O6 = O1); lane 14, 889 (O8); lane 15, 1205 (O9).

synthesize lipid A core and O polysaccharide, it is possible that the mutation affecting synthesis of D-galactan II also affects the normal regulation of D-galactan I chain length. The LPS phenotype seen in strain KD37 also occurs in strains 2482 and 5758, representatives of serotype O2. We have also found LPS of this type occurring at low frequencies among different serotype O1 isolates (11a). Whether these strains reflect a separate group or are simply mutants of *Klebsiella* O1 strains remains to be established.

We have previously shown that high-molecular-weight LPS is responsible for the resistance of *K. pneumoniae* O1 to complement-mediated serum killing (34). *K. pneumoniae* KD37, which synthesizes only D-galactan I, was found to be serum sensitive. The basis for serum resistance is not clarified by the results reported here, since high-molecular-weight LPS contains both D-galactan II and smaller amounts of D-galactan I. The serum resistance phenomenon could be attributed to either polysaccharide and may be a function of either the chain length or the specific structure of the O polysaccharides.

K. pneumoniae O1 is not the first bacterium reported to simultaneously express two O antigens. Perhaps the most complex situation in O serotyping exists in *Salmonella* spp. (24). In some strains, the recognized O antigen is coexpressed (40) with a second structurally distinct polysaccharide, termed T1 antigen (6). In addition to variation in basic O polysaccharide structures, heterogeneity can result from nonessential postpolymerization modifications including acetylation and α-D-glucosylation (for a review, see reference 31). Nonstoichiometric modifications can lead to a multiplicity of O polysaccharide structures within an individual culture (9, 13). A variety of *E. coli* strains appear to synthesize both a neutral and an acidic O antigen (22). Simultaneous expression of two O polysaccharides has also been detected in strains of *Pseudomonas aeruginosa* (28). In *P. aeruginosa* one LPS form constitutes a common antigen, which is similar in some respects to the enterobacterial common antigen detected in many members of the family *Enterobacteriaceae* (30). On the basis of Western blotting analysis of the other galactan LPS serotypes of *Klebsiella* spp., neither of the two D-galactans found in O1 LPS are sufficiently widespread to be considered common antigens.

The observations that *E. coli* transconjugants carrying the *his* region of *K. pneumoniae* O1 express only D-galactan I raises particularly interesting questions about O antigen expression in *Klebsiella* spp. The absence of D-galactan II cannot be explained by a lack of appropriate precursors, since UDP-D-Gal*p* appears to be the precursor for the D-Gal*p* residues in both polysaccharides (11b). The results obtained by analysis of the transconjugants are consistent with the possibility that at least some of the genes which determine the expression of D-galactan II are not closely linked to those for D-galactan I at the classical location for the *rfb* cluster. These genes may be defined by the mutation in *K. pneumoniae* KD37. Interestingly, in *Salmonella* spp., the genes for the T1 polysaccharide do not map at *rfb*. Similarly, the locus involved in α-D-glucosylation is also located outside the *Salmonella rfb* cluster (31). In *Salmonella* spp., α-D-glucosylation genes can be subject to on-off switching in form variation (24, 31). Within a population of *K. pneumoniae* O1, all cells express D-galactan II and can be labeled by using MAb O1-52.6 and immunofluorescence techniques (11a). This would indicate that expression of D-galactan II is not subject to form variation.

Studies are now under way to analyze the *K. pneumoniae rfb* cluster at the molecular level. The characterization of precise structures, the development of MAb probes, and the isolation of mutants such as strain KD37, which is now known to be defective in D-galactan II synthesis, are essential prerequisites for further studies.

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