Carbohydrate Research, 195 (1989) 101–110
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

THE STRUCTURES OF THE LIPOPOLYSACCHARIDE CORE COMPONENTS FROM *Rhizobium leguminosarum* BIOVAR *phaseoli* CE3 AND TWO OF ITS SYMBIOTIC MUTANTS, CE109 AND CE309

RUSSELL W. CARLSON*, FRANCISCA GARCI†,
Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602 (U.S.A.)

DALE NOEL,
Department of Biology Marquette University Milwaukee, WI (U.S.A.)

AND RAWLE HOLLINGSWORTH
Department of Biochemistry, Michigan State University, East Lansing, MI (U.S.A.)

(Received January 11th, 1989; accepted for publication June 3rd, 1989)

ABSTRACT

The structures for the core regions of the lipopolysaccharides (LPSs) from *R. leguminosarum* bv. *phaseoli* CE3 and two symbiotic mutants were determined by g.l.c.–m.s., proton nuclear magnetic resonance spectroscopy (n.m.r.), fast-atom-bombardment mass spectrometry (f.a.b.–m.s.), and by comparison with known structures from the LPS of *R. leguminosarum* bv. *trifolii* ANU843. The core oligosaccharides were separated into two components, P2-2 and P2-3, by gel-filtration chromatography using Bio-Gel P2. The P2-2 oligosaccharide from CE3 is a tetrasaccharide consisting of 3-deoxy-D-*manno*-2-octulosonic acid (Kdo), mannose, galactose and galacturonic acid. The mannosyl residue is α-linked to O-4 of Kdo, and the galactosyl and galactosyluronic residues are α-linked to O-4 and O-6, respectively, of the mannosyl residue. The P2-2 oligosaccharide from mutant CE109 is missing the galactosyluronic residue, while that from mutant CE309 is missing both the galactosyl and galactosyluronic residues. The P2-3 oligosaccharide from CE3 LPS is a trisaccharide consisting of two galactosyluronic residues α-linked to the O-4 and O-7 of Kdo. Fraction P2-3 from mutant CE309 has the same structure as CE3 P2-3. Fraction P2-3 from mutant CE109 contains galacturonic acid and Kdo, but its structure differs from that of CE3 P2-3.

INTRODUCTION

Rhizobia are Gram-negative bacteria that have a nitrogen-fixing symbiotic relationship with legume plants. The lipopolysaccharides (LPSs) from those

*To whom reprint requests should be sent.
†This work was done in partial fulfillment of the requirments of the M.S. degree for FG at Eastern Illinois University in Charleston, Illinois.

0008-6215/89/$ 03.50

bacteria are thought to play a role in this symbiotic relationship. Initial studies were aimed at determining whether or not *Rhizobium* LPSs play a role in the specific recognition between the symbiont and the host cell. These studies presented data showing that LPSs may inhibit lectin-mediated binding of the bacterium to the host root[1-4]. Other reports indicated that the binding of *Rhizobium* to the host root is non-specific[5] and that lectin binding to symbionts was also non-specific[6-8]. Recently, in the case of *R. meliloti*-alfalfa symbiosis, it has been reported that specific attachment is observed only when using a very low concentration of bacteria and that this attachment may be inhibited by LPS[9,10]. Thus, it is still uncertain whether or not LPSs play a role in early recognition events between a *Rhizobium* and its host legume.

While it is uncertain whether or not LPSs play an important role in early symbiotic steps, recent data show that an intact LPS is essential for later symbiotic events. It has been shown that *R. leguminosarum* and *B. japonicum* mutants which have LPSs that lack the O-chain are defective in nodulation[11-13,25]. The *R. leguminosarum* mutants elicit infection threads and nodule formation, but the infection threads abort and the resulting nodules are underdeveloped and devoid of nitrogen-fixing bacteroids[11]. Thus, the presence of a complete LPS is important in order to form a nitrogen-fixing nodule.

