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Structures of the oligosaccharides obtained from the core regions of the lipopolysaccharides of *Bradyrhizobium japonicum* 61A101c and its symbiotically defective lipopolysaccharide mutant, JS314

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ABSTRACT

The only core oligosaccharide released from the lipopolysaccharide (LPS) of *Bradyrhizobium japonicum* 61A101c by prolonged (5 h) mild hydrolysis with acid, and the major core oligosaccharide obtained from its symbiotic and LPS-defective mutant, JS314, was the trisaccharide α-D-Man*p*-(1 → 4)-α-D-Glc*p*-(1 → 4)-2,7-anhydro-α-Kdo*f*. The 2,7-anhydro-3-deoxy-α-D-*manno*-2-octulofuranosonic acid moiety was probably formed during the prolonged mild hydrolysis with acid. A disaccharide core component, also released by mild acid hydrolysis of the mutant LPS, had the structure 4-*O*-Me-α-D-Man*p*-(1 → 5)-Kdo. The Kdo residue in this disaccharide is present as the normal pyranose form and as an anhydro derivative, possibly 4,8-anhydro-3-deoxy-D-*manno*-2-octulosonic acid, which may have formed also during prolonged mild hydrolysis with acid. Mild acid hydrolysis of the LPS of the parent strain does not produce this disaccharide, but 4-*O*-Me-Man is found exclusively in the O-chain fraction released from the parent LPS. Additionally, a small amount of O-chain is found in the mutant LPS. The results imply that the O-chain is attached to the remainder of the LPS through the 4-*O*-Me-Man-Kdo disaccharide component of the core region.

INTRODUCTION

Intact lipopolysaccharides (LPSs) are necessary in order for rhizobia to form nitrogen-fixing nodules with their legume hosts[1–6], and mutants having LPSs that lack their O-antigenic polysaccharide are either defective in the formation of infection threads or in the release of bacteria into the root cells.

The phenotype of each mutant varies, depending on whether the host forms a determinate or an indeterminate nodule. Kijne[7] pointed out that the infection threads of these two types of nodules are distinct. Determinate nodules possess

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narrow infection threads that contain little matrix material, and the bacteria are usually in single file and in close contact with the membrane of the infection thread. Indeterminate nodules have wide infection threads that contain matrix material and, within which, several bacteria can reside. Clover, alfalfa, and pea are examples of hosts which form indeterminate nodules, and bean and soybean are examples which form determinate nodules. For *R. leguminosarum* biovars trifolii and viciae (symbionts of clover and pea, respectively), mutants with defective LPSs form normal infection threads, but are defective in the release of bacteria into the root nodule cells[5,6]. For *B. japonicum* or *R. leguminosarum* bv. phaseoli (symbionts of soybean and bean, respectively), LPS mutants fail to form normal infection threads[3,4]. Rhizobial extracellular polysaccharides are required in order to form fully effective indeterminate nodules, but are not required for the formation of effective determinate nodules[8–15].

In general, *Rhizobium* (and other Gram-negative bacteria) produce LPSs that consist of a polysaccharide O-antigen attached to an oligosaccharide core, and this complex is linked to a lipid A through 3-deoxy-D-*manno*-2-octulosonic acid (Kdo). Other than these general characteristics, *Rhizobium* LPSs can be quite different from those of enteric bacteria. The core oligosaccharides of the LPSs of *R. leguminosarum* strains are composed[16–19] of GalA, Gal, Man, and Kdo, and their structures are probably the same for many strains[16–20]. Recently, 3-deoxy-2-heptulosaric acid has been reported[21] in the LPS of *R. leguminosarum* biovar trifolii strain, *R. meliloti*, and *Agrobacterium tumefaciens*. The lipid A's of *R. leguminosarum* biovars trifolii and phaseoli consist[22] of a disaccharide component comprising GalA and GlcN. Other sugar backbones of *Rhizobium* lipid A's have also been reported, including phosphorylated β-(1 $\rightarrow$ 6)-linked disaccharides of GlcN in the LPSs of *R. meliloti*[23] and in one strain of *R. leguminosarum*[24], GlcNA in the lipid A from another *R. leguminosarum* strain[25], and 2,3-diamino-2,3-dideoxyglucose (DAG) in lipid A's from strains of *Bradyrhizobium japonicum* and *lupini*[26,27a]. The fatty acyl residues of these lipid A's include 3-hydroxymyristate, 3-hydroxypalmitate, 3-hydroxystearate, 3-hydroxypentadecanoate, and 27-hydroxyoctacosanoate[22]. In fact, the presence of the latter fatty acid is common to the LPSs of all strains of *Rhizobiaceae* examined thus far[22] and LPSs which contain 27-OH-C28:0 belong to the α-2 group of Proteobacteria[27b].

Stacey et al.[28] reported a LPS mutant of *B. japonicum* that could not infect its soybean host but would still initiate the division of root cortical cells. Composition analysis and polyacrylamide gel electrophoresis (PAGE) showed that the mutant LPS lacked the O-antigen polysaccharide. We now report the structures of the major LPS core oligosaccharides for the mutant and parent LPSs.

