JOURNAL OF BACTERIOLOGY, Sept. 1996, p. 5529–5532 Vol. 178, No. 18
0021-9193/96/$04.00+0

NOTES

The "Missing" Typical *Rhizobium leguminosarum* O Antigen Is Attached to a Fatty Acylated Glycerol in *R. leguminosarum* bv. trifolii 4S, a Strain That Also Lacks the Usual Tetrasaccharide "Core" Component

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Received 2 May 1996/Accepted 14 July 1996

Rhizobium leguminosarum **bv. trifolii 4S has a lipopolysaccharide O antigen that lacks galactose and many of the typical glycosyl components found in related strains. Here, we show that it also lacks the typical core tetrasaccharide but synthesizes an alternative glycolipid that contains galactose and the typical O-antigen glycosyl components, suggesting that in this strain, the O antigen is transferred to an alternative lipid acceptor.**

The structural and genetic interrelations among the various rhizobial carbohydrate-containing cell surface molecules are not at all well understood. For instance, there are structural (and genetic) links between lipopolysaccharides (LPSs) and chitolipooligosaccharides (10). The same is true for extracellular polysaccharide (EPS) and LPS structures (2, 3, 23, 26, 29, 33). The EPS and LPS structures of *Rhizobium leguminosarum* bv. trifolii 4S have been particularly well characterized and are quite distinct from those of other members of the species. This strain produces an EPS-capsular polysaccharide with a heptasaccharide repeat unit that lacks the typical terminal galactose with the 3-hydroxybutyryl and carboxyethylidene groups (1, 27) found in related strains (12, 13, 17, 18, 24, 25, 28). If the strain is impaired for the synthesis or utilization of galactose, some very important consequences would result for LPS synthesis, since both the core and O-antigen components of these LPSs typically contain galactose (5, 8, 15, 16, 22, 34). It is known that the O antigen of this strain also lacks galactose (30). It also lacks the typical glycosyl components found in the O antigens of related strains. These components include methyl deoxyhexoses, deoxyhexoses, mannose, galactose, glucose, 2-amino-2,6-dideoxy glucose (quinovosamine), uronic acids, heptose, and 2-keto-3-deoxyoctulosonic acid (KDO) (5, 8, 22, 34).

There are two core oligosaccharides in wild-type *R. leguminosarum* bv. trifolii LPS. One is a trisaccharide that contains two molecules of galacturonic acid linked to KDO (7), and the other is a tetrasaccharide in which galactose, mannose, and galacturonic acid are linked to KDO (15, 16). Bacteria that are impaired in the synthesis of this galactose-containing tetrasaccharide seem to be unable to attach an O antigen and, as a consequence, have a rough phenotype (6, 21, 35). The tetrasaccharide, therefore, appears to be the point at which the O antigen is attached to the LPS. The lack of galactose in both the EPS and O antigen indicated that this strain may be an example in which an effective strain did not contain the galactose-containing tetrasaccharide and that an alternative compatible core and O antigen had evolved. A closer look at the cell surface chemistry of this organism was therefore taken.

The LPS of *R. leguminosarum* bv. trifolii 4S was isolated and hydrolyzed, and the lipids were extracted with chloroform (19, 30). The aqueous layer was subjected to Biogel P2 chromatography (35). Three peaks were obtained, with the first corresponding to the O antigen and the third to the typical trisaccharide core component. The second peak, which usually corresponds to the galactose-containing tetrasaccharide, was found by gas chromatography-mass spectrometry (GC-MS) to be devoid of galactose. It was divided into four consecutive fractions which were analyzed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy, and none contained signals for the tetrasaccharide. The spectrum of one subfraction (Fig. 1) contained signals that corresponded to a disaccharide that was composed of mannose and KDO and that was identified in a mutant that was incapable of synthesizing the tetrasaccha-

