JOURNAL OF BACTERIOLOGY, Sept. 1989, p. 4583–4588
0021-9193/89/094583-06$02.00/0

Vol. 171, No. 9

Immunogold Localization of the NodC and NodA Proteins of *Rhizobium meliloti*

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Received 27 January 1989/Accepted 1 June 1989

Monospecific, polyclonal antibodies to the *nodC* and *nodA* gene products of *Rhizobium meliloti* were used in combination with immunogold labeling and transmission electron microscopy to localize the NodC and NodA proteins in cultures of *R. meliloti*. Both NodC and NodA were detected in the cytoplasm and cell envelope in thin sections of free-living rhizobia treated with luteolin, a known inducer of *nod* gene expression; however, only NodC was detected on cell surfaces when immunolabeling was performed with intact induced cells. In view of biochemical data characterizing NodC as an outer membrane protein with a large extracellular domain, the pattern of immunolabeling on thin sections suggests that NodC is produced on free cytoplasmic ribosomes prior to assembly in the membrane. The pattern of NodA labeling on thin sections is consistent with biochemical data detecting NodA in both soluble and membrane fractions of NodA-overexpressing strains of *R. meliloti*.

Bacteria of the genera *Rhizobium* and *Bradyrhizobium* have the special ability to stimulate the formation of nitrogen-fixing root nodules in certain plants, primarily legumes. Some of the genes involved in nodulation (*nod* genes) are physically and functionally conserved among different species of *Rhizobium* and *Bradyrhizobium* and are thus referred to as common *nod* genes (4, 7, 10, 15, 16, 19). *nodA*, *-B*, and *-C* are common *nod* genes essential for root hair curling and initiation of nodule formation (5, 14, 22, 24). Expression of the *nodABC* operon is inducible by plant-secreted flavonoid compounds (11, 17, 23). For *Rhizobium meliloti*, the inducing compound is luteolin, a flavone exuded by the host plant alfalfa (20).

Recent studies have begun to characterize the products of common *nod* genes in an effort to understand their biochemical roles in nodulation. A combination of genetic, biochemical, and physiological methods has shown NodC to be a 46.8-kilodalton (kDa) dimeric, integral outer membrane protein with receptor like structure (12, 13), while the 21.8-kDa NodA protein may be a soluble polypeptide (25) involved in synthesis of a diffusible plant growth factor (26). The use of immunocytochemistry to localize nodulation gene products has not previously been reported, although this approach has been employed effectively for the localization of other nodule constituents, including the oxygen-binding protein leghemoglobin (21), the nodule-specific enzyme uricase (18, 28, 29), and certain components of the peribacteroid and plasma membranes of infected cells (2, 3, 8). In the work reported here, we used immunogold labeling with monospecific, polyclonal antibodies directed against the NodA and NodC proteins to localize these antigens in luteolin-induced *R. meliloti*.

MATERIALS AND METHODS

Bacteria and conditions for induction of nodulation genes. Cultures of *R. meliloti* 1021, used because NodC is expressed approximately 40 times more than in most other wild-type strains (13), were grown in M9 salts supplemented with 0.2% casamino acids and 0.4% glycerol (25). At an OD_{600} of 0.2, 10 μM luteolin was added for 20 to 24 h at 28°C to induce the expression of *nod* genes. Cells attained an OD_{600} of approximately 0.5 by the end of the induction period.

Antibodies. Preimmune serum and polyclonal anti-NodA and anti-NodC antibodies were a generous gift from M. John and J. Schmidt (Max-Planck-Institut für Züchtungsforschung, Cologne, Federal Republic of Germany). Antibodies were isolated from rabbit antiserum and purified by affinity chromatography on antigen-coupled Sepharose (13, 25). Specificity of antibodies was demonstrated by immunoprecipitation of labeled proteins (12, 25) and Western immunoblot analyses of nodule tissue and fractionated *R. meliloti* cells (13, 25). For prolonged storage, antibodies were filter-sterilized, frozen in liquid nitrogen, and kept at −70°C.