EXPERIMENTAL

Bacterial strains.—*Bradyrhizobium japonicum* strains 61A101c and its mutant, JS314, were obtained from Dr. G. Stacey (University of Tennessee, Knoxville);

strain 61A101c was obtained originally from LiphaTech, Inc. (Milwaukee). The development of the mutant strain JS314 has been described[28].

Each strain was grown in the medium developed by Tulley[29] that represses the production of extracellular polysaccharides which interfere with the purification of LPS. Five 1-L starter cultures were prepared and used to start 100-L batches of bacteria that were grown at the fermenter facility at the University of Georgia.

Isolation of the lipopolysaccharides.—The LPSs were extracted using hot phenol–water[30] and were purified[27a] from the phenol layer by dialysis against deionized water, centrifugation, and treatment of the supernatant solution with proteinase K, ribonuclease, and deoxyribonuclease, dialysis, and lyophilization. The LPS was then ultracentrifuged at 100 000*g* for 4 h, and the pellet was suspended in deionized water and lyophilized.

Isolation of the core oligosaccharides from the LPSs.—Each LPS (53 mg from 61A101c, and 140 mg from JS314) was dissolved in deionized water (10 mg/mL), acetic acid was added to 1%, and each solution was heated at 100° for 5 h. Hydrolysis for 5 h, rather than the usual 1 h, was required in order to release the polysaccharide from the lipid A[27a]. The lipid A was removed by centrifugation and the supernatant solution was lyophilized. The oligosaccharide material (40 mg) obtained from JS314 LPS corresponded to 29% of the total LPS, and that (34 mg) from 61A101c LPS corresponded to 64%. Gel-filtration chromatography on Bio-Gel P2 of the oligosaccharides from 61A101c, using aqueous 1% acetic acid, gave P2-1 (29 mg), which was eluted near the void volume, and P2-2 (3.6 mg), which was eluted near the included volume of this column (96% recovery). Likewise, the oligosaccharides from strain JS314 gave P2-1 (8.4 mg) and P2-2 (20.1 mg) with 71% recovery.

Each P2-2 fraction was purified further by HPLC on a column (9 × 250 mm) of DIONEX CarboPac™ PA1 twice for the 61A101c sample and thrice for the JS314 sample, using a 0 → 10% gradient of M NaOAc during 30 min at 5 mL/min. Fractions were collected every 30 s, and each was assayed for hexose, using the phenol–H_2SO_4 method. Appropriate fractions were combined, acetate was removed by passage through DIONEX OnGuard H cartridges, and the solution was freeze-dried.

Glycosyl composition and linkage analysis.—The trimethylsilylated methyl glycosides (methanolic M HCl, 80° for 2 h) and the alditol acetates were prepared as described[31]. The products were identified and quantified by comparison with authentic standards. Standard Kdo was a gift from Dr. J. Ellis (Eastern Illinois University).

For methylation analysis, samples were reduced conventionally with $NaBD_4$ or $NaBH_4$, and methylated or ethylated by the Hakomori methylation procedure as described by York et al.[31]. The carboxymethyl or carboxyethyl groups were reduced using lithium triethylborodeuteride, the products were re-methylated or re-ethylated, and the alditol acetates were prepared. The various products were analyzed by GLC–MS with a Hewlett–Packard 5890/5970 instrument. Some analyses were

performed with a Hewlett–Packard 5985 GLC–MS system. The trimethylsilylated methyl glycosides and ethylated oligosaccharides were analyzed using 30-m and 15-m DB-1 columns (J&W Scientific), respectively. The methylated and ethylated alditol acetates were analyzed using a 30-m SP2330 column (Supelco).

NMR spectra (external DSS) were recorded at 23° on solutions in D_2O with a Bruker AMX 600 or AM500 MHz spectrometer. The samples were deuterium-exchanged twice by dissolving in D_2O and lyophilization. 2D DQF-COSY[32], HOHAHA[33], ROESY[34], and HMQ[35] data sets were collected in the phase-sensitive mode using the TPPI[36] method. The HMBC[37] experiment was applied to the residual HDO signal. The HOHAHA pulse program contained a DIPSI-2[38] spin-lock pulse of 115 ms, and a GARP[39] sequence was used for ^{13}C decoupling during acquisition in the HMQC experiment. For homonuclear experiments, $512t_1$ sets of 2048 data points were collected with 4–16 scans each. The spectral width was set to 3623 Hz and the carrier was placed at the residual HDO signal at δ 4.78. For the HMQC spectrum, a 147×2048 data set was acquired with 128 scans per t_1 block; for the HMBC, a 92×1024 data set was acquired with 512 scans per block. The spectral width in the ^{13}C dimension was set to 120 ppm, with the carrier at δ 64.7, based on external DSS. Data were processed typically with Lorentzian-to-Gaussian weighting functions applied to t_2 and shifted squared-sinebell functions and zero-filling applied to t_1.

RESULTS

Purification of the core oligosaccharides.—Elution of the P2-2 oligosaccharides from the parent strain 61A101c from DIONEX gave a minor peak in the void volume and a single major peak (61A101c-DNX2) in the $0 \rightarrow 10\%$ M NaOAc gradient. Likewise, P2-2 oligosaccharides from the mutant strain JS314 gave major peaks (JS314-DNX2/4) in the acetate gradient together with two other minor peaks, and another minor peak in the void volume of the column. These minor peaks were not characterized.

