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Note

Re-examination of the structures of the lipopolysaccharide core oligosaccharides from *Rhizobium leguminosarum* biovar phaseoli

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Recent reports have shown that intact lipopolysaccharides (LPSs) are necessary in order for rhizobia to form nitrogen-fixing nodules with their legume hosts[1–8]. The data in these reports show that mutants having LPSs that lack their O-antigenic polysaccharide are either defective in infection thread formation or in release of bacteria into the root cells. Other reports show that a structural feature of the LPS may be involved in the differentiation of bacteria to the nitrogen-fixing bacteroid[9–12]. Thus it is important to characterize the structures of *Rhizobium* LPSs and of LPSs from mutants that are defective in symbiotic infection.

In our efforts to determine the complete structure of the LPS from *R. leguminosarum* biovar phaseoli strain CE3, new evidence was obtained that conflicted with the structures already reported[5] for the two core oligosaccharides of this LPS. The new evidence indicated that the 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) residue in the trisaccharide core was linked at O-4 and O-5 rather than at O-4 and O-7, and that the tetrasaccharide Kdo residue was linked at O-5 rather than O-4. These new data made it necessary to re-examine the structures of the two core oligosaccharides. The oligosaccharides were isolated as previously described[5]. Their structures were determined by gas chromatography (g.c.)-mass spectrometry (m.s.) of their partially methylated alditol acetates, g.c.-m.s. of the permethylated oligomers, and n.m.r. spectroscopy.

The trisaccharide core. — The glycosyl composition and ^{1}H-n.m.r. spectrum of this oligosaccharide were as previously reported[5] and show that is it a trisaccharide consisting of two α-linked galactosyluronic residues and one Kdo residue. Pre-reduction of the trisaccharide results in a negative thiobarbituric acid test, indicating that the Kdo residue is at the reducing end of the trisaccharide. Additionally, a positive Kdo test can be observed only after hydrolysis in relatively strong acid (2M H_2SO_4), indicating that the Kdo residue is linked at O-4 and/or O-5. Linkage at O-4 and/or O-5 of Kdo prevents periodate oxidation of Kdo and thereby prevents a positive thiobarbituric acid

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test. The thiobarbituric acid assay was performed as previously described[13]. The stereochemical configurations of the glycosyl residues were determined by analysis of their trimethylsilylated (+)- and(−)-*sec*-butyl glycosides as previously described[14].

^{1}H-N.m.r. analysis was performed on the ammonium salt of the trisaccharide in D_2O at 300K with a Bruker 500-MHz instrument. Resonances were measured relative to sodium 4,4-dimethyl-4-silapentanoate (TSP). Two resonances, at δ 5.12 ($J_{1,2}$ 3.8 Hz) and 5.04 ($J_{1,2}$ 3.8 Hz) may be assigned to the α-anomeric protons of the two galactosyl uronic residues. The H-3 methylene protons of Kdo resonate at δ 2.17 ($J_{a,e} = J_{3,4} = 12.5$ Hz) and δ 1.82 ($J_{a,e}$ 12.5, $J_{3,4}$ 4.3 Hz) as a triplet and a doublet of doublets, respectively. These resonances may be assigned to the H-3 axial (H-3*a*) and H-3 equatorial (H-3*e*) protons of Kdo, respectively. The chemical shifts of H-3*a* and H-3*e*, namely δ 2.17 and 1.82, are reversed from their normal positions for Kdo[15]. Normally, equatorial protons resonate at lower field than axial protons. The reason for this unusual behavior of the Kdo methylene protons in the trisaccharide is not known, however other exceptions have been reported, one of which is an α-L-Rha*p*-(1→5)-Kdo*p* disaccharide from a plant cell-wall component[16] and another being[15] the ammonium salt of monomeric Kdo.

