Antigenic Change in the Lipopolysaccharide of *Rhizobium etli* CFN42 Induced by Exudates of *Phaseolus vulgaris*

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Growth of *Rhizobium etli* CE3 in the presence of exudates from *Phaseolus vulgaris* resulted in a modified lipopolysaccharide (LPS) that no longer reacted with monoclonal antibody JIM28. However, the overall LPS structure appeared not to be greatly altered, as revealed by unchanged mobility in gel electrophoresis and partial or unaltered reactivity with other antibodies. Activity that triggered LPS antigenic conversion was exuded from both seeds and roots, but reactivity with one of the antibodies indicated that the resulting alterations were not identical. Antibody binding to the LPS decreased as a function of the concentration of exudate present during growth of the bacteria. The antigenic change did not occur if purified LPS or nongrowing bacteria were incubated with the exudates. Exudate-induced LPS modification did not require the Sym plasmid.

Additional keywords: legume, root nodules, symbiosis.

Chemical communication between bacterium and plant is crucial in the development of nitrogen-fixing legume root nodules. The best understood example of this communication occurs in the initiation of nodule formation (Brewin 1991; van Rhijn and Vanderleyden 1995). Bacterial *nod* genes are induced by flavonoid compounds produced by the plant (Peters et al. 1986). The induced *nod* genes direct the synthesis of lipooligosaccharide Nod factors (Lerouge et al. 1990) that trigger root hair deformations and cell division in susceptible regions of the root. In order that the bacteria infect and the nodules differentiate normally, other bacterial determinants besides the Nod factors are required. These determinants include certain polysaccharides: β-glucans, acidic exopolysaccharides, Kdo-rich capsular polysaccharides, and lipopolysaccharides (Noel 1992; Petrovics et al. 1993). How these polysaccharides promote infection is unknown.

The requirement for the O-antigen portion of lipopolysaccharide (LPS) has been documented for the symbioses of *Rhizobium leguminosarum*, *Rhizobium etli* CE3, and *Bradyrhizobium japonicum* 110 (Noel 1992; Stacey et al. 1991). On *Trifolium* spp. and *Vicia sativa* hosts, O-antigen-deficient *R.*

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leguminosarum mutants elicit significant infection thread development, but few bacteria are released into host cells (Dazzo et al. 1991; Brink et al. 1990; de Maagd et al. 1989; Priefer 1989). The infection threads of LPS-defective mutants of *R. leguminosarum* bv. *viciae* on *Pisum sativum* are often distended and appear to elicit deposition of compounds characteristic of host-defense reactions. One mutant, which is severely altered in LPS, does not infect at all (Perotto et al. 1994). Mutants of *R. etli* deficient in O-antigen elicit infection threads on *Phaseolus vulgaris* that are grossly distended and cease development within the outer cell layers of the developing nodule (Noel et al. 1986). The resulting nodule, or pseudonodule, has relatively few bacteria, differs anatomically from normal nodules, and lacks nitrogen-fixing activity.

During nodule development on *P. sativum*, *Vicia sativa*, and *P. vulgaris*, the LPSs of three strains of *R. leguminosarum* undergo modifications that have been detected with monoclonal antibodies (VandenBosch et al. 1989; Goosen-de Roo et al. 1991; Sindhu et al. 1990). At least some of the changes in LPS antigenicity can be duplicated ex planta by growth of the bacteria at low pH or low oxygen concentrations (Kannenberg and Brewin 1989). These and certain other conditions also lead to changes in the LPS of *R. etli* CE3, as detected by loss of binding of two monoclonal antibodies (Tao et al. 1992). The LPS of bacteroids of this strain also differs antigenically from the LPS of the bacterial inoculum grown without nutrient limitations at neutral pH (Tao et al. 1992). Since LPS changes during infection, it was of interest to determine whether material exuded from the plant would trigger a change in LPS structure.

RESULTS

Epitope change induced by bean exudate.