The major peaks were re-analyzed and purified on an analytical DIONEX CarboPac™ column, using a gradient of M NaOAc in 100 mM NaOH, 10% for 12 min, then to 20% during 30 min. The results are shown in Fig. 1. Under these conditions, JS314-DNX2 and JS314-DNX4 each gave two peaks (JS314-DNX2-1/2 and JS314-DNX4-1/2, respectively). JS314-DNX3 gave a single peak with a retention time (20.3 min) the same as those of DNX2-2 and DNX4-2. Peaks DNX2-1 and DNX4-1 were eluted at 7 and 8 min, respectively.

Structures of 61A101c-DNX2, JS314-DNX3, JS314-DNX2-2, and JS314-DNX4-2.—Each oligosaccharide consisted of Glc, Man, and Kdo in the ratios 1 : 1 : 1. Fig. 2 shows the ^1H-NMR spectrum for 61A101c-DNX2, which was identical to those of JS314-DNX3, -DNX2-2, and -DNX4-2 and showed that these oligosaccharides were identical. Thus, this oligosaccharide is the major component of small molecu-

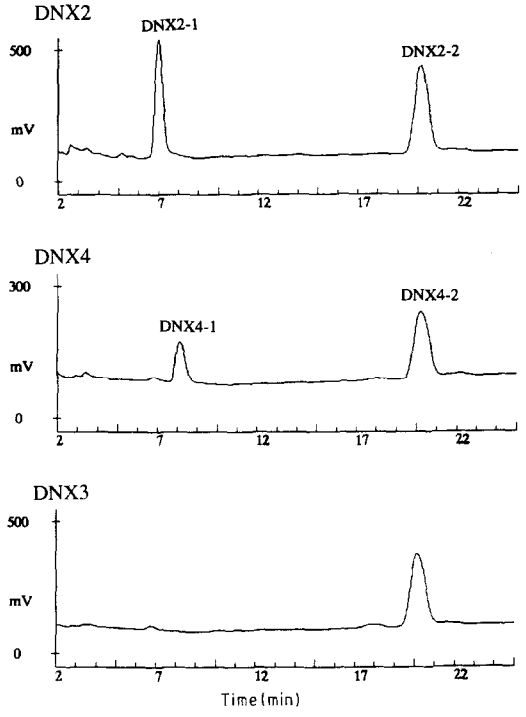


Fig. 1. HPLC on DIONEX, using an acetate gradient in 100 mM NaOH, of the core fractions (DNX2, DNX4, and DNX3) from JS314 isolated by preparative HPLC on DIONEX, using an acetate gradient (see Experimental).

lar weight released by mild acid hydrolysis of the LPSs of the parent (61A101c) and mutant (JS314) strains.

The ^{1}H-NMR spectrum of 61A101c-DNX2 contained resonances for H-1 at δ 5.26 ($J_{1,2}$ 1.9 Hz) and 4.99 ($J_{1,2}$ 3.8 Hz). The former resonance is assigned to the Man residue because of its small $J_{1,2}$ value, and its chemical shift is consistent with an α configuration. The latter resonance is assigned to an α-Glc residue. The resonances centered at δ 2.28 and 2.68 are assigned to the Kdo methylene protons. Their chemical shifts and coupling constants ($J_{3a,3b}$ 14.9, $J_{3a,4}$ 7.5, and $J_{3b,4}$ 2.4 Hz) are consistent with those reported for a Kdo*f* residue found in a disaccharide released from *Salmonella godesberg* LPS[40] and for synthetic Kdo*f*[41]; resonances due to Kdo*p* were not detected. Integration shows that the ratios of the intensities of Glc H-1, Man H-1, and Kdo H-3a,3b resonances were 1:1:2, which is consistent with this oligosaccharide being a trisaccharide.

Methylation analysis of the oligosaccharide (see Experimental) revealed a 1:1 ratio of the partially methylated alditol acetates derived from terminal Man (*m/z* 118, 161, 162, and 205) and 4-linked Glc (*m/z* 118 and 233); the derivative of the Kdo residue was not detected. FABMS of the oligosaccharide gave an $(M - H)^-$

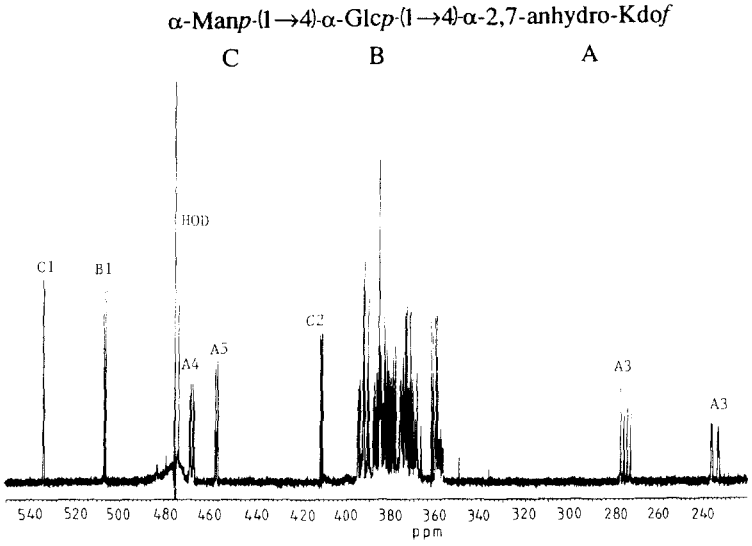


Fig. 2. A resolution-enhanced ^{1}H-NMR spectrum of the trisaccharide core component isolated from the LPS of strain 61A101c.