The n.m.r. spectrum also shows that the Kdo residue, even though it is at the reducing end of this trisaccharide, is present only as the pyranose tautomer. Normally, the ammonium salt of a reducing Kdo residue, not linked at O-5, is present in both the pyranose and furanose forms (65 and 35%, respectively[17]). The H-3 resonances of the major furanose anomer of Kdo occur as two doublets of doublets at ~δ 2.10 ($J_{3,3}$ 14–15,$J_{3,4}$ 2–3 Hz) and 2.58 ($J_{3,3}$ 14–15,$J_{3,4}$ 7 Hz)[17]. There is no indication of these resonances in the trisaccharide, which indicates that the Kdo residue is linked at O-5 and is therefore prevented from forming a furanose structure. The methylation data presented next confirm that one of the two galactosyluronic residues is linked to O-5 of Kdo.

Methylation analysis was performed using potassium methylsulfinyl anion and methyl iodide according to Tacken *et al*[18] with some modifications. The sample was pre-reduced with NaB^2H_4 in D_2O at room temperature for 2 h. The reduced sample was permethylated and the carboxymethyl esters of the Kdo and galactosyluronic residues were reduced with "Superdeuteride" (a M solution of lithium triethylborodeuteride in tetrahydrofuran, Aldrich). The excess of reducing reagent was decomposed with acetic acid, the solvent evaporated under a stream of nitrogen, and the sample desalted using Dowex 50 (H^+) in 1:1 methanol–water. The resulting carboxyl-reduced, permethylated trisaccharide was remethylated and purified using a C_{18} Sep-Pak cartridge[18]. The resulting permethylated trisaccharide was analyzed directly by g.l.c.–m.s., as well as by g.l.c.–m.s. analysis of the partially methylated alditol acetates.

The g.l.c.–m.s. analysis of the partially methylated alditol acetates showed two major components, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol (with two deuterium atoms at C-6) arising from terminally linked galactosyluronic residues, and a 4,5-di-*O*-acetyl-3-deoxy-1,2,6,7,8-penta-*O*-methyloctitol arising from a 4,5-linked Kdo residue at the reducing end of this oligosaccharide. The mass spectrum of the Kdo derivative is shown in Fig. 1A. This spectrum is characterized by the primary fragment-ions, *m/z* 294, 338, and 336 derived from the C1/6, C1/7 and C8/2 moieties, respectively.

Additional small amounts of the partially methylated alditol acetates arising from 4- and 5-linked Kdo, and monomeric Kdo were also observed. These Kdo residues were thought to result from β-elimination of unreduced galactosyluronic residues from C-4 and/or C-5 of Kdo during the second methylation step. This was verified by subjecting the pre-reduced, permethylated trisaccharide to β-elimination according to the procedure of Aspinall and Rosell[19] and remethylation with C^2H_3I. The carboxymethyl group of Kdo was reduced with lithium triethylborodeuteride and the sample was then remethylated as already described, using CH_3I. Partially methylated alditol acetates were prepared and analyzed by g.l.c.–m.s. The result is one major peak consisting of 3-deoxy-1,2,4,5,6,7,8-hepta-*O*-methyloctitol with C^2H_3- at O-4 and O-5. These data, together with the f.a.b.-m.s. and n.m.r. data previously reported[5] and already discussed, prove that trisaccharide consists of two terminal galactosyluronic residues α-linked to O-4 and O-5 of Kdo.

Direct analysis of the pre-reduced permethylated trisaccharide by g.l.c.–m.s. gave results that are consistent with the linkages just described. The ammonia c.i.–m.s. (Fig. 2A) shows the presence of ions of *m/z* 757 ($M + NH_4^+$) and 502; the latter arising from the cleavage of one of the galactosyluronic residues. The e.i.–m.s. (Fig. 3A), shows the "aA" series ions, *m/z* 221 (aA1), 189 (aA2), and 157 (aA3)that are specific for the terminal galactosyl residues having two deuterium atoms at C-6. Also the absence of the "baA" and "ald" series ions indicates a branched structure for the trisaccharide. The mass spectrum is also characterized by the presence of a "v" ion, *m/z* 310 [CD_2OMe–