Immunogold labeling. For postembedding labeling on thin sections of bacteria, cell pellets were treated with 2.5 to 3% glutaraldehyde in 50 mM potassium phosphate buffer, pH 6.8, for 1 h, followed by postfixation for 1 h in 2% osmium tetroxide in the same buffer. Cells probed for NodC were dehydrated in a graded ethanol series and embedded in LR White resin, which was superior to other resins for detection of this protein. Bacteria probed for NodA were embedded in Spurr resin after dehydration in a graded acetone series. Immunogold labeling was carried out by the procedure of VandenBosch (28) and VandenBosch and Newcomb (29). Thin sections on nickel grids were incubated on drops of specific antibody for 1 to 2 h at room temperature, followed by incubation on drops of protein A-gold (15 nm diameter; Janssen Life Sciences Products, Olen, Belgium) for 1 h. Experimental controls included substitution of preimmune serum or buffer for specific antibody. All sections were poststained with 2% aqueous uranyl acetate for 20 min.

For localization of antigens on whole cells, cultures grown to log phase were first washed with 0.5 M NaCl to remove exopolysaccharides that could interfere with binding of antibodies to surface antigens. Immunogold labeling was then carried out by the protocol of Fuerst and Perry (9), except that specimen grids were coated with Formvar and carbon. In place of specific antibody, experimental controls were treated with preimmune serum or phosphate-buffered saline containing 2% bovine serum albumin. Grids from all experiments were observed and photographed in a Hitachi

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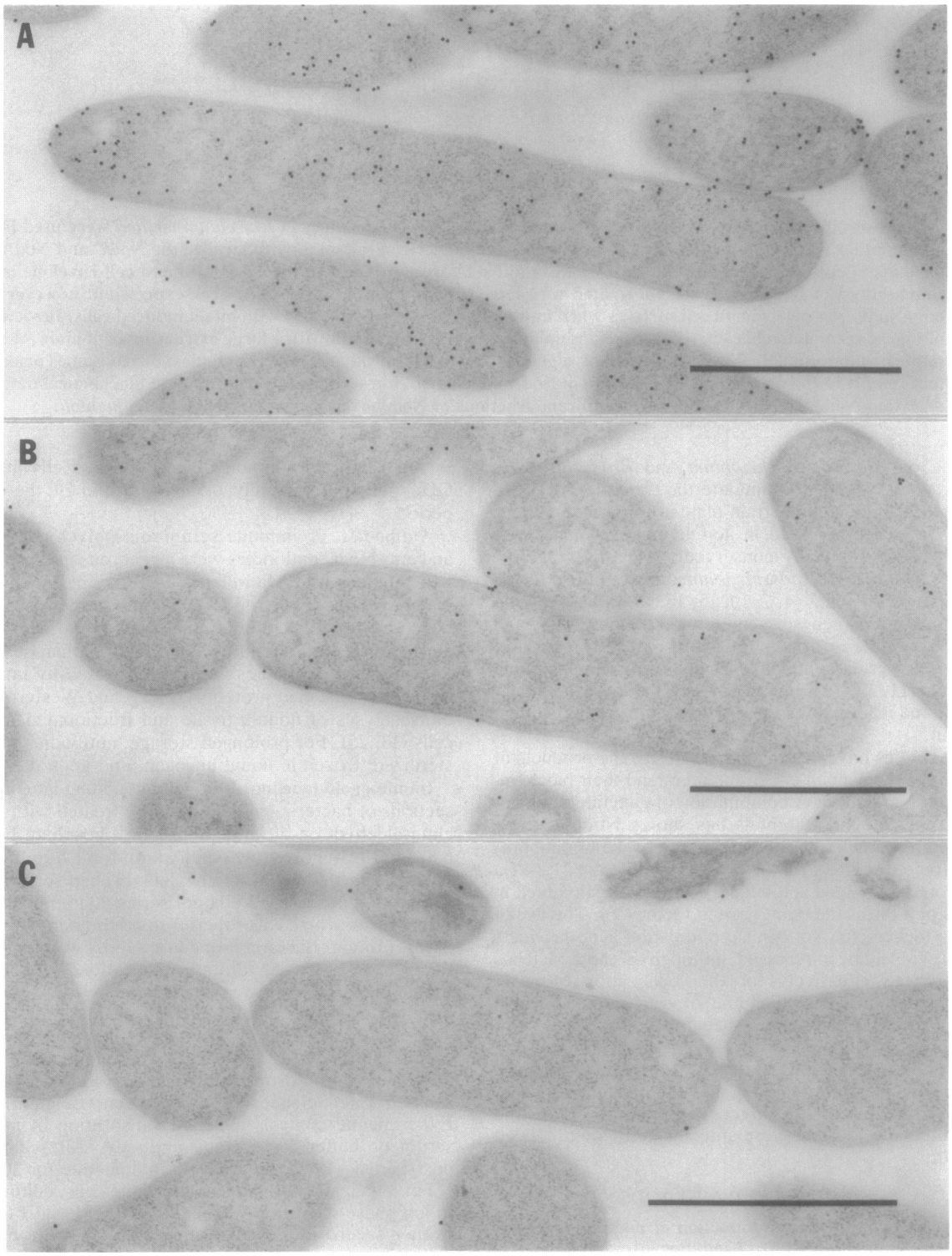

