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IMMUNOGOLD LOCALIZATION STUDIES OF TWO NODULATION GENE PRODUCTS OF *RHIZOBIUM MELILOTI*

A Thesis

Presented for the

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Degree

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Deane L. Johnson

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ABSTRACT

Monospecific, polyclonal antibodies to the *nodA* and *nodC* gene products of *R. meliloti* were used in combination with immunogold labeling and transmission electron microscopy to localize the NodA and NodC proteins in cultures of *R. meliloti* and in mature nodule tissue of alfalfa and soybean plants. 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. Only NodC was detected on the surfaces of cells when immunolabeling was performed with intact induced cells. In view of biochemical data characterizing NodC as an outer membrane protein, the pattern of immunolabeling on thin sections suggests that NodC is produced on free cytoplasmic ribosomes prior to assembly in the membrane. NodA and NodC were both detected in infection threads of mature alfalfa nodules. In similar experiments with nodule tissue from soybean, these proteins were localized in bacteroids; however, quantitation of labeling showed that peroxisomes in uninfected cells were the most heavily labeled structures in thin sections probed for NodC. Evidence was also presented to demonstrate detection of NodA in peroxisomes of soybean nodules.

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I. INTRODUCTION

Bacteria of the genera *Rhizobium* and *Bradyrhizobium* have the special ability to form nitrogen-fixing symbioses primarily with plants of the legume family. Establishment of the symbiosis involves specific interactions between a particular species of *Rhizobium*/*Bradyrhizobium* and a compatible host plant. *Rhizobium meliloti*, for example, infects only alfalfa (*Medicago sativa*) and a few related plants. A successful symbiosis is characterized by the formation of a unique plant organ, the root nodule. Nodulation is a morphologically complex process which involves the regulated expression of genes from both plant and bacterium. Among the *Rhizobium*/*Bradyrhizobium* genes which have been shown to be essential for nodule formation, four genes (*nodA*, -*B*, -*C*, and -*D*) have been found to be highly conserved among different strains (1, 2, 14, 22, 35, 36, 40, 58, 75). Strains mutated for *nodA*, -*B*, or -*C* loci are unable to curl root hairs and stimulate cell divisions in the root, two early events in the process of nodulation. Strains mutated for *nodD* typically show a delay in nodulation. The *nodD* gene is transcribed divergently from the *nodA*, -*B*, and -*C* genes, whose transcription is *nodD*-dependent

and inducible by plant-secreted phenolic compounds (14, 48, 75).

Little is known about the biochemical functions encoded by the nodABC genes, but detailed studies of mutant strains suggest that these loci may determine a structural component of the outer bacterial surface or regulate plant growth by producing or stimulating production of a plant growth factor. One approach to understanding the function of nodulation genes is to characterize their protein products. In recent years the nodA, -B, and -C gene products have been identified, purified, and used to raise antibodies for detection and localization of the NodA, -B, and -C proteins (15, 31, 32, 41, 63). NodC appears to be an integral membrane protein in the outer membrane of the bacterial cell envelope, while NodA and NodB may be present in the cytosol. Much remains to be learned about the structure and function of these proteins, and the use of a variety of techniques is important for a multi-faceted investigation of nod gene products.

Immunogold cytochemistry has recently become a proven method for localizing surface and intracellular antigens on specimens prepared for electron microscopy. The technique has several distinct

advantages. Colloidal gold markers are easily and inexpensively prepared and produce high-resolution, quantifiable staining. Colloidal gold can be stabilized with staphylococcal protein A, a superior reagent for indirect labeling procedures because of its high affinity for immunoglobulins from a broad range of mammalian species. The immunogold technique is also convenient to use because it requires no more than routine processing of specimens for electron microscopy. Different pre-embedding, post-embedding, and whole-mount labeling methods offer a variety of approaches for localizing the antigen of interest. As with other immunocytochemical techniques, however, successful application of immunogold staining is dependent on a highly specific antiserum, low nonspecific staining, and optimal preservation of antigenicity.

Immunogold cytochemistry is a popular technique that is becoming more widely used every day. In studies of the Rhizobium/Bradyrhizobium-legume symbiosis immunogold labeling has been effectively employed for the localization of various nodule constituents, including the oxygen-binding protein leghemoglobin (52), the nodule-specific enzyme uricase (46, 80, 81), and certain components of the

peribacteroid and plasma membranes of infected cells (7, 8, 19). To date, immunogold staining has not been employed in studies of *nod* gene products. The purpose of this thesis is to apply the methods of immunogold cytochemistry for localization of the *nodA* and *nodC* gene products in induced free-living cells of *R. meliloti* and in nodule tissue from *R. meliloti*-infected alfalfa and *B. japonicum*-inoculated soybean plants.

II. SURVEY OF THE LITERATURE

The *Rhizobium/Bradyrhizobium*-Legume Symbiosis

Rhizobia and the Cytology of Nodulation

Rhizobia are Gram-negative, aerobic, soil bacteria which have the ability to invade leguminous plants and stimulate the formation of nitrogen-fixing root nodules. Rhizobia comprise a subgroup of the Rhizobiaceae and are closely related to the tumor-inducing plant pathogen *Agrobacterium*. A formal taxonomic distinction between fast-growing and slow-growing rhizobia was recently instituted with the separation of species into two different genera: *Rhizobium* (2-4 h. doubling time) and *Bradyrhizobium* (7-20 h. doubling time) (33, 69). In addition to rate of growth, other phenotypic distinctions between the two genera include differences in carbohydrate utilization, antibiotic sensitivity, level of nitrogenase activity in free-living cells, arrangement of flagella, guanine + cytosine content of the DNA, and salt tolerance (69).

The symbiotic association of rhizobia with plants is almost exclusively limited to members of the Leguminosae, of which about 90% are capable of being nodulated (9). Only one non-legume, *Parasponia*, has

been reported to be a host for a strain of rhizobia (76). Symbiotic relationships between legumes and nitrogen-fixing bacteria are usually characterized by a marked specificity of interaction, i.e., a single strain of rhizobia may be able to infect certain groups of legumes but not others. For example, R. meliloti nodulates alfalfa (M. sativa); R. trifolii nodulates clover (Trifolium species); and B. japonicum is a symbiont of soybean (Glycine max). In general, the fast-growing Rhizobium strains nodulate only one plant species effectively, whereas slow-growing strains of Bradyrhizobium have a broad host range (54). An exception to this pattern is the extensive host range of Rhizobium species strain NGR 234 which nodulates a variety of tropical and temperate legumes as well as the nonlegume tropical tree Parasponia andersonii (77, 78).

A successful Rhizobium/Bradyrhizobium-legume symbiosis is characterized by the formation of a unique plant organ containing nitrogen-fixing bacteria, the root nodule. Cytological features of the initiation and development of root nodules have been well documented. Initially, rhizobia in the soil are stimulated by plant secretions to multiply and colonize root surfaces. The mechanism of recognition

and attachment is not known, but it appears to be a complex, multi-step process which may involve plant lectins and surface polysaccharides of the bacteria (82). Following attachment to a root hair, infective rhizobia induce the epidermal cell to curl. This creates a new microenvironment where the bacteria proliferate and degrade the host cell wall. The nucleus of the plant cell then migrates to the infection site, and new cell wall material is formed (51). Invagination of the cell wall and continuous synthesis of new wall components creates a tube-like structure, the infection thread, in which invading bacteria multiply and penetrate the cell. Prior to or concurrent with initiation of infection thread synthesis, nodule tissue begins to develop from cells of the root cortex that are stimulated to dedifferentiate and divide. Meanwhile, the infection thread passes through the outer cortical cells and ramifies within the inner root cortex. When the advancing infection threads reach proliferating cells of the incipient nodule, bacteria are released from the tips of the threads and enclosed in a host-derived membrane. At this stage the bacteria differentiate into morphologically and physiologically distinct forms, known as bacteroids, that are capable of actively reducing

atmospheric nitrogen to ammonia and utilizing compounds of host origin as energy sources.

Common Nodulation Genes: nodABC

The stages of nodule development in the legume-Rhizobium/Bradyrhizobium symbiosis are well known at the cellular, structural level, but the biochemical mechanisms for these events are relatively obscure. One approach to understanding the molecular basis of the infection of legumes by rhizobia is to study the genes involved in symbiosis. The goal of genetic studies using strains of Rhizobium/Bradyrhizobium is to identify, sequence and clone bacterial symbiotic genes and subsequently to isolate and characterize the protein products as a means of determining the function of each gene expressed in the symbiosis. During the last eight years bacterial genes involved in the formation of nodules (nodulation or nod genes) have been studied extensively in fast-growing strains of Rhizobium and in some slow-growing strains such as B. japonicum. A number of nodulation genes have already been cloned, and their protein products are beginning to be characterized and localized at the subcellular level.

The first nodulation genes to be clearly identified were four contiguous genes found on large

symbiotic (Sym) plasmids in strains of Rhizobium (1, 22, 35, 40, 58). These genes, designated nodA, nodB, nodC, and nodD, were isolated by various techniques of molecular genetics including transposon mutagenesis, cloning, complementation analysis, and DNA sequencing (14, 30, 36). Figure 1 shows a physical and genetic map of the region of DNA containing nodDABC in R. meliloti. The nodA, -B, and -C genes appear to have a role in the early stages of nodulation because strains mutated at these loci do not induce root hair curling (Hac^- phenotype) or develop visible nodules on compatible hosts (Nod^- phenotype). The regulatory role of the nodD gene is discussed below.

DNA hybridization studies and sequence analysis have shown that the nodABC genes are highly conserved among nearly all species of Rhizobium and Bradyrhizobium tested (22, 42, 47, 58, 60, 64, 75). For example, a comparison of amino acid sequences for the nodA, -B, and -C gene products in R. meliloti and R. leguminosarum shows 58%, 69%, and 70% homology, respectively, and 60%, 82%, and 47% homology, respectively, when R. meliloti and B. japonicum are compared (70, 75). In addition to being physically conserved among species, the nodABC genes appear to be functionally conserved in that cloned genes from one

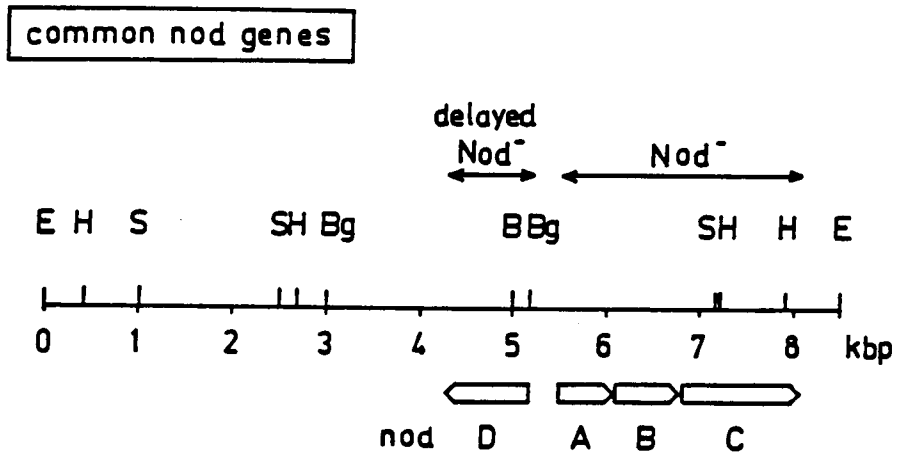


FIGURE 1. Physical and genetic map of the 8.5-kb EcoRI fragment containing nodDABC genes of R. meliloti. Restriction sites: E: EcoRI, H:HinDIII, S:SalI, B: BamHI, Bg:BglII. (From Kondorosi et al. [36])

species can complement mutations in equivalent genes of other species without affecting host specificity (2, 13, 18, 35). Fisher *et al.* (18) performed interspecies complementation studies demonstrating that *nodA*, *-B*, and *-C* are functionally interchangeable between species. Plasmids containing wild-type *nodABC* genes of *R. meliloti* (symbiont of alfalfa) were introduced into Nod^- mutants of *R. trifolii* (symbiont of clover), and the transconjugants were tested for their ability to nodulate alfalfa and clover. The cloned *nod* genes of *R. meliloti* restored a Nod^+ phenotype to *R. trifolii* mutants inoculated on clover but did not confer the ability to infect alfalfa. Similarly, when cloned wild-type *nodABC* genes of *R. trifolii* were introduced into *R. meliloti* Nod^- mutants, the mutants were restored to Nod^+ on alfalfa but remained unable to nodulate clover. Thus *nodABC* are known as common *nod* genes because they determine functions carried out in a common way by different species, in contrast to other *nod* genes which determine functions specific for nodulation of a particular host (2, 35).