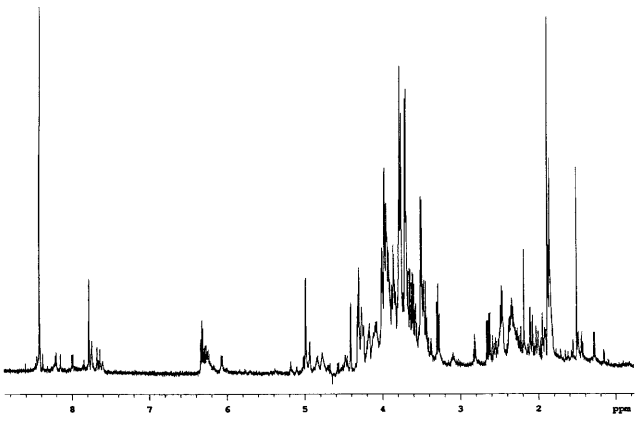

FIG. 1. The ^{1}H NMR spectrum for peak 2 subfraction D. Note the mannose anomeric proton at ~5.0 ppm. Spectra were recorded in D_2O at 25°C.

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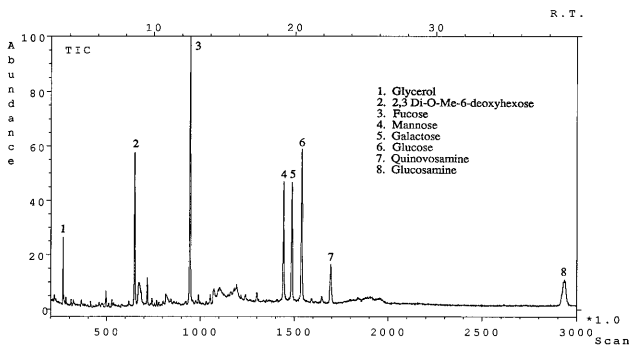

FIG. 2. Total ion chromatogram (TIC) of alditol acetates of the *R. leguminosarum* bv. trifolii 4S extract eluted from a C_{18} column with 100% acetonitrile. The identities of the major peaks were determined by coelution with standards and analysis of mass spectra. R.T., retention time (in minutes).

ride (35). This disaccharide was readily identifiable by the anomeric proton at 5.0 ppm and the other carbohydrate signals. The other signals in the spectra were attributable to 2-deoxy ribose, probably from contaminating DNA. The spectra did contain low-intensity signals, which indicated that another oligosaccharide (but not the tetrasaccharide) might be present.

A more detailed analysis was performed with a membrane extract. *R. leguminosarum* bv. trifolii 4S cells were extracted with 200 ml of chloroform–1-propanol–methanol–water (1:2:2:3) by vigorous stirring for 24 h at 37°C. The membrane extract was dried by flash evaporation, redissolved in 20% acetonitrile, and loaded onto a LiChroprep RP-18 column (8 by 2 cm; EM Separations, Gibbstown, N.J.). Successive elutions were made with 30-ml portions of 20, 50, and 100% acetonitrile in water. The 20% acetonitrile fraction, after being converted to alditol acetates and subjected to GC and GC-MS analyses (30), was found to be composed exclusively of glucose. The proton NMR spectrum indicated that it was a cyclic-β-1,2-glucan (11). The 50 and 100% acetonitrile fractions appeared very similar to each other by NMR spectroscopy. Spectra of the aqueous fraction, from the same mild acid hydrolysis as that for the LPS, contained resonances that indicated the presence of methoxy, deoxy, and amino sugars. These resonances were, specifically, sharp singlets between 3.2 and 3.4 ppm, which indicated relatively large amounts of O methylation, while the signal at ~2.0 ppm indicated the presence of an *N*-acetyl group. Additionally, signals at ~2.6 ppm indicated the presence of KDO. There were also signals between 0.9 and 2 ppm for fatty acid groups. The carbohydrate composition was determined definitively by GC-MS (30). The compositions of the two fractions were virtually identical, and the total ion chromatogram of the last eluting fraction is shown in Fig. 2. The major components were glycerol, 2,3-di-*O*-methyl-6-deoxyhexose, fucose, mannose, galactose, glucose, quinovosamine, and glucosamine. The thiobarbituric acid assay (31) also indicated the presence of KDO in these samples. These carbohydrates are the typical glycosyl components of rhizobial LPS O antigens and have been described in the various *R. leguminosarum* biovars that have been analyzed over the years. The data in Table 1 summarize some of these publications. Most of these glycosyl components are missing in the LPS of strain 4S (30). The predominant fatty acids were typical phospholipid fatty acids, namely stearic and oleic acids (Fig. 3). However, other minor fatty acids which are characteristic of LPSs, such as the 3-hydroxy fatty acids and 27-hydroxyoctacosanoic acid (4, 14, 19), were also present. The possibility that a typical lipid A was the anchor was easily ruled out. First, there was the relatively low abundance of hydroxy fatty acids compared with the abundance of the other fatty acids. Second, GC and GC-MS analyses clearly indicated that galacturonic acid was absent from the samples. Galacturonic acid is a component of lipid A (20). The small amount of hydroxy fatty acids might indicate the presence of a contaminating alternative lipid A. Thin-layer chromatography (9) ruled out the presence of phospholipids.

