Isolation of Monoclonal Antibodies Reacting with the Core Component of Lipopolysaccharide from *Rhizobium leguminosarum* Strain 3841 and Mutant Derivatives

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Monoclonal antibodies reacting with the core oligosaccharide or lipid A component of *Rhizobium* lipopolysaccharide (LPS) could be useful for the elucidation of the structure and biosynthesis of this group of macromolecules. Mutant derivatives of *Rhizobium leguminosarum* 3841 with LPS structures lacking the major O-antigen moiety were used as immunogens, and eight antibodies were selected for further study. All the antibodies reacted with the fast-migrating species known as LPS-2 following gel electrophoresis of *Rhizobium* cell extracts. For four of these antibodies, reactivity with affinity-purified LPS was lost after mild acid hydrolysis, indicating that they probably recognized the core oligosaccharide component. The four other antibodies still reacted with acid-treated LPS and may recognize the lipid A moiety, which is stable to mild acid hydrolysis. The pattern of antibody staining after gel electrophoresis revealed differences in LPS-2 epitope structure between each of the mutants and the wild type. Furthermore, for each of the mutants the antibodies cross-reacted with a minor band that migrated more slowly than LPS-2; we have termed this more slowly migrating form LPS-3. The majority of the antibodies also reacted with LPS from strain CE109, a derivative of *Rhizobium etli* CE3, confirming that the LPS core antigens can be relatively conserved between strains of different *Rhizobium* species. One of the antibodies isolated in this study (JIM 32) was unusual because it appeared to react with all forms of LPS from strain 3841 (namely, LPS-1, LPS-2, and LPS-3). Furthermore, JIM 32 reacted positively with the LPS from many strains of *Rhizobium* tested (excluding the *Rhizobium meliloti* subgroup). JIM 32 did not react with representative strains from *Bradyrhizobium*, *Azorhizobium* or other related bacterial species.

Soil bacteria belonging to three genera (*Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*) establish a symbiosis with legume hosts in a special morphologically defined organ called a root nodule (5, 6, 19, 24). As with all gram-negative bacteria, lipopolysaccharide (LPS) is the major component of the *Rhizobium* outer membrane, but its molecular structure and biological function have not been completely established because of the complexity and variability of these molecules (9, 13). It is well known that lipid A of gram-negative bacteria, or endotoxin, has numerous biological activities on animal cells (3). Although the *Rhizobium* lipid A may be slightly different in structure (2), it is possible that this component of LPS could also have functional roles in symbiosis and other forms of plant-microbe interaction. In order to investigate this possibility, the structure and biosynthesis of core oligosaccharide and lipid A need to be better understood. In the present report, we describe the isolation of monoclonal antibodies reacting with these components; these antibodies can be used as analytical probes to examine structural differences among LPS mole-

cules, both those derived from wild-type strains and those from LPS-defective mutant derivatives.

Evidence for the importance of LPS in plant-microbe interactions comes from several sources. Variations in the structure and composition of LPS have been reported for free-living rhizobia cultured at different oxygen levels, pHs, or ion and salt compositions, with different carbon sources, or with host root extracts (1, 23, 26, 30, 40, 42, 43). Differences between the LPSs from bacteria and bacteroids have also been observed (7, 25, 42). Furthermore, *Rhizobium* mutant derivatives with major defects in the carbohydrate components of LPS cannot establish a normal symbiosis in pea or other legume host plants: either growth of the infection thread is arrested (35) or the infection process and bacteroid development are profoundly altered (17, 26, 36, 38). Recent cytological evidence has shown that a host plant defense response is mounted in peas by LPS-defective mutants of *Rhizobium leguminosarum* (36).

On the basis of the electrophoretic mobility of LPS on polyacrylamide gels, two different classes (LPS-1 and LPS-2) have been defined (11). LPS-1 macromolecules are formed from three components, i.e., lipid A, core oligosaccharide, and O antigen, whereas LPS-2 lacks the O antigen. Lipid A is normally anchored in the outer side of the outer membrane. In *R. leguminosarum*, the carbohydrate moiety of lipid A is composed of galacturonic acid, glucosamine, and amino-deoxygluconic acid (2, 3). These molecules are acylated with diverse hydroxy fatty acids of variable lengths (C_{14} to C_{18}). There is also a unique 27-hydroxy-octacosanoic acid (27-OH $C_{28:0}$) which could span the whole lipid bilayer (3). The core oligo-