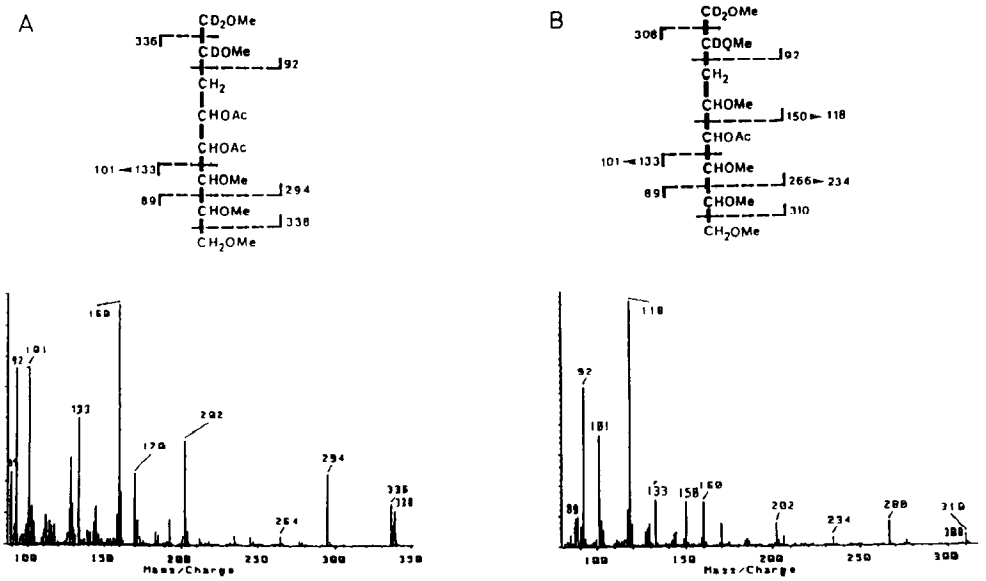

Fig. 1. The e.i.-m.s. fragmentation pattern of (A) 4,5-di-*O*-acetyl-3-deoxy-1,2,6,7,8-penta-*O*-methyloctitol derived from the trisaccharide core and (B) 5-*O*-acetyl-3-deoxy-1,2,4,6,7,8-hexa-*O*-methyloctitol derived from the tetrasaccharide core. Analysis was performed on a Hewlett–Packard 5890/5970 GC/MSD system using a 30-m SP2330 capillary column from Supelco.

CDOMe-CH_2-CH_2-C=O(-CHO)-CHOMe-CHOMe-CH_2OMe] obtained by the quasi-elimination of the monosaccharide unit from the "a^{1}ald"ion at *m/z* 502. G.l.c.–m.s. analysis also indicated the presence of small amounts of the pre-reduced, carboxyl-reduced, permethylated disaccharides arrising from GalA(1→4)Kdo-ol and GalA (1→5)Kdo-ol, which most probably arise through *β*-elimination of unreduced galactosyluronic residues as already described.

