Isolation and Characterization of Mutants of *Rhizobium leguminosarum* bv. *viciae* 248 with Altered Lipopolysaccharides: Possible Role of Surface Charge or Hydrophobicity in Bacterial Release from the Infection Thread

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Effects of alterations in lipopolysaccharide (LPS) structure of *Rhizobium leguminosarum* bv. *viciae* on effective symbiosis and on a number of cell surface characteristics were studied. Tn5 mutants with altered LPSs were screened for their inability to bind monoclonal antibody 3, one of three monoclonal antibodies to the tentative O-antigenic part of the wild-type LPS of strain 248. Ten class I LPS mutants completely lacked the O-antigen-containing LPS species. The class II LPS mutant had a severely diminished amount of an antigenically altered O-antigen-containing LPS. The class III LPS mutant had normal amounts of an altered, O-antigen-containing LPS. Class I and II mutants, but not the class III mutant, showed abnormal nodule development (i.e., blocked in the stage of bacterial release from the infection thread) resulting in nodules in which very few, at the most, plant cells contained bacteroids and which were unable to fix nitrogen. Class I and II mutants were nonmotile and were more sensitive to hydrophobic compounds than the parent strain. The most striking difference between the symbiotically defective class I and II LPS mutants on one hand and the wild-type strain and the class III mutant on the other hand was that the class I and II mutants have a more hydrophobic cell surface and a higher electrophoretic mobility. A role for an O-antigen-containing LPS in bacterial release from the infection thread, through its effects on general physicochemical cell surface characteristics, is proposed.

Gram-negative bacteria belonging to the genus *Rhizobium* (fast-growing rhizobia) are able to infect roots of plants of the family *Leguminosae*, resulting in the formation of nitrogen-fixing root nodules (for a review, see reference 2).

Lipopolysaccharides (LPS) are a component of the rhizobial outer membrane (8). The composition and part of the structure of rhizobial LPS have been determined for a number of strains of different species and biovars (9, 11). Observation of the properties of isolated LPS, such as interaction with lectins (35), as well as the isolation of noneffective mutants with altered LPSs (10, 23, 25) suggests that the LPSs of rhizobia play a role in the establishment of an effective symbiosis.

The LPS mutants earlier reported (10, 23, 25) were selected in nodulation assays for defects in normal nodule development. To study the role of the bacterial LPS in nodulation by *Rhizobium leguminosarum* bv. *viciae*, we set out to isolate different classes of LPS mutants of strain 248. Instead of selecting mutants defective in nodulation, we directly selected mutants with altered LPSs.

Among a number of monoclonal antibodies (MAbs) against outer membrane antigens of strain 248, three (MAb 3, MAb 16, and MAb 24) reacting with the O-antigenic part of the LPS were identified (11a). In this paper we describe the use of MAb 3 for the isolation of 12 mutants with altered LPS and the effect of the mutations on their symbiotic phenotypes. Since the symbiotic behavior of most mutants was severely altered, we also studied the effects of LPS

alterations on the overall properties of the outer membrane. These studies may be important for establishing the exact role of LPS in symbiosis. LPS alterations appeared to affect growth, motility, sensitivity to hydrophobic compounds, surface hydrophobicity, surface electrokinetic potential, and outer membrane protein profiles.

MATERIALS AND METHODS

Transposon mutagenesis. *R. leguminosarum* bv. *viciae* 248 (18) was randomly mutagenized by transposition of Tn5. For this purpose, the parent strain was crossed with *Escherichia coli* 1830 containing Tn5 on the plasmid pJB4JI (4). Conjugation was performed overnight on nitrocellulose filters on tryptone-yeast (TY) (3) agar plates. Bacteria were suspended in water and plated in appropriate dilutions on B⁻ (31) agar plates supplemented with 200 µg of kanamycin per ml to select for Tn5-containing rhizobia.

MAbs. MAb 3, MAb 16, and MAb 24 were obtained by immunizing mice with a cell envelope fraction of strain 248 and fusing spleen lymphocytes with a myeloma cell line. All three antibodies used here were shown to react with the electrophoretically more slowly moving of the two major LPS species of strain 248, i.e., the O-antigen-containing LPS species (11a).