The LPS in detergent extracts of *R. etli* CE3 cultured cells was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and probed by reaction with monoclonal antibodies (Tao et al. 1992). SDS-PAGE separates the LPS of this strain into two mobility classes, LPS I and faster-moving LPS II bands. LPS I differs from LPS II in that it contains the O-antigen, the most distal polysaccharide portion of the complete LPS molecule (Carlson et al. 1987). Previously described monoclonal antibodies JIM26, JIM27, JIM28, and JIM29 bind only to the LPS I molecules and rec-

ognize strain-specific LPS structures (Brink et al. 1990; Tao et al. 1992).

Growth of *R. etli* CE3 in rich medium (TY) containing the exudate from germinating bean (*P. vulgaris* cv. Midnight) seedlings resulted in an LPS structural change that greatly diminished recognition of LPS I by antibody JIM28 and decreased the binding of antibodies JIM27 and JIM29 as well (Fig. 1A). On the other hand, the LPS I mobility in SDS-PAGE and binding to antibody JIM26 were not significantly changed (Fig. 1A). The antigenic change was stable to phenol-water extraction and subsequent purification by sepharose 4B chromatography (data not shown).

Exudate from roots also induced a change in LPS I, which similarly prevented binding with antibody JIM28, decreased binding to antibody JIM29, but did not alter the SDS-PAGE mobility (Fig. 1B). Unlike treatment with seed exudate, however, growth in root exudate did not appear to decrease the recognition of LPS I by JIM27 (Fig. 1B).

The extent of the antigenic conversion, as monitored by decreased binding of JIM28, was dependent on the concentration of seed or root exudate (Fig. 2, lanes 1 to 4; Fig. 3). Based on this measure of activity, the exudate accumulated from the roots of one plant during the standard 6-day period of collection had two to five times higher activity than the exudate released from one seed during the standard 24 h of collection.

Although all data presented in this report are from assays involving SDS-PAGE, the antigen change was demonstrated also by a microplate colorimetric immunoassay and an immunoassay of dot blots of intact cells. Dot blots of intact cells required growth in fivefold higher exudate concentrations than gel or microplate assays to show equivalent effects on antibody binding.

The alteration in O-antigen did not occur in the presence of either exudate if growth of the bacteria was prevented by chloramphenicol (200 µg/ml) or bacitracin (5 mg/ml). Likewise, when purified LPS (from *R. etli* CE3 grown in TY without exudate) was incubated with root or seed exudate in TY broth for 16 to 24 h, its reactivity with antibody JIM28 was not diminished, even after treatment with exudate at concentrations up to 50-fold higher than that needed during growth in culture to trigger the half-maximal change in LPS.

Seed exudate from two other *P. vulgaris* cultivars, Royal Burgundy (purple seeds) and Contender (brown seeds), also exhibited this activity (data not shown). Root exudate from these cultivars was not tested.

Growth of the bacteria at pH below 5.2 results in loss of reactivity of the LPS I with antibodies JIM28 and JIM29 (Tao et al. 1992). Although the addition of bean exudates affected the pH of TY medium, the pH did not become this acidic, and, therefore, presumably was not responsible for the observed antigenic changes. The presence of crude exudate from 1.0 plant per ml of culture, which is higher than the concentrations needed to bring about complete antigenic conversion (Figs. 1 to 3), altered the initial pH of TY medium from 6.6 to 6.4 (seed exudate) or 6.8 (root exudate) and after full growth changed the pH from the value of 8.2 in TY alone to 7.0 (seed exudate) or 8.0 (root exudate).

Properties of the plant material responsible for triggering LPS antigenic modification.

The exudate from germinating seeds was still active after

incubation at 121°C for at least 20 min, or storage at −20°C or 4°C for 3 months. The active material did not extract into hexane or ethyl acetate, and it was only 10% as soluble in n-butanol as in water. It was precipitated by 90% (vol/vol) acetone, but was soluble in 80% (vol/vol) or less acetone in water. Extraction into 75% acetone effected a significant purification by removing the bulk of the material in the exudate as a precipitate. The activity was resistant to ribonuclease A, deoxyribonuclease I, and proteinase K. Through all treatments and partial purification, the activity from germinating seeds that triggered LPS modification was accompanied by an activity that partially inhibited bacterial growth (Fig. 3). Active compounds from roots also were soluble in 75% acetone. Root exudate did not inhibit growth.

Lack of dependence on Sym plasmid.