Recent rules also indicate that LPSs from bacteroids may be altered from those of cultured bacteria. Immunoblots of polyacrylamide gels using monoclonal antibodies show that the LPSs from bacteroids, while still containing an O-chain, have a different banding pattern than the LPSs from cultured bacteria[14,15]. Thus, not only may the O-chain be necessary for nodule development, but subtle changes may occur in the LPS during bacteroid differentiation.

In order to asses the role(s) of LPSs in symbiosis it is important to determine the structures of these molecules. While the O-chains of LPSs from the *R. leguminosarum* strains vary greatly in structure[16,17], the core regions are very similar in their composition and molecular weight[12,18,19]. We have recently determined the structures of two core oligosaccharides from bv. *trifolii* ANU843. One of these oligosaccharides is a trisaccharide consisting of two galacturonic acid residues α-linked to the 4 and 7 positions of a 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) residue[20]. The other is a tetrasaccharide consisting of galactose, galacturonic acid, mannose, and Kdo. The mannose residue is α-linked to O-4 of Kdo, the galacturonic acid residue is α-linked to O-6 of mannose, and the galactose residue, which is acetylated at O-4, is α-linked to O-4 of the mannose residue[26]. Here we describe the structures of the core oligosaccharides from *R. leguminosarum* bv. *phaseoli* CE3 and two transposon-generated symbiotic mutants, CE109 and CE309. Both mutants have been shown to lack the O-chain and are defective in nodule development[11,12]. Genetic analysis has established that in each mutant a single mutation causes both the LPS and symbiotic defects[25].

EXPERIMENTAL

General methods. — The bacteria were grown at 30° in 0.5% tryptone, 0.3% yeast extract and 10mM $CaCl_2$. The LPSs and their core regions were purified as previously described[16,18]. The two core oligosaccharides from each LPS were isolated by further purification using a Bio-Gel P-2 column with 1% acetic acid as the eluant[26]. The core oligosaccharides were converted into their NH_4^+ forms prior to n.m.r. analysis and fast-atom-bombardment mass spectrometry (f.a.b.–m.s.).

Analytical techniques. — Neutral sugars were identified and quantitated by preparation of their alditol acetates[21] and analysis by gas–liquid chromatography (g.l.c.) using a 15-m column of Supelco SP2330 fused silica. Uronic acids were identified by reduction[22] followed by alditol acetate preparation and g.l.c. analysis. Uronic acids and Kdo were also analyzed by colorimetric methods[23,24]. The Kdo assay was modified as previously described[20].

N.m.r. spectra were recorded in D_2O on a Nicolet T-360 instrument at the University of Illinois Molecular Spectroscopy Laboratory. Some spectra were also recorded on a Bruker AM250 instrument. F.a.b.–m.s. was performed on a JEOL HX-110 HF spectrometer using a 2-thioglycerol matrix.

RESULTS AND DISCUSSION

The core structures from the parent strain, CE3. — Gel-filtration chromatography, using Bio-Gel P-2 in 1% acetic acid, separates the O-chain, P2-1, from two core oligosaccharides, P2-2 and P2-3, respectively, Fig. 1. This procedure has been previously described[20,26].

Composition analysis shows that P2-2 is a tetrasaccharide consisting of galacturonic acid, mannose, galactose, and Kdo in the ratio of 1:1:1.1:0.9 (Table I). The value of Kdo may be low due to some degradation of this residue during the assay procedure. The composition was verified by n.m.r. (Fig. 3A and Table II) and f.a.b.–m.s. analyses. Integration of the n.m.r. spectrum results in a GalA-H-1/Man-H-1/Gal-H-1/Kdo-H-3*a* Kdo-H-3*e* ratio of 1:1:1:1:1. The chemical shifts of the anomeric protons indicate that all linkages are α. The n.m.r. assignments given in Table II are based on those obtained for the tetrasaccharide core-component for the LPS from bv. *trifolii* ANU843, the structure of which has already been determined[26] F.a.b.–m.s. analysis results in quasi-molecular ions of $M + H^+ = 739$, $M + NH_4^+ = 756$ and $M + Na^+ = 761$ (Figure 2A), which are consistent with P2-2 being a tetrasaccharide with the composition already described. Previous work has shown that the core region from this strain contains Kdo at the reducing end, as reduction prior to Kdo analysis gives a negative thiobarbituric acid test[12]. Additionally, a negative thiobarbituric acid assay is obtained unless hydrolysis of the core oligomer is performed in relatively strong acid, namely, M H_2SO_4 for 30 min at 100°, prior to periodate oxidation. The ^{1}H-n.m.r. chemical shifts and coupling constants, sequence, quasimolecular ions from f.a.b.–m.s., composition