ion with m/z 543, i.e., 18 amu less than that expected for a trisaccharide composed of Man, Glc, and Kdo. Reduction of the oligosaccharide with borohydride at neutral pH or in M NH_4OH did not result in an increase in the mass of the $(M - H)^-$ ion, indicating that the Kdo residue is not present as a lactone, since lactones can be reduced under these conditions[42], and does not have a reducing residue (see below).

The ^{1}H and ^{13}C assignments for each glycosyl residue, shown in Table I, were made through COSY, HOHAHA (Fig. 3), and HMQC (Fig. 4) 2D NMR experiments. The proton assignments were made from the COSY and HOHAHA spectra.

For the Man residue, the COSY experiment (spectrum not shown) shows that the resonance centered at δ 4.03 ($J_{2,3}$ 3.3 Hz) is coupled to that of H-1 at δ 5.26 and can be assigned to H-2, and that the resonance at δ 3.79 ($J_{3,4}$ 11 Hz) is coupled to that of H-2 at δ 4.03 and can be assigned to H-3. A subspectrum through Man H-2 in the HOHAHA experiment reveals the other Man resonances. In this subspectrum the resonance at δ 4.03 (H-2) was coupled to resonances at δ 3.79 (H-3), 3.63 (H-4), 3.67 (H-5), 3.72 (H-6b), and 3.86 (H-6a). The H-4 and H-5 assignments were made on the basis that the HMQC experiment shows these resonances to be coupled to carbon resonances at δ 69.0 and 76.4, respectively, which are consistent with values reported for C-4 and C-5 of terminal Man residues[43]. The H-4 and H-5 resonances overlapped and it was not possible to measure the relevant coupling constants. However, both the H-6a and H-6b resonances were well resolved and had a typical $J_{6a,6b}$ value (11 Hz). Additionally,

TABLE I

^{1}H and ^{13}C assignments for the trisaccharide component of the core of the LPS of *B. japonicum* 61A101c

	H-1 (C-1)	H-2 (C-2)	H-3 (C-3)		H-4 (C−4)	H-5 (C-5)	H-6a	H-6b (C-6)				
Man	5.26 (103.9)	4.03 (72.9)	3.79 (72.9)		3.63 (69.0)	3.67 (76.4)	3.86	3.72 (63.2)				
Glc	4.99 (99.0)	3.54 (73.7)	3.85 (76.2)		3.61 (79.1)	3.76 (73.5)	3.83	3.77 (62.9)				
	(C-1)	(C-2)	H-3a	(C-3) H-3b	H-4 (C-4)	H-5 (C-5)	H-6 (C-6)		H-7 (C-7)	H-8a	(C-8)	H-8b
Kdo	(173.4)	(106.9)	2.28	2.68 (43.2)	4.60 (76.6)	4.49 (84.7)	3.74 (64.0)		3.52 (78.0)	3.80	3.67 (63.3)	

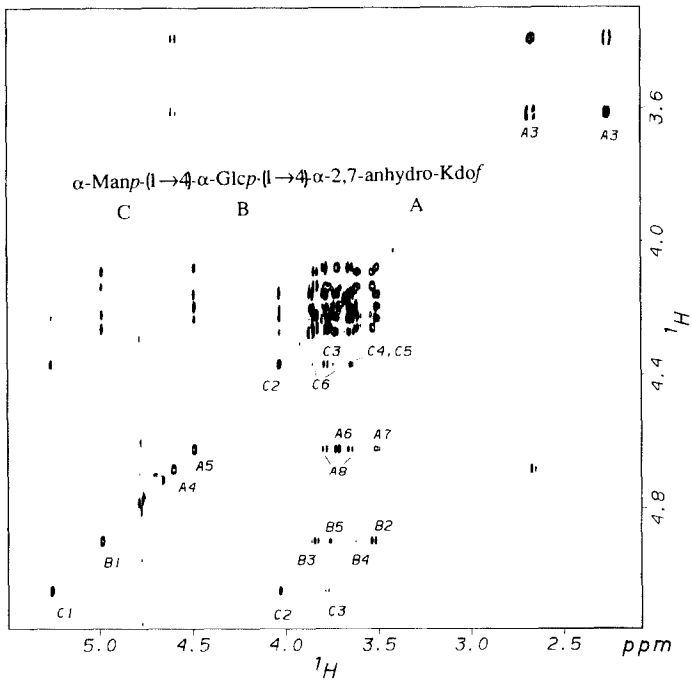


Fig. 3. A homonuclear Hartmann–Hahn (HOHAHA) spectrum of the trisaccharide core component from strain 61A101c LPS.

the HMQC experiment shows that both these resonances are coupled to that of a carbon resonating at δ 63.2. The remaining ^{13}C assignments were made from the HMQC experiment.