Nod⁻ strain CE144, which lacks the Sym plasmid band on Eckhardt-type gels, synthesizes an LPS I that is identical in SDS-PAGE mobility and antigenicity with the LPS I of wild-type strain CE3 (Tao et al. 1992). When this strain was grown
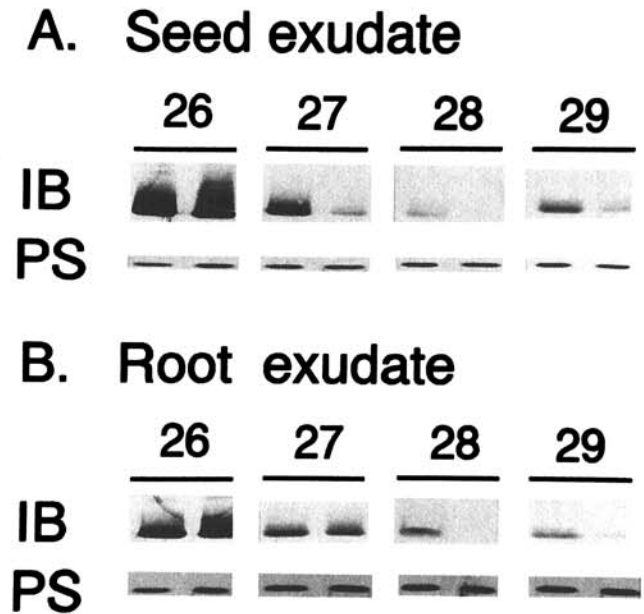

Fig. 1. Antigenic change in LPS I of *Rhizobium etli* CE3 effected by growth in exudate. *R. etli* CE3 cells were cultured in the absence or presence of exudate obtained from (A) seeds or (B) roots of *Phaseolus vulgaris* cv. Midnight. Sodium dodecyl sulfate (SDS) extracts of the harvested bacteria were subjected to SDS–polyacrylamide gel electrophoresis and the gels were electroblotted. The nitrocellulose blot was cut into strips of paired lanes from treated and untreated cultures and reacted with monoclonal antibodies. The residual material in the gel after blotting was stained with periodate-silver. Only the portions of the blots that reacted with antibodies and the same region of the gel (LPS I band) stained with silver are shown. Less LPS was loaded in this experiment and the conditions of electrophoresis gave less resolution of different LPS I subtypes than in Figures 2 and 4. IB, immunoblots of paired lanes reacted with antibodies JIM26, JIM27, JIM28, and JIM29 (as indicated by the numerals 26, 27, 28, and 29, respectively); in each pair the lane on the left was from untreated cells and the one on the right was from cells grown in the exudate from (A) one plant or from (B) 0.5 plant per ml of culture. PS, periodate-silver staining of the LPS I bands that comigrate exactly with the bands stained in the immunoblot; the equivalent intensity of this staining indicates approximately equal loading of cell contents. (Two control lanes that were not blotted also showed that lanes of treated and untreated cells had almost equal LPS contents [not shown]).