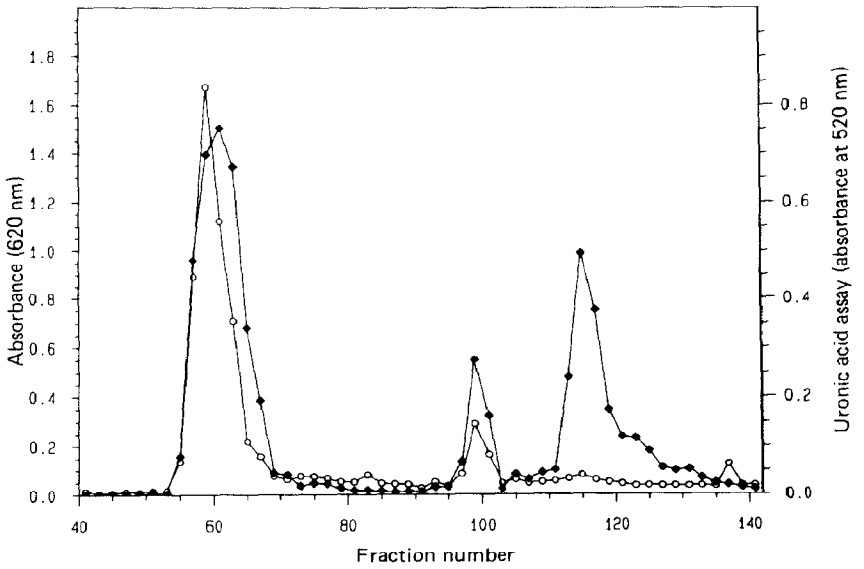


Fig. 1. A Bio-Gel P2 profile showing the separation of the O-chain, P2-1, from the two core oligosaccharides, P2-2 and P2-3, of strain CE3. The column was 1 cm by 1 m, and 1-mL fractions were collected. The column was eluted with 1% acetic acid. The open circles denote hexose and the closed diamonds denote uronic acid.

analysis, and the presence of Kdo at the reducing end and its resistance to the thiobarbituric acid assay are results that are identical to those obtained for the deacetylated tetrasaccharide core from bv. *trifolii* ANU843, the structure of which has been rigorously determined[26]. Therefore, we conclude that the structure of P2-2 from bv. *phaseoli* CE3, Fig. 4A, is the same as that described for the tetrasaccharide core of bv. *trifolii* ANU843. Differences in sequence or in linkages between the CE3 and ANU843 (ref. 26) tetrasaccharides would have resulted in detectable differences in the chemical shifts of the anomeric protons and possibly the Kdo methylene protons.

TABLE I

THE MOLAR COMPOSITION OF THE P2-2 OLIGOSACCHARIDES FROM *Rhizobium leguminosarum* CE3, CE109, AND CE309

Strain	*Mannose*	*Galactose*	*Galacturonate*	*Kdo*[a]
CE3	1.0	1.0	1.1	0.9
CE109	1.0	1.0	n.d.[b]	0.6
CE309	1.0	n.d.	n.d.	0.5

[a]The levels of Kdo are probably underestimated because of partial degradation during the assay procedure, since stronger acid conditions were necessary to detect Kdo (see text). [b]N.d. = none detected.