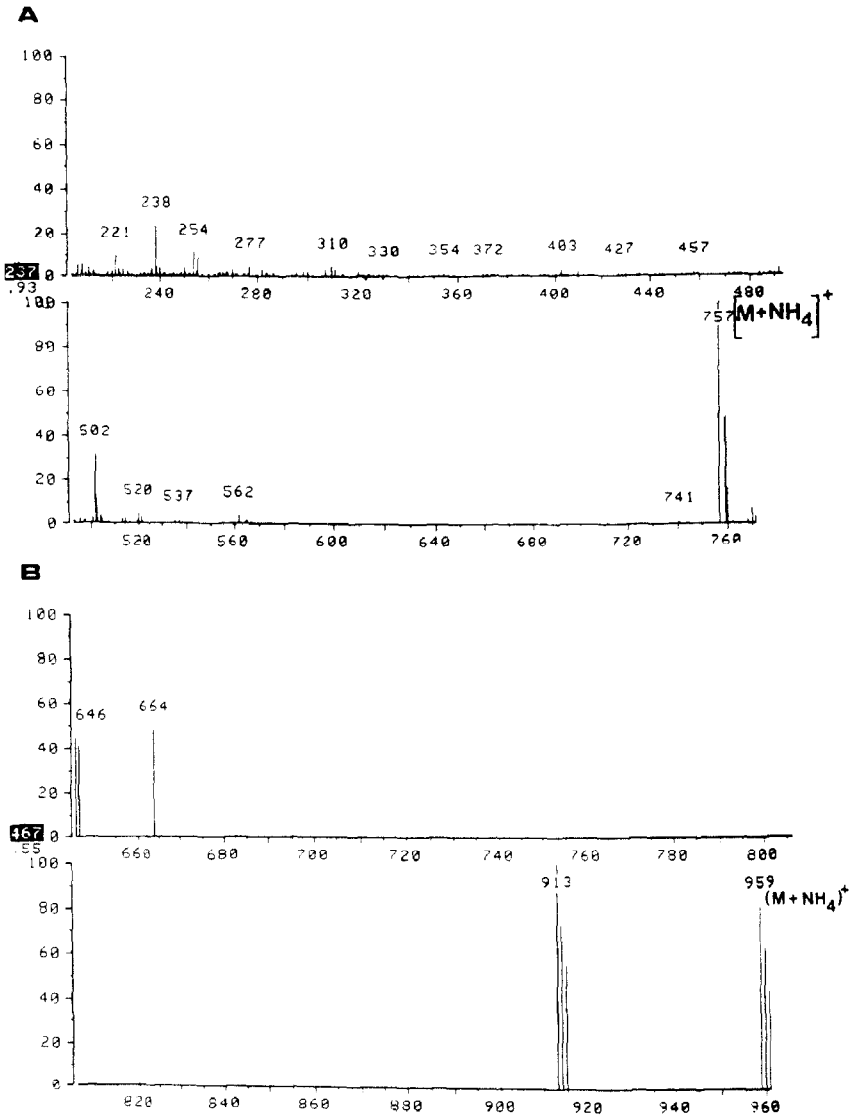

Fig. 2. C.i.-m.s. of the pre-reduced, carboxyl-reduced, and permethylated (A) trisaccharide and (B) tetrasaccharide. Analysis was performed on a Hewlet–Packard 5885 g.l.c.–m.s. system using a 15-m DB-1 column form J&W Scientific.