in the presence of either seed exudate (Fig. 4) or root exudate (data not shown), its LPS I displayed the diminished reactivity with antibody JIM 28 that is characteristic of the wild-type strain. The same result was obtained with strain CFNX89, another mutant derived from *R. etli* wild isolate CFN42 that has been cured of the Sym plasmid (Brom et al. 1992).
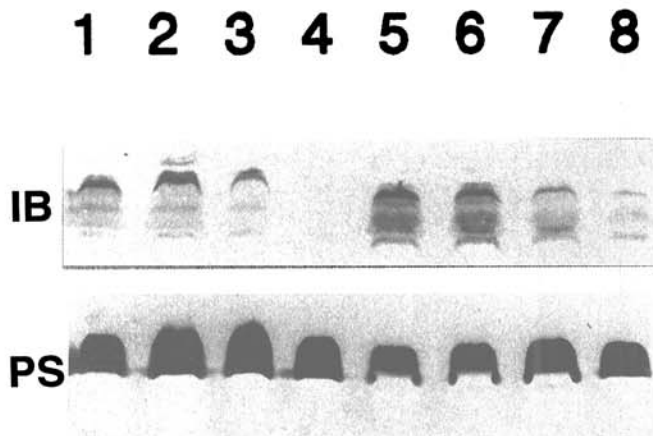
Fig. 2. The concentration-dependent effect of root exudate on *Rhizobium etli* strains CE3 and CE374. Sodium dodecyl sulfate (SDS) extracts of strain CE3 and CE374 grown in various concentrations of root exudate were separated on SDS–polyacrylamide gel electrophoresis, blotted onto nitrocellulose, and the blot was reacted with JIM28. The following portion of the average total exudate from one root was added per ml of culture: zero (lanes 1, 5); 0.005 (lanes 2, 6); 0.05 (lanes 3, 7); 0.50 (lanes 4, 8). Only the portions of the gel and blot containing LPS I are shown. IB, immunoblot reacted with antibody JIM28. PS, periodate-silver staining of residual material in the gel.
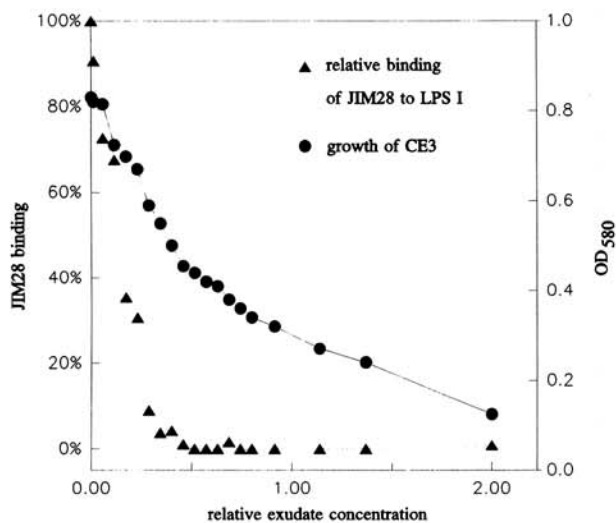
Fig. 3. Concentration dependence of the effects of seed exudate. Various amounts of the exudate were incubated with *Rhizobium etli* CE3 cultures in the standard assay. Before the cultures were harvested for lipopolysaccharide (LPS) analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, growth was measured (by turbidity at 580 nm [OD_{580}]). The extent of LPS antigenic modification (relative binding of antibody JIM28) was measured by image analysis of silver-stained gel bands and immunostained bands on a nitrocellulose blot, as described in Materials and Methods. One unit along the abscissa represents the average amount of exudate from one seedling, in this case having a dry weight of 1.6 mg.

Mutant strain CE374.

Strain CE374 carries mutation *lps-233* in the genetic background of wild-type strain CE3 (Tao et al. 1992). This mutation is in a long stretch of chromosomal DNA (*lps* region α) required for synthesis of LPS I and is the only mutation mapped within this region that does not eliminate or truncate LPS I (Tao et al. 1992; Cava et al. 1990). In the absence of induction, the LPS I of strain CE374 (Fig. 2, lane 5) was somewhat more reactive than the wild type (Fig. 2, lane 1) with antibody JIM28. The CE374 LPS I exhibited partial reactivity with JIM28 after growth in root exudate (Fig. 2, lanes 4 and 8) or seed exudate (data not shown) at a concentration that eliminated binding of this antibody to wild-type LPS I. Likewise, binding of antibody JIM29 to CE374 LPS I is decreased by growth in exudates but not to the extent observed with CE3 (not shown).

In agreement with previous results (Tao et al. 1992), this mutant exhibited deficiencies in symbiosis. It elicited fewer and more widely dispersed nodules, which developed more slowly and had fewer bacteroids than normal. In a representative experiment involving 60 plants per strain, mutant CE374 yielded 19% as many nodules per plant as strain CE3 15 days after inoculation, but after 23 days gave 44% as many as CE3. The mutant-induced nodules after 23 days had 58% as many bacterial CFU as nodules of the same size elicited by the wild type.

Wild-type *lps* region α, carried on low-copy-number recombinant plasmid pCOS109.11 (Cava et al. 1989), was transferred into strain CE374. This transconjugant exhibited the wild-type reactivity with antibodies and response to exudate and had wild-type symbiotic properties (data not shown).