Expression of the *nodABC* Operon

Gene fusion studies have demonstrated that expression of the *nodABC* operon is induced by plant-

secreted factors (29, 43, 59). Free-living rhizobia grown in normal culture media do not produce nodABC transcripts at a detectable level (41). However, induction of nod genes can be monitored in cells containing nod genes fused to the promoterless lacZ gene of E. coli. This type of study has shown that nodA, -B, and -C are induced by treatment of cells with plant root exudate, provided that a functional nodD gene is present (43, 59). Inducers of nodABC expression have been isolated from root and seed exudates of various legumes and identified as hydroxylated flavones or flavanones (12). The stimulatory compound produced by clover, for example, is 7,4'-dihydroxyflavone (50), and that of alfalfa is luteolin (5,7,3',4'-tetrahydroxyflavanone) (48).

Data from sequence analyses indicate that nodD is transcribed divergently from nodABC (14, 75), and nodD:lacZ fusions have shown that nodD is expressed constitutively (29, 43, 59). The nodD gene product appears to bind and promote transcription of nod genes from a 47-bp, reiterated sequence (termed nod box) that occurs 5' to inducible nod operons (12). Using a gel retardation assay Hong et al. (23) showed that fragments of DNA which included the nod boxes of R. leguminosarum formed a protein-DNA complex when

exposed to cell-free extracts of Rhizobium strains, but only if the extract contained a functional nodD gene. Fisher et al. (17) extended this finding to R. meliloti and demonstrated that partially purified NodD displayed specific binding to restriction fragments containing the nod boxes of this species. Moreover, studies of strains containing multiple alleles of nodD have demonstrated that the different alleles recognize flavonoid compounds from different hosts (21, 27). In such strains nodD genes may have a dual role of activating transcription of nodulation genes and recognizing specific flavonoids as the basis for host-specific interactions (21).

Analysis of Common nod Gene Mutants

Although little is known about the molecular basis for nod gene activity, detailed analysis of the physiological and nodulation properties of strains mutated in the common nod region is one approach to elucidating the biochemical mechanisms involved in early nodulation events. For example, strains mutated for nodA, -B, or -C are unable to curl root hairs and stimulate cortical cell divisions in the root, and studies of Rhizobium/Bradyrhizobium-legume interactions have shown that root hair curling requires physical contact between plant and bacterium.

These findings suggest that the common nod genes may determine a structural component of the outer surface of the bacterium and/or regulate plant growth by producing or stimulating production of a plant growth factor. Thus various possibilities for the function of common nod genes are suggested by detailed analysis of mutant strains.

Recently, Dazzo et al. (10, 11) have utilized R. trifolii mutants to investigate the effect of nodulation genes on cell surface interactions of host and bacterium during the initial stages of symbiosis. Mutual recognition of the symbiotic partners is believed to be mediated by cell surface recognition molecules. These are glycoprotein lectins exuded at the tips of root hairs and complementary polysaccharide receptors on the surfaces of the rhizobia. Mutant strains of R. trifolii containing Tn5 insertions in the common nod genes were tested for ability to bind clover lectin and attach to root hairs. Binding by mutant cells to clover lectin in vitro was only ca. 5% of the level of binding by wild-type cells as determined by fluorescence microscopy. None of the mutant strains exhibited polar attachment to root hairs in vivo, in contrast to the typical pattern of binding shown by wild-type cells in which

polar attachment of individual cells follows a phase of random clumping. Scanning electron microscopic examination of root hairs four days after inoculation indicated that nodABC mutants also did not produce the extracellular microfibrils normally seen in association with bacteria irreversibly bound to root hairs in the final phase of attachment.

Other investigators have observed that bacterial surface polysaccharides are cleaved by plant enzymes after the initial attachment of rhizobia to root hairs (67) and propose that root hair curling is stimulated by oligosaccharide products of the enzyme-mediated cleavages (6, 10). In light of this hypothesis, the work of Dazzo et al. suggests that the common nod genes could have an indirect role in producing a signal for root hair deformation if these genes control the production of lectin-binding surface receptors and if recognition and attachment are followed by the degradation of bacterial surface polysaccharides to biologically active oligosaccharides.

Other studies of Rhizobium mutants support the possibility that nodA, -B, and -C are involved in producing a soluble factor that affects the growth of legume roots. van Brussel et al. (79) have studied an

unusual physical effect of inoculating Rhizobium species on Vicia sativa subsp. nigra (common vetch) and the dependence of this phenomenon on functional nodDABC genes. The formation of thick short roots (Tsr phenotype) on V. sativa after inoculation with strains of Rhizobium is uncommon in other legumes nodulated by rhizobia. This macroscopically visible phenotype, characterized by roots that are shorter and at least 50% thicker than uninfected roots, typically occurs before the appearance of nodules. The extreme sensitivity of V. sativa subsp. nigra to the factor(s) causing Tsr makes this plant useful for detecting the presence of the Tsr elicitor(s).

Experiments reported by van Brussel et al. (79) in which the Tsr phenotype was induced by sterile excretion fluids of bacteria and plants grown in co-culture showed that a soluble factor or factors cause Tsr and that a physical interaction between bacterium and plant is not required for expresssion of the phenotype. Furthermore, cultivation of bacteria and plants separately in the sterile supernatant fluids of each other established that a plant product is involved in stimulating synthesis of Tsr factor(s) by rhizobia. Nod$^-$ mutant strains of R. leguminosarum carrying Tn5 insertions in the nodD, -A, -B, and -C

genes were unable to cause Tsr, in contrast to nodulation mutants with genetic lesions in other parts of the Sym plasmid.

Various models can explain the molecular signaling involved in the Tsr phenomenon (79). A plant factor may directly or indirectly induce expression of the nodDABC genes whose role in the synthesis of Tsr factor could be structural or regulatory. On the other hand, the plant factor may not induce the nodDABC genes but still require their activity. For example, the plant factor may be a precursor molecule converted into Tsr factor by a bacterial enzyme that requires nodDABC genes for its activity, or the factor could be a plant enzyme which catalyzes the formation of Tsr factor from a bacterial substrate produced or modified by the nodDABC genes.

Characterization and Localization of the nodC Gene Product

An essential step toward understanding the function of nodulation genes is the characterization and localization of their protein products. Within the last four years genetic, biochemical, and immunological techniques have been utilized to identify and localize the NodC and NodA proteins of R. meliloti. Such studies indicate that the 46.8-kDa NodC protein

is a transmembrane protein with receptor-like structure (31, 32, 63). The first clue that NodC is membrane-associated came from hydrophobicity analysis of its predicted amino acid sequence (75). NodC is highly hydrophobic, a property indicative of an integral membrane protein. The most hydrophobic part of the protein is in its carboxy-terminal region where the arrangement of hydrophobic residues is similar to that of outer membrane proteins of *E. coli*. Another very hydrophobic segment of approximately 20 amino acid residues is present at the amino terminus. This region may correspond to a signal sequence for the initial steps of translocation to the membrane (32).

NodC was first localized in the bacterial outer membrane as a fusion protein expressed in *E. coli*. John *et al.* (31) overproduced 70% of the NodC protein at the carboxyl terminus together with sequences from the amino terminus of the λCI repressor of *E. coli* and injected the purified 46.5 kDa fusion protein into rabbits to raise antibodies. Class G immunoglobulins (IgG's) were purified from the antiserum by chromatography on protein A-Sepharose and used to localize the hybrid protein in the outer membrane of *E. coli* by Western blot analysis of cell fractions. Since the CI repressor is a cytoplasmic protein, it was concluded

that the NodC portion of the hybrid protein was responsible for association with the outer membrane.

Another line of evidence provided further indirect support for NodC as an outer membrane protein. When wild-type strains of R. meliloti and R. trifolii were inoculated onto their host plants in the presence of antibodies against the fusion protein, nodule formation was significantly reduced (approximately 50%) in comparison with control experiments (31). These results suggested that the NodC protein is expressed in the plant environment and is present on the surface of Rhizobium where it is accessible to antibodies. Inhibition of nodulation is presumed to be caused by binding of antibodies to NodC because immunodiffusion tests showed that antibodies against the hybrid protein and the CI repressor did not react with root extracts of alfalfa. In addition, normal numbers of nodules were formed when anti-CI repressor antibodies were used in a control experiment.

Localization of NodC in membranes of R. meliloti was demonstrated by Western blot analysis of cytoplasmic and membrane fractions from a mutant strain which constitutively overexpresses the nodA, -B, and -C genes at a higher level than the wild-type strain induced with plant exudate (63). For this experiment

monospecific polyclonal antibodies against the CI-NodC fusion protein were isolated from the IgG fraction of the antiserum by affinity chromatography on a matrix of the purified protein coupled to Sepharose.

In nodule tissue a truncated form of NodC was detected (32, 63). When antibodies against the fusion protein were used to probe for NodC in mature nodules of M. sativa (alfalfa), a strong 34-kDa polypeptide band was detected in addition to a weak 46.8-kDa band, results suggesting that the nodC protein may be processed to a smaller form during nodule development. When immunoblot analysis was performed to determine the relative amounts of the 34-kDa form in M. sativa nodules of different ages, it was found that the protein increased 2- to 3-fold during the development of nodules in a 36-day period (32). A similar analysis for detecting the NodA protein showed no increase during development. Truncated forms of NodC were also present in bacteroids isolated from M. sativa nodules and in nodules of other legumes infected with different rhizobia. Thus the processing of NodC may play a general and important role during nodule development (32).