For the Glc residue, the COSY experiment shows that H-1 (δ 4.99) is coupled to a proton resonating at δ 3.54, which can be assigned as H-2. The $J_{2,3}$ value of 12 Hz was determined from a subspectrum of the HOHAHA experiment through H-1. This subspectrum shows that H-1 is coupled, in addition to H-2 (δ 3.54), to protons resonating at δ 3.61 and 3.76. The HMQC experiment shows that the resonance at δ 3.61 is coupled to that of a carbon resonating at δ 79.1, consistent with C-4 of a 4-linked Glc residue[43], and, thus, the resonance at δ 3.61 was assigned to H-4. Therefore, the resonance at δ 3.76 was assigned to H-5. The HMQC experiment also showed that resonances at δ 3.77 and 3.83 are coupled to a carbon resonating at δ 62.9 and, therefore, these were assigned to H-6b and H-6a. The remaining ^{13}C resonances were assigned on the basis of the HMQC experiment.

The complete ^1H assignments for the Kdo residue could be made from both the COSY and HOHAHA (Fig. 3) experiments. Coupling constants were determined from the ^1H-NMR spectrum (Fig. 2) and from subspectra of the HOHAHA experiment. The methylene protons, H-3a and H-3b, resonate at δ 2.28 and 2.68,

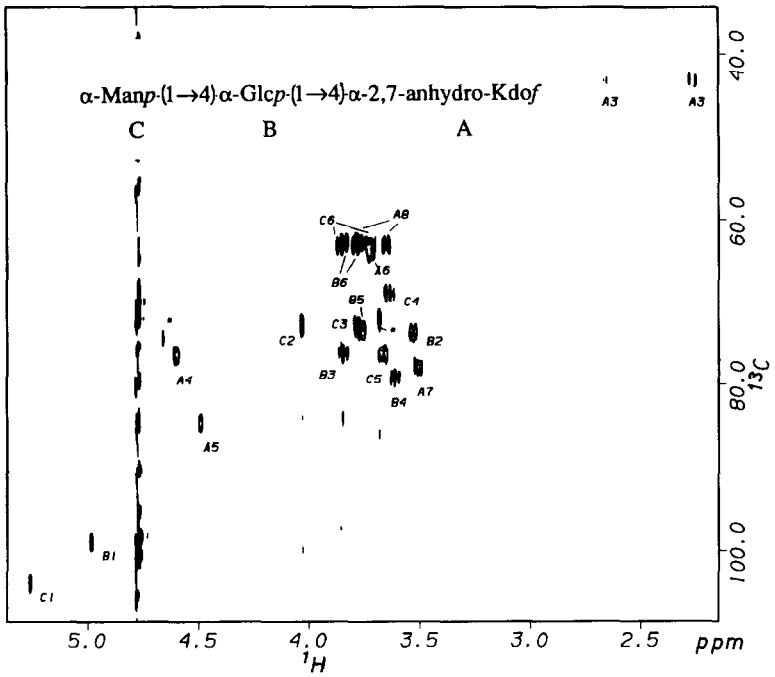


Fig. 4. An HMQC spectrum of the trisaccharide core component from strain 61A101c LPS. The two cross-peaks marked * are due to a small amount of a contaminant.

respectively, and were assigned on the basis of the data reported in the literature for a Kdo*f* residue[40,41,44]. The $J_{3a,3b}$, $J_{3a,4}$, and $J_{3b,4}$ values, discussed above, are consistent with those reported for a Kdo*f* residue[40,41,44]. The HMQC experiment (Fig. 4) shows that both of these protons are coupled to C-3 (δ 43.2). The chemical shift of the C-3 resonance is also consistent with that reported for a Kdo*f* residue[40,41,44]. Both the COSY and the HOHAHA spectra show that H-3a and H-3b are coupled to a proton resonating at δ 4.60 ($J_{4,5} < 1.0$ Hz), which can be assigned as H-4. This proton is coupled to a carbon resonating at δ 76.6 (Fig. 4), a value consistent with that (δ 76.7) reported for C-4 of a 4-linked Kdo*f* residue[40] and indicates that the Kdo*f* residue is 4-linked. The resonance at 4.49 (dd, $J_{4,5} < 1.0$, $J_{5,6}$ 4.6 Hz) is consistent with that reported for H-5 of Kdo*f* residues[41,44]. Because of the small $J_{4,5}$ value, coupling between H-4 and H-5 is not observed in the COSY or HOHAHA spectra. However, the ROESY spectrum (Fig. 5) shows a strong NOE between H-4 and H-5. In addition, H-5 is coupled to a carbon resonating at δ 84.7, which is also consistent with values reported for C-5 of Kdo*f* residues[40,41,44]. Therefore, the resonance at δ 4.49 is assigned to H-5 of the Kdo*f* residue. The HOHAHA spectrum clearly shows coupling from H-5 to the remaining protons of the Kdo*f* residue. The resonance at δ 3.74 can be assigned to H-6 since the COSY spectrum shows that this resonance is coupled to that of H-5. The