DISCUSSION

The exudate does not act directly on purified LPS or the LPS of bacteria that are not growing. Therefore, as with the modifications induced by low pH and low oxygen (Tao et al. 1992), it appears that *R. etli* LPS modification in response to exudate occurs during growth of the bacteria and probably during de novo synthesis of the LPS. Most likely, the plant compounds trigger modifications in bacterial enzyme activity or gene expression. This interaction between the symbiotic partners therefore differs from one reported by Dazzo et al. (1982), in which capsular material isolated from *Rhizobium trifolii* 0403 or present on heat-killed cells was altered antigenically by exudate from clover roots. Furthermore, this effect of clover exudate was ascribed to plant enzymes, whereas the properties of the exudate material responsible for the changes in *R. etli* LPS are not typical of enzymes.

Bean roots and germinating seeds both release potent inducers of the *nod* genes of *R. etli* and *R. leguminosarum* bv. *phaseoli* (Hungria et al. 1991a, 1991b; Hungria and Phillips 1993). Most of the *nod*-inducing activity from colored seeds is due to anthocyanins, whereas the *nod*-inducers from roots are mainly flavanones and an isoflavone (Hungria et al. 1991a, 1991b). The identity of the inducers of the LPS modification will be the subject of another report, but preliminary analysis indicates that fractions of seed exudate that trigger LPS modification are rich in particular anthocyanins, whereas the active compounds of root exudate appear to be different than not only the seed inducers but also the major root-derived *nod*-inducers (D. M. Duelli, unpublished).

Analysis of the structural change in LPS that accompanies the antigenic change also is underway and will be reported elsewhere. Whereas growth of *R. etli* at low pH reportedly alters the content of two O-methylated LPS sugar residues (Bhat and Carlson 1992), sugar composition determined in the same way, after hydrolysis of the LPS in strong acid at 121°C, has not revealed an obvious change in the LPS of *R. etli* CE3 grown in the presence of seed exudate (K. D. Noel, unpublished). Differences in reactivity toward JIM27 antibodies (Fig. 1) indicate that the structural alteration induced by the root compounds is not identical to that induced by seed exudate. Even at root exudate concentrations five times higher than those tested in Figure 1, there was no change in JIM27 binding (K. D. Noel and V. J. Neumann, unpublished). This result is consistent with different inducers being released by the root and seed.

The induction of the LPS modification does not appear to require any of the Sym plasmid genes, which include all known copies of the gene presumed to be required for *nod* induction, *nodD* (Girard et al. 1991, Vasquez et al. 1991). These results suggest that *R. etli* is capable of responding to exuded plant components by at least one pathway that does not require *nodD*. *nodD*-Independent regulation has been suggested previously in rhizobial responses to plant flavonoids leading to resistance to phytoalexins (Parniske et al. 1991; Kape et al. 1992). In *B. japonicum* 110, even the *nod* genes can be induced (partially) by plant signals in the absence of *nodD* (Sanjuan et al. 1994).

Another difference between the induction of LPS modification and *nod* induction is that *R. etli nod* genes are induced at approximately 100-fold lower concentrations of exudate (Hungria et al. 1991a) than those required to effect changes in LPS. At these higher concentrations, compounds from the seed were inhibitory to growth, but LPS changes were not merely associated with growth retardation. The root exudate and partially purified fractions from the root exudate were not growth-inhibiting at concentrations that resulted in complete antigenic conversion. Coumesterol, a flavonoid found in bean extracts and exudates, inhibits growth of this *R. etli* strain (Eisenschenk et al. 1994) but does not induce a change in LPS (data not shown). The need for relatively high concentrations of inducing compounds may be due to the relatively long period of incubation used in these experiments, during which time the inducers may degrade spontaneously or biotically. It was presumed, but not tested, that the incubation period would have to be long enough to allow substantial dilution of the original LPS with LPS synthesized under inducing conditions. The concentrations needed at a given time to modulate enzyme activity or gene expression might be considerably lower.