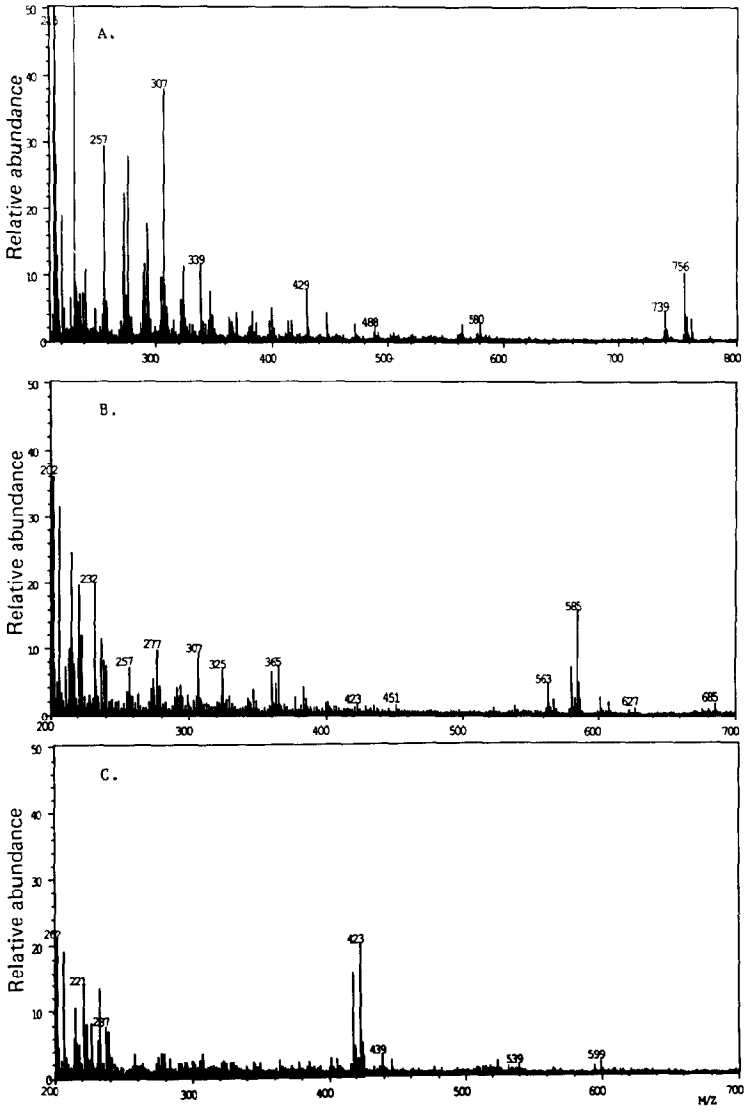


Fig. 2. F.a.b.–m.s. spectra of the P2-2 oligosaccharides from strain CE3 (A), CE109 (B), and CE309 (C).

Composition analysis of fraction P2-3 from CE3 indicates that it is composed of only galacturonic acid and Kdo. N.m.r. analysis, Fig. 5A and Table III, shows that there are two anomeric protons and two Kdo methylene protons in the ratio of 1:1:1:1. The n.m.r. assignments given in Table III are based on those for the trisaccharide core-component from bv. *trifolii* ANU843 (ref. 20). These results indicate that P2-3 is a trisaccharide consisting of two galactosyluronic and one Kdo residues. This is supported by f.a.b.–m.s. data, which give M + H^+ and M + Na^+ ions of 613 and 635, respectively. The Kdo residue is at the reducing end of this