Mutant screening. Individual kanamycin-resistant colonies were transferred from the selection plates described above onto large yeast-mannitol-B (YMB) (31) agar plates containing 200 µg of kanamycin per ml, and the plates were incubated overnight. Bacteria were transferred to nitrocellulose filters (Ba85; Schleicher & Schuell, Inc., Dassel,

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Federal Republic of Germany) by gently layering the filters over the agar plates and removing them after they were completely wetted. Excess bacteria were washed off with running tap water, and the filters were blocked for 1 h with phosphate-buffered saline (PBS)-Tween (0.1% Tween-20 in 10 mM sodium hydrogen/dihydrogen phosphate–0.9% sodium chloride, pH 7.4) containing 2% dried, defatted milk powder. Subsequently, the filters were incubated for 1 h with MAb 3 in PBS-Tween, washed for 30 min in PBS-Tween, and incubated for 1 h in horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin serum (Nordic, Tilburg, The Netherlands), diluted 1:2,000 in PBS-Tween. After incubation with the second antibody, filters were washed for 30 min in PBS-Tween and developed with 0.015% hydrogen peroxide and 0.06% 3,3',5,5'-tetramethylbenzidine in a buffer consisting of 25% ethanol, 0.2% diethylsulfosuccinate, 3.6 mM citric acid, and 7.7 mM sodium hydrogen phosphate, pH 5.0. Mutants, which did not develop a color in this reaction, were picked up from the YMB plates and purified twice on B⁻ agar containing 200 µg of kanamycin per ml.

Cell envelope isolation and SDS-polyacrylamide gel electrophoresis. For cell envelope isolation, bacteria were grown overnight in TY medium to an A_{620} of 0.2 to 0.5. Cell envelope isolation by sonication and differential centrifugation was performed as described elsewhere (13). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described by Lugtenberg et al. (20). Before electrophoresis, samples of cell envelopes were heated for 10 min at 95°C. Proteins were stained with fast green FCF. For visualization of LPS, samples were heated for 10 min at 95°C, cooled to 60°C, incubated with 0.2 mg of proteinase K per ml for 1 h at 60°C, and diluted 15-fold with sample buffer without β -mercaptoethanol. After electrophoresis, LPSs were stained by silver staining (29).

Western blotting (immunoblotting) and immunodetection of LPS and flagellar antigens. For detection of LPS antigens, cell envelope components, isolated as described above, were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose by electroblotting (28).

For detection of flagellar antigens, cells of TY cultures of parent and mutant strains with an A_{620} of 0.3 to 0.5 were harvested by centrifugation for 20 min at $3,000 \times g$ at 4°C. Culture supernatant was kept apart. Cell-bound flagella were isolated by shearing and ultracentrifugation as described by G. Smit, (Ph.D. thesis, Leiden University, Leiden, The Netherlands, 1988). Sheared cells were suspended in the original culture volume of water and mixed with concentrated sample buffer for electrophoresis. Proteins of sheared cells, culture supernatant, and isolated flagella were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose by electroblotting. Subsequently, the blots were incubated with MAbs or a rabbit antiserum against intact strain 248 cells (11a). The blots were treated further as described above, except that alkaline phosphatase-conjugated rabbit anti-mouse or goat anti-rabbit immunoglobulin serum was used as the second antiserum and color development was done as described by Ey and Ashman (15).

Spot immunoassays. Bacteria were grown in TY medium, harvested, washed once in PBS, and suspended to an A_{620} of 0.3. Drops of 1 µl were spotted onto nitrocellulose and incubated with antibodies and developed as described above.

Nodulation assays. Nodulation ability and nitrogen fixation on *Vicia sativa* and *Vicia hirsuta* plants was tested as

described elsewhere (32). Twelve days after infection, the occurrence of hair curling and infection thread formation was observed by using light microscopy. Nitrogen fixation was assayed 3 weeks after infection.

