4-1-1992

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Published version. *Journal of Bacteriology*, Vol. 174, No. 7 (April 1992): 2222-2229. Permalink.

JOURNAL OF BACTERIOLOGY, Apr. 1992, p. 2222–2229
0021-9193/92/072222-08$02.00/0

Vol. 174, No. 7

Rhizobium leguminosarum CFN42 Lipopolysaccharide Antigenic Changes Induced by Environmental Conditions

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Received 17 September 1991/Accepted 24 January 1992

Four monoclonal antibodies were raised against the lipopolysaccharide of *Rhizobium leguminosarum* bv. phaseoli CFN42 grown in tryptone and yeast extract. Two of these antibodies reacted relatively weakly with the lipopolysaccharide of bacteroids of this strain isolated from bean nodules. Growth ex planta of strain CFN42 at low pH, high temperature, low phosphate, or low oxygen concentration also eliminated binding of one or both of these antibodies. Lipopolysaccharide mobility on gel electrophoresis and reaction with other monoclonal antibodies and polyclonal antiserum indicated that the antigenic changes detected by these two antibodies did not represent major changes in lipopolysaccharide structure. The antigenic changes at low pH were dependent on growth of the bacteria but were independent of nitrogen and carbon sources and the rich or minimal quality of the medium. The Sym plasmid of this strain was not required for the changes induced ex planta. Analysis of bacterial mutants inferred to have truncated O-polysaccharides indicated that part, but not all, of the lipopolysaccharide O-polysaccharide portion was required for binding of these two antibodies. In addition, this analysis suggested that O-polysaccharide structures more distal to lipid A than the epitopes themselves were required for the modifications at low pH that prevented antibody binding. Two mutants were antigenically abnormal, even though they had abundant lipopolysaccharides of apparently normal size. One of these two mutants was constitutively unreactive toward three of the antibodies but indistinguishable from the wild type in symbiotic behavior. The other, whose bacteroids retained an epitope normally greatly diminished in bacteroids, was somewhat impaired in nodulation frequency and nodule development.

Bacteria of the genus *Rhizobium* fix nitrogen within the confines of root nodules on host legumes. These unique organs are the result of a developmental program induced by the bacteria in a susceptible region of the root. The bacteria infect as nodules develop. Infection culminates in bacterial release into certain plant cells, where the bacteria proliferate and assume the forms known as bacteroids (15, 26). The conversion from bacteria to bacteroids involves many changes, including the induction of nitrogen fixation. It is becoming clear that some of these changes are responses to decreased oxygen concentrations (9, 12). Other aspects of the bacteroid physical environment may provide inducing cues as well.

Intact lipopolysaccharide (LPS), a major component of the outer membrane of gram-negative eubacteria (28), is required for infection by certain rhizobia (16, 26). In *Rhizobium leguminosarum*, the LPS is composed of a novel lipid A (2) attached to a trisaccharide and a tetrasaccharide, whose structures are conserved in all strains so far tested (5), and an O-polysaccharide portion whose structure varies from strain to strain (4, 6) (Fig. 1). The O-polysaccharide, the immunodominant portion of the outer membrane (6), extends furthest into the bacterial environment. *R. leguminosarum* mutants deficient in O-polysaccharide are incapable of complete infection of the legume host. Infections on *Phaseolus vulgaris* (bean) by such mutants abort early in nodule development, usually within root hairs (19). On *Vicia* and *Trifolium* hosts, infections by such mutants break down at later stages of nodule development in which bacteria are being released to become bacteroids (3, 10, 21).

Recently, it was found that an LPS epitope of the bacteroid form of a pea-nodulating *R. leguminosarum* strain was absent or inaccessible in the LPS of free-living cells of this strain grown at pH 7. However, this epitope was present in free-living bacterial cells grown at pH 5 or at low oxygen concentration (14, 29, 33). Subsequently, it was reported that the LPS of a bean-nodulating strain also differed between its bacteroid and free-living state (24). If such changes are required to sustain infection, they could account for the requirement for O-polysaccharide in nodule development.

These considerations prompted an investigation of LPS structural variation in *R. leguminosarum* CFN42. The genetics and structure of the LPS of this strain and the requirement for its O-polysaccharide during infection of bean plants are known in some detail from previous work (3, 5–8, 19). Evidence to be presented indicates that the LPS of strain CFN42 exhibits at least minor antigenic differences in the bacteroid state. Growth free of the plant also resulted in LPS alterations, not only under low pH or low oxygen, but also under two other stress conditions, low phosphate availability and high temperature.

The main tools in this analysis were monoclonal antibodies whose epitopes were altered or masked during growth under the conditions studied. As a step toward understanding the structural basis of the changes, previously isolated Lps mutants were used to define the relative positions of the epitopes and LPS structures required for alterations induced at low pH. The accompanying report by Bhat and Carlson (1) extends this analysis to the LPS chemical changes that occur during symbiosis and growth at low pH.