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TABLE 1. *Rhizobium* strains

Strain	Derivation	Nodulation phenotype ^a	Reference
<i>R. leguminosarum</i>			
bv. <i>viciae</i>			
3841	300 <i>str</i>	Fix ⁺	50
B659	3841 <i>lps::Tn5</i>	Inv ⁻	39
B660	3841 <i>lps::Tn5</i>	Fix ⁻	36
B633	B574 <i>lps</i> (NTG)	Fix ⁻	26
B655	3841 <i>lps::Tn5</i>	Fix ⁻	26
B656	3841 <i>lps::Tn5</i>	Fix ⁻	26
B661	3841 <i>lps::Tn5</i>	Fix ⁻	26
B778	B659 Rev-1	Fix ⁺	This work
B779	B659 Rev-2	Fix ⁺	This work
<i>R. etli</i>			
CE3	CNF42 Str ^r	Fix ⁺	35
CE109	CE3 <i>lps::Tn5</i>	Fix ⁻	35

^a Fix⁺, pink nodules that reduce acetylene; Fix⁻, white nodules that do not reduce (or reduce very little) acetylene; Inv⁻, empty (uninvaded), small white nodules with no signs of cell or tissue invasion.

saccharide component is structurally conserved among strains and is built from a trisaccharide and a tetrasaccharide, with galacturonic acid, mannose, galactose, and 3-deoxy-D-manno-octulonic acid constituents (10). The O-antigen region is composed of a strain-specific oligosaccharide repeating unit which projects beyond the outer membrane and is the most antigenic component of the cell wall (9).

Three different experimental approaches have been used to define the structural relationships of LPS macromolecules: biochemical analysis of fractions from wild-type strains, comparative analysis of LPSs from mutant strains, and immunochemical analysis with monoclonal antibodies as submolecular probes. The O-antigen component of the LPS from *R. leguminosarum* 3841 has been extensively studied by genetic analysis and by using monoclonal antibodies (26), but there is no information about the core and lipid A regions for this strain. In this paper, we describe the use of LPS-defective mutants that lack the major O-antigen component in order to isolate a new series of monoclonal antibodies reacting preferentially with the LPS-2 class of molecules. The majority of these antibodies seem to react with the core oligosaccharide, while others may recognize a component of the lipid A moiety of LPS. These antibodies have proved to be useful in comparing the LPS structures of mutant strains and have allowed the identification of a new form of LPS macromolecule which is immunologically related to LPS-2 but has a molecular weight more similar to that of LPS-1. One of the monoclonal antibodies reacts with an epitope that is common to both LPS-1 and LPS-2 from all strains of *R. leguminosarum* that were examined. This antibody may also be useful as a taxonomic or diagnostic probe for the LPSs of strains belonging to certain species of *Rhizobium*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used are listed in Table 1. Bacteria were normally grown for 2 to 4 days on agar medium containing either tryptone-yeast extract or yeast-mannitol medium (46), depending on the strain. *Azorhizobium caulinodans* was grown on a complex medium described by Dreyfus et al. (20), and *Agrobacterium tumefaciens* was grown on Lennox medium (28). For detailed biochemical analysis of LPSs from *R. leguminosarum* 3841 and its mutant derivatives, the bacteria were grown on complete tryptone-yeast extract medium as described previously (42). Strains B655, B656, B660, and B661 induce the process of nodulation on peas. However, strain B660 formed relatively few nodules, while the other strains formed a higher-than-normal number of root nodules on host plants. The nodules formed were altered,

and only a few host cells were colonized by bacteria (36). Strain B659 has a rough colony morphology and induces the formation of very small white nodule-like structures that do not show signs of intracellular or intercellular infection thread development (39). Strains B778 and B779 are revertants of mutant B659, isolated from pink nodules that arose occasionally when inoculated pea seedlings were cultivated at elevated temperatures (constant 20°C). These B659 derivatives had wild-type colony morphology and, when retested on pea seedlings, were able to induce pink nitrogen-fixing nodules.