Since glycerol could be freed by acid hydrolysis, it follows that it had to be linked glycosidically to the reducing terminus of the oligosaccharide chain, where it could serve as a membrane anchor through acylation by fatty acids. This configuration is the usual arrangement of glycerol-containing glycolipids. A structure that is consistent with the data is shown in Fig. 4. Such a structure could arise by the simple transfer of the

TABLE 1. LPS carbohydrate compositions of various *R. leguminosarum* bv. trifolii strains

Component	LPS (% dry wt)[a]						Total carbohydrate (%) for new strain 4S lipid[b]
	0403	Ar-3	Coryn KL	K-8	162S7	2S	
3-*O*-methylhexose	nr	nr	nr	nr	0	1.1	0
2-*O*-methyl-6-deoxyhexose	8	1.4	2.1	6.4	4.5	0	0
3-*O*-methyl-6-deoxyhexose	nr	nr	nr	nr	0	3.6	0
2,3-di-*O*-methyl-6-deoxyhexose	nr	nr	nr	nr	nr	nr	13
3,4-di-*O*-methyl-6-deoxyhexose	nr	nr	nr	nr	0.7	0	0
3-*N*-methyl-3-amino-3,6-dideoxyhexose	15	nr	nr	nr	0	0	0
Rhamnose	4	40.3	0.2	1.9	0.6	7.5	0
Fucose	13	20.2	0.8	7.2	4.0	7.5	28
Mannose	7	0.9	7.9	3.2	1.9	1.7	11
Galactose	tr	0	4.9	2.8	1.1	2.2	12
Glucose	4	26.6	28.4	7.1	0.9	1.0	17
Heptose	21	0	1.0	0	0	0	0
Hexosamines	nr	0.8	2.1	1.2	1.5	1.6	5
Deoxyhexosamines	nr	nr	nr	nr	nr	nr	4
Uronic acids	27	2.2	11.7	5.3	2.2	7.0	0
2-Keto-3-deoxy-octulosonic acid	nr	1.3	2.9	4.1	7.2	2.7	+

[a] Data for strain 0403 are taken from reference 5, data for strains Ar-3, Coryn KL, and K-8 are taken from reference 34, and data for strains 162S7 and 2S are taken from reference 8. nr, none reported; tr, trace.

[b] Results from this study. +, detected but not quantitated.