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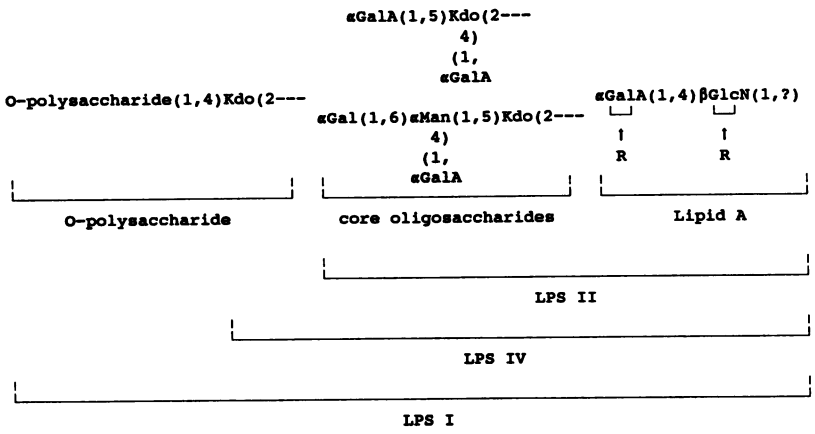

FIG. 1. *R. leguminosarum* CFN42 (CE3) LPS structure. Four major fragments are released by mild hydrolysis at Kdo residues (as denoted by – – –). The O-polysaccharide (also called O-antigen or O-chain) is composed of fucose, glucuronic acid, and 3-*O*-methylrhamnose residues, with lesser amounts (about one residue each per LPS molecule) of tri-*O*-methylfucose, mannose, a 2-amino-2,6-dideoxyhexose, and 2-*O*-methylrhamnose (1, 3, 6). The R groups are hydroxy fatty acids esterified or amide linked to the disaccharide of the lipid A portion (2). The relative arrangement of the conserved core oligosaccharides and the strain-specific O-polysaccharide is unknown, but wild-type LPS II molecules (see Fig. 2) contain only core residues, whereas LPS I molecules contain core and O-polysaccharide (6). LPS IV molecules of CE121 (see Fig. 5) contain core residues as well as fucose, mannose, a 2-amino-2,6-deoxyhexose, and 3-*O*-methylrhamnose (27). The compositions of LPS III and LPS V (see Fig. 5) have not been analyzed. Abbreviations: Kdo, 3-deoxy-D-manno-octulonic acid; GlcN, glucosamine; GalA, galacturonic acid; Gal, galactose; Man, mannose.

MATERIALS AND METHODS

Bacterial strains. All strains (Table 1) were derived from *R. leguminosarum* CFN42 (22). Strain CE144 was isolated by screening for symbiotically defective mutants (18) following nitrosoguanidine mutagenesis of erythromycin-resistant strain CE8. Mutant strain CE359 was isolated after Tn*5* mutagenesis by screening colony blots (33) for mutant derivatives that reacted with JIM29 antibodies after growth at low pH. Mutant CE367 was isolated after four cycles of subculturing at pH 5.0 (in YGM medium [see below] containing streptomycin, kanamycin, and nalidixic acid) after strain CE3 had been mutagenized with Tn*5-gusA1* (23). Strains CE109, CE121, and CE166 were isolated as Ndv^- mutants (eliciting nodules defective in development) after random Tn*5* mutagenesis (7, 19). Strains CE346, CE350, CE356, CE357, CE358, CE360, and CE374 were constructed by localized mutagenesis of cloned *lps* DNA (8).

TABLE 1. Bacterial strains

Strain[a]	Characteristics[b]	Reference or source
CE3	*str-1* Lps^+ LPS I Ndv^+ Fix^+	19
CE109	*str-1 lps-109*::Tn*5* LPS III Ndv^-	19
CE121	*str-1 lps-121*:: Tn*5* LPS IV Ndv^-	7
CE144	*ery-1* Lps^+ LPS I $pSym^-$ Nod^-	P. Elias
CE166	*str-1 lps-166*::Tn*5* LPS I, Ndv^-	7
CE346	*str-1 lps-21*::Tn*5* LPS I* Ndv^+ Fix^+	8
CE350	*str-1 lps-3*::Tn*5* LPS III Ndv^-	8
CE356	*str-1 lps-7*::Tn*5* LPS IV Ndv^-	8
CE357	*str-1 lps-5*::Tn*5* LPS IV Ndv^-	8
CE358	*str-1 lps-2*::Tn*5* Ndv^-	8
CE359	*str-1 lps-359* Tn*5*[c] LPS V Ndv^-	This work
CE360	*str-1 lps-6*::Tn*5* LPS V Ndv^-	8
CE367	*str-1 lps-367* Tn*5-gusA1*[c] LPS I Ndv^+ Fix^+	This work
CE374	*str-1 lps-233*::Tn*5* LPS I Ndv^+ Fix^+	8

[a] All strains are derived from wild isolate *R. leguminosarum* CFN42 (22).
[b] *str-1*, *ery-1*, and *lps* mutations alter streptomycin sensitivity, erythromycin sensitivity, or LPS; LPS I, I*, III, IV, and V indicate SDS-PAGE bands exhibited by the strain in addition to LPS II; Ndv^-, elicits incomplete nodule development.
[c] It has not been demonstrated that the Tn*5* or Tn*5-gusA1* insertion is responsible for the *lps* mutation of this strain.

Growth of bacteria. Except as noted, *R. leguminosarum* strains were grown at 30°C in shaken liquid medium. TY medium contained 0.5% tryptone (Fischer Scientific), 0.2% yeast extract (Fischer Scientific), and 10 mM $CaCl_2$. The pH of this medium was 6.7 before growth and 8.3 to 8.6 after full growth. Low-pH minimal medium (YGM), based on Y_{mod} (14) with some modifications, contained 0.4 mM $MgSO_4$, 1.25 mM K_2HPO_4, 0.11% Na-glutamate, 0.4% glucose, 4 mM NH_4Cl, 1 mM $CaCl_2$, 0.15 mM $FeCl_3$, 1 μg of biotin per ml, 1 μg of thiamine per ml, 1 μg of pantothenic acid per ml, and 40 mM MES [2-(*N*-morpholino)ethanesulfonic acid] (Sigma Chemical Co.) buffering the medium between pH 4.8 and 5.0. For some experiments, this medium was adjusted to pH 7.0 by titration with NaOH. Where specified, another minimal medium (Y), which employs succinate and glutamate as the carbon and nitrogen sources (30), was used. Microaerobic growth was obtained by injecting measured volumes of air into sealed tubes with liquid cultures under N_2 atmosphere. Well-sealed culture tubes were put into an anaerobic jar and shaken for 2 days at 30°C. For potassium phosphate starvation, the normal 1.25 mM phosphate concentration of minimal Y medium was decreased by adding less potassium phosphate. Even a moderate decrease (to 125 μM) resulted in slower growth.