In order to assess the symbiotic importance of the subtle modifications of LPS structure induced by exudate and certain other conditions (Tao et al. 1992), mutants specifically unable to undergo these modifications would be very useful. It was suggested previously that strain CE374 might be such a mutant (Tao et al. 1992). Its LPS is recognized by antibody JIM28 after growth under three conditions that in the wild type result in LPS that is not recognized by this antibody. These conditions include low oxygen concentrations, the bacteroid state (Tao et al. 1992), and, as shown here, the presence of bean exudate. However, it turns out that this strain does alter its LPS in response to low oxygen and bean exu-

date, but less oxygen or more exudate is required to eliminate JIM28 binding. Furthermore, even under normal growth conditions, the LPS I of this strain differs from the wild type. It reacts more intensely with antibody JIM28 on immunoblots and is relatively enriched in a minor band that migrates slightly faster than the majority of LPS I (Fig. 2). Therefore, the difference in reactivity with JIM28 and partial symbiotic deficiency of this mutant may be due to a minor structural difference in its LPS that is independent of the response to plant factors. All of the mutant properties probably are due to mutation *lps-233*, because wild-type copies of this DNA restore all aspects of the wild-type phenotype. Aside from the possibility that the mutation may affect something else in addition to LPS and regardless of whether it alters the response to exudate, the properties of this mutant lend support to the idea that subtle differences in LPS structure may have important effects on the ability of *R. etli* to infect its host.

Growth of *Rhizobium fredii* USDA205 in apigenin or soybean root extract results in altered LPS SDS-PAGE banding patterns and LPS sugar composition (Reuhs et al. 1994). It is possible that such changes in LPS in response to host factors may occur in most rhizobia, and they may be important in facilitating the infection of the plant.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Strain CE3 is a streptomycin-resistant, symbiotically proficient derivative (Noel et al. 1984) of wild isolate *R. etli* CFN42 (Segovia et al. 1993). Strain CE144 was isolated by nitrosoguanidine mutagenesis of an erythromycin-resistant, Fix⁺ derivative of CFN42 (Tao et al. 1992). Strain CFNX89, obtained from Susana Brom, is a derivative of strain CFN42 that lacks pCFN42d, the Sym plasmid (Brom et al. 1992). Strain CE374 was isolated by homogenotization in CE3 after localized Tn*5* mutagenesis of plasmid pCOS109.11 (Cava et al. 1990; Tao et al. 1992). Plasmid pCOS109.11 is cosmid vector pLAFR1 into which has been inserted 30 kb of *R. etli* DNA, including the *lps* region α (Cava et al. 1989; Noel 1992). Bacterial cultures were grown in slanted tubes shaken at 250 rpm (Lab-Line orbital shaker 3525, Melrose Park, IL)
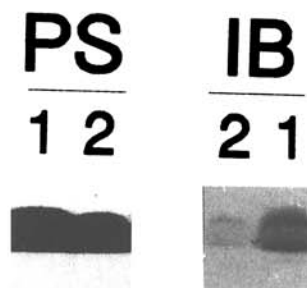

Fig. 4. The effect of bean exudate on Sym-plasmid deletion strain CE144. After growth in the absence (lanes 1) or presence (lanes 2) of seed exudate from *Phaseolus vulgaris* cv. Midnight, sodium dodecyl sulfate (SDS) extracts of CE144 cells were applied to SDS–polyacrylamide gel electrophoresis (PAGE). PS: Periodate-silver staining of the portions of SDS-PAGE lanes containing residual LPS I after blotting. IB: Nitrocellulose blot of these gel regions reacted with antibody JIM28.

at 30°C in TY liquid medium containing tryptone, yeast extract, and calcium chloride (VandenBosch et al. 1985).

Seed exudate.