The most recent attempts to characterize and localize the NodC protein have used genetic and

biochemical techniques to define a membrane-anchor domain and determine direction of orientation in the membrane. To find out whether the large hydrophobic segment near the carboxyl terminus of NodC serves as a membrane anchor, John *et al.* (32) fused a portion of the *nodC* gene coding for the large hydrophobic region to sequences from the amino terminus of the λ*c*I repressor gene and expressed the fused gene in *E. coli*. Analysis of cytosol and membrane proteins by immunoblotting using anti-CI repressor antibodies showed that the hybrid protein was localized in the inner and outer membrane fractions of *E. coli*, while the complete CI repressor was detected in the cytosol. Thus the carboxy-terminal hydrophobic segment of NodC appears to be necessary and sufficient for insertion into the membrane (32).

Transmembrane orientation of NodC was confirmed by surface-specific radiolabeling and proteolysis experiments (32). Lactoperoxidase-catalyzed iodination was used to radiolabel the surface proteins of induced and uninduced cells of *R. meliloti*. The cells were then lysed and subjected to immunoprecipitation with monospecific anti-NodC antibodies followed by SDS-PAGE. A 46-kDa protein band in the gel pattern of induced cells demonstrated that NodC reacts with

extracellular lactoperoxidase and is thus a surface protein.

In another experiment, treatment of induced cells by chymotrypsin followed by Western blot analysis of the NodC protein also showed that NodC is present on the cell surface (32). After 90 min. of proteolysis the 46-kDa band representing NodC almost disappeared, while induced cells not treated with chymotrypsin (control) showed no change in the level of NodC. Thus the degradation of NodC in induced cells indicates that the protein is exposed to the extracellular medium since proteases cannot penetrate biological membranes.

Direction of orientation of NodC in the membrane was deduced from the results of the proteolysis experiment. Because the 46-kDa NodC band disappeared during exposure of cells to chymotrypsin and no truncated forms were detected, the investigators deduced that protease treatment destroyed all the antigenic determinants recognized by anti-NodC antibodies. Such determinants are most likely to be located in regions containing charged and polar amino acid residues upstream from the large hydrophobic region (24), so this portion of the protein would have to be extracellular in order to be sensitive to

protease digestion. These conclusions suggest a model for the domain structure of the NodC protein which includes a large extracellular domain of 295 amino acids, a membrane-anchor domain of 65 amino acids, and a short intracellular domain (45 amino acids) at the carboxyl terminus (32). (See Figure 2.) Computer searches of the Genbank and EMBL data libraries have revealed no significant homology of NodC with other proteins; however, the proposed domain structure has striking similarities with various cell surface receptors. Furthermore, an unusual cysteine-rich cluster is present in the extracellular domain of NodC, and similar features have been found in mammalian cell-surface receptors with very diverse functions.

In relation to the idea that NodC may function as a receptor, John et al. (32) report the discovery that NodA and the 23-kDa NodB protein are involved in synthesizing an unidentified, low molecular weight diffusible factor which stimulates cell division of plant protoplasts. They speculate that the NodC protein is a cell-surface receptor which participates in transducing the diffusible growth factor from the bacterial cell to the plant. Whatever its role, NodC appears to function in the membrane as a dimer (32);

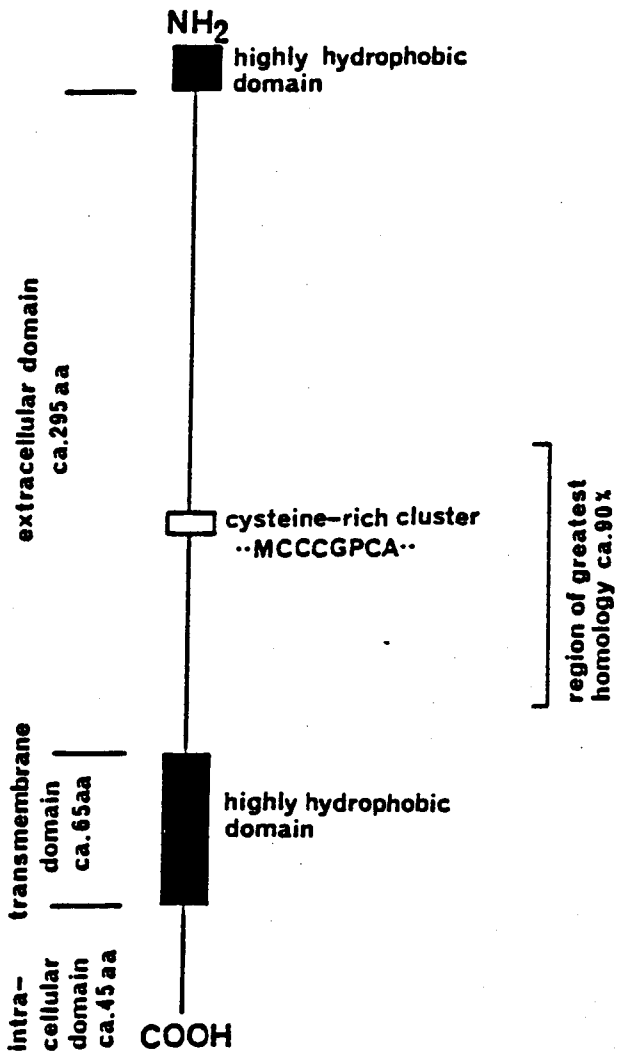


FIGURE 2. Domain structure of the NodC protein as proposed by John et al. (32). Bracketed area is approximately 90% conserved among different species of Rhizobium and Bradyrhizobium.

when membranes of R. meliloti were treated with a protein cross-linker and the proteins analyzed by SDS-PAGE followed by immunoblotting using anti-NodC antibodies and autoradiography, only one major 92-kDa product resulted.

Characterization and Localization of the nodA Gene Product

In contrast to NodC, the data indicate that NodA is not an outer membrane protein. But whether NodA is a soluble polypeptide in the cytoplasm or a protein associated with the inner membrane is not yet clear. Its characterization began with analysis of sequence data. A hydrophobicity plot of the deduced amino acid sequence showed that NodA has hydrophobic regions but lacks a hydrophobic amino-terminal leader peptide similar to that of NodC (75). In NodC the leader peptide is believed to correspond to a signal sequence for initiating translocation across the membrane (32). Sequence analysis of the open reading frame for NodA and NodB showed a four base-pair overlap of the stop codon of A and the translational start site for B (41). Overlap of reading frames is often associated with translational coupling of the protein products, but coupling of NodA and -B has not yet been demonstrated at the biochemical level.

For initial localization experiments, a segment of DNA containing nodA was cloned and subcloned into overexpressing strains of E. coli maxicells (15, 41). The 21-kDa protein product was identified by correlation with the 21.8-kDa polypeptide predicted from the DNA sequence. Purified NodA was then used to generate antiserum for immunodetection of the antigen in R. meliloti. Western blots with anti-NodA antibodies demonstrated that NodA is present in wild-type cells of R. meliloti and in nodB or nodC defective strains. Cell fractionation and immunoblotting localized NodA in the membrane fraction of strains which express the gene product at low levels. In overproducing strains, however, NodA was found in both membrane and soluble fractions.

Different results were obtained in more recent studies which employed a gene fusion strategy to overexpress a hybrid version of NodA in E. coli minicells. Using a procedure similar to that reported in studies of NodC, Schmidt et al. (63) fused approximately 92% of NodA at the carboxyl end to a portion of the λCI repressor at the amino terminus and expressed the fusion protein in a lac repressor overproducing strain of E. coli. Synthesis of the NodA hybrid protein had no effect on bacterial growth,

but microscopic examination of cells in stationary phase showed cell walls bulging from the presence of inclusion bodies in the cytoplasm. The hybrid protein was isolated from the inclusion bodies, purified in the presence of SDS, and injected into rabbits to raise antiserum. Monospecific polyclonal antibodies were prepared by antigen affinity chromatography and used to detect and localize NodA in *E. coli* and *R. meliloti*. Cell fractionation, SDS-PAGE, and immunoblotting localized the NodA protein in the cytosol of a strain of *E. coli* minicells containing a cloned *nodA* gene. In the same manner NodA was localized in the cytosol of a strain of *R. meliloti* which constitutively expresses the *nodABC* operon. Immunodetection also revealed NodA in nodules of *M. sativa*.

In other experiments *R. meliloti* was inoculated onto the host plant *M. sativa* in the presence of antibodies against the NodA fusion protein (63). Normal nodulation occurred, and there was no difference in the number of nodules formed in comparison with control experiments, results suggesting that NodA is not present in the outer membrane where binding by antibodies would presumably interfere with nodulation. Instead, NodA appears to be located within the cell where it is not accessible to immunoglobulins.

John et al. (32) report that NodA and NodB are present in nodules in essentially constant amounts during the development of nodules over a 36-day period. Whether the detection of NodA in nodules is due to continuous synthesis of the protein throughout nodule development or due to its accumulation at an early phase remains to be elucidated. Its presence in mature nodules suggests the possibility that NodA is involved in later stages of symbiosis in addition to its role in early nodulation events (63).

The Immunogold Labeling Technique

For studying the molecular organization and function of cellular components, biochemical analysis alone provides limited information regarding the precise distribution of cellular constituents. Immunocytochemical techniques have been developed which complement biochemical localization studies by utilizing the specificity of antigen-antibody interactions to detect molecular constituents in tissue sections prepared for electron microscopy. These techniques offer the advantage of examining molecular structures in situ. One such technique which is relatively new and becoming increasingly popular is immunogold labeling.

Historical Perspective

The field of immunolabeling for electron microscopy was initiated in 1959 with the introduction of ferritin-conjugated antibodies as probes for in situ localization of antigens (66). This technique represented a significant advance in the study of the molecular structure of cells and tissues (55). A second major landmark of ultrastructural immunolabeling occurred in 1966 when the detection of antigens by enzyme-conjugated antibodies was demonstrated (44). Techniques such as immunoperoxidase labeling became widely used. In 1971 Faulk and Taylor (16) introduced colloidal gold as a specific marker for whole antisera, and three years later Romano and colleagues (57) applied colloidal gold as a marker for isolated antibodies.

The immunogold technique was an improvement over earlier methods for immunolabeling because of the high electron density and particulate nature of the label (55). In the electron microscope a colloidal gold marker is more opaque than the iron core of ferritin, and the granular nature of gold particles permits a quantitation of labeling not possible with immunoenzyme techniques which give rise to amorphous reaction products. In 1975 use of the immunogold

staining technique was extended to the scanning electron microscope by Horisberger and colleagues (26). Since then, many studies have reported the use of colloidal gold markers for labeling surface and intracellular antigens with pre- and post-embedding techniques on ultrathin sections and for marking surface antigens on intact cells, bacteria, viruses, and isolated subcellular structures (55). For these studies gold particles have been coated with macromolecules such as lectins, immunoglobulins, and staphylococcal protein A. The protein A-gold technique has become especially popular since its introduction by Romano and Romano (56) and is now widely used for the localization of both surface and intracellular antigens.

Preparation of Colloidal Gold Markers

One of the advantages of using colloidal gold probes for immunocytochemistry is that they are easily prepared by the reduction of an aqueous solution of tetracloroauric acid with a reducing agent such as sodium citrate (71). Negative charges on the gold particles stabilize the resulting colloid but render it susceptible to coagulation by electrolytes. Macromolecules such as immunoglobulins or protein A are added to coat the gold particles and provide

stability against coagulation. Although the binding of macromolecules to colloidal gold is not well understood, adsorption can be achieved very simply under correct conditions of pH, reagent concentration, and ionic strength (25). Evidence indicates that macromolecules adsorb as a monolayer through a non-covalent binding process and remain firmly attached and biologically active for months.