Testing of sensitivity to antibiotics, detergents, and hydrophobic dye. Sensitivities of parent and mutant strains to SDS, Triton X-100, rifampin, and crystal violet were compared by judging growth of individual colonies on TY agar plates supplemented with twofold increasing concentrations of these compounds.

Motility. Motility of wild-type and mutant strains was determined by judging their abilities to swarm during growth on B⁻ plates containing 0.3% agar.

Cell surface hydrophobicity and electrophoretic mobility measurements. For both measurements, early-stationary-phase cells which had been washed twice in PBS were used. Cell surface hydrophobicity was measured as the contact angle between a drop of 0.1 M NaCl and a bacterial layer collected on a microfilter by procedures described elsewhere (33). Data are the means of six independent measurements. Electrophoretic mobility was measured by laser Doppler velocimetry as described elsewhere (34). Bacteria were suspended in 0.01 M PBS, pH 6.9.

Reisolation of bacteria. Bacteria were reisolated from nodules or nodule-like structures. Root parts were excised from 4-week-old plants and surface-sterilized by immersion for 15 to 30 s in 96% ethanol and subsequently for 2 min in 0.5% HgCl₂–0.5% HCl followed by six washes in sterile water. Nodules were crushed in sterile B⁻ medium, and bacteria were plated on B⁻ agar.

Microscopic studies. Nodules and nodule-like structures were fixed in 2.5% glutaraldehyde and subsequently in 1% osmium tetroxide, both in 0.1 M sodium cacodylate (pH 7.2), at room temperature and embedded in Epon. After being stained by toluidine blue, 6-µm sections were studied by light microscopy. For electron microscopy, ultrathin sections were stained with uranyl acetate and lead citrate by the method of Reynolds (26).

RESULTS

Mutant isolation. Random insertion mutants of Tn5 in the genome of *R. leguminosarum* bv. *viciae* 248 were isolated as described in Materials and Methods. Screening of approximately 1,600 mutant colonies with MAb 3 yielded 12 mutants which did not react with the antibody, most probably because of an alteration in LPS structure. These mutants are listed in Table 1, together with their characteristics, which are discussed below. Analysis by Southern blotting showed that insertion of Tn5 had occurred at different sites (not shown) and that these mutations were therefore induced independently.

LPS profiles of wild-type and mutant strains. LPS profiles of wild-type and mutant strains were compared after electrophoresis of proteinase K-treated cell envelopes and subsequent silver staining. The wild-type profile (Fig. 1, lane 1) consists of two major LPS species. The mutants could be categorized into three classes on the basis of their LPS profiles (Fig. 1). The largest group, having a LPS profile designated class I, consisted of mutants that completely lacked the more slowly moving LPS species (Fig. 1, lane 2). This group consists of mutants RBL1471, RBL1472, RBL1473, RBL1474, RBL1475, RBL1476, RBL1477, RBL1481, RBL1482, and RBL1483. A single mutant, RBL1479, contained a small amount of a more slowly moving LPS species, which has a somewhat higher electro-

TABLE 1. Characteristics of *R. leguminosarum* bv. *viciae* 248 mutants described in this study

Strain	LPS profile class ^a	MAbs 16 and 24 reactions ^b	Culture characteristics ^c	Sensitivity to hydrophobic compounds ^d	Motility	Contact angle ^e	Electrophoretic mobility ^f	Symbiotic phenotype ^g
248	Wild type	+	++++, ****	—	+	31	−1.4	nod ⁺ fix ⁺
RBL1471	I	—	++, *	+++	—	42	−2.8	nod ⁺ fix [−]
RBL1472	I	—	++++, *	++	—	45	−2.9	nod ⁺ fix [−]
RBL1473	I	—	++++, ****	++	—	45	−3.5	nod ⁺ fix [−]
RBL1474	I	—	++++, *	+++	—	48	−3.2	nod ⁺ fix [−]
RBL1475	I	—	++++, **	+++	—	43	−3.2	nod ⁺ fix [−]
RBL1476	I	—	++, ****	+++	—	44	−3.2	nod ⁺ fix [−]
RBL1477	I	—	++++, *	+++	—	45	−3.0	nod ⁺ fix [−]
RBL1481	I	—	++++, ****	+++	—	42	−3.5	nod ⁺ fix [−]
RBL1482	I	—	++++, ****	+++	—	48	−3.6	nod ⁺ fix [−]
RBL1483	I	—	+, ****	+++	—	44	−3.0	nod ⁺ fix [−]
RBL1479	II	+	++++, ****	+	—	42	−2.6	nod ⁺ fix [−]
RBL1478	III	+	++++, ****	—	+	30	−1.3	nod ⁺ fix ⁺