Monoclonal antibodies. The antibodies used are described in Table 2. AFRC MAC 57 reacts with a constitutive epitope expressed on the LPS-1 component of *R. leguminosarum* 3841 (7, 25). MASM-1 is a 3-deoxy-D-manno-octulosonic acid (Kdo)-specific monoclonal antibody recognizing *Rhizobium* LPS-2 (29, 50). JIM 32-39 were obtained in three different fusion experiments by the method described by Bradley et al. (4). A mixture of mutant strains with different alterations in their LPSs was used as an immunogen: B633, B655, B656, and B659 (Table 1). More than 50 rat hybridoma cell lines which produce monoclonal antibodies reacting with LPS from bacteria listed in Table 1 were isolated. By using strain 3841 and mutant derivatives, the antibodies were screened by dot immunoassay (50) with goat anti-rat immunoglobulin G (IgG) conjugated to horseradish peroxidase.

Antigen isolation and characterization. LPS was isolated by a modification of the method described by Wood et al. (50). Cells of strains B660 or B659 (approximately 10¹⁰ cells per ml of water) were broken by sonication. Cell debris was removed by centrifugation, and the released LPS was bound overnight to a lipid A affinity column of matrix-bound polymyxin B (1 ml of Detoxi-Gel [Pierce] [34]). The column was washed with 50 ml of water to remove unbound LPS, and the bound LPS was released with 10 ml of an aqueous solution of deoxycholate (1% [wt/vol]) or a solution of deoxycholate (0.5% [wt/vol]) and salt (0.5 M). The presence of LPS in fractions of the eluate was probed for in dot immunoassays. The LPS-containing fractions were pooled and concentrated by lyophilization. A fraction of the LPS was subjected to mild acid hydrolysis by boiling for 1 h at 100°C in 1% (vol/vol) aqueous acetic acid. The hydrolyzed LPS was dried down and redissolved in chloroform-isopropanol-water (10:10:1).

Characterization of epitopes. The sensitivity of the LPS epitopes to periodate oxidation and protease treatment was tested as described previously (37, 42). Sugar inhibition studies were performed by the standard dot immunoassay protocol but with supplementary sugars included in the incubation buffer to test for disruption of the antigen-antibody binding reaction: D-galacturonic acid (GalA), D-(+)-galactose (Gal), D-(+)-mannose (Man), D-(+)-glucosamine (GlcN), and 2-amino-2-deoxy-D-gluconic acid (GlcAN), all at 100 mM and pH 7.7, and Kdo at 14 mM. Purified LPSs from strains B660 and B659 were applied to nitrocellulose sheets as aliquots of 1 or 0.5 µg. Before use, each of the monoclonal antibodies was preincubated at room temperature in the respective sugar solution at pH 7.7 for 30 min. As a positive control for inhibition, the characterized antibody MASM-1, specific for Kdo (29), was included.

Taurodeoxycholate-polyacrylamide gel electrophoresis and immunoblotting. Electrophoretic analysis of LPS was performed as described by Kannenberg et al. (26) on 15% (wt/vol) polyacrylamide gels with 0.4% (wt/vol) taurodeoxycholate as a detergent. Gels were loaded with approximately 10 µg of protein per lane. LPS bands on gels were visualized by a carbohydrate-specific silver staining procedure described by Wood et al. (50). LPS fractions separated on gels were electroblotted onto Immobilon polyvinylidene difluoride membranes (Millipore), using a Bio-Rad Transblot apparatus at 60 V for 3 h at 4°C; transfer buffer was 20 mM Tris-HCl containing 5% (vol/vol) methanol (pH 8.3). In order to compare the cross-reactivities of different monoclonal antibodies with LPS species from the same strain, samples were fractionated on polyacrylamide gels with only one continuous well across the slab. Blotted sheets were cut longitudinally into sections, and each one was tested by incubation with a different antibody solution. Following treatment with primary antibody, immunostaining was carried out with goat anti-rat IgG conjugated to alkaline phosphatase (Sigma, Poole,

TABLE 2. Monoclonal antibodies

Monoclonal antibody	Isotype	Antigen(s) recognized ^a	Reference(s)
AFRC MAC 57	Rat IgM	LPS-1	7
JIM 32	Rat IgG2b	LPS-1, LPS-2	This work
JIM 33	Rat IgG2a	LPS-2	This work
JIM 34	Rat IgM	LPS-2	This work
JIM 35	Rat IgM	LPS-2, lipid A	This work
JIM 36	Rat IgG2c	LPS-2 of B659	This work
JIM 37	Rat IgM	LPS-2	This work
JIM 38	Rat IgM	LPS-2, lipid A	This work
JIM 39	Rat IgG2c	LPS-2 of B659	This work
MASM-1	Mouse Ig	LPS-2	29, 50

^a From *R. leguminosarum* 3841 or derivatives.

