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Root Colonization of Different Plants by Plant-Growth-Promoting *Rhizobium leguminosarum* bv. trifolii R39 Studied with Monospecific Polyclonal Antisera

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Monospecific polyclonal antisera raised against *Rhizobium leguminosarum* bv. trifolii R39, a bacterium which was isolated originally from red clover nodules, were used to study the colonization of roots of leguminous and nonleguminous plants (*Pisum sativum*, *Lupinus albus*, *Triticum aestivum*, and *Zea mays*) after inoculation. Eight weeks after inoculation of soil-grown plants, between 0.1 and 1% of the total bacterial population in the rhizospheres of all inoculated plants were identified as *R. leguminosarum* bv. trifolii R39. To characterize the associative colonization of the nonleguminous plants by *R. leguminosarum* bv. trifolii R39 in more detail, a time course study was performed with inoculated roots of *Z. mays*. *R. leguminosarum* bv. trifolii R39 was found almost exclusively in the rhizosphere soil and on the rhizoplane 4 weeks after inoculation. Colonization of inner root tissues was detected only occasionally at this time. During the process of attachment of *R. leguminosarum* bv. trifolii R39 to the rhizoplane, bacterial lipopolysaccharides were overexpressed, and this may be important for plant-microbe interaction. Fourteen weeks after inoculation, microcolonies of *R. leguminosarum* bv. trifolii R39 were detected in lysed cells of the root cortex as well as in intracellular spaces of central root cylinder cells. At the beginning of flowering (18 weeks after inoculation), the number of *R. leguminosarum* bv. trifolii R39 organisms decreased in the rhizosphere soil, rhizoplane, and inner root tissue.

Rhizobiaceae have the unique ability to induce nitrogen-fixing nodules on the roots or stems of leguminous plants. These symbiotic interactions are host specific (32, 35). Nodule development consists of several stages determined by different sets of genes located both in the host plant and in the bacterial symbiont (10). However, *Rhizobiaceae* also have the ability to form nonspecific associative interactions with roots of other plants without forming nodules (23). Whereas the symbiotic interactions have been studied in detail, not much is known about the associative interactions of *Rhizobiaceae* with different nonleguminous plants.

Associative root colonization has been studied with various nonsymbiotic bacteria, mainly those, such as *Pseudomonas*, *Azospirillum*, or *Bacillus* (known as associative root colonizers), which are able to grow rapidly with easily degradable substrates such as monomeric carbohydrates or organic acids (18, 19). In some cases these associative interactions between plant roots exert growth-stimulating effects (21). Although the extent of plant growth stimulation is not as high by far as it is in symbiotic systems, associative interactions are of great interest because many crops show an increase in yield after inoculation (12). Several mechanisms of plant growth stimulation have been proposed, including the involvement of phytohormones (8, 13, 33), improved supply with limiting nutrients and water (22), and enhanced nitrate reductase activity and nitrogen fixation (5). These bacteria have specific mechanisms to interact with the root surface and/or the interior (3, 11, 24, 26, 34, 36).

The strain *Rhizobium leguminosarum* bv. trifolii R39, isolated from red clover nodules, stimulated the growth of different crops, such as wheat and maize, repeatedly in greenhouse and field experiments (11), probably by auxine and cytokinine production (12). In axenic systems *R. leguminosarum* bv. trifolii R39 colonized not only the rhizoplane but also the cortex and root cap intercellular spaces (36). A rifampin-resistant mutant of *R. leguminosarum* bv. trifolii R39 colonized the rhizospheres of different crops in greenhouse and field experiments and gave significant increases in yield (11, 37).

In situ localization of bacteria by using antibiotic-resistant bacteria was not possible, and the fitness of those mutants in an ecosystem might be reduced. The aim of this study was to localize and quantify the *R. leguminosarum* bv. trifolii R39 wild-type strain in various inoculated leguminous and nonleguminous plants (pea, lupine, maize, and wheat) by using different monospecific polyclonal antisera and immunological techniques (26, 29). Since intensive purification and validation of antisera and antibodies are key prerequisites for the reliable use of immunological methods, parts of these data are also included in this work.

MATERIALS AND METHODS

Bacterial strains. The bacterial strain *R. leguminosarum* bv. trifolii R39 was originally isolated from red clover nodules (11) and characterized as *R. leguminosarum* by 23S rRNA sequencing (data not shown). All other bacterial strains were obtained from the German Collection of Microorganisms (DSM), Braunschweig, Germany, and the Belgian Coordinated Collection of Microorganisms (LMG), Ghent, Belgium. For all cultivation steps NB medium (Merck, Darmstadt, Germany) was used.

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Plant cultivation and bacterial inoculation. *Lupinus albus* cv. Lublanc, *Pisum sativum* cv. Grapis, *Zea mays* cv. Felix, and *Triticum aestivum* cv. Naxo were used for a screening experiment. Seeds of these plants were inoculated with 10^4 *R. leguminosarum* bv. trifolii R39 bacteria per seed. The bacteria were grown overnight in NB medium, washed twice in phosphate-buffered saline (PBS), and resuspended in 0.9% NaCl. Plants were grown in pots with loamy sand (soil I) for 8 weeks under greenhouse conditions with a soil humidity of 40 to 60% water capacity and a temperature of 15 to 22°C during the day and 8 to 12°C at night.

Long-term experiment. Plexiglas containers (height, 30 cm; inner diameter, 14.4 cm) were packed up to a height of 25 cm with air-dried soil from the tilled horizon of a cambisol derived from loess (soil II). The bulk density of the soil was adjusted to 1.35 g cm^{-3}. The containers were sealed at the bottom by a bottom lid carrying a nylon membrane filter (pore size, 0.45 μm). After rewetting of the soil over 10 days by constant irrigation with 300 ml of 0.01 M $CaCl_2$ solution per day, a partial vacuum of 10 kPa was applied at the bottom of the containers to keep unsaturated water conditions in the soil. During the experiment, the containers were irrigated with a syringe twice each day with 0.01 M $CaCl_2$ solution. Twenty milliliters of the irrigation solution was fed in the morning, and 30 ml was fed in the evening. Four seed grains of *Z. mays* were placed in each container. No fertilizer was applied. The temperature was kept at 20 ± 1°C. The illumination, supplied by three lamps (Osram Company, Berlin, Germany; type HQI-E 250 W/D) for 12 h a day, achieved 30 W m^{-2} at the top of the soil surface.