Heat shock. Bacteria were grown in 5 ml of TY or YGM medium at 28°C overnight. Aliquots of 0.025 ml were diluted into tubes containing 5 ml of TY medium, incubated at 28°C for 1 h, and then shifted to 39°C. Cells were harvested at different times during growth, and induction of a heat shock response was monitored by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein profile.

Generation of monoclonal antibodies. Rats (male LOU/iap) were immunized with supernatant suspensions obtained after sonication of *R. leguminosarum* CE3 cells cultured on TY slants. Immunization, generation of hybridomas from rat

myeloma line IR983F, and screening of antibodies were performed as described previously (24). Hybridoma clone JIM26 was selected by virtue of the reaction of its antibodies with Lps mutant strain CE109 as well as wild-type strain CE3. JIM27 and JIM29 antibodies were immunoglobulin (Ig) class IgG2c; JIM26 and JIM28 were IgM.

Bacteroid preparation. Nodules were harvested from bean plants 15 to 17 days after inoculation with bacteria and crushed vigorously in a tube on ice. Ice-cold extraction buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, pH 7.5) was added (0.3 ml/100 mg of wet nodules), and after further maceration, the sample was centrifuged at 400 × *g* for 1 min. The supernatant suspension was centrifuged at 15,000 × *g* for 5 min at 4°C. The resulting ("bacteroid") pellet was washed three times by adding 1.0 ml of cold extraction buffer and centrifuging for 1 min at 15,000 × *g*.

SDS-PAGE. Washed bacterial cells were extracted by heating in SDS buffer for 3 min at 100°C. After centrifugation to remove insoluble debris, the extract was subjected to SDS-PAGE on 15% (wt/vol) acrylamide slab gels (3, 7). LPS was stained with Bio-Rad silver reagents after periodate treatment (7). In most cases, this staining was done on residual material in the gel after transfer to a nitrocellulose blot.

Immunoblots. Dot immunoblots were modified slightly from a procedure described previously (14). Bacteria were harvested by centrifugation of 1.5 ml of fully grown cultures and resuspended in 1 ml of buffer containing 10 mM Tris-Cl, 5 mM $MgCl_2$, and 10 mM 2-mercaptoethanol at pH 7.5. Two microliters of resuspended culture per dot was applied to 0.45-μm-pore-size nitrocellulose sheets (Schleicher & Schuell). For gel blots, the contents of SDS-polyacrylamide gels were electroblotted to nitrocellulose (6). Blots were incubated in TSG (50 mM Tris-HCl, 0.2 M NaCl, 0.5% gelatin, pH 7.4) containing monoclonal antibodies for 34 h at room temperature, washed five times with TSG (with 15-min incubations between replacements of TSG), incubated with peroxidase-conjugated anti-rat IgG (Sigma Chemical Co.) for 2 h, and finally washed five times with TSG (5 min each time). The blots were developed with 4-chloro-1-naphthol (Sigma Chemical Co.) in hydrogen peroxide (6).

Immunocompetition assay. Strain CE3 was grown in 5 ml of TY medium overnight at 30°C. This culture was applied directly to nitrocellulose sheets in aliquots of 2 μl per dot. The spotted nitrocellulose was dried overnight at room temperature. Different amounts of competitor (purified O-antigen or LPS) were preincubated with the monoclonal antibodies in TSG for about 36 h at 37°C. As controls, the antibodies were preincubated without competitor. The dot blots were incubated with the antibodies (preincubated with or without competitor) for 24 h at 30°C, washed, and developed with peroxidase-conjugated anti-rat IgG as described above. Maximum sensitivity was obtained by diluting the monoclonal antibody to almost the limit of detection.

Plant tests. *R. leguminosarum* CE3 and mutant derivatives were inoculated onto *P. vulgaris* cv. Midnight (Johnny's Selected Seeds, Albion, Maine), and the inoculated plants were grown as described previously (7). Nitrogenase activity was measured by acetylene reduction (7). Bacteroid-bacterium populations were counted by colony formation on TY agar after serial dilutions of the contents of nodules crushed into TY liquid after surface sterilization (19).

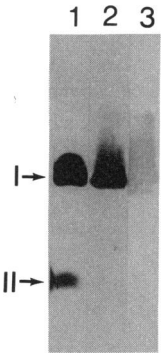

FIG. 2. SDS-PAGE of purified CE3 LPS (lanes 2 and 3) or the contents of TY-grown CE3 cells extracted into SDS buffer (lane 1). Lanes 1 and 2 (from different gels) were stained by the periodate-silver technique. Lane 3 is a blot of lane 2 reacted with JIM28 antibody. Arrows indicate the positions of LPS I and LPS II. Lane 2 was stained after the gel was electroblotted to nitrocellulose. (This was the case also in Fig. 3, 4, and 8). Since LPS II of the wild type occurs at much lower concentration than LPS I (6), LPS II often is not detected by staining the gel after blotting.