FIG. 1. Comparison of the LPS profiles of *Rhizobium* wild-type strains with those of mutant derivatives, following polyacrylamide gel electrophoresis and staining with sodium metaperiodate and silver ions in order to visualize LPS components. Lanes 1 to 6, *R. leguminosarum* 3841 B659 (3841 *lps::Tn5*), B660 (3841 *lps::Tn5*), B661 (3841 *lps::Tn5*), B778 (B659 Rev-1), and B779 (B659 Rev-2), respectively; lanes 7 and 8, *R. etli* CE3 and CE109 (CE3 *lps::Tn5*), respectively.

United Kingdom). Goat anti-mouse IgG conjugated to alkaline phosphatase was used in conjunction with MASM-1.

Cross-reactivity of JIM 32 antibody with LPSs from a panel of strains. Cell suspensions of roughly 10^7 cells per ml were diluted 1:10, 1:100, and 1:1,000. Aliquots (1.5 μ l) were dotted onto nitrocellulose sheets, and the sheets were developed with JIM 32 antibody by a procedure described elsewhere (23).

RESULTS

Characterization of LPS-defective mutants by gel electrophoresis. In order to isolate monoclonal antibodies reacting with core LPS, it was necessary to exclude LPS-1 components from the immunogen. As a preliminary to immunizing rats and screening for monoclonal antibodies, several LPS-defective mutants of *R. leguminosarum* 3841 were characterized. Figure 1 shows the LPS profiles of strain 3841 and mutant derivatives after polyacrylamide gel electrophoresis. The LPS from strain 3841 (carrying wild-type LPS) was resolved into the fast-migrating band of LPS-2, which represents the incomplete form of LPS, and several bands of lower mobility that presumably are LPS-1.

The fast-migrating LPS-2 species was expressed by mutants B661, B660, and B659 (Fig. 1). As expected, these mutants apparently failed to produce significant amounts of high-molecular-weight forms of LPS-1, similar to those seen in the wild-type strain, 3841. (However, some new bands were revealed and were particularly obvious under the staining conditions used here. Samples of B659 and B660 yielded faintly staining bands that migrated slightly more slowly than the LPS-1 bands of 3841. Because these bands did not react with AFRC MAC 57 or with any of the other antibodies recognizing LPS-1, it is probable that they are not related to LPS, and they have not been studied further.) On the other hand, the sample of B661 yielded a broad band migrating just behind the LPS-2 component; this material reacted with AFRC MAC 57 and presumably represents a truncated form of LPS-1. The two revertants of B659 (B778 and B779) showed LPS profiles that were similar, but not identical, to that of the wild-type strain, 3841. Compared with that in 3841 and its mutants, a more complete loss of LPS-1 bands was observed in strain CE109 (Fig. 1), the LPS mutant derivative of *R. etli* CE3 (12, 35).

Characterization of antibodies and mutants by immunoblotting. After hybridoma cell supernatants were screened for reactivity with 10 LPS-defective mutant derivatives of strain 3841 (26), eight monoclonal antibodies (designated JIM 32 to JIM 39) were selected for further study on the basis of their differential staining patterns. Evidence that these antibodies

recognize cell surface antigens was obtained by using antibody-fluorescein isothiocyanate conjugates for immunofluorescence studies with intact cells (data not shown). Moreover, by dot immunoassay it was shown that antibody-antigen binding was resistant to predigestion with protease but was sensitive to periodate oxidation, indicating that carbohydrate components were involved.

Figure 2 shows the pattern of antibody staining on immunoblots following polyacrylamide gel electrophoresis. The staining patterns of the antibodies were compared with those for AFRC MAC 57 and MASM-1, which recognize LPS-1 and LPS-2, respectively (26). Six antibodies (JIM 32, JIM 33, JIM 34, JIM 35, JIM 37, and JIM 38) all reacted with fast-migrating bands from strains 3841, B660, and B661 (Fig. 2). These same bands also stained with MASM-1 and presumably correspond with LPS-2. JIM 36 and JIM 39 reacted with a component that comigrated with the LPS-2 component of strain B659 but not with LPS-2 of the wild-type, 3841, or of the other mutants tested. JIM 32 was unique in that this antibody seemed to recognize components comigrating with both LPS-1 and LPS-2 bands for all strains of *R. leguminosarum* tested (Fig. 3).