FIG. 1. Localization of the *nodC* gene product on thin sections of *R. meliloti* embedded in LR White resin. (A) Cells induced with luteolin were labeled with anti-NodC antibody followed by protein A-gold (15 nm diameter). (B) Uninduced cells were grown concurrently with those in panel A and immunolabeled in the same manner. (C) As a control, induced cells were incubated with preimmune serum and protein A-gold. Bars, 1 μm.

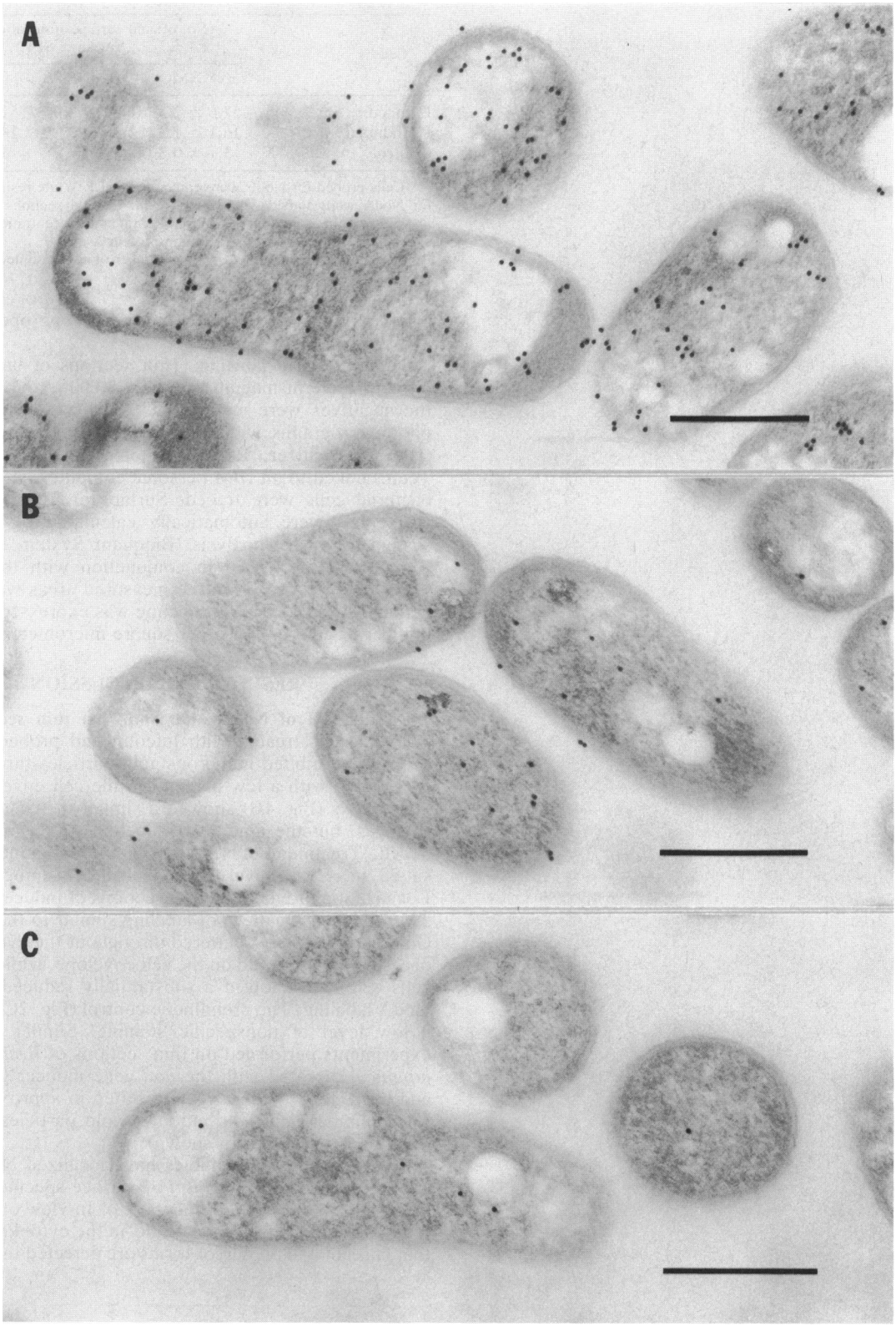

FIG. 2. Localization of the *nodA* gene product on thin sections of *R. meliloti* embedded in Spurr resin. (A) Luteolin-treated cells were labeled with anti-NodA antibody followed by protein A-gold. (B) Uninduced cells grown in parallel with induced cells were labeled as in panel A. (C) As a control, induced cells were treated with preimmune serum and protein A-gold. Bars, 0.5 μm.