^a See Fig. 1 and text.^b Reaction with MAb 16 and MAb 24 in a spot immunoassay.^c Growth in TY medium (+) and B[−] medium (*): +++++ and ****, wild-type growth rate and stationary optical density; +++ and ***, intermediary growth rate and wild-type stationary optical density; ++ and **, intermediary growth rate and low stationary optical density; + and *, low growth rate and low stationary optical density.^d Sensitivity to SDS, Triton X-100, crystal violet, and rifampin. Symbols: +++, twice as sensitive as wild type to rifampin and SDS, 2.5 times as sensitive to crystal violet, and 4 times as sensitive to Triton X-100; ++, twice as sensitive as wild type to Triton X-100 and rifampin and 2.5 times as sensitive to crystal violet; +, sensitive to crystal violet only.^e Contact angle of a drop of 0.1 M NaCl on a layer of bacteria (degrees). Average standard deviation, $\pm 2^\circ$.^f Electrophoretic mobility in 0.01 M PBS, pH 6.9, expressed as 10^{-8} m/V per s. Average standard deviation, $\pm 0.2 \times 10^{-8}$ m/V per s.^g Nodulation ability (Nod) and nitrogen fixation (Fix) as observed on *V. sativa* and *V. hirsuta*.

phoretic mobility than the wild type has (Fig. 1, lane 3). This LPS profile was designated class II. Another mutant, RBL1478, had a more slowly moving LPS species in amounts similar to that of the wild-type strain, but with an increased electrophoretic mobility compared with both that of the wild type strain and that of the class II mutant (Fig. 1, lane 4). This LPS profile was designated class III.

Reaction with antibodies against LPS of strain 248. The reactions of the three available antibodies directed against wild-type LPS, MAb 3, MAb 16, and MAb 24, were tested in a spot immunoassay on cells of the mutant strains and by immunodetection on Western blots of cell envelope preparations (Fig. 2 and Table 1). Figure 2, lanes 1 show the reactions of MAb3, MAb 16, and MAb 24 with the slowly migrating LPS species of the parent strain 248. Lanes 2, 3, and 4 of Fig. 2 show the remaining reactions of the LPSs of LPS classes I, II, and III mutants, respectively. As could be expected, because MAb 3 was used for mutant selection, none of the isolated mutants reacted with MAb 3. The class I LPS mutants reacted neither with MAb 16 nor with MAb

24, whereas the class II and III LPS mutants still reacted with MAb 16 and MAb 24. These results were confirmed by the spot immunoassays of whole cells and by the observation that MAb 16 and MAb 24, but not MAb 3, were still able to agglutinate class II and III LPS mutants (results not shown). The results indicate that the antigenic determinant for MAb 3 has disappeared in all mutants, whereas the antigenic determinants for MAb 16 and MAb 24 are retained in the mutants with class II and III LPS profiles and are therefore different from that of MAb 3.

Culture characteristics. Growth characteristics of parent and mutant strains were compared in liquid TY and B[−] medium (Table 1). Mutants with class I LPS profiles showed a large variety in growth rates compared with the parent strain, in both media. Most mutants had lower growth rates in one or both media, with generation times up to twice that of the parent strain. The final optical density was often lower than that of the parent strain. However, some mutants had normal growth characteristics, in TY medium (RBL1472 and

FIG. 1. Silver-stained LPS profiles of proteinase K-treated cell envelopes, separated by SDS-polyacrylamide gel electrophoresis. Lanes: 1, wild-type *R. leguminosarum* bv. *viciae* 248; 2, class I LPS mutants; 3, class II LPS mutant; 4, class III LPS mutant.