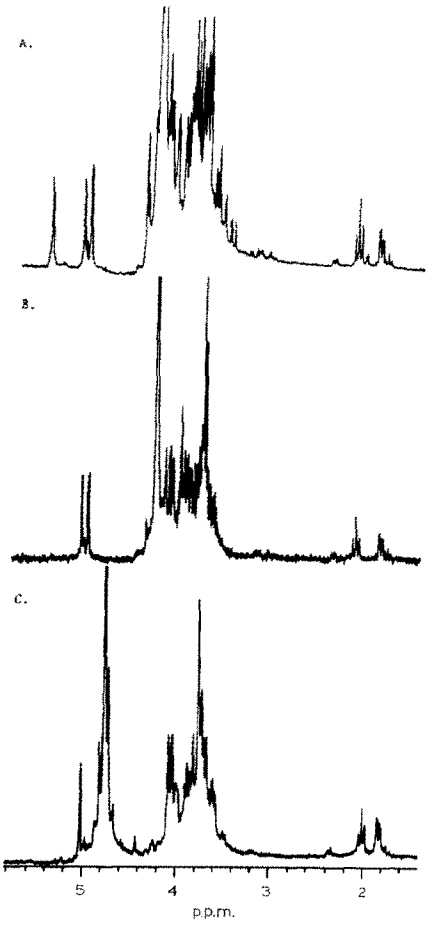


Fig. 3. N.m.r. spectra of the P2-2 fractions from (A) CE3, (B) CE109, and (C) CE309 LPSs. The spectra of the CE3 and CE109 were taken at 353K and that of CE309 at 298K. All spectra were obtained with a Nicolet T-360 instrument.

trisaccharide and is resistant to the thiobarbituric acid assay, as already described for the trisaccharide. These results are identical to those obtained for the trisaccharide core from bv. *trifolii* ANU843 (ref. 20). Thus, it can be concluded that the structure of CE3 P2-3, shown in Fig. 4B, is the same as that of the ANU843 trisaccharide core and consists of two galactosyluronic residues α-linked to the 4 and 7 position of Kdo.

The structures of the core oligosaccharides from the mutants CE109 and CE309. — The P2-2 fraction from CE109 consists of galactose, mannose, and Kdo (Table I) in the ratio of 1:1:0.6. Again, the level of Kdo given in Table I is low because of degradation of this residue during the assay procedure. The composition results are confirmed by n.m.r. analysis (Table II). Integration of the n.m.r.

TABLE II

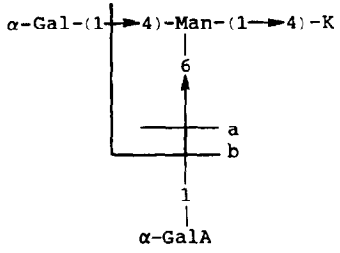
CHEMICAL SHIFTS[a] FOR THE P2-2 CORE OLIGOSACCHARIDES FROM BV. *phaseoli* CE3, CE109, AND CE309 LPSs

Strain	*Glycosyl residue*	*Proton*	*Chemical shift* (J)
CE3	Galacturonate	H-1	5.32 (3.6)
	Mannose	H-1	4.98 (2.0)
	Galactose	H-1	4.90 (4.1)
	Kdo	H-3*a*	2.06 (12.6)
		H-3*e*	1.81 (12.6, 4.5)
CE109	Mannose	H-1	4.98 (1.6)
	Galactose	H-1	4.92 (4.0)
	Kdo	H-3*a*	2.10 (12.6)
		H-3*e*	1.80 (12.6, 4.5)
CE309	Mannose	H-1	5.02 (<1.0)
	Kdo	H-3*a*	2.01 (12.4)
		H-3*e*	1.83 (12.2, 4.3)

[a]The spectra were recorded with a Nicolet T-360 instrument at 353K. For these spectra the chemical shifts are given relative to HOD at 4.25 p.p.m. (determined relative to TSP).

spectrum of CE109 P2-2 shows that the ratio of Gal-H-1/Man-H-1/Kdo-H-3*a*/Kdo-H-3*e* is 1:1:1:1. Furthermore, the n.m.r. spectrum also verifies that the galactosyluronic residue is missing, in that the resonance at 5.32 p.p.m. is absent. F.a.b.–m.s. analysis of CE109 P2-2 gives quasi molecular ions of $M + H^+ = 563$, $M + NH_4^+ = 580$, and $M + Na^+ = 585$ (Fig. 2B), which are consistant with a Gal/Man/Kdo

A.