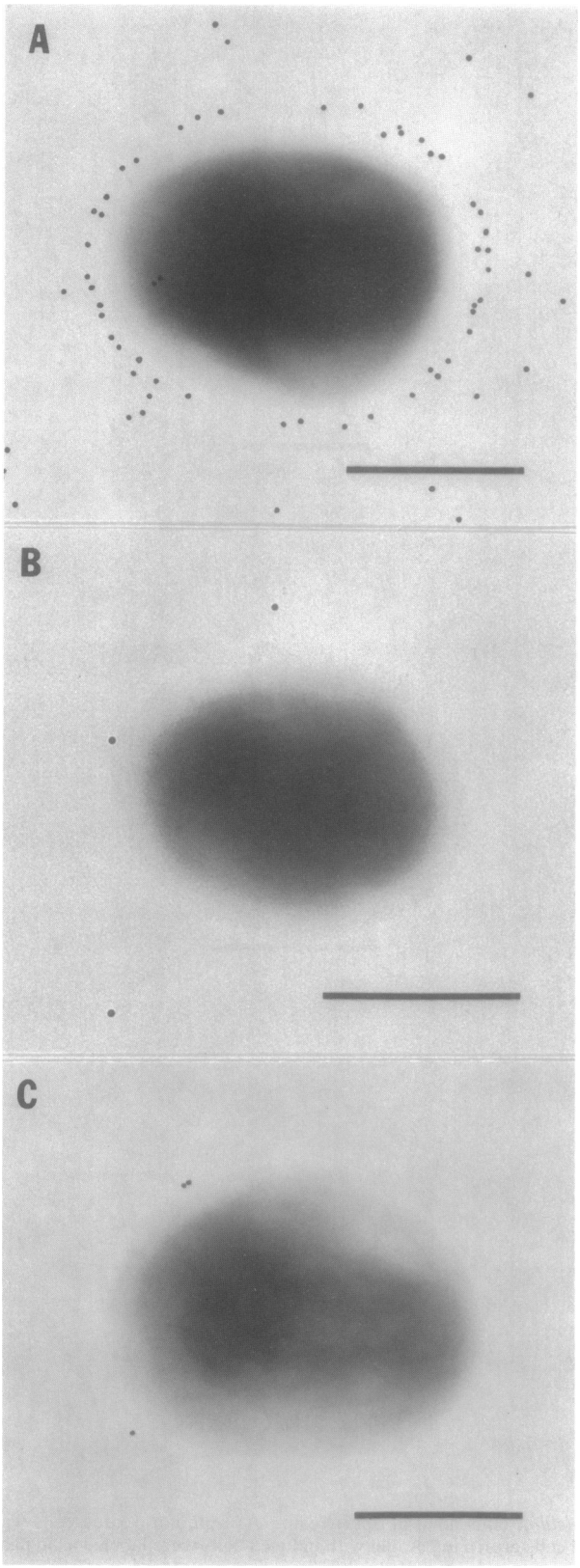

TABLE 1. Density of immunogold labeling of NodC and NodA on thin sections of *R. meliloti*

Cells[a]	No. of gold particles/μm^2 of cell profile (mean ± SE)[b]	
	NodC	NodA
Induced	57.6 ± 2.3 (81)	51.9 ± 2.7 (64)
Uninduced	16.1 ± 1.2 (82)	14.7 ± 1.1 (56)
Control	3.7 ± 0.3 (97)	3.4 ± 0.5 (55)

[a] Cells probed for NodC were embedded in LR White resin. Cells probed for NodA were embedded in Spurr resin. Control sections of induced *R. meliloti* were incubated on preimmune serum at a concentration equal to that of the antiserum and then treated with protein A-gold.

[b] The number in parentheses is the number of cells counted.

(Tokyo, Japan) H-600 electron microscope operated at 75 kV.

Quantitation of labeling. Thin sections of bacteria were photographed at magnifications of 8,000× to 17,000×, and the negatives were enlarged to 20,000× to 43,000×. The photomicrographs were then placed on a digitizing tablet (HIPAD Digitizer; R & M Biometrics, Inc., Nashville, Tenn.) linked to an IBM personal computer, and profiles of bacterial cells were traced. Surface areas (in square micrometers) were automatically calculated by a software system for image analysis (Bioquant System IV; R & M Biometrics, Inc.) used in conjunction with the digitizing tablet. Gold particles on the measured areas were counted manually, and density of labeling was expressed as average number of gold particles per square micrometer.

RESULTS AND DISCUSSION

Localization of NodC and NodA on thin sections of *R. meliloti*. Cells treated with luteolin and probed for NodC (Fig. 1A) exhibited numerous gold particles throughout the cytoplasm, with a few present on the cell envelope. Uninduced cells (Fig. 1B) showed a similar distribution of gold particles, but the amount of labeling was significantly reduced. Treatment of sections with preimmune serum resulted in a low level of nonspecific staining (Fig. 1C). Localization of NodA on thin sections of induced cells (Fig. 2A) produced a pattern of labeling similar to that of NodC. Gold particles were scattered throughout the cytoplasm and occasionally appeared on the cell envelope, while uninduced cells (Fig. 2B) showed a substantially reduced amount of NodA labeling. The preimmune control (Fig. 2C) resulted in a low level of nonspecific staining. Similar localization experiments performed on thin sections of *Bradyrhizobium japonicum* treated with the *nod* gene inducer genistein (1) and probed for both proteins resulted in approximately the same number and distribution of gold particles as seen in induced *R. meliloti* (not shown).