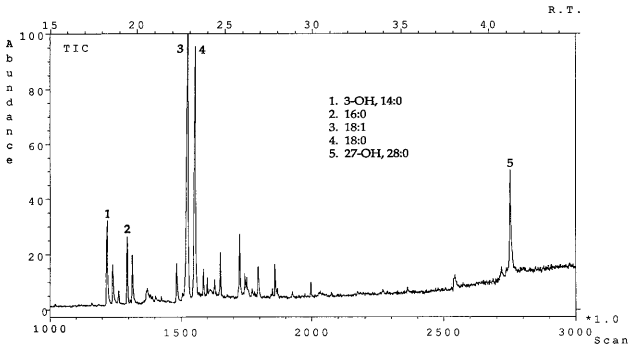

FIG. 3. Total ion chromatogram (TIC) of fatty acid methyl esters. The identities of the major peaks were determined by coelution with standards as well as analysis of mass spectra. R.T., retention time (in minutes).

activated LPS O-antigen repeat from the carrier lipid to diacylglycerol, as indicated, in the absence of the usual acceptor core oligosaccharide. It is quite possible that a fair amount of this hybridization of molecular structure occurs in normal strains, even in capsule formation. In the event that the carbohydrate chain in the glycolipid becomes further polymerized by the addition of more O-antigen chains, the resulting polymer would have the usual carbohydrate composition of LPSs (including the presence of KDO) as well as some of the characteristic fatty acids. Such a hybrid LPS could restore the proper functionality of LPS-EPS-defective strains. The LPS-like molecule whose production is controlled by the *lpsZ* gene

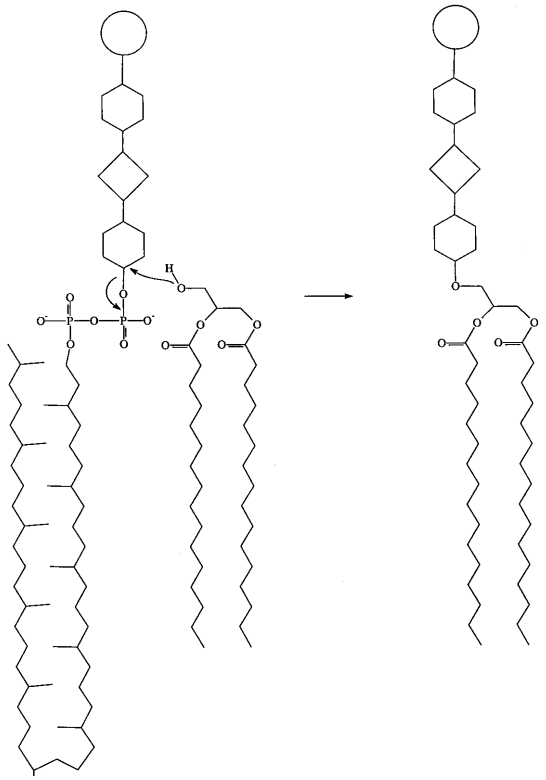

FIG. 4. Possible mechanism of transfer of the LPS O-antigen repeat unit from the carrier lipid to diacylglycerol. The product is a chimeric lipid. The O-antigen repeat might be polymerized by successive transfers to a hydroxyl group on the end sugar residue.

in *Rhizobium meliloti* (32, 33) fits the profile of such a hybrid molecule. The discovery of this class of LPS-like, lipid-linked oligosaccharides in *R. leguminosarum* bv. trifolii 4S is important, because it brings this strain in line with others. The results also demonstrate that the synthesis of a core tetrasaccharide is not a prerequisite for a strain to be effective in nodulation, contrary to earlier observations (6, 35).

This work was supported by grant DE-FG02-89ER 14029 from the U.S. Department of Energy to R.I.H. The GC-MS data were obtained at the Michigan State University Mass Spectrometry Facility, which is supported in part by grant DRR-00480 from the Biotechnology Research Technology Program, National Center for Research Resources, NIH. The NMR data were obtained on instrumentation that was purchased in part with funds from NIH grant 1-S10-RR04750, NSF grant CHE-88-00770, and NSF grant CHE-92-13241.

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