Reactivity with purified LPS. To confirm that the antibodies reacted with LPS-2, we isolated the LPSs from the strains B660 and B659. Table 3 shows that the same antibodies that reacted with the LPS-2 bands of 3841, B661, and B660 in immunoblots (Fig. 2) also reacted with purified LPS from B660, indicating that they indeed recognize LPS-2 of this strain. JIM 36 and JIM 39 did not recognize the LPS-2 from B660 but these antibodies recognized the LPS isolated from B659, further

FIG. 2. Immunoblots showing reactivities of monoclonal antibodies with LPS antigen bands derived from *R. leguminosarum* 3841, B659, B660, and B661, following polyacrylamide gel electrophoresis. Bacterial extracts were fractionated on a slot gel (with no individual wells) and transferred by electroblotting onto sheets of polyvinylidene difluoride membrane (Immobilon). Subsequently, the sheets were cut into strips, treated with individual monoclonal antibodies, and stained with anti-rat IgG-alkaline phosphatase conjugate. Lanes: 1, JIM 32; 2, AFRC MAC 57; 3, JIM 36; 4, JIM 33 and JIM 37; 5, JIM 34; 6, JIM 35 and JIM 38; 7, MASM-1. The positions of bands corresponding to LPS-1 and LPS-2 are indicated, as is a novel component termed LPS-3 (double arrowheads). Note that the results for JIM 37, JIM 38, and JIM 39 were similar to those presented for JIM 33, JIM 35, and JIM 36, respectively.

FIG. 3. Immunoblot showing LPSs from a variety of *Rhizobium* strains stained with JIM 32 antibody. Lane 1, 3841; lane 2, B659; lane 3, B660; lane 4, B661; lane 5, B778; lane 6, B779; lane 7, CE3; lane 8, CE109. (For further details, see the legend to Fig. 1.)

indicating that the LPS-2 component of this mutant has a different antigen structure. As shown in Table 3, the reactivity towards some antibodies was lost after mild acid pretreatment, confirming their reactivity with the LPS-2 core oligosaccharide. Interestingly, the reactivities of the JIM 32, JIM 34, JIM 35, and JIM 38 antibodies were not lost by this treatment, indicating that these antibodies may react with a lipid A component which is not hydrolyzed under these conditions.

Sugar inhibition studies. To test which sugar of the LPS core region could be involved in the epitopes recognized by these monoclonal antibodies, a sugar competition assay was carried out with purified LPSs from strains B660 and B659. The sugars used in this experiment were selected because they are known to be components of the core oligosaccharide and lipid A of *R. leguminosarum* biovar *viciae* (2, 10). As a positive control, it was shown (Table 4) that Kdo was an effective inhibitor of MASM-1 binding (29). Galacturonic acid was an effective competitor for all the newly isolated antibodies. In addition, reactivity with JIM 33 and JIM 35 was reduced by glucosamine. Incubation with galactose, mannose, amino-deoxygluconic acid, and Kdo did not inhibit binding of JIM 32 to JIM 39. (However, for JIM 33 and JIM 35, some small reduction in signal was repeatedly observed with Gal, Man, and GlcAN, and this phenomenon is currently under further investigation.)

Use of antibodies for structural studies of LPS. When JIM 32, JIM 33, JIM 34, and JIM 35 antibodies were used on

TABLE 3. Reactivities of monoclonal antibodies with *Rhizobium* LPSs

Monoclonal antibody	Source of LPS	Purified LPS ^a	Purified LPS after mild acid treatment
MASM-1	B660	+	—
JIM 32	B660	+	+
JIM 33	B660	+	—
JIM 34	B660	+	+
JIM 35	B660	+	+
JIM 37	B660	+	—
JIM 38	B660	+	+
JIM 36	B660	—	NT
JIM 39	B660	—	NT
JIM 36	B659	+	—
JIM 39	B659	+	—

^a Aliquots containing approximately 1 µg of affinity-purified LPS (or acid-treated material) were applied to nitrocellulose sheets and immunostained with different antibodies. +, positive reaction; —, negative reaction. NT, not tested.