The experiment was carried out over 4 months after seeding. One day after the cotyledons appeared, seeds of these plants were inoculated with 10^5 *R. leguminosarum* bv. trifolii R39 bacteria per seed by the same procedure as described above.

Extraction of bacteria from root material. Rhizosphere soil and roots were separated by shaking the root in PBS for 10 min at 4°C. The rhizosphere soil was separated by a filtration step with a Whatman filter. One gram of washed root or rhizosphere soil was mixed with 10 ml of sterile 0.1% sodium cholate solution and treated ultrasonically (50 W; 7 min). Afterwards, 0.25 g of polyethylene glycol (Boehringer, Mannheim, Germany) and 0.2 g of chelating resin (Sigma, Munich, Germany) were added and incubated for 2 h at 4°C. The suspension was filtrated through a 5-μm-pore-size filter (Millipore, Frankfurt, Germany). The extracted bacteria were centrifuged (5,000 × *g*; 10 min) and fixed in a 4% paraformaldehyde solution at 4°C overnight; this was followed by a centrifugation step and resuspension in carbonate buffer (50 mM; pH 9.6). To determine the number of bacteria in the root tissue, the roots were incubated for 5 min in a 1% chloramine T solution (2) and washed overnight in PBS at 4°C.

Production and purification of the polyclonal antiserum. The polyclonal antiserum pab 200 (raised in 6-month-old female New Zealand White rabbits) was produced by Scholz et al. (30) with whole cells of *R. leguminosarum* bv. trifolii R39, which were killed by heat treatment. The serum was cleaned with a protein A column (Bio-Rad, Munich, Germany) (to give pab $200_{prot\ A}$) and purified from nonspecific antibodies by using a batch incubation system with 5 ml of a washed overnight culture of *Rhizobium meliloti* DSM1021 (optical density at 436 nm = 20) as the antigen and 0.1 mg of pab $200_{prot\ A}$. After 4 h of incubation at room temperature, the bacteria coupled with the nonspecific antibodies were centrifuged (4,000 × *g*; 20 min). The supernatant (pab 200_{pur}) was used for further purification. To obtain a monospecific polyclonal antibody which reacts exclusively with lipopolysaccharides (LPS) of *R. leguminosarum* bv. trifolii R39 (pab 200/I), an affinity chromatography column (Bio-Rad) (7) was prepared by using a total protein extract of *R. leguminosarum* bv. trifolii R39 (27). The unbound antibodies were collected and used for further experiments. To obtain a monospecific polyclonal antibody which reacts exclusively with proteins of *R. leguminosarum* bv. trifolii R39 (pab 200/II), an affinity chromatography column was prepared with an LPS extract of *R. leguminosarum* bv. trifolii R39 (27). The unbound antibodies were collected and used for further experiments.

Immunoassays. Conventional immunoassays were performed in 96-well PVC microtiter plates (Flow, Meckenheim, Germany) as described by Schloter et al. (29) with a peroxidase-coupled antirabbit secondary antibody (Amersham, Braunschweig, Germany) and ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] (Boehringer) as substrate. Quantitative chemoluminescence immunoassays were performed in 96-well white PE microtiter plates (Merlin, Hersel, Germany) with a peroxidase-coupled antirabbit secondary antibody and luminol (Amersham) as a substrate as described by Schloter et al. (29).

Characterization of the antigenic determinants. Outer membrane proteins (OMP) and LPS were obtained from an overnight culture of *R. leguminosarum* bv. trifolii R39 as described by Schloter et al. (27). Two-dimensional (2D) gel electrophoresis (for OMP) was performed with isoelectric-focusing gels (ampholytes, pH 3 to 10) (Sigma) in the first dimension and with sodium dodecyl sulfate-polyacrylamide (10 to 22%) gradient gels in the second dimension. 1D gel electrophoresis (for LPS) was performed with sodium dodecyl sulfate-polyacrylamide (10 to 22%) gradient gels as described by Laemmli (20). The gels were transferred by electroblotting onto nitrocellulose membranes (Bio-Rad) for Western blotting or were stained with silver nitrate (9). Immunodetection on the blotted membranes was performed in combination with peroxidase-coupled antirabbit secondary antibody and with 4-chloro-1-naphthol as the substrate to develop the blots (7).

Epifluorescence microscopy. The antibodies were coupled with the fluorochrome fluorescein isothiocyanate (FITC) (Sigma) as described by Goding (6) and Schloter et al. (28). Staining of roots was performed in petri dishes as described by Schloter et al. (28). For determination of the total microflora, staining with DAPI (4′,6-diamidino-2-phenylindole) was used (1). To prevent fading of the fluorochromes, an antifading reagent containing 100 mg of paraphenylenediamine in 10 ml of PBS (pH 9) and 90 ml of glycerin was used. A confocal laser scanning microscope (LSM 310; Carl Zeiss, Oberkochen, Germany) was used to record optical sections. The instrument was equipped with an Ar ion laser (488 and 514 nm) and an HeNe laser (543 nm). Objective lenses of 40×/1.3, 63×/1.4, and 100×/1.4 were used. The instrument was capable of recording two fluorescence channels simultaneously. A 3D software package made it possible to render roots and bacteria in different angles.

Electron microscopy. Bacterial suspensions of an overnight culture and root segments of the lateral zone were fixed overnight with 3% paraformaldehyde and 0.1% glutaraldehyde buffered in PBS (pH 7.4). After being washed with 50 mM NH_4Cl in PBS, the samples were dehydrated with ethanol up to 80% and embedded in L.R. white resin (The Resin Company, London, Great Britain) with polymerization at 60°C for 24 h. For in situ localization studies, ultrathin cuts were prepared and treated with the polyclonal antiserum and a secondary antirabbit antibody, which was coupled with gold particles (5-nm-diameter) (Amersham) (15). The specimens were examined in a transmission electron microscope (EM10; Carl Zeiss).

TABLE 1. Cross-reactivities of pab $200_{prot\ A}$ and pab 200_{pur} in ELISA with whole cells of different bacteria as antigens

Bacterial strain	Signal strength (OD_{405})[a] with:	
	pab $200_{prot\ A}$	pab 200_{pur}
Rhizobium leguminosarum bv. trifolii R39	++	++
Agrobacterium tumefaciens DSM30205	−	−
Alcaligenes eutrophus DSM516	−	−
Arthrobacter citreus DSM20133	−	−
Acetobacter pasteurianus DSM3509	−	−
Azospirillum brasilense Sp7 DSM1690	−	−
Bacillus polymyxa DSM365	−	−
Burkholderia cepacia DSM50180	+	−
Escherichia coli K-12 DSM423	0	−
Klebsiella pneumoniae DSM30104	0	−
Ochrobactrum anthropi LMG2136	0	−
Paracoccus denitrificans DSM1408	0	−
Rhizobium meliloti DSM1021	+	−
Rhizobium leguminosarum DSM30132	+	−
Rhizobium trifolii DSM30149	+	−
Rhizobium lupini DSM30140	+	−

[a] ++, >2.0; +, 1.0 to 2.0; 0, 0.1 to 1.0; −, <0.1. OD_{405}, optical density at 405 nm.