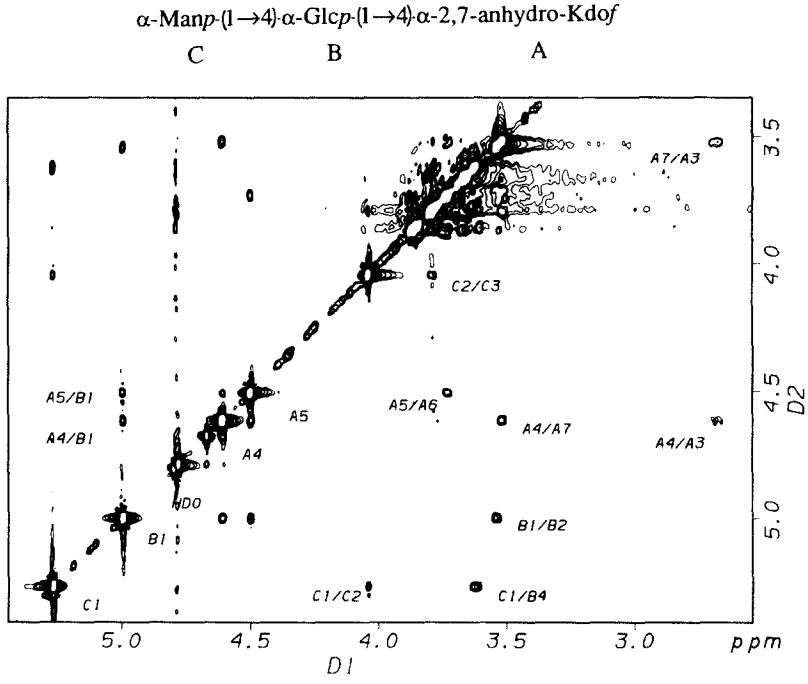


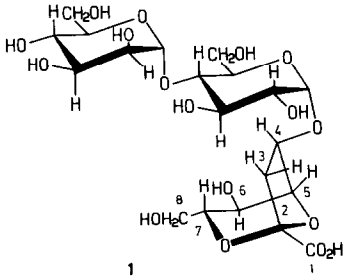
Fig. 5. A ROESY spectrum of the trisaccharide core component from strain 61A101c LPS.

$J_{6,7}$ value (12 Hz) is consistent with this assignment. In addition, a subspectrum through H-5 of the HOHAHA spectrum shows that the resonance at δ 3.74 (H-6) is next in intensity to that of H-5. The resonance at δ 3.52 is assigned to H-7 since the COSY spectrum shows that it is coupled directly to H-6. The subspectrum through H-5 of the HOHAHA spectrum shows that this resonance (δ 3.52, H-7) has a complex coupling pattern, as would be expected due to coupling with H-6,8a,8b. The remaining two Kdo*f* resonances in the HOHAHA spectrum are assigned to H-8a and H8b (δ 3.67 and 3.80, respectively, $J_{8a,8b}$ 13 Hz). In addition, the HMQC spectrum shows that both of these protons are coupled to a carbon resonating at δ 63.3, which is consistent with values reported for C-8 of Kdo[40,41]. The assignments for C-6 and C-7 from the HMQC spectrum are δ 64.0 and 78.0, respectively. The downfield position of the C-7 resonance suggests that this carbon, as with C-4, is involved in a linkage. The assignments for C-1 and C-2 were obtained from the HMBC spectrum (data not shown) and are δ 173.4 and 106.9, respectively. The chemical shift of the C-2 resonance is downfield from that (δ 105.4) reported for a reducing Kdo*f* residue[40], but is consistent with that (δ 106.7 or 107.1) reported for C-2 of the methyl glycoside of Kdo*f*[41]. These data suggest that C-2 of the Kdo*f* residue is involved in a linkage.

The ^{1}H- and ^{13}C-NMR data for the Kdo*f* residue are consistent with those reported for a 2,7-anhydro-3-deoxy-D-*manno*-2-octulofuranosonic acid which is

formed on prolonged heating of Kdo in dilute acid[44]. A small planar W $J_{3b,5}$ value (0.9 Hz) is consistent with values reported for this structure[44]. Also, in this 2,7-anhydro-Kdo*f* structure, NOEs between H-3 and H-7, and between H-4 and H-7 would be expected and are observed in the ROESY spectrum (Fig. 5). The FAB-mass spectrum of this trisaccharide reveals a molecular weight 18 less (i.e., minus H_2O) than would be expected for a trisaccharide with a reducing Kdo*f* residue and is indicative of a lactone or an anhydro derivative. The fact that the Kdo*f* residue could not be reduced under conditions known to reduce Kdo lactones or reducing Kdo residues suggests that the Kdo*f* is 2,7-anhydro-3-deoxy-α-D-*manno*-2-octulofuranosonic acid; the β anomer cannot form such a structure. The result is a bicyclic Kdo*f* residue in which the furanose ring adopts an E_o conformation, and the 6-membered anhydro ring, C-2–O-7–C-7–C-6–C-5–O-5, is in a normal chair conformation[44]. If the Kdo*f* residue exists as a 2,7-anhydro derivative, it will not be oxidized by periodate. Periodate oxidation and borohydride reduction of the trisaccharide followed by analysis of trimethylsilylated methyl glycoside derivatives changed the ratios of Man, Glc, and Kdo from 1 : 1 : 1 in the oligosaccharide to 0 : 0.08 : 1.