B.

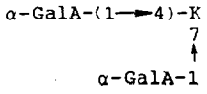


Fig. 4. Structures of the P2-2 and P2-3 core oligosaccharides from bv. *phaseoli* CE3, CE109 and CE309. A. The structures of the P2-2 oligosaccharides. Case "a" indicates that the galactosyluronic residue is missing in CE109 P2-2, while case "b" indicates that both the galactosyluronic and galactosyl residues are missing in CE309 P2-2. B. The structure of the P2-3 trisaccharide from CE3 and CE309.

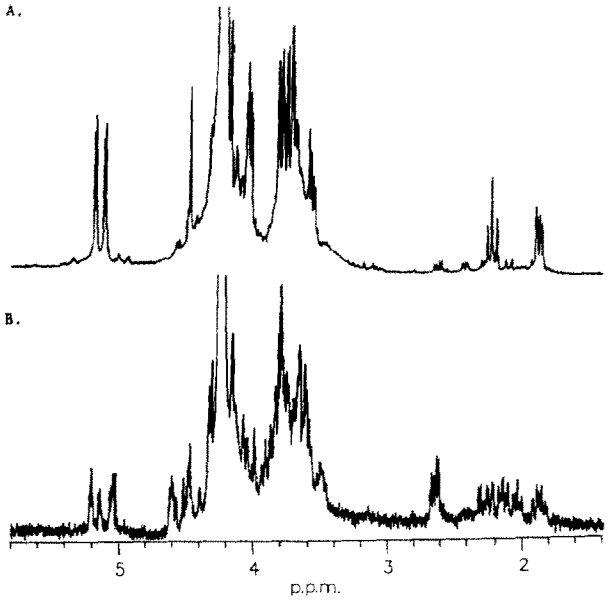


Fig. 5. The n.m.r. spectra of the P2-3 oligosaccharides from (A) CE3 and (B) CE109. The spectra were obtained with a Nicolet T-360 instrument at 353K.

trisaccharide. From these data, and by comparison with the structure of CE3 P2-2, we conclude that CE109 P2-2 is a trisaccharide consisting of a mannosyl residue α-linked to O-4 of Kdo with a galactosyl residue α-linked to O-6 of mannose, Fig. 4A case "a".

Analysis of CE309 P2-2 shows that it consists of only mannose and Kdo in 1:0.5 ratio (Table I). The level of Kdo is low for the reasons already described. The composition results are confirmed by n.m.r. analysis, Table II and Fig. 3C. Integration of the mannosyl H-1 and the Kdo H-3 protons show that they are in the ratio

TABLE III

CHEMICAL SHIFTS[a] FOR THE P2-3 CORE OLIGOSACCHARIDES FROM BV. *phaseoli* CE3 AND CE309 LPSs

Strain	*Glycosyl residue*	*Proton*	*Chemical shift* (J)
CE3	Galacturonate	H-1	5.14 (3.8)
	Galacturonate	H-1	5.06 (3.5)
	Kdo	H-3*a*	2.11 (12.6)
		H-3*e*	1.88 (12.6, 4.4)
CE309	Galacturonate	H-1	5.14 (3.7)
	Galacturonate	H-1	5.06 (3.4)
	Kdo	H-3*a*	2.11 (12.6)
		H-3*e*	1.88 (12.8, 4.4)

[a]The spectra were recorded with a Nicolet T-360 instrument at 353K. For these spectra, the chemical shifts are given relative to HOD at 4.25 p.p.m. (determined relative to TSP).

of ~1:1:1, indicating a Man/Kdo disaccharide. N.m.r. analysis also shows one H-1 resonance at 5.02 p.p.m. with a coupling constant of <1.0 Hz, attributable to the α-linked mannosyl residue. The H-1 resonances for galactosyluronic and galactosyl residues are absent, as expected. The H-3*e* and H-3*a* resonances of Kdo occur at 1.83 and 2.01 p.p.m., respectively. F.a.b.–m.s. analysis shows quasi-molecular ions of $M + NH_4^+ = 418$ and $M + Na^+ = 423$, Fig. 2C, confirming a Man/Kdo disaccharide. From these results, and by comparison with CE3 P2-2 and CE109 P2-2, we conclude that the CE309 P2-2 core is disaccharide consisting of a mannosyl residue α-linked to O-4 of Kdo, Fig. 2A case "b".