FIG. 2. Western blots of cell envelope components (separated by SDS-polyacrylamide gel electrophoresis) of *R. leguminosarum* bv. *viciae* 248 (lanes 1), class I LPS mutant RBL1483 (lanes 2), the class II LPS mutant (lanes 3), and the class III LPS mutant (lanes 4), incubated with MAb 3 (A), MAb 16 (B), and MAb 24 (C). Because of the low sensitivity of detection by MAb 24, the reaction with the LPS of the class II mutant is hardly visible (panel C, lane 3).

RBL1477), in B⁻ medium (RBL1476, RBL1481, RBL1482, and RBL1483), or in both media (RBL1473). The mutants with class II and III LPS profiles (RBL1479 and RBL1478, respectively) had normal growth characteristics in both media. When observed by phase-contrast microscopy, TY cultures of class I and II LPS mutants showed a strongly increased tendency to grow in clumps, with very few single cells.

Sensitivity to hydrophobic compounds. To test the effect of LPS alterations on the function of the outer membrane as a permeability barrier for potentially lethal hydrophobic compounds, sensitivity to a number of these compounds was tested (Table 1). All class I LPS mutants, and to a lesser extent the class II LPS mutant RBL1479, were somewhat more sensitive to the tested compounds than the parent strain. The class III LPS mutant RBL1478 showed no detectable changes in any of the tested properties. The growth-inhibiting concentration of SDS was lowered from 0.02% for the parent strain to 0.01% for the class I LPS mutants, with the exception of RBL1474 and RBL1475. The latter two strains, as well as the class II and III LPS mutants, had the same sensitivity as the parent strain. The inhibitory concentration of Triton X-100 was lowered from 0.4% for the parent strain to 0.2% for RBL1474 and RBL1475 and to 0.1% for the other class I LPS mutants, whereas the class II and III mutants were as resistant as the parent strain. The inhibitory concentration of crystal violet was lowered from 1 µg/ml for the parent strain to 0.4 µg/ml for all class I LPS mutants as well as for the class II LPS mutant. The inhibitory concentration of rifampin was lowered from 4 µg/ml for the parent strain to 2 µg/ml for all class I LPS mutants but not for the class II and III LPS mutants.

Motility. When tested on soft-agar plates, neither of the mutants with class I and II LPS profiles was able to swarm, indicating that they are nonmotile. Mutant RBL1479 (class III LPS profile) had normal motility (Table 1).

To define the cause of the defect in motility in the mutants more precisely, we studied the production and localization of the major flagellar protein in cultures of parent and mutant strains. Cultures were separated into the following fractions: sheared cells, flagella isolated by shearing and ultracentrifugation, and spent culture medium. The flagellar protein, with a relative molecular mass of 35 kilodaltons (kDa), was visualized by immunoblotting by using an antiserum against intact cells of the parental strain 248. In an earlier study we had demonstrated that the flagellar protein subunit is a major antigen recognized by this antiserum (11a). Results for the parent strain and mutant strain RBL1479 are shown in Fig. 3A and B, respectively. Most of the flagellar subunit protein is found in the fraction containing the intact flagella, isolated by shearing and ultracentrifugation (Fig. 3, lanes 3) both in cultures of the parent strain (Fig. 3A) and in those of all mutant strains, of which RBL1479 is shown as an example (Fig. 3B). A small amount, possibly representing detached flagella or nonassembled flagellin, was found in the spent culture medium (Fig. 3, lanes 2), and even less was found in the cellular fraction (lanes 1). No differences in localization or production of the 35-kDa flagellar protein were found between any of the mutants and the parental strain. Therefore, assembly of the major flagellar subunit protein into flagella is most probably unaffected by the alteration in LPS structure of the mutants.