The sequence of the trisaccharide was determined from the ROESY and HMBC experiments. The ROESY spectrum (Fig. 5) shows NOE between the Man H-1 (δ 5.26) and Glc H-4 (δ 3.61). Thus, the terminal α-Man residue is 4-linked to the Glc residue. This conclusion was confirmed by the HMBC experiment (data not shown) in which a coupling was observed between Man H-1 and Glc C-4. The ROESY spectrum also shows NOE between Glc H-1 and H-4 and H-5 of the Kdo*f* residue. Since the Kdo is a furanose residue, the α-Glc residue must be 4-linked to Kdo*f*. The HMBC experiment (data not shown) confirmed this conclusion, in that coupling was observed between the Glc H-1 and Kdo*f* C-4, but not C-5. Also, the downfield position of the Kdo*f* C-4 resonance (discussed above) is consistent with a linkage at this position. Thus, all of the data support the structure **1** for this trisaccharide.



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Structures of the JS314-DNX2-1 and -DNX4-1 oligosaccharides.—Each of these oligosaccharides consisted of 4-*O*-Me-Man and Kdo residues. FABMS gave $(M - H)^-$ ions with *m/z* 413 for DNX2-1 and *m/z* 395 for DNX4-1. These results are consistent with DNX2-1 being a disaccharide and DNX4-1 being a lactone or an

anhydro derivative of DNX2-1. After reduction with $NaBH_4$, FABMS of DNX2-1 gave an $(M - H)^-$ ion with m/z 415, whereas DNX4-1 $(M - H)^-$ gave ions with m/z 395 and 397, indicating that partial reduction of DNX4-1 had occurred.

^{1}H-NMR spectroscopy of DNX2-1 revealed a single signal for H-1 (δ 5.02, $J_{1,2}$ 1.7 Hz), which can be assigned to a 4-*O*-Me-Man residue. There is also a sharp singlet at δ 3.50 due to OMe. H-3*ax* and H-3*eq* of Kdo resonate at δ 2.01 ($J_{3eq,3ax} = J_{3ax,4} = 12.5$ Hz) and 1.84 ($J_{3eq,4}$ 4.8 Hz), respectively. These data are consistent with those reported[42] for reducing 5-linked Kdo*p* residues. Since Kdo is the reducing moiety of this disaccharide and there are no resonances indicative of a furanose form, the Kdo residue is probably 5-linked.

The DNX2-1 disaccharide was re-reduced with $NaBD_4$, ethylated, carboxyethyl-reduced with lithium triethylborodeuteride, and re-ethylated, and the product was analyzed by GLC–MS. The partially ethylated alditol acetates were

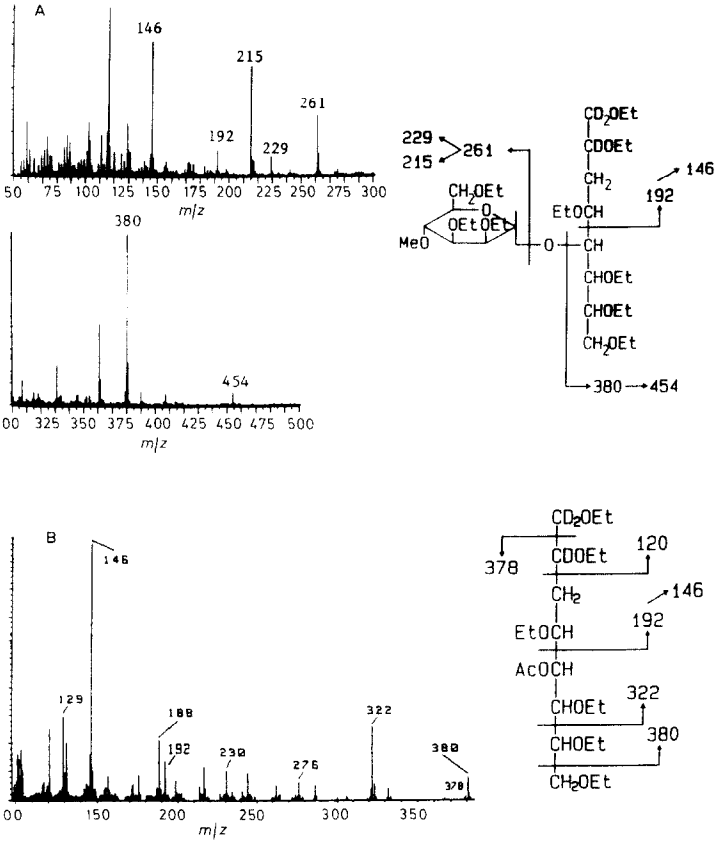
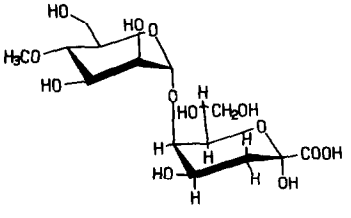


Fig. 6. (A) The EI-mass spectrum of pre-reduced, carboxyl-reduced, ethylated DNX2-1 released from JS314 LPS by prolonged mild hydrolysis with acid. (B) The EI-mass spectrum of the partially ethylated alditol acetate of the Kdo residue derived from pre-reduced, carboxyl-reduced DNX2-1.