RESULTS

Characterization of the polyclonal antiserum. (i) Purification and cross-reactions of the antiserum. The cross-reactions of the protein A-purified antiserum (pab $200_{prot\ A}$) with different soil bacteria are shown in Table 1. The antiserum gave a high cross-reactivity in enzyme-linked immunosorbent assay (ELISA) tests with whole cells of other *Rhizobium* strains as antigens. For further purification of the serum, *R. meliloti* DSM1021 was chosen as the antigen. The cross-reactions of the purified antiserum (pab 200_{pur}) were significantly reduced (Table 1). As this serum still contained a mixture of antibodies binding to LPS and proteins of *R. leguminosarum* bv. trifolii R39 (see Fig. 2 and 3), the antiserum was further purified with a total protein extract of *R. leguminosarum* bv. trifolii R39 (to give pab 200/I) or an LPS extract of *R. leguminosarum* bv. trifolii R39 (to give pab 200/II).

To determine a wider range of potential cross-reactivities with other, mainly noncultivatable soil bacteria, pab 200/I and pab 200/II were labeled with FITC and used in situ with root samples from the rhizospheres of noninoculated *P. sativum* and *L. albus* roots. Staining of the total bacterial population was performed with DAPI. For detection, confocal laser scanning microscopy was used. No FITC signal was observed with any of

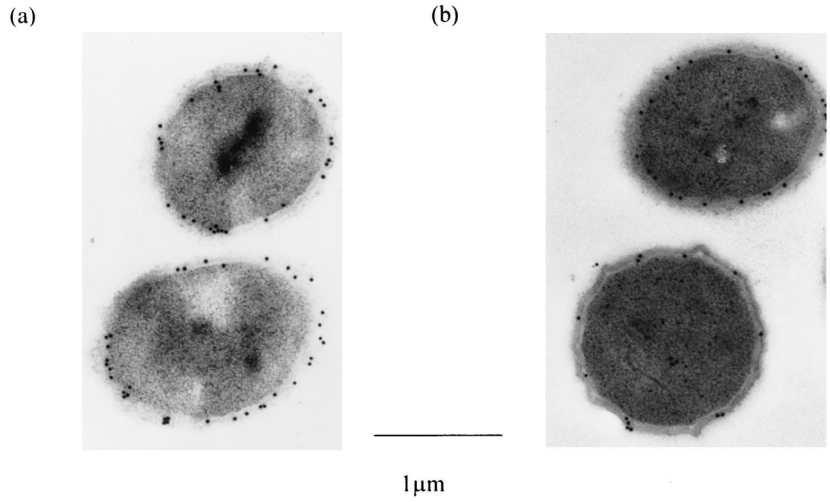

FIG. 1. Localization of the polyclonal antibody epitopes by immunogold labeling of *R. leguminosarum* bv. trifolii R39 cells and TEM (magnification, ×64,000). (a) pab 200/I; (b) pab 200/II.

the noninoculated plants (data not shown). For further experiments, the affinity-purified antisera pab 200/I and pab 200/II were used.

(ii) Characterization of the antigenic determinants. To localize and enumerate the antigenic determinants, an overnight culture of *R. leguminosarum* bv. trifolii R39 was embedded in resin, and ultrathin sections were prepared and treated with pab 200/I and pab 200/II, which were coupled with gold particles. Figure 1a and b show transmission electron microscopy (TEM) pictures of the bacteria with gold-coupled pab 200/I and pab 200/II, respectively. It is obvious that both antisera bind to the outer membrane of *R. leguminosarum* bv. trifolii R39. The numbers of antigenic determinants per cell were similar for the two sera (1,000 epitopes/cell). As a control, *R. leguminosarum* DSM30132 was used in the same way. There was no binding of gold particles observed after treatment with pab 200/I or pab 200/II (data not shown).

To describe the antigenic determinants in more detail, OMP or LPS were separated by electrophoresis. After blotting onto nitrocellulose membranes, incubations with the antisera pab 200_{pur}, pab 200/I, and pab 200/II were performed. The application of pab 200_{pur} gave a signal with an 80-kDa protein and with low-molecular-weight LPS (Fig. 2b and 3b). pab 200/I specifically bound to low-molecular-weight LPS (same signal as in Fig. 3b; no signal with OMP). pab 200/II, in contrast, showed a specific reaction with the 80-kDa protein (same signal as in Fig. 2b; no signal with LPS).

(iii) Validation of the antiserum for a quantitative immunoassay. The validation of the antisera for a quantitative immunoassay is shown in Fig. 4. With an overnight culture of *R. leguminosarum* bv. trifolii R39, quantitative detection of at least 10^4 bacteria/ml was possible with pab 200/I and pab 200/II by using an ELISA based on chemiluminescence. In contrast, the detection limit with pab $200_{prot\ A}$ was about 10^6 bacteria/ml, due to high cross-reactivities.

To use the antiserum for a direct quantification of *R. leguminosarum* bv. trifolii R39 from the rhizosphere, the numbers of antigens per cell surface have to be constant under laboratory conditions as well as in the rhizosphere, because this technique compares the signal of a known bacterial number from an overnight culture with the signal of the bacteria from a root extract. Therefore, axenic maize plants grown in sterilized soil (soil II, autoclaved five times at 2×10^5 Pa for 30 min) were inoculated with 10^8 cells of *R. leguminosarum* bv. trifolii R39. After 3 weeks, the bacteria were reextracted, embedded in resin, ultrathin sectioned, and treated with the antisera coupled to gold particles. Figure 5 shows the location of the immunoreactive species. The number of immunoreactive epitopes

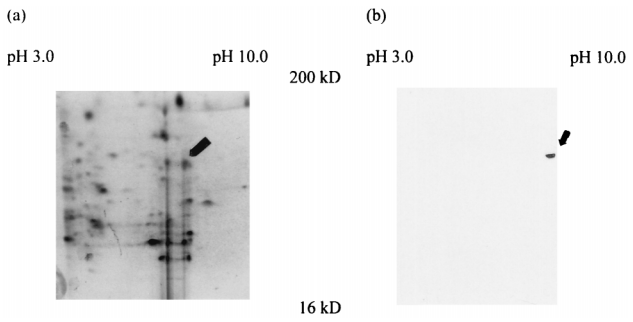

FIG. 2. Biochemical characterization of the antigenic epitopes for the *R. leguminosarum* bv. trifolii R39-specific polyclonal antibody. (a) 2D fingerprint of OMP of *R. leguminosarum* bv. trifolii R39. The gel was stained with silver. The estimated molecular masses of the standard proteins are shown. (b) Western blot of the gel shown in panel a with pab 200_{pur}. For detection, 4-chloro-1-naphthol was used. The 80-kDa protein is marked with arrows.