Mutant RBL1481 lacks a soluble, cellular 55-kDa antigen of the parent strain. The experiment designed for detection of the flagellar antigen, described above, revealed that one mutant, RBL1481 (Fig. 3C), almost completely lacks a major

FIG. 3. Western blots of components (separated by SDS-polyacrylamide gel electrophoresis) of culture fractions of *R. leguminosarum* bv. *viciae* 248 (A) and its LPS mutants RBL1479 (B) and RBL1481 (C), incubated with antiserum against cell of strain 248. Fractions were applied in amounts that are equivalent to an equal culture volume: sheared cells (lanes 1), culture supernatant (lanes 2), and flagella isolated by shearing of cells (lanes 3). The closed arrow indicates the main flagellar subunit protein; the open arrow indicates the cellular 55-kDa antigen, almost completely lacking in RBL1481.

antigen with an M_r of approximately 55,000, which is detected with the antiserum in the cellular fractions of the parent strain and all the other mutant strains described here (compare lanes 1 of Fig. 3). Earlier experiments have shown that this antigen is located in the soluble fraction (cytoplasm and periplasm) of the cell (14; R. A. de Maagd, unpublished results).

Effects of alterations in LPS structure on membrane protein profiles. To determine the effects of mutations leading to alterations in LPS structure on the overall outer membrane architecture, protein profiles of isolated cell envelopes of parent and mutant strains were compared by SDS-polyacrylamide gel electrophoresis (Fig. 4). Compared with the profile of the parent strain (Fig. 4, lane 1), protein profiles of mutants with class I LPS (RBL1472, shown in lane 2 as an example) and class II LPS (RBL1478, lane 3) show differences in the lower part of the gel. The staining intensity of bands, corresponding to an outer membrane protein with an M_r of 18,000 and, to a lesser extent, an outer membrane protein with an M_r of 22,000 (12), is increased relative to the staining intensity of the other outer membrane proteins, in both types of mutants. This change is not found in strain RBL1479 (class III LPS) (Fig. 4, lane 4). The weak, nondiscrete staining between the two bands mentioned above, in the parent strain and RBL1479 (Fig. 4, lanes 1 and 4), is not visible in the mutants with class I and II LPS profiles (lanes 2 and 3). The position of this region is identical to that of the LPS species with lower electrophoretic mobility in silver-stained gels (not shown). Therefore, this staining, and the disturbance of the protein bands in this region that coincides with it, is probably caused by the presence of the LPS species with low electrophoretic mobility.

Cell surface hydrophobicity and electrophoretic mobility. We have investigated the role of the O-antigenic moiety of the LPS of strain 248 on the overall surface hydrophobicity and electrokinetic surface potential (which represents the

FIG. 4. Protein profiles of isolated cell envelopes of strain 248 (lane 1), RBL1472 (class I LPS) (lane 2), RBL1479 (class II LPS) (lane 3), and RBL1478 (class III LPS) (lane 4). Arrows indicate outer membrane proteins with M_r s of 18,000 and 22,000 (see text).

amount of electrical charge on the surface). This was done by comparing contact angles and electrophoretic mobility of the wild-type strain with those of the mutant strains (Table 1).

The contact angle, which increases with increasing surface hydrophobicity, was substantially increased in mutants with class I and class II LPS profiles. The class II mutant RBL1479 shows an increase of 11° , while in the class I LPS mutants the increase in contact angle varied between 11° and 17° . The class III LPS mutant, RBL1478, gave a contact angle that did not differ significantly from that of the wild-type strain. These results show that (almost) complete absence of the O-antigenic part of the LPS results in a substantial increase of surface hydrophobicity.

Electrophoretic mobility, which increases with surface electrokinetic potential, was significantly increased in all class I and II LPS mutants. Class II LPS mutant RBL1479 showed an increase of 1.4×10^{-8} m/V per s compared with strain 248, while the increase in the class I LPS mutants varied between 1.4×10^{-8} and 2.2×10^{-8} m/V per s. Again, the class III LPS mutant RBL1478 did not differ significantly from the wild-type strain.