The stability of a colloidal gold conjugate is greatest with proteins that have high solubility near their isoelectric points in solutions of low salt concentration. Staphylococcal protein A meets this condition better than IgG molecules, which are characterized by low solubility at their isoelectric points (71). Protein A is a 42-kDa cell wall component of strains of Staphylococcus aureus. One of the functions of protein A in vivo is to help bacterial cells evade phagocytosis by host macrophages. By binding the F_c region of Class G immunoglobulins, protein A interferes with the stimulation of macrophages by immunoglobulins. Purified protein A contains two equivalent antibody-binding regions. Its affinity for immunoglobulins of several mammalian species, especially human and rabbit IgG, is very high--on the order of the binding affinity observed

for antigen-antibody reactions (55). Other factors that make protein A useful as a conjugate of colloidal gold include its high resistance to denaturing agents and the long shelf life (at least one year) of protein A-gold complexes (55).

The size of the gold marker is dependent on the type of microscopy and the degree of magnification desired (25). For transmission electron microscopy gold particles from 5 to 20 nm in diameter are commonly used. As a rule, the density of labeling increases as the particle size decreases, but smaller particles may be less easily recognized on post-stained sections at low magnifications. For scanning electron microscopy, the most convenient particle size is approximately 50 nm, although the use of gold particles as small as 22 nm has been reported.

General Aspects of Post-Embedding Immunogold Labeling

Post-embedding immunogold labeling has been widely employed for the localization of both internal and surface antigens in cells and tissues that have been fixed, embedded, and thin-sectioned for electron microscopy. With this technique a two-step, indirect mode of labeling is more convenient and may give a stronger signal than a direct mode of labeling in which sections are treated in one step with gold-

tagged specific antibody (5, 71). The indirect method involves treatment of thin sections with specific antibody, followed by treatment with a colloidal gold conjugate that binds the immune complex formed in Step 1. Figure 3 is a schematic representation of the indirect immunolabeling procedure using a protein A-gold marker. While either an anti-species antibody tagged with gold particles or a protein A-gold conjugate may be used in the second step, protein A-gold has been found to give denser labeling (25). Protein A-gold also provides more flexibility as a marker because protein A has a high binding affinity for IgG's from several different mammalian species, and thus a single preparation of protein A-gold can be used to detect a variety of specific antibodies raised in any of several species of experimental animal.

The general scheme of post-embedding immunogold labeling requires the production of antibodies and the preparation of tissues or cells for electron microscopy prior to performing the immunolabeling procedure. Specific antibodies are produced by injection of highly purified antigen into an experimental animal, usually a rabbit. The resulting antiserum is assessed for immunological specificity by techniques such as the immunodiffusion test of Ouchterlony (34).

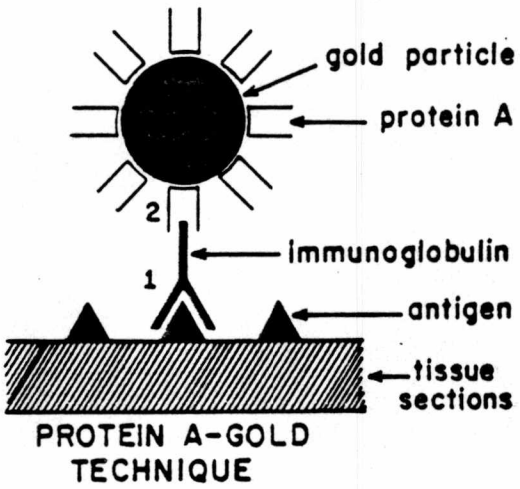


FIGURE 3. Principles of the two-step protein A-gold technique. In Step 1, an immunoglobulin interacts specifically with the antigen present at the surface of the tissue section. In Step 2, a molecule of protein A, adsorbed to the surface of a gold particle, binds the F_C domain of the antibody. The gold particle allows the immune complex to be viewed in the electron microscope. (From Bendayan [5])

Ideally, specific antibody is isolated from the immunoglobulin fraction and purified by antigen-affinity chromatography to reduce nonspecific background staining during the immunolabeling procedure.

The tissues or cells to be probed for antigenic sites must be prepared for microscopy by chemical fixation, dehydration in an organic solvent, and embedding in plastic. The staining precedure is carried out on thin sections, approximately 100 nm thick, attached to nickel or gold specimen grids. Figure 4 presents a brief summary of the general scheme of post-embedding immunogold labeling.

Problems and Limitations of the Method

Effective use of the technique of post-embedding immunogold labeling depends in part on a proper appreciation of the problems and limitations inherent in the method. Obviously, specificity of the antibody is an important consideration. Any indication that the antibody preparation reacts with other cellular constituents besides the antigen of interest raises uncertainties in interpretation of the data. Proof of specificity of the antibody for a proteinaceous antigen can be achieved by various techniques including immunodiffusion, immunoelectrophoresis, radioimmunoassay, and immunohistochemistry (5, 53). Experimental

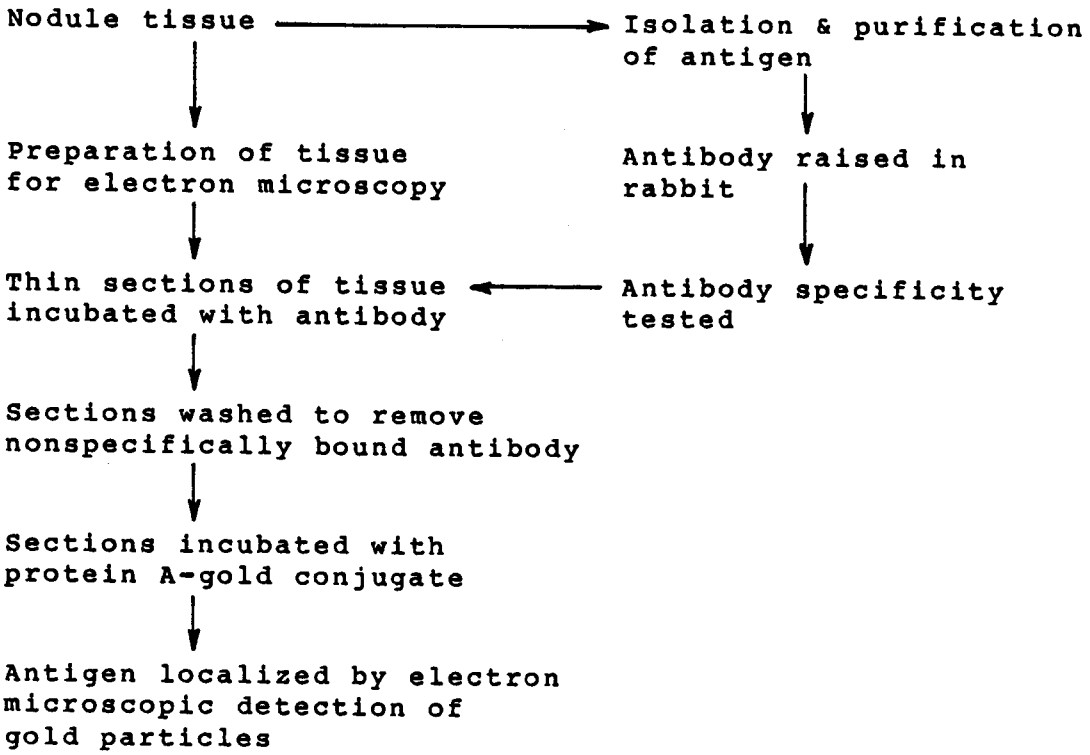


FIGURE 4. Scheme for immunogold localization of nodule-specific proteins. (Adapted from Knox and Clarke [1978])

controls that verify the specificity of the antigen-antibody interaction are discussed below.

Other problems inherent in post-embedding immunogold labeling are nonspecific background staining and loss of antigenicity during tissue processing. Nonspecific staining must be evaluated by performing a battery of control experiments in parallel with the actual labeling experiment. There are three types of controls most commonly reported in the literature (5, 71): (1) Substitution of normal, pre-immune serum for specific antiserum demonstrates the degree of staining which is due to interactions of nonspecific IgG's with the tissue sections. (2) Incubation of sections on the gold-tagged reagent without prior incubation on specific antibody reveals the amount of nonspecific labeling which is due to the colloidal gold conjugate. (3) Treatment of sections with antibody preadsorbed with an excess of antigen or homogenate of antigen-containing tissue verifies the specificity of the antigen-antibody interaction.

In addition to nonspecific interactions of immunoreagents with tissue sections, procedural artifacts can contribute to background labeling. Adequate rinsing of the sections between steps of the procedure is essential for removing nonspecifically adsorbed and

excess unbound immunoreagents (5). Concentration of the antiserum is another factor affecting background labeling; high dilutions of the antiserum are recommended as a precaution against low-affinity binding of cross-reacting antibodies (39). Since cross-reacting antibodies are usually of a lower titre than the desired antibodies, dilution effectively minimizes their contribution to nonspecific binding.

Another significant problem in post-embedding immunolabeling is preservation of antigenicity in specimens prepared for electron microscopy. Antigenicity may be adversely affected by every stage of the preparative process. Chemical fixatives introduce intra- and inter-molecular cross-links which may inhibit antigen-antibody interactions (49). Dehydration of specimens in organic solvents affects the structural integrity and conformation of proteins (73). Heat-curing during embedding can cause additional damage. Furthermore, the matrix of the embedding material obscures antigenic sites and obstructs the entry of antibodies into the sections. Because different antigens vary in their sensitivity to these procedures, appropriate conditions of fixation, dehydration, and embedding have to be determined for each particular antigen (5). In situations where

the antigen is present in low concentrations or is particularly sensitive to fixation and embedding, localization techniques other than post-embedding immunogold labeling may be called for.

Detection of Surface Antigens

Immunogold labeling of surface antigens for transmission electron microscopy can be accomplished by pre-embedding and whole-mount labeling systems. With pre-embedding techniques specimens are immunostained prior to fixation, embedding, and sectioning. For example, unicellular organisms or isolated subcellular structures can be labeled in suspension and then examined in thin section after embedding. The immune complex is thus formed and labeled before antigenic sites are destroyed by subsequent steps of the procedure. The disadvantage is that this technique requires large amounts of immunoreagents (61) and is not useful for blocks of tissue because antibodies do not readily penetrate membranes (4).

Another method for labeling surface antigens is whole-mount labeling. With this technique unfixed or mildly fixed cells are adsorbed onto coated specimen grids, immunolabeled, and examined in the electron microscope without further manipulation. This method is simple and quick and consumes very little of the

sample or the immunoreagents (4). It has been especially useful for immunolabeling small structures such as viruses and bacterial pili (3). The disadvantage of this technique is that it is limited to detection of surface antigens of single-celled organisms, viruses, and subcellular structures.