TABLE 4. Binding of antibodies in the presence of monosaccharides

Antibody	LPS source	Effect of sugar in competition ^a						
		GalA	Gal	Man	GlcN	GlcAN	Kdo	None (blank)
MASM-1	B661	+	+	+	+	+	±	+
JIM 32	B661 + B659	±	+	+	+	+	+	+
JIM 33	B661	—	+	+	±	+	+	+
JIM 34	B661 + B659	±	+	+	+	+	+	+
JIM 35	B661 + B659	—	+	+	±	+	+	+
JIM 37	B661	—	+	+	+	+	+	+
JIM 38	B661 + B659	±	+	+	+	+	+	+
JIM 36	B659	—	+	+	+	+	+	+
JIM 39	B659	—	+	+	+	+	+	+

^a Dot immunoassays were used to probe for antibody binding to LPS in the presence of dissolved monosaccharides. Loading was done in triplicate with approximately 1 and 0.5 µg of LPS. Antibodies were incubated in the presence of sugars at 100 mM (14 mM for Kdo). +, positive reactivity; ±, significantly reduced or trace amounts of reactivity; —, no reactivity.

immunoblots of mutant B660, they reacted not only with the fast-migrating LPS-2 band but also with a pair of more slowly migrating bands (Fig. 2). We tentatively consider this more slowly migrating component to be a previously unrecognized form, LPS-3. Although clearly enhanced in the B660 sample, these LPS-3 bands were also faintly visible after immunostaining of the other LPS-defective mutants.

Strain B659 yielded a pattern of staining on immunoblots that was very different from the staining patterns for 3841 and the other LPS-defective mutants. First, the LPS-2 band of strain B659 was resolved into a doublet which was stained by JIM 36 and JIM 39 but not by JIM 33 or MASM-1. Second, staining of B659 with JIM 32 revealed a prominent LPS-3 component which was also apparently stained by AFRC MAC 57, an antibody which has hitherto been associated exclusively with the LPS-1 component.

Use of antibodies in *Rhizobium* strain recognition. LPS-2 and the core region of LPS-1 are believed to be relatively conserved in the genus *Rhizobium*, and thus most of the newly isolated antibodies were shown to cross-react with the LPSs from *Rhizobium etli* CE3 and CE109 when tested on immunoblots (data not shown). The exceptions were JIM 36 and JIM 39, which had previously been shown not to react with the LPS-2 component of *R. leguminosarum* 3841 (Fig. 2) or with any of its mutant derivatives except for the unusual mutant strain B659. In summary, the reactivity of the panel of antibodies seems to indicate that the antigen structure of LPS-2 is conserved, at least between the strains of *Rhizobium* tested.

When tested against a wider variety of plant-associated bacteria (Table 5), it was found that JIM 32 reacted with antigenic determinants on the cell surfaces of strains from the genus *Rhizobium* and to a certain extent with bacteria from the genus *Phyllobacterium* but not with LPSs from species of *Bradyrhizobium* or from other gram-negative bacteria tested. This finding suggests that monoclonal antibodies with specificity for the LPS core could be useful as taxonomic markers to distinguish certain species within the genus *Rhizobium*.

DISCUSSION

Several laboratories have isolated monoclonal antibodies with specificity for *Rhizobium* LPS (7, 16, 25, 26, 42, 45), but these have all proved to be specific for LPS-1, which is the dominant antigenic form of LPS in wild-type rhizobia. In order to develop monoclonal antibodies with specificity for LPS-2, a