RESULTS

Reaction of antibodies with LPS of strain CE3 cultured in TY liquid. Antibody-producing hybridomas were generated after immunization of rats with extracts of wild-type *R. leguminosarum* CE3 that had been grown on TY agar slants. The antibodies of four purified hybridoma cell lines (designated JIM26, JIM27, JIM28, and JIM29) were chosen for study. SDS-PAGE separates the LPS of strain CE3 into two major mobility classes, LPS I and LPS II (Fig. 2). All four antibodies reacted with an antigen in SDS extracts of CE3 that on SDS-PAGE comigrated with LPS I (Fig. 3). After treatment of the extracts with protease, the reactive antigen still comigrated with LPS I (data not shown). Furthermore, all four antibodies reacted with LPS that had been purified by chromatography after phenol-water extraction (6) (Fig. 2, lanes 2 and 3). Therefore, the main, or probably the sole,

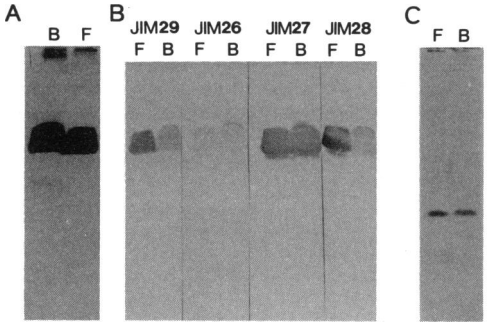

FIG. 3. Antibody reactions and SDS-PAGE mobility of the LPS I of bacteroids (wild-type strain CE3) and free-living bacteria (CE3 cultured in TY liquid). (A) Periodate-silver-stained SDS-PAGE of SDS extracts from bacteroids (lane B) and free-living bacteria (lane F). (Only one of the four duplicate sets of lanes is shown.) The intensely stained bands are LPS I. (B) Immunoblots from this gel reacted with the indicated monoclonal antibodies. (C) Lanes from a different gel to which less-concentrated samples were applied to show the equivalent electrophoretic mobilities of bacteroid LPS I and the LPS I of bacteria grown in TY.

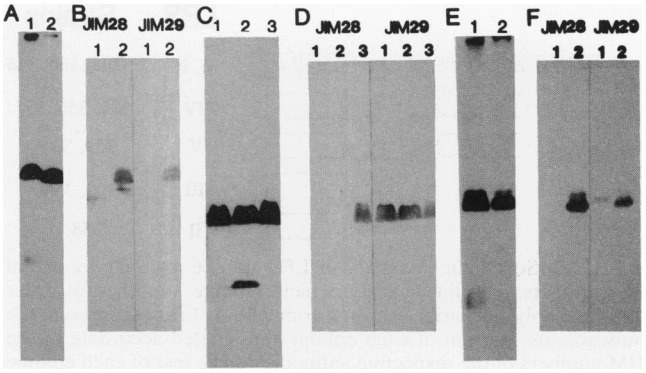

FIG. 4. SDS-PAGE analysis of wild-type CE3 cells grown at low pH, low oxygen concentration, or high temperature. Immunoblots of these gels (B, D, and F) were reacted with JIM28 and JIM29 antibodies, and residual material in the gels after blotting was silver stained (A, C, and E). (A and B) Cells were grown in YGM medium at pH 5.0 (lanes 1) or pH 7.0 (lanes 2). (C and D) Cells were grown in TY medium with 5% (lanes 1), 1% (lanes 2), or 20% (lanes 3) O_2 in the gas phase. (E and F) Cells were grown in TY medium for 20 h after shift to 39°C (lanes 1) or maintained at 28°C (lanes 2).

antigen of strain CE3 for each antibody was LPS I, the form of LPS that contains O-polysaccharide (6) (Fig. 1).

Binding of JIM29 antibody to O-polysaccharide isolated from CE3 cells grown at pH 7.2 (see the accompanying report by Bhat and Carlson [1]) was measured by an immunocompetition assay. Preincubation of JIM29 antibodies with O-polysaccharide inhibited their subsequent binding to CE3 cells dot-blotted onto nitrocellulose, although 160-fold more O-polysaccharide than intact LPS (based on hexose content) was required to eliminate detectable binding. In serial twofold dilution series, the lowest concentrations that inhibited binding by 1 μl of JIM29 antibody preparation were 1,400 and 9 ng of hexose equivalents of O-polysaccharide and LPS, respectively. Insufficient O-polysaccharide was available to test the other antibodies in this manner.

LPS I of bacteroids. CE3 bacteroid LPS I had the same SDS-PAGE mobility as the LPS I of CE3 bacteria grown ex planta in TY medium (Fig. 3C). The bacteroid LPS I reacted as strongly as LPS I of TY-grown bacteria with monoclonal antibodies JIM26 and JIM27 (Fig. 3B), antibodies from three hybridoma fusions not further characterized, and polyclonal rabbit antisera prepared against CE3 cells grown on TY medium. However, JIM28 and JIM29 antibodies reacted relatively weakly with the bacteroid LPS (Fig. 3B).

LPS of bacteria grown at low pH. Growth of the wild-type (CE3) bacteria ex planta at pHs below 5.2 also resulted in LPS that reacted well with JIM26 and JIM27 antibodies (data not shown) but very weakly, if at all, with JIM28 and JIM29 antibodies (Fig. 4B). In addition, LPS I from bacteria grown at low pH migrated slightly slower than LPS I of bacteroids and free-living bacteria grown at higher pHs (Fig. 4A) (1). These changes in antigenicity and electrophoretic mobility occurred at low pH regardless of whether the bacteria were grown with glucose, succinate, or glutamate as the carbon source and regardless of whether MES was used to achieve low pH. Growth in minimal YGM medium buffered at pH 7 with MES resulted in LPS that behaved antigenically and electrophoretically like LPS of TY-grown bacteria.