Unless otherwise indicated, exudate was obtained from *Phaseolus vulgaris* cv. Midnight Black Turtle Soup (Johnny's Selected Seeds, Albion, ME, and AgriSales, Twin Falls, ID). In procedure A (Figs. 3, 4) (modified from that described by Mulligan and Long [1985]), seeds were sterilized by soaking in full-strength chlorine bleach (5% hypochlorite) for 15 min, rinsed 5 times with sterile water, and allowed to imbibe water in 15 ml of sterile water per 70 seeds in a glass dish (14 cm diameter) in the dark at 30°C. Ten milliliters of sterile water was added after 24 h. Two hours after the second addition of water, the dish was shaken at 40 rpm (Bellco Orbital Shaker, Vineland, NJ) for 15 min and the liquid was collected. In procedure B (Fig. 1) (modified extensively from that of Hungria et al. [1991a]), after sterilization and rinsing as described above, 100 seeds in an Erlenmeyer flask were flooded with 50 ml of sterile water, continuously aerated by bubbling with filtered air, and shaken at 30 rpm on a reciprocating shaker (Eberbach, Ann Arbor, MI). Exudate was collected after 24 h, subsequent to adding 50 ml of additional water to compensate for imbibition and evaporation. After collection, the exudate was lyophilized, dissolved in 6.2 ml of water, and 18.6 ml of acetone was added. The precipitate was removed by centrifugation at $17,000 \times g$ at 4°C for 30 min. Acetone was removed from the solution by evaporation in a stream of filtered air and the concentrated solution was lyophilized. The dried material was dissolved in 1.5 ml of water. The exudates from either procedure were sterilized by filtration through 0.8μ/0.2 μ polysulfone PF Acrodiscs (Gelman Sciences, Ann Arbor, MI) and used immediately or stored at −20°C. During all collection steps and storage, the material was covered to protect it from light. Procedure B resulted in substantially higher activity per seed than procedure A.

Root exudate.

After removal of the seed coats, 2-day-old seedlings were rinsed in sterile water before being planted in plastic growth pouches (Vaughan's Seed Co., Downer's Grove, IL). Four beans were planted per pouch containing 25 ml of sterile water for 6 days. Every other succeeding day 10 ml of water was added per pouch. After the sixth day, 10 ml of water was added, the shoots were cut off, the bags were sealed and shaken in an upright position for 1 h at room temperature, and then the liquid in the bags was removed. After filtration through Whatman #1 paper and then nitrocellulose (0.45 μ pore size), the liquid was lyophilized. Water was added to give a solution of the combined exudate from five plants per ml and the solution was sterilized by filtration through 0.8μ/0.2 μ Acrodiscs PF. Mock preparations obtained from pouches that were not planted showed little relative activity unless they had been autoclaved. Therefore, all data shown was from use of pouches that were not autoclaved before planting. Root exudates that caused LPS antigenic conversion also were obtained by growth of plants in plastic containers by a method similar to that of Hungria et al. (1991b). Crude root and seed exudates were tested for microbial contamination before filtration by spreading 0.5 ml on TY agar plates and incubating at 30°. In the rare instances in which 10 or more colonies were detected, the exudate was discarded.

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. The isolation of the hybridoma clones has been described (Tao *et al* 1992). JIM27 and JIM29 antibodies were immunoglobulin class IgG2c; JIM26 and JIM28 were IgM.

Assay of LPS modification.

For tests of the effects of exudate, freshly and fully grown bacteria were diluted 1:50 into 1.5 ml of liquid TY containing a given aliquot of exudate and shaken at 30°C for 18 to 24 h. Pelleted and washed bacterial cells from these cultures were extracted in SDS buffer at 100°C. After centrifugation to remove insoluble debris, the extracts were subjected to SDS-PAGE. LPS in the resolving gel was stained by a periodate-silver treatment modified from that of Cava et al. (1989) by omitting the oxidizer used in protein staining (Elmar Kannenberg and John Kim, personal communication). For Figures 1, 2, and 4, this staining was carried out on residual material in the gel after electroblot transfer to nitrocellulose. After being air dried, nitrocellulose blots were incubated in two additions of TSG (50 mM Tris-HCl, 0.2 M NaCl, and 0.5% gelatin, pH 7.4) for 20 min each and then for 16 to 24 h in TSG containing monoclonal antibodies diluted 1:600, washed 5 times with TSG, incubated in TSG with 1,500-fold diluted anti-rat IgG or anti-rat IgM conjugated with alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) for 4 h, washed 4 times with TSG, and developed for 3 to 5 min in 0.12 g of 5-bromo-4-chloro-3-indolyl phosphate per liter and 0.10 g of nitroblue tetrazolium per liter in 100 mM 2-amino-2-methyl-1,3-propanediol (AMPD) and 1 mM $MgSO_4$ (brought to pH 9.6 with HCl). SDS-PAGE and all subsequent steps were at room temperature.