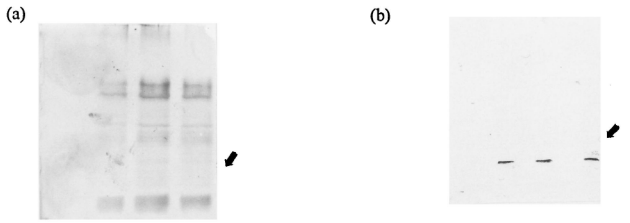

FIG. 3. Biochemical characterization of antigenic epitopes for the *R. leguminosarum* bv. trifolii R39-specific polyclonal antibody. (a) 1D fingerprint of LPS of *R. leguminosarum* bv. trifolii R39. The gel was stained with silver. (b) Western blot of the gel shown in panel a with pab 200_{pur}. For detection, 4-chloro-1-naphthol was used. The low-molecular-weight LPS is marked with arrows.

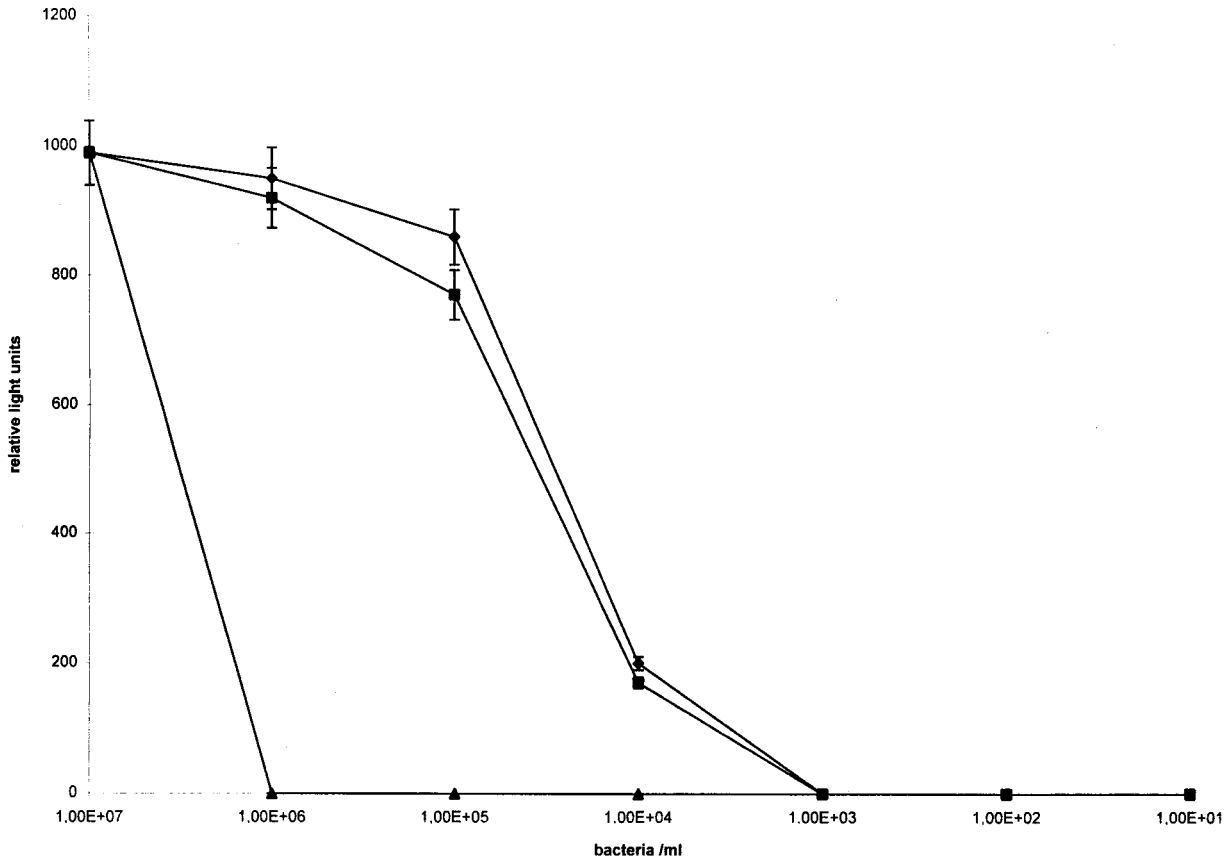

FIG. 4. Validation of pab $200_{prot\ A}$ (▲), pab 200/I (■), and pab 200/II (●) by using a peroxidase-coupled antirabbit secondary antibody and chemiluminescence for the quantification of *R. leguminosarum* bv. trifolii R39. Dilutions of *R. leguminosarum* bv. trifolii R39 were subjected to the immunoassay. The light counts were measured in a microtiter plate luminometer. Error bars indicate standard deviations.

per cell can be obtained from averaging the numbers in many photomicrographs. The antigenic determinants from the reisolates are shown in Fig. 5a (pab 200/I) and b (pab 200/II). The numbers of antigenic determinants per cell surface for the isolated bacteria and bacteria from an overnight culture were identical for pab 200/II (compare Fig. 1b and 5b). In contrast, with pab 200/I (directed against LPS), the number of antigenic determinants per cell surface for the isolates was much higher