To test whether these LPS changes were due to abiotic chemical reactions at low pH, CE3 cells were grown overnight at pH 7.0, harvested, and then incubated for 36 h in low-pH medium without growth by omitting the N source or after adding chloramphenicol. All four antibodies reacted strongly with dot immunoblots of cells treated in this manner. These incubation conditions also did not affect the LPS mobility on SDS-PAGE. Incubation of purified LPS for 36 h at low pH likewise did not affect its antigenicity or mobility on SDS-PAGE.

TABLE 2. Summary of LPS I reaction with monoclonal antibodies after growth of wild type (CE3) under various conditions

Condition	Reaction[a] with monoclonal antibody:				SDS-PAGE mobility[b]
	JIM26	JIM27	JIM28	JIM29	
Normal[c]	+	+	+	+	N
Bacteroid	+	+	±	±	N
pH <5.2	+	+	−	−	S
Low $[O_2]$	+	+	−	+	N
Low $[O_2]$, pH 5.0	+	+	−	−	S
125 μM phosphate	+	+	+	−	N
25 μM phosphate	+	+	−	−	N
39°C	+	+	−	±	N

[a] +, reaction; −, no reaction; ±, weak reaction.
[b] N, normal mobility; S, somewhat slower (Fig. 4).
[c] TY or minimal medium at pH 7 to 8 shaken at 30°C; with glucose, glutamate, or succinate as the carbon source.

Effect of other physiological stress conditions on wild-type LPS structure. Growth at low oxygen concentrations caused the loss of reaction with JIM28, but not JIM29, antibodies (Fig. 4D). The resulting LPS I had the same mobility on SDS-PAGE as LPS I from cells grown under normal aeration.

A type of heat shock was induced by growth at the maximum growth temperature for this strain (39°C) following growth at 28°C. LPS I produced at the higher temperature reacted very weakly or not at all with JIM28 and JIM29 antibodies but was not altered in SDS-PAGE mobility (Fig. 4E and F).

Growth at low phosphate concentration yielded LPS I that reacted with JIM28 but not JIM29, or neither the JIM28 nor the JIM29 antibody, depending on the severity of the starvation (Table 2). SDS-PAGE mobility of LPS I was not affected.

LPS I synthesized under all these stress conditions reacted as strongly as LPS I synthesized in TY medium with JIM26 and JIM27 antibodies. Table 2 summarizes the changes in antigenicity of wild-type LPS I under the various conditions tested, including the combination of low pH and low oxygen.

Lack of dependence on the Sym plasmid. Strain CE144 is Nod^- and lacks the Sym plasmid band on an Eckhardt-type gel (13) (data not shown). The epitope changes induced by low pH, high temperature, low phosphate, and low oxygen concentration all occurred in this strain.

Mutants lacking LPS I or having altered LPS I. As a means of determining the relative location of the antibody epitopes, we tested the antibodies against TY-grown mutant derivatives of strain CE3 (Table 1) in which LPS I is altered, truncated, greatly decreased, or absent (7, 8) (Fig. 5). Strain CE346 has a major SDS-PAGE band (LPS I*) that migrates somewhat faster than LPS I (Fig. 5, lane 3). All four antibodies reacted strongly with this LPS I* band. In the other previously isolated mutant strains, the major bands comigrated with wild-type LPS II (Fig. 5, lanes 1 and 4 to 7);

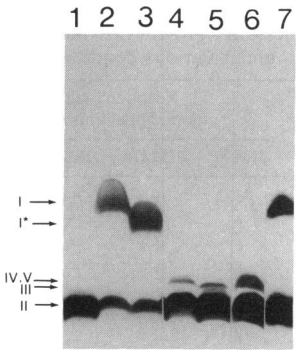

FIG. 5. LPS bands of mutant strains. SDS extracts of bacteria cultured in TY medium or, for lane 6, YGM medium were subjected to SDS-PAGE, and the gels were stained by the periodate-silver technique. Lanes: 1, CE358; 2, CE3 (wild type); 3, CE346; 4, CE121 (LPS IV); 5, CE350 (LPS III); 6, CE359 (LPS V); 7, CE166. Lane 7 is from a separate gel. Arrows indicate the positions of LPS I, LPS I*, LPS II, LPS III, LPS IV, and LPS V.

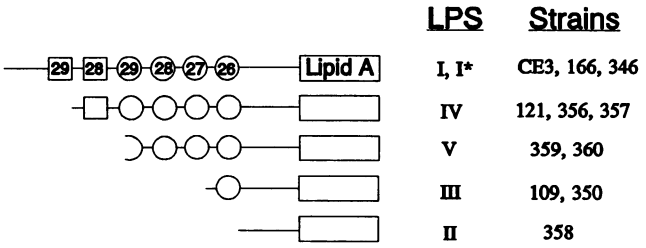

FIG. 6. Schematic summary of LPS epitope mapping by use of Lps mutants. The linear scale depicts relative hypothetical order along the polysaccharide outward from lipid A. Circles represent the outwardmost portion of each epitope (designated according to the JIM numbers of the respective antibodies). The rest of each epitope may overlap with other epitopes. The outermost portion for JIM29 is shown as a half circle in LPS V because this antibody reacts weakly with mutants CE359 and CE360. The relative order of epitopes 27 and 28 is arbitrary. Squares represent sites whose absence results in the LPS not being modified at low pH to prevent binding of the indicated antibody (28, JIM28; 29, JIM29). A given modification could occur at one of these sites, within the epitope itself, or elsewhere.

i.e., the major LPSs of these strains presumably lack O-polysaccharide residues (6). Regardless of the strain tested (including the wild type), LPS II bands did not react with any of the antibodies. Lack of reaction against LPS II was not due to poor transfer or loss of LPS II during blotting; the presence of LPS II on such blots has been revealed in similar experiments by intense reaction with antiserum raised against LPS II (6).