The composition of CE309 P2-3 is identical to that of the parent P2-3 and consists of GalA/Kdo = 2:1, indicating that it is also a trisaccharide. Its n.m.r. spectrum (not shown) is identical to that of CE3 P2-3, Fig. 5A. The chemical shifts and coupling constants of the anomeric protons and Kdo methylene protons are given in Table III and are, within error, the same as those for CE3 P2-3. We conclude from these results that CE309 P2-3 is a trisaccharide with the same structure as that of the parent molecule, shown in Fig. 4B.

The CE109 P2-3 core oligosaccharide gives a very complex n.m.r. spectrum (Fig. 5B) in comparison with the spectrum for the CE3 P2-3 trisaccharide. We have not yet determined the structure of this component, but it is obviously different from that of the CE309 and CE3 P2-3 oligosaccharides. It consists of galacturonic acid and Kdo in ~1:1 ratio and does not have neutral sugars. Its structure is under further investigation.

Summary. — Two core oligosaccharide components are found in bv. *phaseoli* CE3 LPS. These components are altered in mutants CE309 and CE109. The CE109 P2-2 oligosaccharide is missing the galactosyluronic residue and also has an altered P2-3 oligosaccharide, while the CE309 P2-2 fraction lacks both the galactosyl and galactosyluronic residues and has a normal P2-3 structure. The fact that the core oligosaccharides of these mutants are altered undoubtedly explains why the LPSs from these strains lack the O-chain; the O-chain cannot be added to the defective core. This result also raises the possibility that the O-chain is dispensable in eliciting proper nodule development, but intact core is essential. An investigation of mutants having an intact core but lacking the O-chain is needed to test this possibility.

It has been demonstrated that many *R. leguminosarum* bv. *phaseoli* strains are closely related to bv. *trifolii* and bv. *viceae* strains. This close relationship is reflected in the similarity of their LPS core structures. The P2-3 trisaccharide core from CE3 is identical in structure to the P2-3 components from bv. *trifolii* and bv. *viceae* LPSs (ref. 20, Carlson unpublished data). The P2-2 tetrasaccharide core from CE3 is identical to P2-2 from bv. *trifolii* ANU843 except that it lacks an acetyl group at O-4 of galactose. Present evidence also indicates that the P2-2 tetrasaccharide from a strain of bv. *viceae* is identical in structure to that from CE3 (Carlson, unpublished data).

ACKNOWLEDGMENT

This work was supported by grants from NSF (DCB8406697) and NIH (RO1 GM39583-02) to RWC and from the USDA (87-CRCR-1-2409) to KDN. This work was also supported in part by Department of Energy Grant DE-FG09-87ER13810 as part of the USDA/DOE/NSF Plant Science Centers program. We thank Debra Turowski of Marquette University for growing large batches of bacteria and preparing the LPS extracts.