TABLE 5. Reactivities of JIM 32 with rhizobia and other bacteria

Species	Strain ^a	Reactivity with JIM 32 ^b	Reference or source
<i>R. leguminosarum</i>			
<i>bv. viciae</i>	3841	+	50
	3624	+	This laboratory
	3849	+	This laboratory
	B556	+	50
<i>bv. phaseoli</i>	8002	+	27
	B715	+	This laboratory
<i>bv. trifolii</i>	6710	+	22
	RCR5	+	This laboratory
<i>R. etli</i>	CE3	+	35
<i>R. tropici</i>	CIAT899	—	33
	CFN299	—	33
<i>R. loti</i>	NZP 2235	—	This laboratory
	NZP 2037	—	This laboratory
<i>Rhizobium</i> sp.	NGR234	—	44
<i>R. meliloti</i>	AK631	—	21
<i>R. fredii</i>	HH103	—	18
<i>A. tumefaciens</i>	C58 (NAL)	—	8
	ACH5	—	8
<i>Agrobacterium rhizogenes</i>	LBA9402	—	8
	A4	—	8
<i>Phyllobacterium</i> spp.	Strain 17	(+)	P. J. J. Hooykaas
	Strain 19	(+)	P. J. J. Hooykaas
<i>Bradyrhizobium japonicum</i>	USDA 59	—	LMG ^c
	USDA 123	—	LMG
	ATCC 10324	—	LMG
	61-A-101	—	47
<i>Bradyrhizobium elkanii</i>	USDA 76	—	LMG
	USDA 31	—	LMG
<i>Bradyrhizobium</i> sp. (<i>Arachis</i>)	NC92	—	48
<i>Bradyrhizobium</i> sp. (<i>Lupinus</i>)	ATCC 10319	—	DSM ^d
	ISLU 16	—	15
	ISLU 65	—	31
	Hig 5 ₂	—	31
	NZP 2257	—	LMG
<i>Photobacterium</i> spp.	USDA 4362 ^e	—	USDA ^f (51)
	MKAa3	—	USDA (49)
<i>Azorhizobium caulinodans</i>	LMG 6465	—	LMG
<i>Pseudomonas syringae</i> pv. <i>glyciniae</i>	428	—	T. Denny
<i>Escherichia coli</i>	803	—	This laboratory

^a Strains were grown for 2 to 4 days on complex media.^b Cell suspensions were serially diluted, and aliquots were dotted onto nitrocellulose sheets and tested for reactivity with JIM 32 antibody. +, positive reaction; —, negative reaction; (+), intermediate reaction.^c LMG, Laboratorium voor Mikrobiologie, University of Ghent, B-9000 Ghent, Belgium.^d DSM, German Collection of Microorganisms, Munich, Germany.^e This strain was formerly called BTAl1.^f USDA, U.S. Department of Agriculture.

different approach was needed. The antigens were derived from a range of LPS-defective mutants because these strains are apparently devoid, or almost devoid, of LPS-1. We selected eight hybridoma cell lines which produced monoclonal antibodies (JIM 32 to JIM 39) reacting with LPS-2 from a panel of LPS-defective mutants.

Reactivity of the antibodies with LPS-2 was demonstrated in several ways. As a first step, the carbohydrate nature of the antigen was established by showing that it was proteinase resistant and sensitive to periodate oxidation. Second, it was shown in immunoblots that the newly isolated antibodies reacted with bands that cross-reacted with the well-characterized monoclonal antibody MASM-1, which reacts with LPS-2 of *R. leguminosarum* 3841 (50). Furthermore, six of the antibodies reacted with affinity-purified LPS-2 isolated from the LPS-defective mutant B660, a strain which apparently lacks the normal LPS-1 component (Fig. 2). (The two exceptions, JIM 36 and JIM 39, reacted with the LPS-2 fraction purified from mutant B659.) Four of the antibodies (JIM 33, JIM 34, JIM 36, and JIM 39) lost their reactivities with purified LPS-2 after it had been subjected to mild acid hydrolysis, as would be expected for epitopes in the core oligosaccharide region, which is attached to the lipid A moiety via an acid-labile Kdo linkage (10, 52). The other antibodies (JIM 32, JIM 34, JIM 35, and JIM 38) still reacted with hydrolyzed LPS-2, suggesting that they may react with the lipid A moiety. JIM 32 antibody was unique in that it seemed to react not only with LPS-2 in all mutants tested but also with LPS-1 in wild-type strains (Fig. 2). Thus, JIM 32 apparently recognizes an epitope that is common to both the LPS-1 and LPS-2 forms, as well as to LPS-3 (see below).

Further evidence that the panel of antibodies recognized the LPS-2 moiety of LPS came from a study of the competitive effects of sugars on antibody-ligand binding (Table 4). All antibodies were inhibited by galacturonic acid, a sugar which is part of the LPS-2 core oligosaccharide and the lipid A moiety (10). JIM 33 and JIM 35 were also inhibited by glucosamine, a component restricted to lipid A (2, 10). Similarly, the reactivity of JIM 38 was inhibited by galacturonic acid; GalA is a component of the LPS core oligosaccharide as well as lipid A, but because antigen-antibody binding was resistant to pretreatment with mild acid, it is more probable that the GalA of lipid A is a component of the epitope recognized by this antibody.