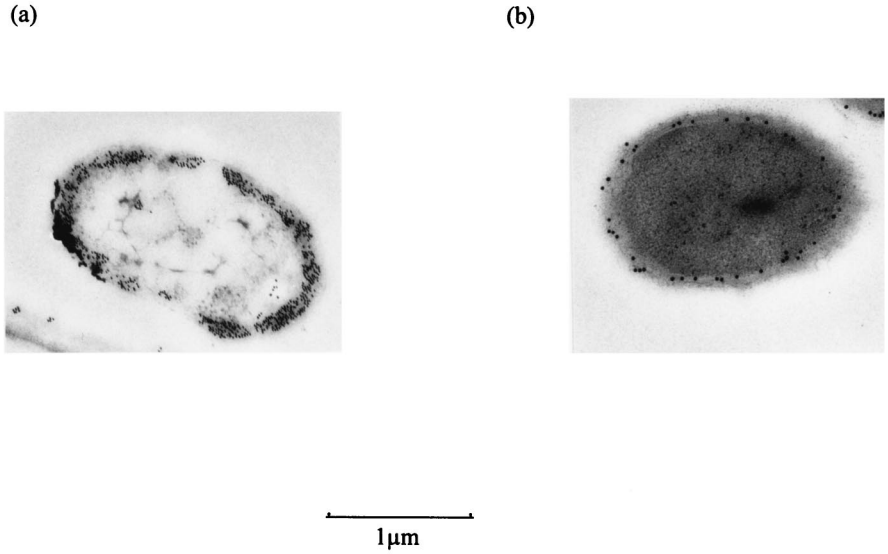

FIG. 5. Comparison of the number of antigens per cell surface of reisolates of *R. leguminosarum* bv. trifolii R39 by immunogold labeling and TEM (magnification, ×65,000) with pab 200/I (a) and pab 200/II (b).

TABLE 2. Colonization of 8-week-old leguminous and nonleguminous plant roots inoculated with *R. leguminosarum* bv. trifolii R39 in greenhouse experiments (soil I)

Plant	Sample[a]	Total no. of bacteria[b] (DAPI counts) in:		Significance level (total bacterial numbers)[c]	No. of *R. leguminosarum* bv. trifolii R39 bacteria[b,d] in:	
		Inoculated plants	Noninoculated plants		Inoculated plants	Noninoculated plants
Lupine	RS	8.5	9.2	**	5.8	<4.0
	R	7.8	8.3	*	5.8	<4.0
	IR	5.9	6.0	NS	4.8	<4.0
Pea	RS	8.3	9.0	**	5.8	<4.0
	R	7.3	8.1	*	5.8	<4.0
	IR	5.8	6.3	NS	4.8	<4.0
Maize	RS	8.0	8.4	*	5.0	<4.0
	R	7.0	7.5	*	5.5	<4.0
	IR	6.1	6.0	NS	4.0	<4.0
Wheat	RS	7.7	8.3	*	5.0	<4.0
	R	6.5	7.0	*	4.5	<4.0
	IR	5.0	6.0	**	<4.0	<4.0

[a] RS, rhizosphere soil; R, washed root; IR, surface-sterilized root.
[b] Results are expressed as log CFU per gram of rhizosphere soil or root and are mean values for three different plants.
[c] Assuming a normal distribution and homogeneous variances, the mean values for three different inoculated and noninoculated plants were tested by the Student-Newman-Keuls test. **, highly significant; *, significant; NS, not significant.
[d] Quantification was with pab 200/II by ELISA.

than that for the bacteria from laboratory culture (compare Fig. 1a and 5a).

Root colonization of different plants. (i) Quantification of root colonization. Root colonization by the inoculated strain *R. leguminosarum* bv. trifolii R39 was quantified by ELISA with pab 200/II. Rhizosphere soil, washed roots, and surface-sterilized roots of *P. sativum*, *L. albus*, *T. aestivum*, and *Z. maize* were examined 8 weeks after inoculation, and the total bacterial population was quantified as DAPI counts. The results are shown in Tables 2 and 3. The total number of bacteria was significantly reduced after inoculation in all three fractions (rhizosphere soil, washed root, and inner root tissue) of all four plant species. The only exception was the inner root tissue of maize, which showed no significant change in colonization of total bacteria after inoculation with *R. leguminosarum* bv. trifolii R39. Overall, the leguminous plants were colonized in higher numbers than the nonleguminous plants (Table 3). According to the ELISA data, all four plant species were colonized by *R. leguminosarum* bv. trifolii R39. The inoculated strain was detected in all three fractions except in the inner root tissue of wheat. The ratio between the number of *R. leguminosarum* bv. trifolii R39 bacteria and the total number of bacteria did not differ significantly in all four plant species.

TABLE 3. Colonization of 8-week-old leguminous and nonleguminous plant roots inoculated with *R. leguminosarum* bv. trifolii R39 in greenhouse experiments (soil I)

Sample[a]	No. of *R. leguminosarum* bv. trifolii R39 bacteria[b] in:		Significance level[c]
	Leguminous plants	Nonleguminous plants	
RS	8.4	7.9	*
R	7.5	6.8	*
IR	5.6	5.6	NS

[a] RS, rhizosphere soil; R, washed root; IR, surface-sterilized root.
[b] Quantification was with pab 200/II by ELISA. Results are expressed as log CFU per gram of rhizosphere soil or root and are mean values for three different plants.
[c] Assuming a normal distribution and homogeneous variances, the mean values for three different inoculated and noninoculated plants were tested by the Student-Newman-Keuls test. **, highly significant; *, significant; NS, not significant.

(ii) In situ localization. To localize *R. leguminosarum* bv. trifolii R39 on the root surface and to estimate the total number of bacteria, confocal laser scanning microscopy was used. *R. leguminosarum* bv. trifolii R39 was specifically labeled with the FITC-coupled pab 200/I, and staining of the total bacterial population was performed with DAPI. Figure 6 shows segments of the root tip, with green-marked *R. leguminosarum* bv. trifolii R39 cells, blue-marked DAPI-labeled bacteria, and red autofluorescence of the root. It was possible to detect *R. leguminosarum* bv. trifolii R39 on the root surfaces of all four investigated plant species, mainly in the root hair zone, forming microcolonies. The ratio between the numbers of bacteria labeled with the antibody and DAPI-stained bacteria was about 1% in all four plant species. This value agrees with the quantitative data obtained.