Some of the mutants have minor LPS bands in addition to LPS II (7) (Fig. 5 and Table 1). The less abundant band of CE166 comigrates with wild-type LPS I; this band reacted strongly with all four antibodies. The other strains have bands that migrate almost as fast as LPS II: LPS III, LPS IV, and LPS V (Fig. 5). The minor SDS-PAGE band (LPS III) of strains CE109 and CE350 reacted only with JIM26 antibody. The minor LPS band (LPS IV or V) of strains CE121, CE356, CE357, CE359, and CE360 reacted with all four antibodies, although the binding of JIM29 antibody to the LPS V of CE359 and CE360 was relatively weak.

Strain CE346, which exhibits the abundant LPS I* band instead of wild-type LPS I, nodulated bean plants normally. Like the LPS I of wild-type bacteroids, the LPS I* of CE346 bacteroids reacted very weakly with JIM28 and JIM29 antibodies. All the other Lps mutants mentioned above are defective in infection and, hence, do not give rise to bacteroids that could be tested.

Lps mutants also were tested by immunoblots (mainly by dot blots) after growth at low pH. The resulting LPS I of strain CE166 and the LPS I* of strain CE346 exhibited the same antigenic changes and retarded SDS-PAGE mobility as wild-type LPS I. However, unlike wild-type LPS I, the minor SDS-PAGE LPS bands of CE360 and CE359 (LPS V) reacted with JIM28 and JIM29 antibodies after growth at low pH. The minor LPS molecules of CE121, CE356, and CE357 (LPS IV) reacted with JIM29, but not JIM28, antibodies after growth at low pH. These results are interpreted schematically in Fig. 6. (Further explanation is presented in the legend to this figure and in the Discussion section.)

The *lps* mutations of all these strains, except CE166, map within a long stretch of the chromosome (*lps* region α) that has been described previously (8) (Fig. 7). Strain CE359 (isolated in this study) could be complemented to Lps$^+$ by a plasmid carrying this *lps* region from the wild type, but when *lps* region α harbored the mutation of strain CE360 (*lps-6*), it did not complement strain CE359 to Lps$^+$. Therefore, the *lps* mutations of strains CE359 and CE360 are located in *lps* complementation group C (8). The mutations of strains CE121, CE356, and CE357 were shown previously to map near each other in *lps* complementation group D (8). Therefore, SDS-PAGE mobility of the minor LPS band, antigenic properties, and *lps* complementation group were perfectly correlated in these Lps mutants.

Mutants with abundant, but antigenically abnormal, LPS I. Strain CE374 which harbors mutation *lps-233*:Tn*5* (Fig. 7), was isolated by localized mutagenesis of chromosomal *lps* region α of strain CE3 (8). Its LPS resembled wild-type LPS after growth at low pH, low phosphate, or in TY medium. However, at low oxygen concentrations and in the bacteroid state, it retained strong reaction with JIM28 antibody (Fig. 8). It elicited somewhat fewer and smaller nodules than the wild type (Table 3), and the nodules were scattered throughout the root system rather than being clustered near the tap root. In fact, at earlier times than the date of sampling for Table 3, the deficiencies of strain CE374 in nodule number and nodule size were even more pronounced. The viable

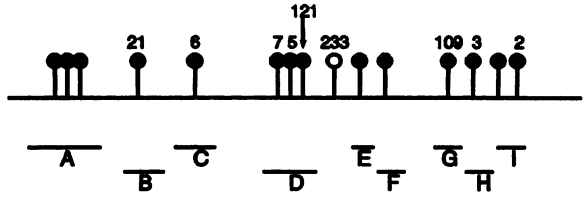

FIG. 7. Large chromosomal *lps* region α of strain CFN42. The ball-and-stick symbols depict the relative locations of Tn*5* insertions that affect LPS structure and define nine complementation groups that span 18 kb of DNA (8). Filled circles indicate insertions that result in the loss of the LPS I SDS-PAGE bands. Only mutations relevant to this study are specified by number. Mutation *lps-359* of CE359 is in group C, but its relative position has not been determined. Results of the present study establish insertion 233 as an *lps* mutation, but whether it defines a new complementation group has not been investigated.

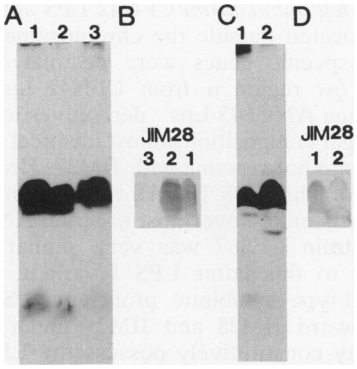

FIG. 8. Mutant CE374 cultured cells and bacteroids analyzed by SDS-PAGE and immunoblots. (A and B) CE374 grown in TY medium with low (5%) oxygen (lanes 1), TY medium (lanes 2), or Y medium with low (25 μM) phosphate (lanes 3). (C and D) Bacteroids of nodules inoculated with CE374 (lanes 1) or CE3 (lanes 2).

bacteroid-bacterium population per nodule was lower than that of the wild-type-induced nodules, even in a nodule sample skewed toward the larger CE374 nodules (Table 3, footnote *c*). Nitrogenase activity per nodule wet weight was higher than that of the wild type, but because of lower total nodule mass, the mutant contributed only about one-half the normal activity per plant.