REFERENCES

1 G. KATO, Y. MARUYAMA, AND M. NAKAMURA, *Agric. Biol. Chem. (Tokyo)*, 44 (1980) 2843–2855.
2 G. KATO, Y. MARUYAMA, AND M. NAKAMURA, *Agric. Biol. Chem. (Tokyo)*, 44 (1979) 1085–1092.
3 W. KAMBURGER, *Arch. Microbiol.*, 121 (1979) 83–90.
4 E. M. HRABAK, M. R. URBANO, AND F. B. DAZZO, *J. Bacteriol.*, 148 (1981) 691–711.
5 S. G. PUEPPKE, *Plant Physiol.*, 75 (1984) 924–928.
6 I. J. LAW AND B. W. STRIJDOM, *Soil. Biol. Biochem.*, 9 (1977) 79–84.
7 S. G. PUEPPKE, T. G. FRUEND, B. C. SCHULTZ, AND H. P. FREEDMAN, *Can. J. Microbiol.*, 26 (1980) 1489–1497.
8 P. P. WONG, *Plant Physiol.*, 65 (1980) 1049–1052.
9 G. C. ANOLLES AND G. FAVELUKES, *Appl. Environ. Microbiol.*, 52 (1986) 377–382.
10 A. LAGARES AND G. FAVELUKES, *Nitrogen Fixation: Hundred Years After, Proceedings of the 7th International Congress on N=Nitrogen Fixation*, Gusav Fischer, New York, 1988, pp. 477.
11 K. D. NOEL, K. A. VANDENBOSCH, AND B. KULPACA, *J. Bacteriol.*, 168 (1986) 1392–1401.
12 R. W. CARLSON, S. KALEMBASA, D. TUROWSKI, AND K. D. NOEL, *J. Bacteriol.*, 169 (1987) 4923–4928.
13 V. PUVANESARAJAH, F. M. SCHELL, D. GERHOLD, AND G. STACEY, *J. Bacteriol.*, 169 (1987) 137–142.
14 N. J. BREWIN, E. A. WOOD, A. P. LARKINS, G. GALFRE, AND G. W. BUTCHER, *J. Gen. Microbiol.*, 132 (1986) 1959–1968.
15 N. J. BREWIN, D. J. BRADLEY, E. A. WOOD, S. PEROTTO, K. A. VANDENBOSCH, AND G. J. BUTCHER, *Int. Congr. Nitrogen Fixation, 7th, Univ. Cologne*, March 1988.
16 R. W. CARLSON, R. E. SANDERS, C. NAPOLI, AND P. ALBERSHEIM, *Plant Physiol.*, 62 (1978) 912–917.
17 L. P. T. M. ZEVENHUIZEN, I. SCHOLTEN-KOERSELMAN, AND M. A. POSTHUMUS, *Arch. Microbiol.*, 125 (1980) 1–8.
18 R. W. CARLSON, *J. Bacteriol.*, 158 (1984) 1012–1017.
19 R. W. CARLSON, R. SHATTERS, J. DUH, E. TURNBULL, B. HANLEY, B. G. ROLFE, AND M. A. DJORDJEVIC, *Plant Physiol.*, 84 (1987) 421–427.
20 R. W. CARLSON, R. L. HOLLINGSWORTH, AND F. B. DAZZO, *Carbohydr. Res.*, 176 (1988) 127–135.
21 P. ALBERSHEIM, D. J. NEVINS, P. D. ENGLISH, AND A. KARR, *Carbohydr. Res.*, 5 (1967) 340–345.
22 W. F. DUDMAN, L.-E. FRANZEN, J. E. DARVILL, M. MCNEIL, A. G. DARVILL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 117 (1983) 141–156.
23 N. J. BLUMENKRANTZ AND B. ASBOE-HANSEN, *Anal. Biochem.*, 54 (1973) 484–489.
24 A. WIESSBACH AND J. HURWITZ, *J. Biol. Chem.*, 234 (1958) 705–709.
25 J. R. CAVA, P. M. ELIAS, D. A. TUROWSKI, AND K. D. NOEL, *J. Bacteriol.*, 171 (1989) 8–15.
26 R. L. HOLLINGSWORTH, R. W. CARLSON, F. GARCIA, AND D. A. GAGE, *J. Biol. Chem.*, 264 (1989) 9294–9299.
27 V. L. CROW, B. D. W. JARVIS, AND R. N. GREENWOOD, *Int. J. Sys. Bacteriol.*, 31 (1981) 152–172.