Time course of root colonization of maize plants. (i) Quantification of root colonization. To characterize the associative colonization of a nonleguminous plant by *R. leguminosarum* bv. trifolii R39 in more detail, a time course study was performed with inoculated maize roots. The inoculum was 10-fold higher than in the experiments described above. Each month bacterial colonization of three inoculated and noninoculated maize plants was subjected to quantitative ELISA with pab 200/II. The data are shown in Table 4. The total numbers of bacteria in the inoculated and noninoculated plants increased significantly during a 14-week period in all three fractions. When flowering occurred (18 weeks after inoculation), the total number of bacteria was decreased compared to that at 14 weeks. *R. leguminosarum* bv. trifolii R39 was detected in all three fractions of maize after inoculation over the 18-week period. The highest numbers were obtained 9 weeks after inoculation. Mainly in the inner root tissue, the number of *R. leguminosarum* bv. trifolii R39 bacteria was high compared to the total bacterial counts. Up to 60% of the bacteria were identified as *R. leguminosarum* bv. trifolii R39. At 18 weeks after inoculation, the numbers of *R. leguminosarum* bv. trifolii R39 bacteria

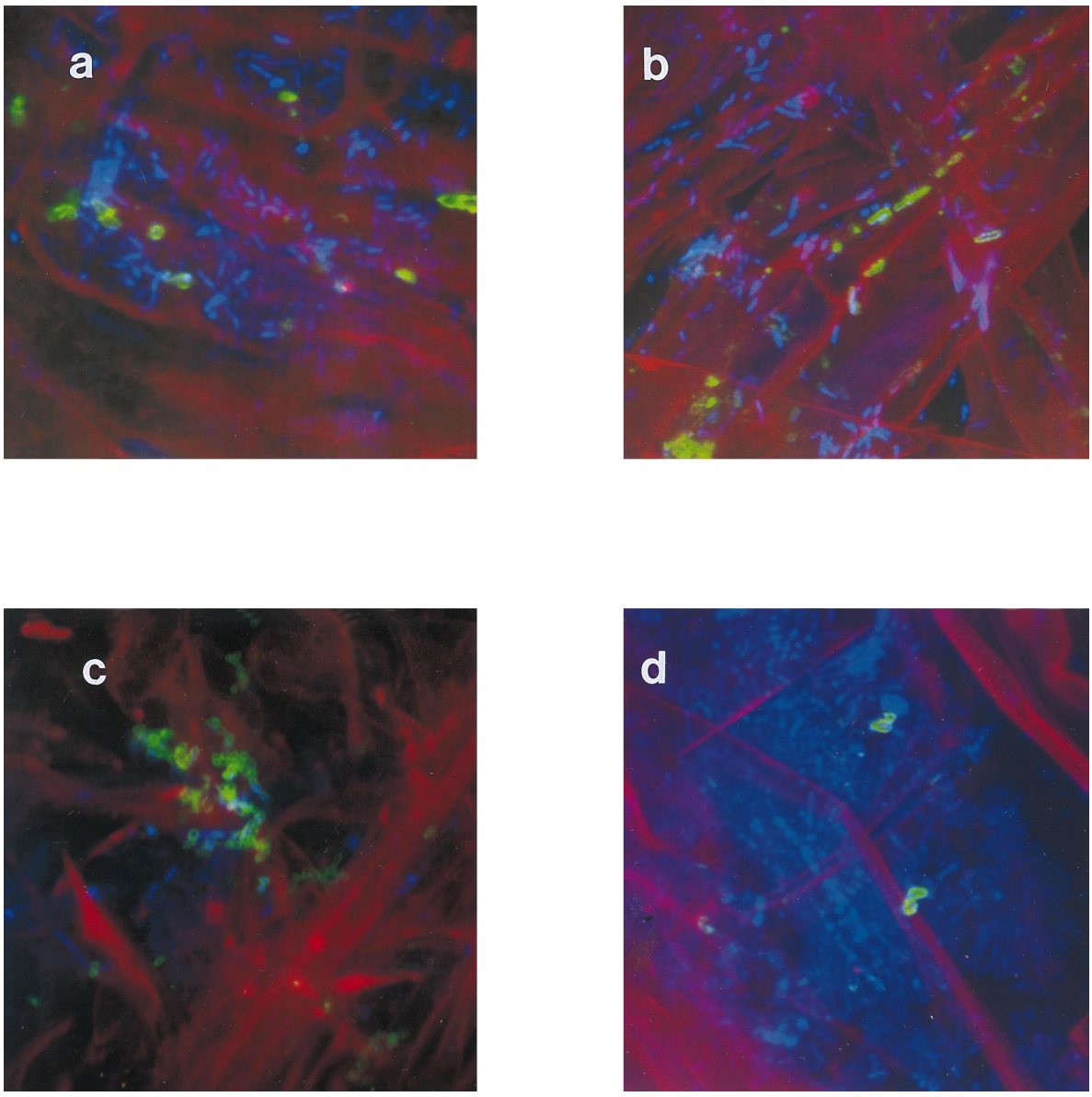

FIG. 6. In situ localization of *R. leguminosarum* bv. trifolii R39 in different 8-week-old inoculated leguminous and nonleguminous plants and autochthone bacteria by using FITC-coupled pab 200/I and confocal laser scanning microscopy. *xy* scan pictures (magnification, ×1,000) of 8-week-old lupin (a), pea (b), maize (c), and wheat (d) root surfaces are shown. The fluorescence of the polyclonal antibody was excited with an HeNe ion laser at 488 nm and detected with a long-pass filter of 515 nm (green fluorescence). The autofluorescence of the root was excited with an Ar laser at 543 nm and detected with a long-pass filter of 590 nm (red fluorescence). DAPI was used for nonspecific staining of bacteria. The DAPI fluorescence (counterstain for total bacteria) was excited with a UV laser at 340 nm and detected with a long-pass filter of 390 nm (blue fluorescence).

TABLE 4. Colonization of inoculated 4-, 9-, 14-, and 18-week-old maize roots by *R. leguminosarum* bv. trifolii R39 and autochthone bacteria in greenhouse experiments (soil II)

Plant development (age [wk], ht [cm], no. of leaves)	Sample[a]	Total no. of bacteria[b] (DAPI counts) in:		Significance level (total bacterial numbers)[c]	No. of *R. leguminosarum* bv. trifolii R39 bacteria[b,d] in:	
		Inoculated plants	Noninoculated plants		Inoculated plants	Noninoculated plants
4, 15, 3	RS	7.0	7.9	*	6.3	<4.0
	R	7.0	8.3	*	5.7	<4.0
	IR	5.0	5.4	NS	4.6	<4.0
9, 40, 5	RS	7.7	8.6	**	5.8	<4.0
	R	7.6	7.9	*	5.8	<4.0
	IR	6.0	6.3	NS	5.7	<4.0
14, 105, 8	RS	8.3	9.0	**	5.3	<4.0
	R	7.4	8.4	*	5.7	<4.0
	IR	6.3	6.4	NS	5.0	<4.0
18 (beginning of flowering)	RS	7.3	7.5	NS	4.5	<4.0
	R	7.7	7.7	NS	4.6	<4.0
	IR	5.0	5.6	NS	4.0	<4.0

[a] RS, rhizosphere soil; R, washed root; IR, surface-sterilized root.
[b] Results are expressed as log CFU per gram of rhizosphere soil or root and are mean values for three different plants.
[c] Assuming a normal distribution and homogeneous variances, the mean values for three different inoculated and noninoculated plants were tested by the Student-Newman-Keuls test. **, highly significant; *, significant; NS, not significant.
[d] Quantification was with pab 200/II by ELISA.

decreased significantly in all three fractions. Compared to the autochtone microflora, the numbers of *R. leguminosarum* bv. trifolii R39 bacteria were less than 1% in all fractions.