Strain CE367 was isolated after a subculturing scheme designed to enrich for mutant bacteria that adapt more rapidly to low pH conditions, followed by screening for mutants that differed antigenically from the wild type. The LPS I of this mutant was abundant and had the wild-type SDS-PAGE mobility, but it did not react with JIM27, JIM28, or JIM29 antibodies after growth at either low pH or in TY medium. Its LPS I was not changed in SDS-PAGE mobility after growth at low pH, and its bacteroids did not react with JIM27, JIM28, or JIM29 antibodies. The *lps* mutation of this strain was not complemented by a plasmid carrying chromosomal *lps* region α of the wild type. It has not been tested with DNA cloned from the other known *lps* regions. Strain CE367 had wild-type symbiotic proficiency.

DISCUSSION

In the conversion from free-living bacteria to the bacteroids of pea and vetch, at least three *R. leguminosarum* strains undergo changes in LPS structure (14, 20, 29, 33) and outer membrane proteins (10). The chemical composition of pea bacteroid LPS of one strain indicates, however, that there is no major difference in neutral sugars and fatty acids (20). This conclusion is consistent with the observed changes in the LPS structure of *R. leguminosarum* CFN42 (CE3) during nodulation of bean plants. Although recognition by JIM28 and JIM29 antibodies was greatly decreased, gel electrophoresis and reaction with other antibodies suggested that the structure remained substantially intact.

In pea nodules infected with *R. leguminosarum* 300, a particular LPS epitope is present on the bacteroids in the bulk of the infected zone but is not detected on bacteroids in the periphery of this zone (29). Similar variation depending on location of the bacteroids in bean nodules would explain why the bacteroid preparations in this study reacted weakly, rather than not at all, with JIM28 and JIM29 antibodies. If the changes are truly bacteroid specific, it also may be pertinent that bacteroids were not purified in the present study to eliminate contaminating bacteria that had not yet converted to bacteroids.

Based on analysis of the above epitope of *R. leguminosarum* 300, it was conjectured (14) that antigenic alterations in bacteroid LPS might be responses to the microaerobic (32) and potentially acidic nature of the bacteroid environment. In support of this idea, growth of strain CE3 at low pH in the present study resulted in antigenic differences resembling those of bacteroids. In addition, the *lps* mutation of strain CE374 affected loss of the JIM28 epitope at low oxygen concentration and in the bacteroid. However, LPS I synthesized at low pH ex planta migrated more slowly on SDS-PAGE than bacteroid LPS I. Microaerobiosis, alone or in combination with low pH (Table 2), also did not lead to exactly the same LPS structure as that of CE3 bacteroids. Instead, the closest match to the bacteroid LPS antigenicity was produced ex planta by severe phosphate starvation (Table 2). However, it is still possible that the bacteroid LPS structure is cued by a combination of factors that has not been tested yet, including factors unique to nodule development.

Given the results of this and two previous studies (14, 24), it is clear that the LPS of this species undergoes modifications as part of the adaptation to several environmental stresses. These stresses include conditions (low pH, phosphate starvation, and high temperature) that induce the *vir* (virulence) regulon of the plant pathogen *Agrobacterium tumefaciens* (31). Although *Agrobacterium* spp. and *Rhizobium* spp. clearly respond to conditions more specific to the plant environment, such as the presence of phenolic and flavonoid compounds, some of the above stress conditions may be part of the overall signalling that indicates to these bacteria that they are within a particular host environment. By analogy, low iron, low pH, and other environmental stresses activate the virulence regulons of many animal pathogens (11). Viewed in this way, the LPS changes observed in this and the previous studies could be part of the overall adaptation to the host. However, it is also possible that gram-negative eubacteria in general, irrespective of symbiotic interactions, modify their LPSs in ways that best cope with changed environments and that with the proper analytical tools, such changes will be found in other bacteria. This possibility is consistent with the observation that each condition tested in this study resulted in a different effect on the LPS (Table 2 and Fig. 4). Note also that it is not yet obvious what advantages such changes provide *R. leguminosarum*. For instance, mutants CE359 and CE360 did not

TABLE 3. Symbiotic parameters of strain CE374[a]

Strain	Nodules/ plant	Nodule mass[b] (mg)	10^6 Bacteria/ nodule[c]	Nitrogenase activity[d] (μmol of C_2H_4 min^{-1})	
				Plant^{-1}	g of nodule^{-1}
CE3	62 ± 24	1.79 ± 0.15	160 ± 100	2.6 ± 0.9	23 ± 2
CE374	38 ± 9[e]	1.05 ± 0.17[f]	77 ± 38[e]	1.4 ± 0.7[e]	35 ± 6[f]

[a] Plants inoculated with strains CE3 (wild type) and CE374 were compared 19 days after inoculation of 2-day-old seedlings.
[b] Wet mass.
[c] CFU from surface-sterilized and crushed nodules. The mean mass of the nodules picked for CE374 was 85% of the mean mass of the nodules chosen for CE3; i.e., the sample was skewed toward the larger CE374 nodules.
[d] Acetylene reduction normalized in two ways: per plant and per wet nodule mass.
[e] These values are statistically different from the corresponding values of the wild-type strain (CE3) at the 95% confidence level according to the *t* test.
[f] Different from the wild-type values at the 99% confidence level.

lose JIM28 or JIM29 epitopes at low pH, but appeared to grow as well as the wild type at low pH.