Symbiotic phenotypes. Nodulation ability and effectiveness of LPS mutants were compared with those of the wild-type strain by using *V. sativa* and *V. hirsuta* as host plants. The class III LPS mutant, RBL1478, was indistinguishable from the wild-type strain in all aspects. All class I LPS mutants and the class II LPS mutant, RBL1479, had altered symbiotic phenotypes. On *V. hirsuta* roots, they formed small nodules which were unable to fix nitrogen. On *V. sativa*, variable results were obtained. All class I and II LPS mutants were able to form only occasionally a single white nodule on the upper ends of lateral roots. All class I and II LPS mutants induced nodule-like structures, ring-form thickenings on the upper ends of lateral roots. All mutants appeared to be able to induce root hair curling and infection thread formation. Electron microscopy study of nodules or nodule-like structures showed that *V. hirsuta* nodules of class I and II LPS mutants had no or only a few infected cells, indicating a defect in bacterial release. Nodule-like structures on *V. sativa* roots showed meristem induction but

contained no infected cells. Nodules of class III LPS mutant RBL1478 showed a normal morphology.

Closer study by electron microscopy of several *V. hirsuta* nodules showed that, although arrest of normal nodule development occurred approximately at the stage of bacterial release from infection threads, there is variation in the exact stage of arrest. Some nodules contained normally developed infection threads but no or only a few infected, bacteroid-containing plant cells. Some nodule sections showed abnormally developed infection threads (Fig. 5). Among a majority of empty nodule cells, a few cells contained large masses of convoluted infection threads, filled with many bacteria (Fig. 5A). Although most of these infection threads were surrounded by infection thread walls, some release of bacteria in host plant cell cytoplasm occurred (Fig. 5B). However, no infected cells with more than a few bacteroids were detected.

Reisolation of bacteria from nodules. To test the possibility of occurrence of secondary mutations or reversions in the LPS mutants under selective pressure by plant factors during infection, bacteria of strains RBL1471, RBL1473, RBL1481, RBL1483, RBL1479, and RBL1478 were reisolated from nodules or nodule-like structures from *V. sativa* and *V. hirsuta*. Reisolated bacteria had retained the same LPS profiles as the original strains used for infection. Reisolation of RBL1473 and RBL1481 from *V. sativa* was unsuccessful.

DISCUSSION

In this article we have described the isolation and characterization of *R. leguminosarum* bv. *viciae* 248 mutants with altered LPSs. The method that we have used has two advantages. (i) Screening with a MAb against LPS allowed us to screen a large number of mutants in a simple way and to obtain a variety of mutants with altered LPSs, which is supported by the localization of Tn5 in different restriction fragments. (ii) Our method does not preselect mutants with a symbiotic defect. In this way we have obtained 10 mutant strains which all lack the O-antigenic part of the wild-type LPS (class I). This allows the conclusion that the presence of this part of the wild-type LPS of *R. leguminosarum* bv. *viciae* is essential for normal nodule development.

The function of the O-antigenic part of the LPS may be a direct interaction with a plant receptor, such as a lectin, as an essential stage in the establishment of a successful symbiosis (5, 16, 19, 35). That would suggest the presence of a specific, essential structure in the O-antigen part, which would be lacking in our class I and II LPS mutants but present in the class III LPS mutant as well as in the parent strain. The fact that our class III LPS mutant has a normal symbiotic phenotype shows that an intact antigenic determinant for MAb 3 is not an essential feature for normal nodule development. In our opinion, the very large variety in LPS structure of *R. leguminosarum* strains (9, 11) makes it unlikely that such a structure is present.

Motility. Class I and II mutants were nonmotile. This result is consistent with earlier results from our laboratory (Smit, Ph.D. thesis) which showed that selection for nonmotile mutants gives not only flagellum-lacking mutants but also mutants lacking the O-antigenic part of the LPS. Loss of motility in itself does not cause loss of symbiotic effectiveness, as flagellum-lacking mutants are still able to nodulate effectively (Smit, Ph.D. thesis). The results described in this paper show that at least the transport across the cell envelope and assembly of the 35-kDa major subunit into flagella

FIG. 5. Infection stage of nodule cells of *V. hirsuta* with RBL1474 (class I LPS). (A) Part of a nodule cell. In the host cell cytoplasm (PC), large masses of infection threads (IT) are filled with bacteria. N, Host cell nucleus. Bar, 5 μ m. (B) Detail of nodule cell with a few developing bacteroids, close to an infection thread (IT). PC, Host cell cytoplasm; PBM, peribacteroid membrane; BC, bacteroid cytoplasm; ITW, infection thread wall. Bar, 1 μ m.