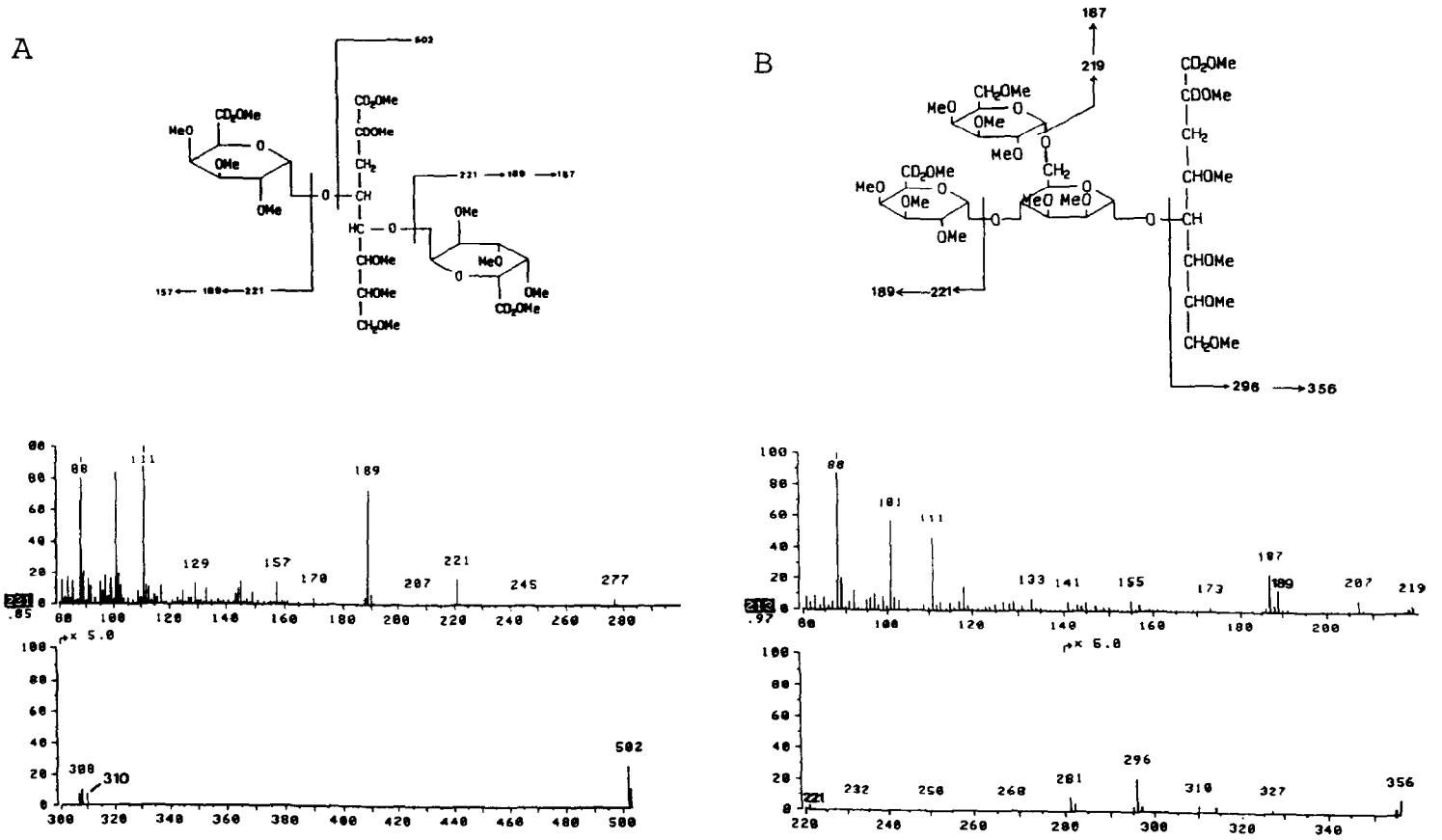

Fig. 3. E.i.-m.s. of the pre-reduced, carboxyl-reduced, and permethylated (A) trisaccharide and (B) tetrasaccharide. Analysis was performed using the same instrument and column described in the legend to Fig. 2.

The data described here are consistent with the following structure of the trisaccharide .

α-D-GalA*p*(1→5)-Kdo*p*
4
↑
1
α-D-GalA

The tetrasaccharide core. — Composition and n.m.r. analysis were as described in a previous report for this molecule [5], showing that it consists of GalA:Gal:Man:Kdo in a 1:1:1:1 ratio and that the GalA, Gal, and Man residues are all α-linked. The need for strong acid hydrolysis in order to observe a positive thiobarbituric acid assay, and a negative thiobarbituric acid test after pre-reduction indicates that the Kdo residue is linked at O-4 and/or O-5 and is located at the reducing end of the tetrasaccharide. Analysis by f.a.b.–m.s. as previously reported[5] gave a molecular ion of 738 ($M + H^+ = 739$), consistent with this molecule being a tetrasaccharide. The stereochemical configurations of the glycosyl residues were determined as already described for the trisaccharide.

^{1}H-N.m.r. analysis was performed as already described for the trisaccharide. The spectrum was identical that previously reported for this molecule[5] showing three resonances for α-anomeric protons at δ 5.32 ($J_{1,2}$ 3.6 Hz), 4.98 ($J_{1,2}$ 2.0 Hz) and 4.90 ($J_{1,2}$ 4.1 Hz). These resonances have been assigned to the galactosyluronic, mannosyl, and galactosyl residues, respectively[5]. The H-3*e* and H-3*a* protons of Kdo resonate at δ 1.81 ($J_{a,e}$ 12.6, $J_{3,4}$ 4.5 Hz) and 2.06 ($J_{a,e} = J_{3,4} = 12.6$ Hz). As for the trisaccharide, described above, the chemical shifts of H-3*e* and H-3*a* are reversed from their usual positions. There are no resonances indicative of a Kdo furanose structure, which implies that the Kdo residue cannot form this tautomer because of linkage at O-5. The methylation and g.l.c.–m.s. data described next confirm that the Kdo residue is linked at O-5.