Applications of Immunogold Cytochemistry for Localization of Constituents of the *Rhizobium*/ *Bradyrhizobium*-Legume Symbiosis

Immunogold staining has great potential as a tool for localizing molecular constituents of the *Rhizobium*/ *Bradyrhizobium*-legume symbiosis. To date, several nodule components have been localized with immunogold techniques. Robertson *et al.* (52) used immunogold staining on thin sections of pea nodules to show that leghemoglobin, a myoglobin-like protein unique to nitrogen-fixing root nodules, is present in the cytoplasm and nucleus of infected cells. Two groups using a variety of different embedding media for immunogold labeling have independently localized the nodule-specific enzyme uricase in peroxisomes of uninfected cells of soybean nodules (46, 80). VandenBosch and Newcomb (81) also applied the technique to a developmental study of the appearance and distribution of uricase during nodule formation.

Other investigators have utilized immunogold techniques with monoclonal antibodies in studies analyzing the structure and biogenesis of the peribacteroid membrane (7, 8, 19). To date, there have been no reports of the localization of nodulation gene products of rhizobia using immunogold labeling, but for future studies immunogold cytochemistry is certain to be a valuable tool for advancing our understanding of nodulation gene products and other aspects of the molecular basis for the Rhizobium/Bradyrhizobium-legume symbiosis.

III. MATERIALS AND METHODS

Plants

Seeds of alfalfa (*M. sativa* (L.) cultivar Buffalo) and soybean (*G. max* (L.) Merr. cultivar Essex) obtained from D. R. Mayo Seed Co., Knoxville, Tenn. were surface-sterilized and aseptically germinated in the dark. Roots of three-day-old seedlings were inoculated with 1.0 ml of a bacterial suspension containing 10^8-10^9 cells per ml of either *R. meliloti* strain 1021 for inoculation of alfalfa or *B. japonicum* strain USDA 110 for inoculation of soybean. Seedlings were grown in sterile vermiculite for 28 da. under greenhouse conditions and maintained on a plant nutrient solution devoid of nitrogen.

Bacterial Strains and Conditions for Induction of Nodulation Genes

R. meliloti strain 1021 was used in experiments localizing nodulation genes because it expresses the NodC protein approximately 40 times more than other wild-type strains (32). Cultures were grown in M9 salts supplemented with 0.2% casamino acids and 0.4% glycerol (63). At $O.D._{600}$ = 0.2, cells were treated with 10 μM luteolin for 20-24 h. at 28°C to induce the expression of nodulation genes. Cells attained an

optical density (600 nm) of approximately 0.5 by the end of the induction period. Uninduced cells were grown concurrently with the induced sample but were not treated with luteolin. For immunolabeling of rifampicin-treated *R. meliloti*, rifampicin at a concentration of 150 µg/ml was added to induced and uninduced cultures at the end of the 24-h. induction period. After 4 h. of incubation with rifampicin, cultures were harvested and processed for electron microscopy.

For immunogold localization of surface antigens of *B. japonicum*, wild-type strain USDA 110 was grown in a yeast extract-mannitol medium (38) for 48 h. at 30°C to obtain a log-phase culture. As a negative control wild-type *R. meliloti* strain 1021 was grown in parallel in TA broth containing (per liter) 10 g bacto-tryptone, 1 g bacto-yeast extract, 5 g NaCl, 1 mM $MgSO_4$, and 1 mM $CaCl_4$.

Preparation of Root Nodules for Electron Microscopy

Root nodules were harvested from legumes 28 days after inoculation. Whole nodules or halves of nodules sliced longitudinally were fixed in 2.5-3% (v/v) glutaraldehyde (Polysciences, Inc., Warrington, Pa.) in 50 mM potassium phosphate buffer (pH 6.8) for 90

min. and post-fixed for 1 h. in 2% (w/v) osmium tetroxide (Polysciences, Inc.) in the same buffer. Fixation and subsequent preparative steps were carried out at room temperature. The samples were then dehydrated through either a graded ethanol series (30%, 50%, 70%, 90%, 100% [2X]), 30 min. per step, for embedding in LR White acrylic resin (available in pre-mixed form from London Resin Co. Ltd., through Ernest F. Fullam, Inc., Latham, N.Y.) or a graded acetone series (25%, 50%, 75%, 95%, 100% [2X]), 30 min per step, for embedding in Spurr's epoxy resin (Spurr 1969; components available from Ladd Research Industries, Inc., Burlington, Vt.). Infiltration of nodules in LR White resin was carried out as follows: 1:1 (resin:ethanol) for 1 h., 2:1 for 1 h., 3:1 for 1 h., and 100% resin for 30 min., overnight, and for 1 h. Nodule pieces were then placed in gelatin capsules with a fresh change of LR White and polymerized at 55°C for 24 h. Infiltration of Spurr's-embedded nodules was carried out by the following schedule: 1:1 (resin:acetone) for 1 h., overnight in 2:1, 3:1 for 2 h., overnight in 100% resin. Following a fresh change of resin, samples were oriented in flat molds and polymerized at 70°C for 24 h.

Thin-sectioning was performed on a Sorvall Porter-Blum ultra-microtome MT-1 outfitted with a diamond knife or on a Reichert Om-U3 ultra-microtome outfitted with glass knives. Sections 90-150 nm thick were mounted on uncoated 400-mesh nickel grids (The Lanum Co., Homewood, Ill.). Adhesion of LR White sections was enhanced by pre-treatment of grids with 10% glacial acetic acid, followed by rinses with water and acetone.

Preparation of Bacteroids and Bacteria for Electron Microscopy

Bacteroids from mature soybean nodules were isolated by differential centrifugation according to Sundaresan et al. (72) and carried through all fixation and embedding steps in the same manner as bacteria. Bacterial cultures (15-25 ml volume) were harvested by centrifugation in 30 ml Corex tubes (Corning Glass Works, Corning, N.Y.) for 10 min. at 8800 x g. The supernatant was removed, and the cell pellet was treated with 2.5-3% glutaraldehyde in 50 mM potassium phosphate buffer (pH 6.8) for 1 h., followed by post-fixation for 1 h. in 2% osmium tetroxide in the same buffer. Fixation and all subsequent steps were carried out at room temperature. Before each

change of solution, bacteria were pelleted by centrifugation at 5000 x g for 5 min.

Samples to be embedded in LR White resin were rapidly dehydrated in a graded ethanol series (5 min. treatments in 30%, 50%, 70%, 95%, and 15 min. in 100% [2X]) and infiltrated with resin as follows: 1:1 (resin:ethanol) for 30 min., 3:1 for 30 min., overnight in 100% LR White. After a fresh change of resin for 1 h., samples were pelleted in gelatin capsules, capped tightly, and heat-cured at 55°C for 24 h.

For embedding in Spurr's resin, samples were dehydrated in a graded acetone series (25%, 50%, 75%, 100% [2X]), 15 min. per step, and infiltrated with resin as follows: 1:1 (Spurr's:acetone) for 30 min., 2:1 for 30 min., 3:1 for 30 min., overnight in 100% Spurr's. Samples were pelleted in BEEM capsules (Ladd Research Industries, Inc.), overlaid with fresh Spurr's, and polymerized at 70°C for 24 h. Blocks of Spurr's- and LR White-embedded bacteria were thin-sectioned as described above.

Antibodies

Monospecific polyclonal antibodies against the Nod A and Nod C inducible nodulation proteins were a gift from Drs. M. John and J. Schmidt (Max-Planck-

Institut für Züchtungsforschung, Köln, Federal Republic of Germany) and were raised in rabbits as described in John *et al.* (31) and Schmidt *et al.* (63). A rabbit was injected with 1 mg of purified antigen mixed with an equal volume of complete Freund's adjuvant. Twenty-one days later a second injection of 1 mg antigen in incomplete Freund's adjuvant was administered, and 10 days after the second injection the rabbit was bled to obtain antiserum. Rabbit immunoglobulin (IgG) was purified from the antiserum by ammonium sulfate precipitation followed by affinity chromatography on protein A-Sepharose. Monospecific antibodies were isolated from the IgG fraction by affinity chromatography on purified antigen coupled to Sepharose. For prolonged storage antibodies were filter sterilized, frozen on liquid nitrogen, and kept at -70°C. Drs. John and Schmidt also provided pre-immune serum from the same rabbit used to raise antibody.

Antibodies against *B. japonicum* strain USDA 110 were provided by Dr. G. Stacey (Department of Microbiology, University of Tennessee, Knoxville). Unpurified antiserum resulting from injection of a rabbit with an emulsion of whole bacterial cells in Freund's adjuvant was stored in 2 ml aliquots at

-20°C. Just prior to use the antiserum was thawed and filtered (0.2 μm diameter of pore) to remove solids.

Immunogold Labeling of Thin Sections

Post-embedding immunogold labeling was carried out according to the procedure of VandenBosch (80) and VandenBosch and Newcomb (81). Prior to labeling, thin sections on nickel grids were treated with 0.56 M aqueous sodium metaperiodate (Sigma Chemical Co., St. Louis, Mo.) for 1 h. to unmask antigenic sites bound by osmium. In this step and throughout the procedure, grids were floated on drops of solution with the side of the grid containing sections placed in contact with the reagent. Next, two 10-min. washes with distilled water were performed by agitating grids on a mechanical aliquot mixer (Ames Co., Division of Miles Laboratories, Inc., Elkhart, Ind.). Grids were then floated on drops of 1% (w/v) bovine serum albumin (BSA; Sigma Chemical Co., catalog no. A-7030) in TBST buffer (Tris-buffered saline plus Tween, containing 10 mM 3-amino-2-(hydroxymethyl)-1,3-propanediol [Tris]-HCl, pH 7.2, 500 mM NaCl and 0.3% (v/v) Tween-20 [polyoxyethylene sorbitan monolaurate; Bio-Rad Laboratories, Richmond, Cal.]) for 15 min. and then transferred, without rinsing, to 25-30 μl droplets of specific antibody diluted, if necessary, in 1% BSA in

TBST. Incubation on antibody was for 1-2 h. at room temperature in sealed plastic petri dishes containing water-soaked filter paper to create a humid environment. Samples were rinsed and agitated 3 times for 10 min. each time in TBST buffer, then incubated for 1 h. on 25-30 µl drops of Protein A-gold (15 nm diameter; Janssen Life Sciences Products, Olen, Belgium, available from Boehringer Mannheim Biochemicals, Indianapolis, Ind.) diluted 1:10 in TBST. Grids were washed 3 times for 5 min. each time in distilled water, rinsed briefly with a jet of water from a spray bottle, blotted on filter paper, and allowed to air dry. Experimental controls included substitution of pre-immune serum or buffer for specific antibody. All sections were post-stained with 2% aqueous uranyl acetate for 20 min. before observation in a Hitachi (Tokyo, Japan) H-600 electron microscope operated at 75 kV. Specimens were photographed using DuPont Cronar Ortho-S Litho COS-7 film.