Since LPS II of strains CE3 and CE121 has intact core oligosaccharides (6, 27) but does not bind any of the antibodies, the monoclonal antibodies used in this study recognize LPS only if a portion of the CFN42 O-polysaccharide is present. In accord with this conclusion, previous work has shown that these antibodies do not bind to the LPS of *R. leguminosarum* ANU843 and 128C53 (3), which have the same core oligosaccharides (5). Use of purified O-polysaccharide in immunocompetition assays also supported the notion that at least part of the JIM29 epitope was in the O-polysaccharide portion of the LPS. Only the part of the O-polysaccharide structure found in LPS IV of mutant strain CE121 (Fig. 1 and 6) is required for binding any of the four antibodies. Based on sugar composition data (27), LPS IV retains roughly one-fourth of the O-polysaccharide. JIM26 antibody may require the least extent of the O-polysaccharide, since it is the only one that reacts with the LPS III of mutant strains CE109 and CE350. However, the LPS of CE109 appears to be altered in the core oligosaccharides as well as in the O-polysaccharide (5).

As depicted in Fig. 6, the data suggest that portions of the O-polysaccharide more distal to lipid A than the epitopes themselves are required for the pH-induced change(s). Perhaps the chemical modifications occur at these more distal sites and mask, rather than alter or eliminate, the epitope structures. Alternatively, putative modification enzymes might require structure distal to the residues they modify. It seems that the greatest extent of O-polysaccharide is required to prevent binding of JIM29 antibody after growth at low pH (Fig. 6), because LPS IV undergoes modification at low pH to prevent binding of JIM28, but not JIM29, antibodies. A similar requirement for distal O-polysaccharide structures to perform LPS changes required (hypothetically) during symbiosis would explain why the O-polysaccharide is required for symbiosis, even if the chemical changes occur in the inner, conserved portions of the LPS.

To establish the physiological significance of these changes, it is crucial to isolate mutants, such as strain CE374, that retain the LPS I structure but do not alter the LPS during growth under a condition that normally induces a change. In addition to its LPS antigenic difference in the bacteroids and ex planta under low oxygen, mutant CE374 elicited fewer nodules, which were widely scattered, rather than clustered. Moreover, its nodules had fewer bacteria and somewhat less mass. Therefore, the LPS structural change eliminated by this mutation may facilitate both nodulation and infection. However, in the absence of further information, it is also possible that the LPS antigenic difference and the symbiotic deficiencies were independent manifestations of the mutation. In any case, this mutant is definitely not as deficient in infection as mutants which are deficient in O-polysaccharide. Therefore, additional factors must be sought to explain the infection phenotype of the latter mutants. However, a requirement for the modification lacking in mutant CE374 might explain the scattered distribution of nodules elicited by O-polysaccharide-deficient mutants. With regard to the physiology of symbiotic nitrogen fixation, it is interesting that the deficits in these two parameters of symbiosis were partially compensated on a total plant basis by the greater specific nitrogenase activity per bacterium (very likely a consequence of having fewer nodules to compete for available photosynthate [17, 25]).

Strain CE367 provided different insights. It was mutated in a gene that is necessary for modifications that provide the complete *R. leguminosarum* CFN42 LPS antigen. The gene affected is located outside the chromosomal *lps* region α. Such strain-specific genes were postulated after it was shown that *lps* region α from CFN42 functioned in *R. leguminosarum* ANU843 Lps^- derivatives to give an LPS I that had a sugar composition almost identical to that of strain CFN42, despite not reacting with JIM27, JIM28, and JIM29 antibodies (3). This LPS I variant was sufficient to restore Fix^+ symbiosis on a clover host of strain ANU843 (3). The LPS I of strain CE367 was very similar, or identical, antigenically to this latter LPS I variant, and, likewise, allowed wild-type symbiotic proficiency. Since it lacked reactivity toward JIM28 and JIM29 under all conditions tested, it may constitutively possess the LPS feature that distinguishes the bacteroid from the inoculant grown in TY medium. However, this question is best answered by chemical analysis. A possibly similar mutant of *R. leguminosarum* is symbiotically proficient as well (33).

Strains CE166 and CE346 have revealing phenotypes regarding the requirement for LPS in symbiosis. One has abundant, but structurally altered, LPS I that provides wild-type symbiotic function (8). The other has greatly decreased LPS I that may be identical structurally to wild-type LPS I (7); this mutant (CE166) is as symbiotically defective as mutants which lack LPS I (7). Like the LPS of CE346, the LPS I molecules of CE166 were antigenically identical to the wild type under all conditions tested; therefore, the most obvious explanation for the symbiotic deficiency of CE166 still relates to its deficiency in the amount of LPS I.

In summary, the LPS structure of strain CE3 is changed subtly in response to various environmental conditions, including the bacteroid environment. SDS-PAGE analysis and comparisons of various mutant LPS forms suggest that a portion of the O-polysaccharide must be present for the modifications to occur, but this analysis could not assign the locations of the chemical changes. The immunochemical approach of this study also does not show how many separate structural changes result from altered growth conditions. One chemical change triggered by a given condition could affect more than one epitope, and different chemical changes arising in response to different conditions could affect the same epitope. The accompanying report by Bhat and Carlson (1) represents a start toward resolving these questions by showing that at least two chemical changes in the O-polysaccharide are induced by growth at low pH and in the bacteroid state.

ACKNOWLEDGMENTS

This work was supported by grant 90-37120-5755 from the Competitive Research Grants Office of the U.S. Department of Agriculture to K.D.N. and NATO travel grant 30.0405.87 to N.J.B. and K.D.N.

We thank J. P. Knox and J. Peart for help with the preparation of monoclonal antibodies, Jeanett Perez-Lesher for help in analyzing the symbiotic phenotype of strain CE374, Ramadas Bhat for purified CE3 O-polysaccharide, and Russell Carlson for helpful discussions and criticism of the manuscript.

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