Use of LPS-2-specific antibodies as probes on immunoblots has revealed the presence of some more slowly migrating cross-reacting material in mutant strains such as B660 and B661 (Fig. 2). This represents a hitherto-unnoticed form of LPS that we have tentatively termed LPS-3. On the basis of its reactivity with monoclonal antibodies, there are several reasons for regarding LPS-3 as a new form of LPS that is distinct from the LPS-1 group. First, it does not express the constitutive wild-type O-antigen epitope recognized by AFRC MAC 57, which is characteristic of LPS-1 (7). Second, LPS-3 molecules are antigenically related to LPS-2, and free Kdo seems to be a structural component, as revealed by immunostaining with MASM-1 (Fig. 2). Third, the relative amount of LPS-3 seems to be opposite to that of LPS-1, as revealed by immunostaining of immunoblots (Fig. 2): mutant B661 (which makes a truncated form of LPS-1) expressed less LPS-3 than the other mutants that lacked LPS-1 completely. The significance of the LPS-3 component is still unknown; it could be that the pathway for synthesizing this hidden species is amplified when it is difficult to make LPS-1, as for instance in the LPS mutants described in this work. It is not clear whether the wild-type strain, 3841, synthesizes LPS-3, because it would have comigrated with the LPS-1 bands (23, 26, 42, 50). However, Fig. 2

demonstrates some very weak reactivity for the antibodies JIM 34 and MASM-1 with samples of 3841 in the region where LPS-3 would be expected, indicating that there may be a very reduced level of expression of the LPS-3 form in 3841 compared with what is observed with some of the LPS-defective mutants.

The pattern of reactivity of monoclonal antibodies with LPS-2 from mutants B660 and B661 was the same as for 3841 (Fig. 2), indicating that there are no important differences between the core and lipid A components of these strains. However, several structural peculiarities in the LPS-2 of strain B659 have been discovered, and they are of interest because this mutant is one of the few *Rhizobium* LPS-defective mutants that is unable to invade the host nodule (39). The LPS-2 of strain B659 has an antigenic configuration different from that of the wild type. It failed to react with JIM 33 but reacted with two antibodies (JIM 36 and JIM 39) which did not react with wild-type material. Furthermore, immunostaining with JIM 36 revealed that the LPS-2 component was resolved into a doublet band in this mutant, and the failure of MASM-1 to react indicates the absence of the normal Kdo-containing epitope in LPS-2. These results therefore suggest that the core component of B659 is structurally abnormal. Moreover, the abnormal structure of LPS-2 is also reflected in the antigenic properties of the presumptive LPS-3 material of B659 (Fig. 2), which reacts with AFRC MAC 57 although previously this antibody was known only to associate with LPS-1 forms.

Because LPS-2 and the core region of LPS-1 are strain-invariant structures (10), antibodies recognizing these components would be expected to show a broad spectrum of cross-reactivity with other *Rhizobium* strains. This was demonstrated by the identical reactivities of the antibodies towards the LPS of *R. leguminosarum* and *R. etli*. Furthermore, it was found that the epitope recognized by JIM 32 is well conserved between *R. leguminosarum* and *R. etli* (Fig. 3), but was not found in other strains of *Rhizobium* that are more distantly related, e.g., *R. loti*, *R. tropici*, *R. meliloti*, *R. fredii* (14), or *Rhizobium* sp. strain NGR 234 (32) or in all *Agrobacterium* strains tested (Table 5). (The observed cross-reactivity with the LPS of *Phyllobacterium* spp. was unexpected and requires further investigation.) These observations largely support recent findings about the phylogenetic relatedness of *Rhizobium* strains (14, 32, 41) and also confirm the generalization that the LPS core structure is relatively invariant in bacterial evolution. Thus, these antibodies may serve as useful taxonomic and diagnostic markers.

In future work, it will be of interest to relate LPS forms and substructures to their biological functions. We therefore hope that the panel of monoclonal antibodies with specificity for the LPS core region (LPS-2) of *Rhizobium* bacteria will be a useful resource for the analysis of LPS structure and biosynthesis.

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