(ii) Localization of *R. leguminosarum* bv. trifolii R39 on inoculated maize roots. To verify the high numbers of *R. leguminosarum* bv. trifolii R39 organisms 2 and 3 months after inoculation in the inner root tissue, ultrathin sections of the inoculated maize roots were treated with immunogold-coupled antibodies (pab 200/I). Figure 7 shows ultrathin cuts of a 4-week-old maize root. Most of the labeled bacteria were found in the rhizoplane in close contact to the root. Almost no penetration of *R. leguminosarum* bv. trifolii R39 to the inner root tissue was observed. Marked cells were detected only on the root surface and in lysed epidermal and cortex cells. In contrast, 8 weeks after inoculation, a high number of labeled bacteria were located in the inner root tissue. The bacteria formed microcolonies in intracellular spaces of the central root cylinder and inside cells of the xylem (Fig. 8). Infected cells of the central cylinder were mostly lysed.

Interestingly, the number of gold particles was significantly higher on labeled bacteria located at the root surface than on bacteria found in the root interior (compare Fig. 7 and 8). As pab 200/I binds to LPS of *R. leguminosarum* bv. trifolii R39, LPS was obviously overexpressed during the attachment of *R. leguminosarum* bv. trifolii R39 to the root surface. With pab 200/II, which reacts with an OMP, the number of gold particles per cell surface was constant in cells from the rhizoplane and from the inner root tissue (data not shown).

DISCUSSION

To use immunological methods for the localization and quantification of bacteria in complex habitats, the antibodies must meet at least four quality criteria: (i) no cross-reaction with other bacteria, (ii) stability of the antigenic determinant in situ, (iii) high affinity to the antigen, (iv) localization of the antigenic determinant on the cell surface (for a review, see reference 25). After several purification steps, the antisera pab 200/I and pab 200/II showed no cross-reaction in ELISA with the other bacteria tested. The affinity of pab 200/I and pab 200/II was sufficient for the techniques applied (data not shown). By the immunogold technique, it could be shown that the antigenic determinants of both antibodies are localized on the cell surface. The biochemical characterization of the antigenic determinant showed that both purified antisera were monospecific; pab 200/I reacts with LPS, and pab 200/II reacts with an 80-kDa OMP. The experiments to verify the stability of the expression of these antigenic determinants clearly showed that the antigenic LPS epitope of pab 200/I is overexpressed if *R. leguminosarum* bv. trifolii R39 cells are reisolated from the rhizoplane. In contrast, the OMP as recognized by pab 200/II is expressed in the same amount regardless whether the bacteria are from pure culture or are reisolates from the rhizosphere. As the number of antigens per cell surface is relatively high, the quantitative immunoassay has a detection limit of about 10^4 cells per ml of extract. Similar results were obtained by other groups (for a review, see reference 14).

All of the inoculated leguminous and nonleguminous plants tested were colonized by *R. leguminosarum* bv. trifolii R39. Many authors have described how certain bacteria can colonize a variety of plant roots (19, 21). Quantification data demonstrate that the number of inoculated *Rhizobiaceae* is about 1% of the total bacterial counts 8 weeks after inoculation. Nevertheless, the number of bacteria per gram of root differs significantly among plant species. Leguminous plant roots were colonized more efficiently than nonleguminous plants. Correspondingly, the numbers of *R. leguminosarum* bv. trifolii R39 bacteria were higher in the leguminous plants. It is known that roots of leguminous plants are colonized associatively by different rhizosphere bacteria in higher numbers than are nonleguminous plants (36, 37). The higher root surface area of the leguminous plants or higher root activity and exsudation are possible reasons for the higher colonization.

When plant roots were colonized by *R. leguminosarum* bv. trifolii R39, an antagonistic effect on the autochtone microflora