Immunogold Labeling of Whole Bacterial Cells

Localization of antigens on the surfaces of whole cells was carried out by the procedure of Fuerst and Perry (20). A 20 ml broth culture of cells grown to log phase was centrifuged for 10 min. at 8800 X g. For experiments localizing NodC and NodA the pellet

was washed with 0.5 M NaCl to remove exopolysaccharides that could interfere with binding of antibodies to surface antigens. The pellet was resuspended in 1% glutaraldehyde in 0.1 M Sorensen's phosphate buffer (0.1 M sodium phosphate [dibasic], 0.1 M potassium phosphate [monobasic], pH 7.2) for 5 min. at room temperature. Cells were then centrifuged and washed once in phosphate buffered saline (PBS; 137 mM NaCl, pH 7.3, 2.7 mM KCl, 4.3 mM $Na_2HPO_4/7\ H_2O$, 1.4 mM KH_2PO_4) and resuspended in PBS to an absorbance at 540 nm of 0.7 (ca. 10^8-10^9 cells per ml). Nickel specimen grids coated with formvar (Tousimis Research Corp., Rockville, Md.) and carbon were placed on drops of cell suspension, coated side down, for 2 min. and then successively on drops of the following reagents: PBS-0.02 M glycine for 10 min., PBS-2% BSA for 5 min., and 30-60 min. on 25-30 µl drops of antibody diluted, if necessary, in PBS-2% BSA. The experimental controls were treated with pre-immune serum or PBS-BSA rather than specific antibody. Each grid was then washed by floating it on a series of 4 drops of PBS-2% BSA, 1 min. per drop. Next, grids were transferred to 25-30 µl drops of goat anti-rabbit IgG-gold (10 nm; Janssen Life Sciences Products) or protein A-gold (15 nm), diluted 1:10 in PBS-2% BSA, for 30 min., and then

rinsed with distilled water 3 times for 5 min. each time, blotted on filter paper, and allowed to air dry. Specimens were observed and photographed as described above.

Quantitation of Labeling

To quantitate the density of immunogold labeling on thin sections of bacteria and nodule tissue, the sections were photographed in a Hitachi H-600 electron microscope at magnifications of 8,000-17,000 X, and the negatives were enlarged and printed at total magnifications of 20,000-43,000 X. The micrographs 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 and plant cell structures were traced. Surface area in μm^2 was automatically calculated by a software system for image analysis used in conjunction with the digitizing tablet (Bioquant System IV, R & M Biometrics, Inc.). Gold particles on the measured areas were counted manually, and the density of labeling was expressed as average number of gold particles per μm^2.

IV. RESULTS

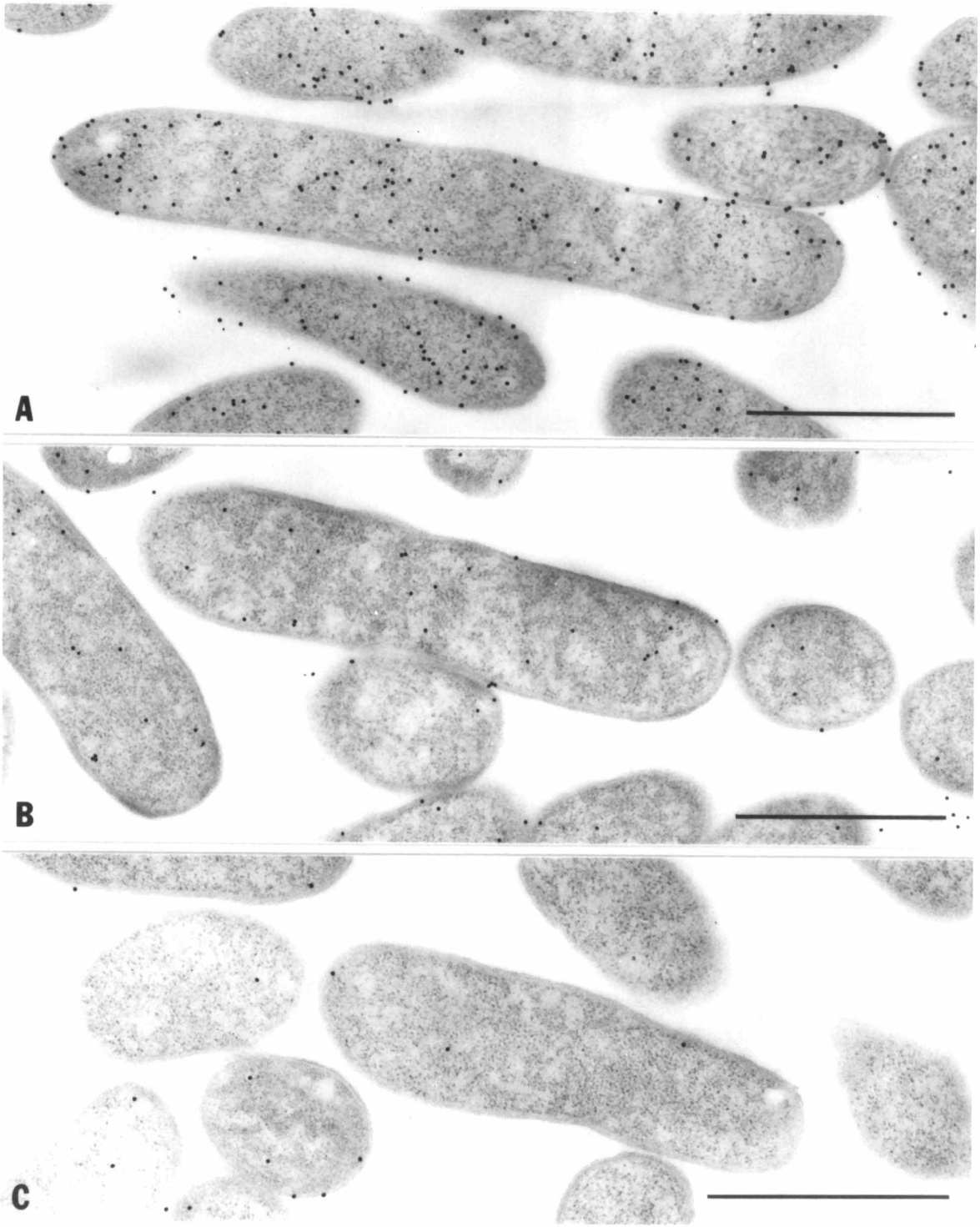
Localization of NodC and NodA in Free-living, Induced Cells of R. meliloti

For immunogold localization of NodC and NodA in cultures of R. meliloti, two different labeling methods were employed: (1) post-embedding labeling on thin sections of cells for detection of intracellular and surface antigenic sites and (2) whole-mount labeling for detection of surface antigenic sites only. Common nod genes are known to be inducible by plant-secreted compounds (29, 43, 59), and therefore localization experiments were performed on cultures treated with luteolin, the inducer secreted by alfalfa. As a type of negative control, uninduced cells were grown in parallel and probed for the nodA and nodC gene products.

Localization of NodC on Thin Sections of R. meliloti

For this experiment cells were embedded in LR White resin since preliminary experiments indicated that LR White was superior to Spurr's resin for detection of NodC. On cells treated with luteolin and probed for NodC (Figure 5-A), numerous gold particles appeared throughout the cytoplasm, and a few were present on membranes or periplasmic spaces;

FIGURE 5. Localization of the nodC gene product on thin sections of R. meliloti embedded in LR White resin. All bars = 1 μm. (A) Cells induced with 10 μM luteolin for 20-24 h. were labeled with anti-NodC antibody followed by protein A-gold(15nm) (PAG). x32,400. (B) Uninduced cells were grown concurrently with those in (A) and immunolabeled in the same manner. x32,000. (C) As a control induced cells were incubated with preimmune serum and PAG. x37,000.


A
B
C

extracellular spaces were virtually free of label. Uninduced cells (Figure 5-B) showed a similar distribution of gold particles in the cytoplasm and on membranes, but the amount of labeling was reduced. Quantitation of labeling (Table 1) revealed that uninduced cells were labeled less heavily than the induced sample by about a factor of 4. Background labeling obtained when sections of induced cells were treated with preimmune serum rather than anti-NodC antibody was approximately 4 times less than the labeling on uninduced cells (Figure 5-C and Table 1).

Localization of NodA on Thin Sections of R. meliloti

For this experiment cells were embedded in Spurr's epoxy resin since NodA labeling was heavy in preliminary experiments using this embedding medium. Induced cells (Figure 6-A) were heavily labeled throughout the cytoplasm and contained a few gold particles on the cell envelope. In the uninduced sample (Figure 6-B) the gold marker had the same distribution, but labeling was reduced by a factor of 3.5 compared to the luteolin-treated sample (Table 1). Treatment of sections of induced cells with preimmune serum resulted in a low amount of nonspecific background labeling (Figure 6-C and Table 2). Quantitation revealed that density of labeling on the three

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

Treatment[a,b]	Gold Particles per μm^2 of Cell Profile (Mean ± SE)			
	NodC		NodA	
Induced cells	57.56 ± 2.27	(81)[c]	51.85 ± 2.74	(64)
Uninduced cells	16.06 ± 1.17	(82)	14.67 ± 1.05	(56)
Control[d]	3.72 ± 0.33	(97)	3.39 ± 0.46	(55)

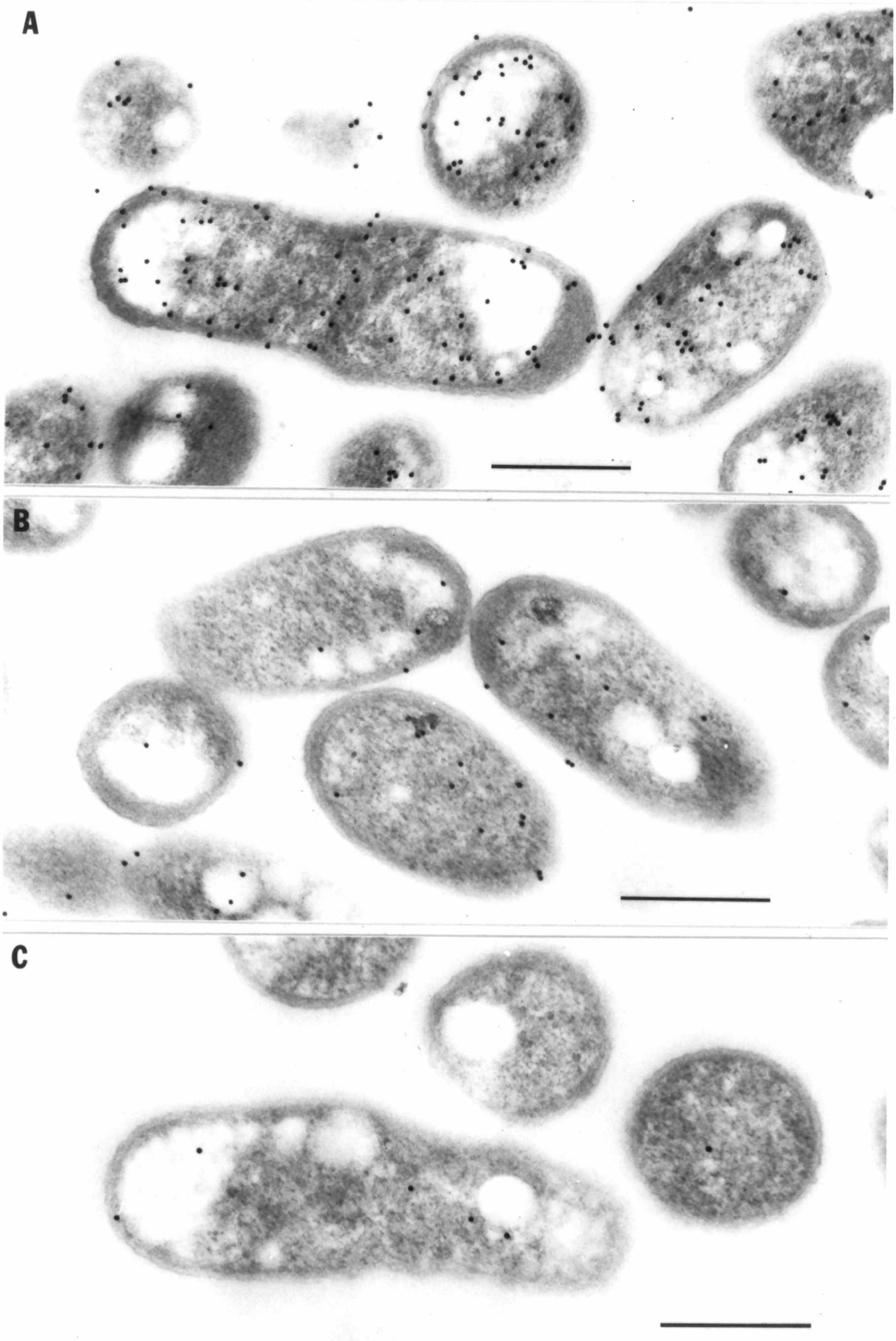
[a]Cells probed for NodC were embedded in LR White resin.