The tetrasaccharide was pre-reduced, methylated, carboxyl reduced, remethylated, and analysed by g.l.c.–m.s. as described for the trisaccharide. 5-*O*-Acetyl-3-deoxy-1,2,4,6,7,8-hexa-*O*-methyloctitol was the only methylated derivative of Kdo. The e.i.-m.s. of this derivative, (Fig. 1B) shows the characteristic fragment ion *m/z* 118 derived from *m/z* 150, which correspondends to the C1/4 moiety with the loss of methanol. This result is consistent with the tetrasaccharide containing a 5-linked reducing Kdo residue. The other ions include *m/z* 266, 310, and 308 derived from C1/6, C1/7, and C8/2 moieties, respectively. These data show that the Kdo residue is linked at O-5, and not at O-4 as reported earlier[5]. The other methylated alditol acetate derivatives obtained were derived from terminal galactose, terminal galacturonic acid (co-eluting with the galactose derivative after carboxyl reduction) and 4,6-linked mannose.

A small amount of the partially methylated alditol acetate of 6-linked mannose was also observed during g.l.c.–m.s. analysis. This could be explained by β-elimination of some unreduced galactosyluronic methyl ester form the 4-position of mannose

during the second methylation procedure. This was verified by a β-elimination experiment performed as already described for the trisaccharide. The g.l.c.–m.s. analysis, after β-elimination and ethylation, showed that the partially methylated alditol acetate derived from a 4,6-linked mannosyl residue was replaced by that derived from a 6-mannosyl residue having an ethyl group at O-4. This result shows that the galactosyluronic residue is linked at O-4 of mannose, not at O-6 as reported earlier[5].

In addition to β-elimination, the small amount of a 6-linked mannosyl residue may be explained, in part, by the presence of a trisaccharide that consists of a modified tetrasaccharide in which the galactosyluronic residue is missing. The presence of such a trisaccharide is supported by f.a.b.–m.s. data, which show an ion of m/z 563 ($M + H^+$) (data not shown). In fact, such a trisaccharide is a major component of the LPS from bv. phaseoli CE109[5], an LPS mutant of strain CE3.

The pre-reduced, carboxyl-reduced, and permethylated tetrasaccharide was also analyzed directly by g.l.c.–m.s. The data obtained were consistent with the tetrasaccharide structure as described. The ammonia c.i.-m.s. (Fig. 2B) shows a quasimolecular ion of m/z 959 ($M + NH_4^+$). The e.i.-m.s. (Fig. 3B) shows ions of m/z 296 and 356 (the "ald" and "aldJ$_1$" ions derived from Kdo-ol), m/z 219 and 187 (derived from terminal galactose), and m/z 221 and 189 (derived from the carboxyl-reduced terminal galactosyluronic residue). Ions at the higher mass range were not observed because of limitations of the mass spectrometer.

These results are consistent with the tetrasaccharide having the structure:

α-D-GalA*p*(1→4)-α-D-Man*p*(1→5)-Kdo*p*
6
↑
1
α-D-Gal*p*

Summary. — We stated previously[5] that the trisaccharide contains two galactosyluronic residues linked to O-4 and O-7 of Kdo, based on the identity of the n.m.r. spectrum with that of an *R. leguminosarum* biovar trifolii trisaccharide[20]. However, the n.m.r., methylation, and g.l.c.–m.s. data presented here clearly show that the galactosyluronic residues are linked to the O-4 and O-5 (not O-7) of Kdo. Re-examination of the linkages of the bv. trifolii trisaccharide by methylation and g.l.c.–m.s. shows that it also has the two galactosyluronic residues linked to O-4 and O-5 of Kdo (data not shown). The earlier report indicating that Kdo was linked at O-4 and O-7 was based exclusively on an incorrect interpretation of the n.m.r. spectrum[20]. In fact the n.m.r. data, which show that the reducing Kdo residue is exclusively present as a pyranose structure, strongly indicate that Kdo must be linked at O-5 and are thus consistent with the structure described here.