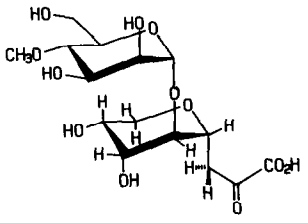
then prepared and analyzed by GLC–MS. Fig. 6A shows that the disaccharide consists of 4-*O*-Me-Man 5-linked to Kdo*p*. Fig. 6B shows the EI-mass spectrum of the partially ethylated alditol acetate of the 5-linked Kdo*p* residue.

The foregoing data show that DNX-2-1 consists of 4-*O*-Me-α-D-Man 5-linked to Kdo*p*.

FABMS, discussed above, indicates that DNX4-1 is an anhydro derivative of DNX2-1. FABMS of DNX4-1, after reduction with $NaBH_4$ under alkaline conditions, results in a change in the mass of the $(M-H)^-$ ion from 395 to 397, indicating that the parent compound was not a lactone. However, the increase in 2 amu indicates that reduction had occurred consistent with an anhydro derivative in which C-2 was not involved. ^{1}H-NMR spectroscopy of DNX4-1 shows that the resonances of the Kdo methylene protons are shifted downfield from their normal positions to δ 3.30 ($J_{3a,3b}$ 17.2, $J_{3a,4}$ 2.1 Hz) and 2.91 ($J_{3b,4}$ 9.5 Hz). In addition, the chemical shift of the H-1 resonance of the 4-*O*-Me-Man residue is shifted downfield to δ 5.19 ($J_{1,2}$ 1.7 Hz) from that (δ 5.02) for DNX2-1. The large $J_{3a,3b}$ value is characteristic of a methylene group which is adjacent to a carbonyl group. Such downfield chemical shifts and large J_{gem} values have recently been reported for 5-acetamido-4,8-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulosonic acid (*N*-acetyl-4,8-anhydroneuraminic acid), one of several products resulting[45] from prolonged heating of *N*-acetylneuraminate at pH 2.0. Thus, the Kdo residue in DNX4-1 is hypothesized to be present as 4,8-anhydro-3-deoxy-D-*manno*-2-octulosonic acid. The involvement of C-4 in the 4,8-anhydro ring may also explain the downfield shift from δ 5.02 to 5.19 for the H-1 resonance of the 4-*O*-Me-Man residue, which, based on the data for DNX2-1, is 5-linked to the Kdo residue. The structure for DNX2-1 and the proposed structure of DNX4-1 are shown in **2** and **3**, respectively.



2 (DNX2-1)



3 (DNX4-1)

Analysis of the O-antigen polysaccharides from strains 61A101c and JS314.—Mild acid hydrolysis of both 61A101c and JS314 LPSs resulted in polysaccharides (61A101c P2-1 and JS314 P2-1, respectively) that were eluted in the void volume of the Bio-Gel P2 column. Much smaller amounts of P2-1 were obtained from strain JS314 LPS than from 61A101c LPS. 61A101c P2-1 contained glycosyl residues typical of the LPS O-chain for this strain[28], namely, 3-*O*-Me-Rha, 2,3-di-*O*-Me-Rha,

Fuc, FucN, QvN, and 4-*O*-Me-Man. These sugars were also present in small proportions in P2-1 from strain JS314. However, JS314 P2-1 contained sugars typical of the EPS for these strains, namely, Rha and 4-*O*-Me-GlcA, in relatively large proportions indicating that this P2-1 fraction was largely EPS. The results indicate that mutant strain JS314 can synthesize the O-chain polysaccharide, and produces a very small amount of the complete LPS (O-chain-core-lipid A).

DISCUSSION

The results described above show that the major oligosaccharide released by mild acid hydrolysis from *B. japonicum* 61A101c LPS and from the LPS of its mutant, JS314, is a trisaccharide consisting of Man, Glc, and Kdo with structure **1**. An unusual feature of this trisaccharide is that the Kdo residue is present as 2,7-anhydro-α-D-*manno*-2-octulofuranosonic acid which is formed most likely during the hydrolysis with aqueous 1% acetic acid (5 h, 100°). The same compound was formed[44] during treatment of Kdo with aqueous 1% formic acid at 100° for 75 min. It is also probable that the 4,8-anhydro-Kdo residue in DNX4-1 is formed during the mild acid hydrolysis procedures. Similar anhydro derivatives of *N*-acetylneuraminic acid occur[45] during prolonged acid hydrolysis at pH 2.0.

The results presented above show that the polysaccharide P2-1 released from the parent LPS, as well at the small amount from the mutant LPS, contains sugars that are typical of the O-chain, as well as 4-*O*-Me-Man. No 4-*O*-Me-Man-Kdo disaccharides are released by mild acid hydrolysis of the parent LPS. These results suggest that the O-chain is linked to the 4-*O*-Me-Man-Kdo moiety of the intact LPS. The fact that the mutant JS314 can make a small amount of LPS that contains the O-chain implies that the mutation (*a*) causes a decrease in O-chain synthesis or (*b*) affects the enzyme which transfers the O-chain to the 4-*O*-Me-Man-Kdo moiety of the core region of the LPS. Any such mutation may be somewhat "leaky" and permit the synthesis of a small amount of LPS with O-chain. The exact nature of this mutation remains to be determined.

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