For quantitation of relative reactivity with JIM28 (e.g., Fig. 3), the washed bacterial pellet from a volume of culture, calculated from its OD_{580} to have cell numbers equivalent to 1.5 ml at an OD_{580} of 0.1, was extracted with 20 μl of SDS sample buffer. Four microliters of each sample was applied to a minigel. Relative intensities of periodate-silver staining of LPS I and the immunostaining by JIM28 in electroblots of parallel lanes were determined with an Ambis Optical Imaging System, using Ambis core software, version 4.20 (Ambis, Inc., San Diego, CA). The relative binding of JIM28 for a given sample was calculated as the ratio of the intensity of immunostaining to the intensity of silver staining.

For a colorimetric immunoassay, bacterial cultures were grown in a 96-well microplate in the presence of exudate and antibiotics to which strain CE3 is resistant (200 μg of streptomycin and 30 μg of nalidixic acid per ml). Bacteria were harvested by centrifugation of the microplate at $1,900 \times g$ for 10 min and washed twice with water. The bacterial pellet in each well was suspended with a toothpick in 11 μl of 10% SDS and, 15 min later, 100 μl of 40% methanol/10% acetic acid was added. After 15 min, the plate was centrifuged and the precipitates were washed once with 100 μl of 40% methanol/10% acetic acid, followed by one wash with 0.05 M sodium carbonate-bicarbonate pH 9.6. The resulting precipitates were incubated under vacuum at 65°C for 1 h. To each well 300 μl of TSG was added and the plate was shaken for 1 h. Monoclonal antibodies diluted 1:200 in TSG were added and

the plate was shaken overnight. After washing the wells 5 times with 100 µl of TSG, 100 µl of anti-rat IgG conjugated with alkaline phosphatase (diluted 1:2,000 in TSG) was added for a 4-h incubation. After 5 washes with TSG, 100 µl of AMPD buffer was added and the turbidity was measured at 405 nm. p-Nitrophenylphosphate was added (to 1 mg/ml) and the absorbance at 405 nm was measured every 5 min for 30 min. Unless specified otherwise, all incubations were at room temperature on a Bellco Orbital Shaker at 40 rpm. Negative controls for subtraction of nonspecific background adsorption of antibodies included wells to which bacteria were not added and wells in which mutant CE367 bacteria were grown. This mutant produces an O-antigen that is not recognized by JIM27, JIM28, and JIM29 antibodies (Tao et al. 1992).

In the dot blot assay, cultures incubated to full growth in the presence of exudate were diluted 1:200 in TY and 100-µl aliquots were applied under vacuum to nitrocellulose in a Biodot apparatus (BioRad Laboratories, Richmond, CA). After the nitrocellulose had air dried, it was processed as described above for the gel blots, except that 0.02% sodium azide was present during the incubation with the first antibody.

Treatment with hydrolytic enzymes.

Before being added to bacteria, the exudate was treated for 2 h with proteinase K (Sigma, 0.3 U/ml) at 37°C, deoxyribonuclease I (from bovine pancreas, Sigma type II, 2.6 U/ml) at 25°C or ribonuclease A (bovine pancreas, Sigma, 2.6 U/ml) at 37°C. Incubations with deoxyribonuclease and proteinase K were buffered with 50 mM Tris-HCl at pH 5.0 and pH 7.5, respectively, and 5 mM $MgCl_2$ was included in the deoxyribonuclease digestion. To show that the enzymes were active in the presence of the exudate, 2 µg CE3 genomic DNA, 4 µg yeast RNA (Sigma, type XI), and 300 µg bovine serum albumin were added to parallel incubations with exudate of deoxyribonuclease, ribonuclease, and proteinase, respectively, and the degradation of these substrates was verified by SDS or urea PAGE.

Nodulation tests.

Nodulation of P. vulgaris cv. Midnight was tested in plastic growth pouches as previously described (Tao et al. 1992 and references cited therein).

ACKNOWLEDGMENTS

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