We also stated[5] that the tetrasaccharide is comprised of a mannosyl residue α-linked to O-4 of Kdo and that the galactosyluronic and galactosyl residues are α-linked to the O-4 and O-6, respectively, of the mannosyl residue. Again this structure was largely based on the identity of its n.m.r. spectrum with that of a bv. trifolii

tetrasaccharide[21]. However, we have recently shown that the bv. trifolii tetrasaccharide glycosyl linkages are the same as those described here for the bv. phaseoli CE3 tetrasaccharide[22], namely that the mannosyl residue is α-linked to O-5 (not O-4) of Kdo and the galactosyluronic and galactosyl residues are α-linked to O-4 and O-6 (not O-6 and O-4), respectively, of the mannosyl residue.

The results described here also imply that the defective core oligosaccharide from the symbiotic mutant bv. phaseoli CE309 [5] consists of a mannosyl residue α-linked to O-5 (not O-4) of Kdo, and the defective oligosaccharide from mutant bv. phaseoli CE109 [5] consists of the mannosyl residue α-linked to O-5 (not O-4) of Kdo with a galactosyl residue α-linked to O-6 (not O-4) of the mannosyl residue. The corrected structures for these bv. phaseoli strains are shown in Fig. 4.

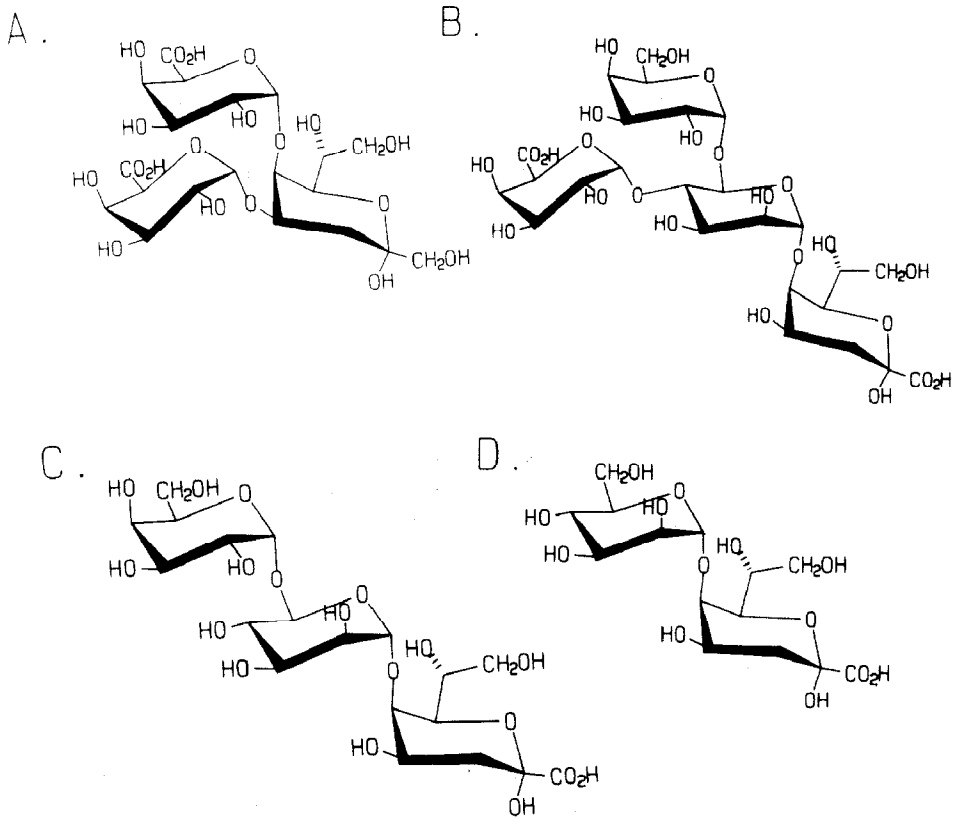

Fig. 4. Structures of the (A) trisaccharide and (B) tetrasaccharide for bv. phaseoli CE3, and (C) modified tetrasaccharide for mutant CE109 and (D) mutant CE309. The mutant structures published previously [5] are incorrect.

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