[b]Cells probed for NodA were embedded in Spurr's resin.

[c]Number in parentheses is number of cells counted.

[d]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.

FIGURE 6. Localization of the nodA gene product on thin sections of R. meliloti embedded in Spurr's resin. All bars = 0.5 µm. (A) Cells induced with 10 µM luteolin for 22 h. were labeled with anti-NodA antibody followed by PAG(15nm). x47,000. (B) Uninduced cells grown in parallel with induced cells were labeled as in (A). x50,000. (C) As a control induced cells were treated with preimmune serum and PAG. x51,000.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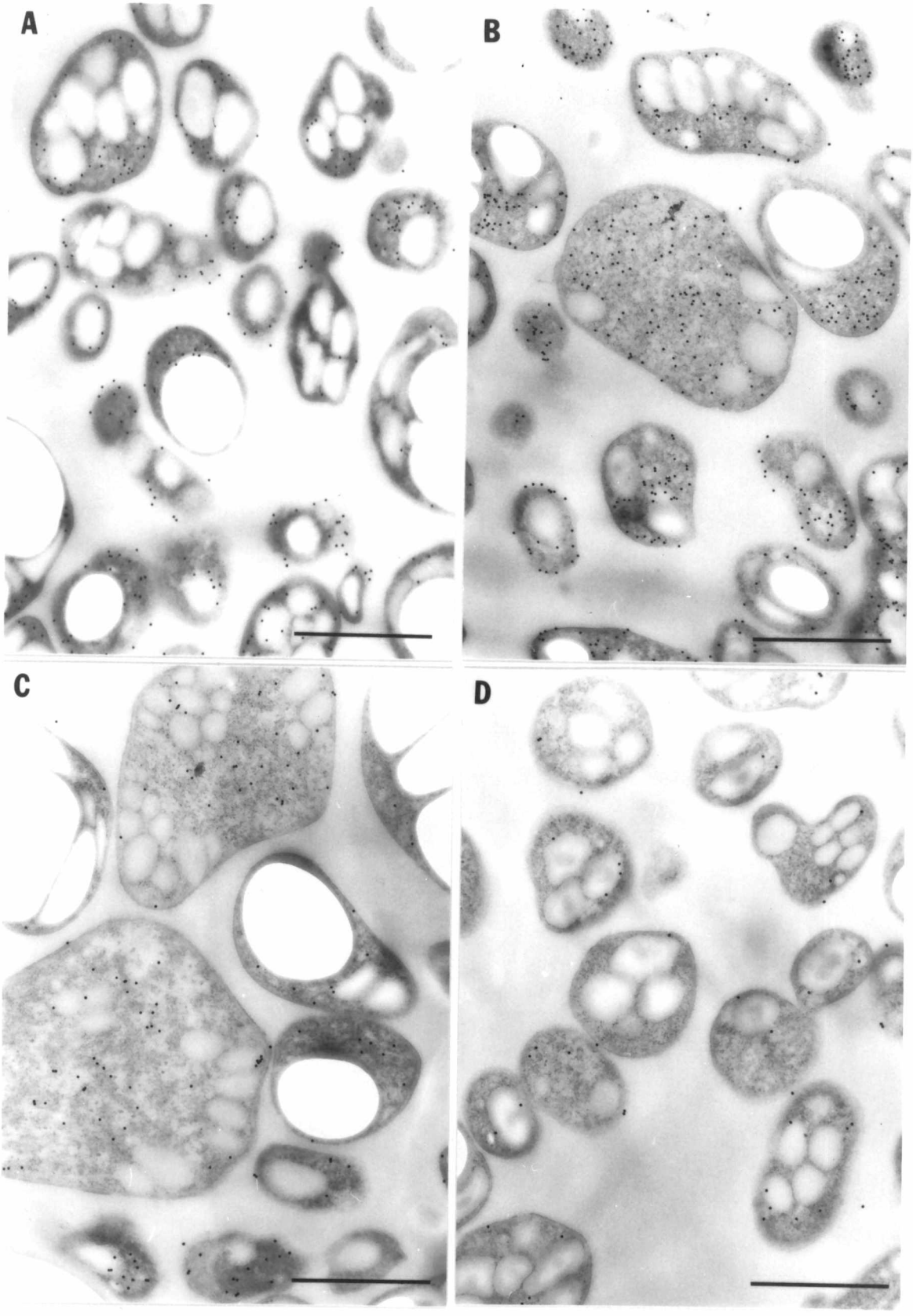
samples probed for NodA was roughly equivalent to the numbers obtained for NodC labeling (Table 1). For example, induced cells probed for NodC had about 58 gold particles/μm^2 and about 52/μm^2 for NodA.

Immunogold Labeling of Rifampicin-Treated Cells

Because biochemical studies have localized NodC in the outer membrane of the cell envelope, it was of interest to probe for this protein on the surface of R. meliloti. An experiment using the antibiotic rifampicin to "chase" NodC into its final location in the induced cell was devised. It was reasoned that treatment of induced cells with rifampicin would stop transcription and thus with time eliminate precursor forms of the protein. Only mature forms already processed and routed to their destination would be available for detection and localization with immunolabeling.

On sections probed for NodC, heavy labeling was present in the cytoplasm of both induced and uninduced rifampicin-treated R. meliloti (Figures 7-A and 7-B); gold particles also appeared on or near membranes. A control sample of induced cells not exposed to rifampicin showed much less labeling than the two samples treated with rifampicin (compare Figure 7-C to Figures 7-A and 7-B). The preimmune control showed a low

FIGURE 7. Immunogold localization of NodC on thin sections of rifampicin-treated R. meliloti. All bars = 1 μm. (A) Induced cells treated with rifampicin were probed for NodC using anti-NodC antibody and PAG(15nm). x23,000. (B) Rifampicin-treated uninduced cells were immunolabeled for NodC as in (A). x23,000. (C) Detection of NodC in induced cells not treated with antibiotic. x23,000. (D) Nonspecific background labeling on uninduced, rifampicin-treated cells incubated with preimmune serum and PAG. x23,000.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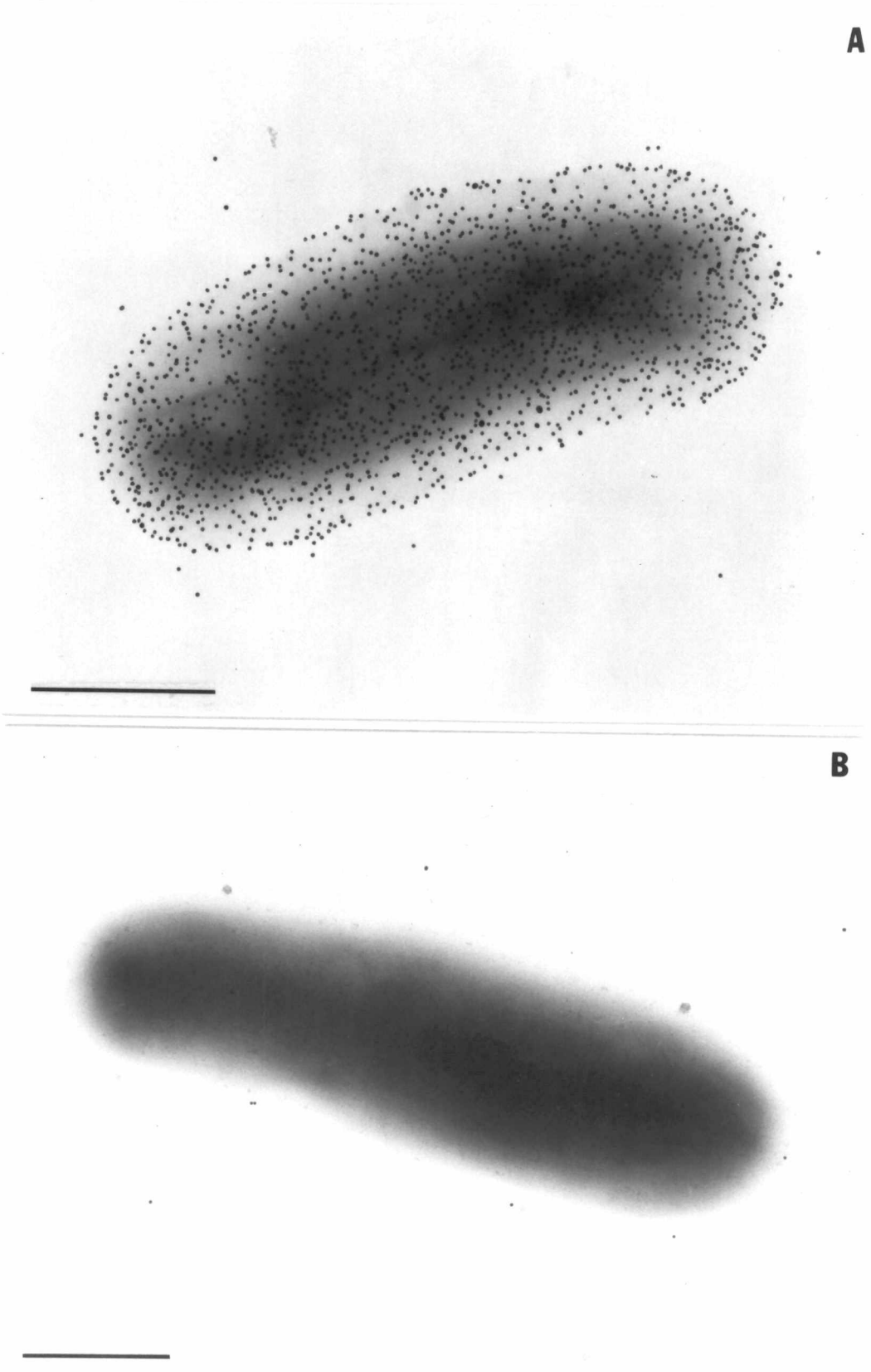
D