Recent biochemical studies have localized NodC on the surface of induced *R. meliloti* by surface-specific radiolabeling and proteolysis experiments (13). In view of these data, immunogold localization of NodC in the cytoplasm suggests that nascent and precursor forms are detected there and that

FIG. 3. Immunogold labeling with anti-NodC or anti-NodA antibodies on whole cells of luteolin-induced *R. meliloti*. (A) NodC labeling. (Results more representative of allover surface labeling are not shown because electron density of cells obscured the gold particles.) (B) NodA labeling. (C) Labeling after treatment with preimmune serum and protein A-gold. Bars, 0.5 μm.

NodC is translated on free cytoplasmic ribosomes prior to assembly in the outer membrane. Localization of NodA in the cytoplasm and cell envelope is consistent with biochemical studies reported by Egelhoff and Long (6), in which the *nodA* product was detected in both membrane and soluble fractions of NodA-overproducing strains of *R. meliloti*. There is no biochemical or other evidence that NodA is a surface antigen, and therefore the immunogold data suggest that NodA is translated on free ribosomes in the cytoplasm and then associates with a part of the cell envelope interior to the extracellular face of the outer membrane.

The density of gold particles was roughly equivalent in each case of NodC and NodA labeling (Table 1). The approximately fourfold increase in labeling of luteolin-treated cells compared with uninduced cultures relates favorably to results from induction studies with *lacZ* fusions to *nod* genes. For example, Mulligan and Long (17) found a two- to threefold increase in expression of a *nodC-lacZ* fusion after exposure of cells to plant exudate. Labeling of NodA or NodC in uninduced cells was approximately four times greater than background labeling in the preimmune control (Table 1), a result that may reflect low-level constitutive expression of *nodA* and *nodC* and thus demonstrates the sensitivity of the immunogold technique.

Localization of NodC on the surface of whole cells. Whole-mount immunogold labeling was used to probe for surface antigenic sites by using antibodies to the *nodC* and *nodA* products. As a result, NodC was localized at the surface of intact, induced *R. meliloti* (Fig. 3A), while NodA was not detectable as a surface antigen (Fig. 3B). These results support genetic and biochemical data which have characterized NodC as a transmembrane protein with a large extracellular domain (12, 13, 25). Localization of NodC, but not NodA, as a surface antigen also correlates with published experiments in which only anti-NodC antibodies were capable of inhibiting nodulation when added to *R. meliloti* cells upon inoculation of alfalfa (12, 25). The relatively low level of detection of NodC as a surface antigen in both immunolabeled whole cells and thin sections may reflect its actual distribution or, alternatively, a lack of affinity of the antibody to the native, multimeric form of the protein (13). For example, Tommassen et al. (27) have shown that immunogold localization of the inducible outer membrane protein PhoE of *Escherichia coli* requires antibody directed against mature PhoE trimer.

In conclusion, elucidation of the functions of *nod* gene products will be advanced by the knowledge of their precise cellular locations. The extremely sensitive method of immunogold labeling was used to localize the gene products of two common nodulation genes, *nodC* and *nodA*. The immunogold data support and extend previous biochemical characterization of NodC as an integral outer membrane protein and suggest that its translation occurs on cytoplasmic ribosomes. While NodA was not detected on cell surfaces in these experiments, results indicate that some NodA protein associates with an interior part of the cell envelope after translation in the cytoplasm. Recent evidence suggests that the *nodAB* gene products are involved in synthesis of a phytohormonelike factor capable of stimulating plant cell division (26). Location of NodA at the interior membrane surface would facilitate transport of the factor from the cell. The work reported here demonstrates the utility of the immunogold technique for subcellular localization of nodulation gene products and suggests future applications of the method for localization of other Nod proteins, studies which could be instructive for elucidating their biochemical functions.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grants from the National Institutes of Health (GM 33494 to G.S. and E.R. and GM 40183 to G.S.) and the Department of Agriculture (87-CRCR-1-2512 and 86-CRCR-1-2120 to G.S.).

We thank Jürgen Schmidt and Michael John for their generous gift of antibodies and valuable comments on the manuscript and Dick Williams, Mary DeBram, and Steve Burman for excellent technical assistance. Special thanks to Dominique Goyeau for providing *R. meliloti* cultures.

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