occurs normally in LPS mutants. Thus, the inability to swim must be a result either of the inability to transport or assemble a minor, though essential, component of flagella or of the inability to propel the otherwise intact flagella. Alternatively, the aggregation observed in class I and II LPS mutants may inhibit motility. Lack of motility as a result of LPS mutations has been described for deep rough mutants of *Salmonella typhimurium* (1) and heptoseless mutants of *E. coli* K-12 (17). However, at least in the first case, this lack of motility was a result of failure to synthesize flagella and therefore is of a nature different from that of the lack of motility in our LPS mutants.

Outer membrane architecture and permeability. Comparison of membrane protein profiles of the parent strain and LPS mutants has shown that the mutations leading to class I and II LPS profiles slightly increase the relative amount of two outer membrane proteins. However, these changes are minor compared with the almost complete disappearance of certain major outer membrane proteins in deep rough mutants of *E. coli* (17, 30) and *S. typhimurium* (1).

The outer membrane lipid bilayer is considered the main permeation pathway for hydrophobic compounds, such as dyes, detergents, and antibiotics, and the presence of lipopolysaccharide in its outer layer is considered an important determinant of resistance to these compounds (21, 24). Deep rough mutants of *E. coli* and *S. typhimurium* are highly sensitive to hydrophobic compounds compared with the parent strain (21, 24). In our experiments, mutants with class I LPS profiles had become two to four times more sensitive to the tested compounds than the parent strain. In our opinion, these results obtained with membrane protein profiles and sensitivity to hydrophobic compounds are not comparable to the gross changes observed in deep rough mutants of other species. However, effects of these slight changes on symbiotic function of the bacterium cannot be ruled out.

Cell surface hydrophobicity and electrokinetic potential. Another surface feature of the rhizobial cell which is apparently dependent on LPS structure is the surface hydrophobicity. All class I and II LPS mutants, which (almost) completely lack O-antigen-containing LPS, have a substantially increased hydrophobicity, whereas the class II LPS mutant remains practically unaltered in this respect (Table 1). This result shows that the sugars of the O-antigenic part of the LPS make the surface of cells of strain 248 more hydrophilic. Differences in hydrophobicity within class I may reflect differences in the structure of the remaining LPS of these mutants. The O-antigenic part of the LPS also seems to shield the negative charges on the outer membrane, giving the wild type and the class III LPS mutant lower absolute surface potentials and thus lower electrophoretic mobilities than those of class I and II LPS mutants (Table 1). Again, differences within class I may reflect differences in the structure of the remaining LPS. Both the changes in surface hydrophobicity and those in surface potential are thus correlated with the loss of (most of) the O-antigen-containing LPS. Similar changes in physicochemical properties of the cell surface in LPS mutants compared with wild-type cells have also been observed in *S. typhimurium* (22, 27). Such changes may well occur upon loss of the O antigen of any rhizobial strain and may be independent of the exact structure of the wild-type LPS. Both properties of the bacterial surface may affect processes during host plant infection which rely on surface contacts and which could be disturbed in the mutant strains. This is particularly interesting because nodule development in the case of class I and II LPS mutants

appears to be arrested in the stage of bacterial release. Bacterial release may be considered endocytosis of bacteria from the infection thread by plant cells and may therefore be heavily dependent on correct membrane interactions. Furthermore, a physical association between peribacteroid membrane and LPS from the bacteroid, which may also be dependent on bacterial surface hydrophobicity, has been demonstrated (6). In this case, the exact chemical structure of the O antigens would be less important. This is consistent with the heterogeneity in chemical structure of rhizobial LPS found by others (9, 11). It is also consistent with the observation by Brink et al. (7) that replacement of O antigens of *R. leguminosarum* bv. *trifolii* ANU843 by those of another strain, or even by unnatural O antigens, restores the wild-type nodulation phenotype.

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