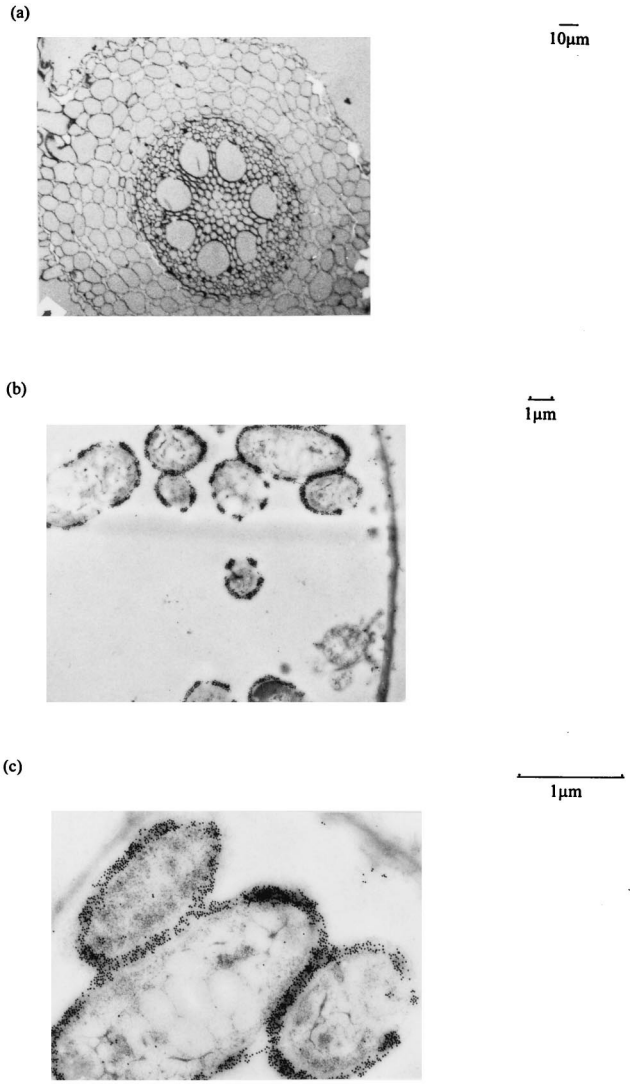

FIG. 7. Localization of *R. leguminosarum* bv. trifolii R39 on the rhizoplane of inoculated maize plants 4 weeks after inoculation with immunogold-labeled pab 200/I. (a) Overview of a section through a maize root (root hair zone) (magnification, ×400). The location of a microcolony of labeled bacteria on the rhizoplane is marked with an arrow. (b) TEM picture of a microcolony of immunogold-labeled bacteria on the rhizoplane (magnification, ×5,700) (same location as marked in panel a). (c) TEM picture of a microcolony of immunogold-labeled bacteria on the rhizoplane (magnification, ×24,000) (same location as that marked in panel a).

was observed in all experiments. So far, nothing is known about the mechanisms of this antagonism.

Between the 4th and the 14th weeks, the numbers of *R. leguminosarum* bv. trifolii R39 organisms increased in all fractions. At the beginning of flowering (18 weeks after inoculation), the number of *R. leguminosarum* bv. trifolii R39 organisms decreased in all fractions. The reduction of root activity during the flowering and maturation phase of the maize plants could be an explanation for the decrease in associated bacteria. A significant shift of *R. leguminosarum* bv. trifolii R39 from the rhizoplane towards the inner root tissue of maize was observed.

LPS seem to play an important role in the initial colonization of plant roots by *R. leguminosarum* bv. trifolii R39, since LPS were overexpressed mainly in the rhizoplane and not in the inner root tissue. Possibly, the attachment process has similarities to the first steps in colonization of *Rhizobiaceae* in symbiotic interaction. First, signal molecules of the host plant root induce the expression of *nod* and *nol* genes in *Rhizobium* in conjunction with the bacterial activator NodD protein. In a second step, LPS Nod factors are produced by the bacterial Nod protein (for a review, see reference 31). The Nod factors induce various plant reactions, such as root hair deformation (35).

R. leguminosarum bv. trifolii R39 was clearly able not only to colonize the rhizoplane but also to penetrate through the endodermis layer and colonize the inner root tissue. Pectinase activity was found in *R. leguminosarum* bv. trifolii R39 by using the cetyltrimethylammonium bromide method of Jayasankar and Graham (16) (not shown). Plant cells which were colo-

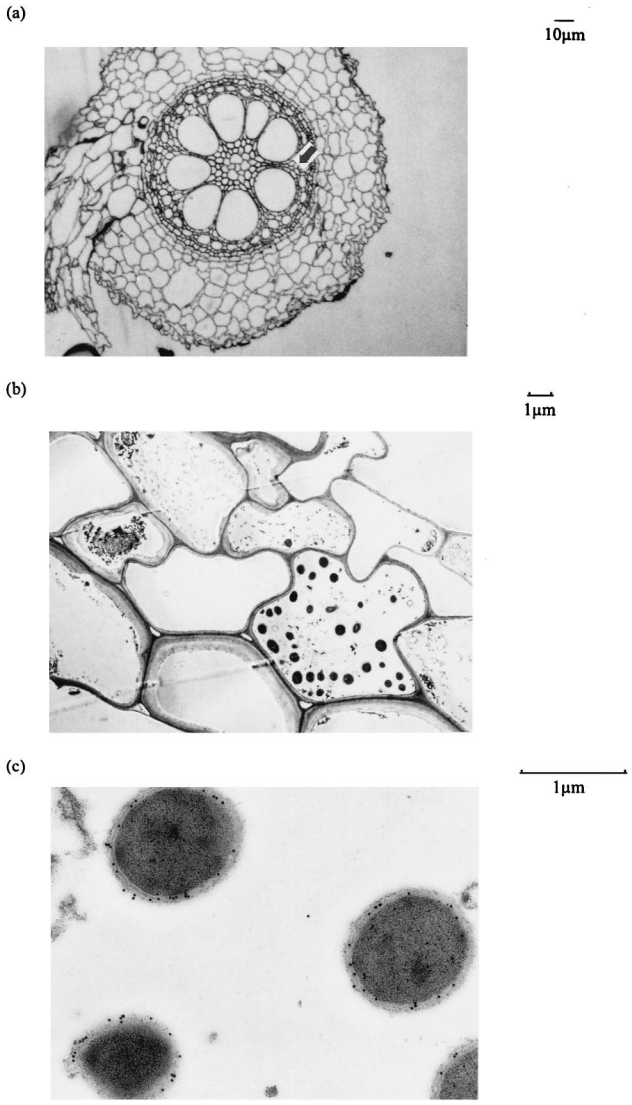

FIG. 8. Localization of *R. leguminosarum* bv. trifolii R39 in the rhizoplane of inoculated maize plants 8 weeks after inoculation with immunogold-labeled pab 200/I. (a) Overview of a section of a maize root (root hair zone) (magnification, ×400). A colonized cell in the xylem is marked with an arrow. (b) TEM picture of a microcolony of immunogold-labeled bacteria colonizing a cell of the xylem (magnification, ×5,700) (same location as marked in panel a). (c) TEM picture of a microcolony of immunogold-labeled bacteria colonizing a cell of the xylem (magnification, ×24,000) (same location as marked in panel a).

nized were mostly lysed. Microcolonies of *R. leguminosarum* bv. trifolii R39 were also found in intracellular spaces, which is known to occur with many other endophytic bacteria (17). This is remarkable, because only close contact of bacteria with the plant root surface or inner root tissue provides reproducible plant growth stimulation effects. So far it is not known if *R. leguminosarum* bv. trifolii R39 is able to invade the plant shoot, as has been shown for plant-endophytic bacteria like *Herbaspirillum* spp. and *Acetobacter diazotrophicus* in sugar cane (4).

This work clearly indicates that *R. leguminosarum* bv. trifolii R39 is able to colonize efficiently plant roots of leguminous and nonleguminous plants. It is able to compete successfully with the autochtone microflora on the root surface. Since these interactions show plant-growth-stimulating effects (12), more interest should be focused on these plant-bacterium associations. Especially, environmental factors which influence plant-microbe interactions as well as the effect of inoculation on the autochtone microflora of the rhizosphere should be considered in more detail.

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