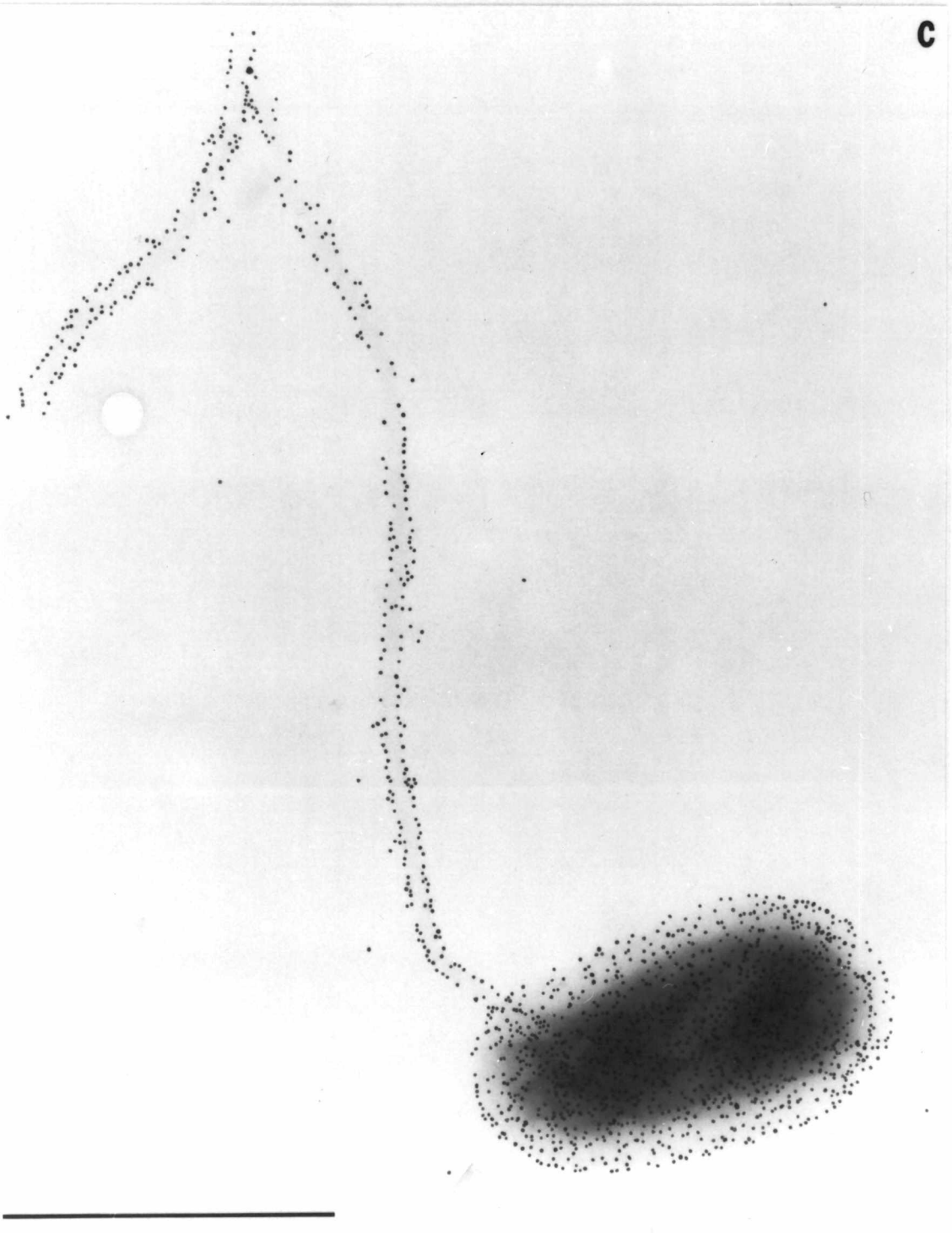
level of background labeling (Figure 7-D). During harvesting and fixation of cells for this experiment, problems associated with proper handling of the cells arose and could have subjected them to cold shock and/or anaerobiosis, problems which may account for the misshapen forms and large holes in the cells pictured. Nevertheless, it appears that rifampicin treatment enhanced expression of NodC, as demonstrated by heavy labeling on the uninduced rifampicin-treated sample (Figure 7-B).

Localization of NodC on the Surface of Induced R. meliloti by Whole-Mount Immunolabeling

As another approach to testing for the localization of NodC on surfaces of R. meliloti, whole-mount immunogold labeling was applied to cells adsorbed to coated specimen grids. Initially, a preliminary experiment was performed to test a recently published protocol (20). Anti-B. japonicum antiserum and a colloidal gold marker were used to detect antigenic sites on the surface of B. japonicum. Figures 8-A and 8-C show that the experiment resulted in very heavy labeling on the cell envelope and on the single sub-polar flagellum that is characteristic of B. japonicum. As a negative control, R. meliloti was immunolabeled with the same reagents (Figure 8-B).

FIGURE 8. Whole-mount immunogold labeling for detection of surface antigens. All bars = 0.5 μm. (A) Whole cells of *B. japonicum* were adsorbed to coated grids and incubated with anti-*B. japonicum* antiserum followed by goat anti-rabbit IgG-gold(10 nm). x57,000. (B) As a control *R. meliloti* was treated with anti-*B. japonicum* antiserum and the same gold marker. x48,000. (C) Labeling of the surface and flagellum of a *B. japonicum* cell immunolabeled as in (A). x52,000.


A
B


C

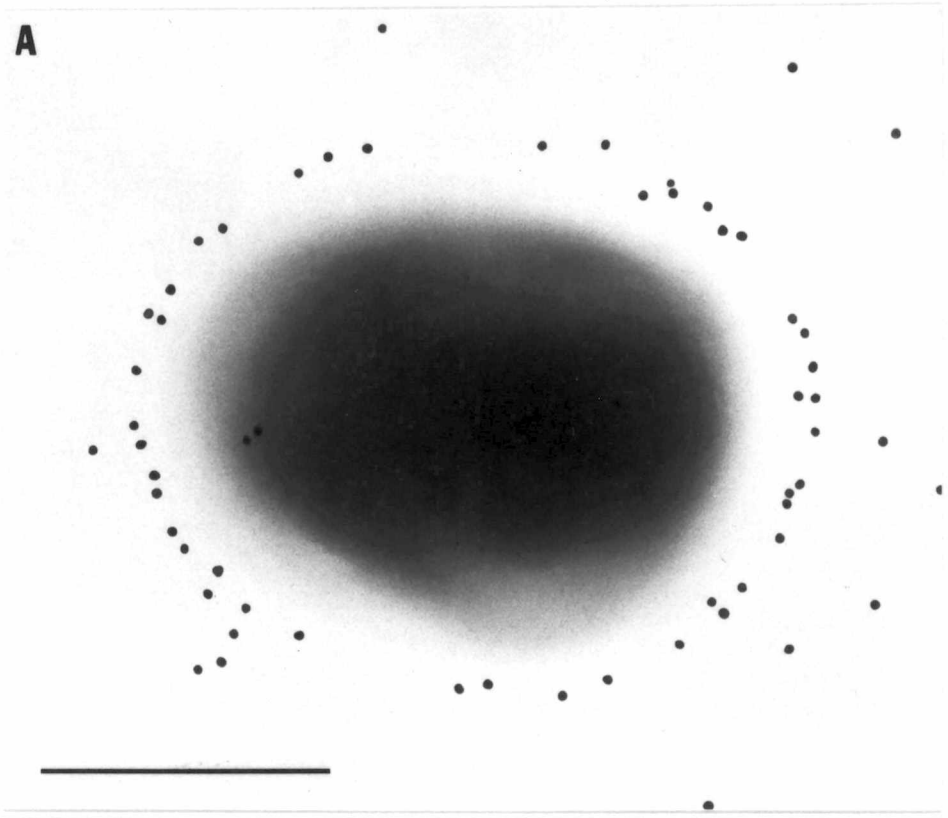
The sparse labeling that resulted verifies the specificity of the antibody for surface antigens of *B. japonicum*. These results demonstrate the usefulness of whole-mount immunogold labeling for detecting surface antigens.

The same procedure was utilized with induced cells of *R. meliloti* to probe for NodC and NodA on the bacterial surface. Figure 9-A shows an outline of gold particles on the periphery of a NodC-labeled cell. This distinct pattern of surface labeling was not present on induced cells probed for NodA (Figure 9-B). Instead, surface labeling of cells treated with anti-NodA antibody was at a very low level, comparable to that of the experimental control for background labeling (Figure 9-C).

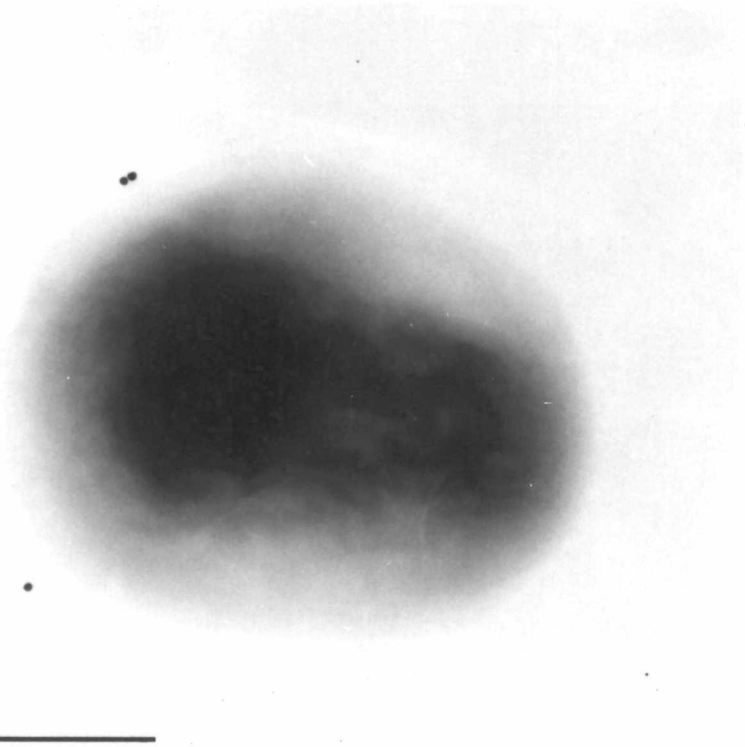
Localization of NodA and NodC in Nodule Tissue

Immunogold labeling of thin sections of nodules was performed to localize the *nodA* and *nodC* gene products at the subcellular level in plant tissue. Western blot analyses have localized NodC in mature nodules of various legumes and in bacteroids isolated from *M. sativa* nodules (32, 63). Similarly, NodA has been detected in mature nodules of *M. sativa* (63). The immunogold technique was employed to corroborate

FIGURE 9. Immunogold labeling using anti-NodC and anti-NodA antibodies on whole cells of luteolin-treated *R. meliloti*. All bars = 0.5 µm. (A) Surface labeling on an induced cell incubated with anti-NodC antibody and PAG(15nm). x76,500. (B) A very low level of surface labeling is typical of induced cells treated with anti-NodA antibody and PAG. x87,000. (C) Treatment of induced cells with preimmune serum and PAG produced a very low number of gold particles on cell surfaces. x72,000.


A
B

C



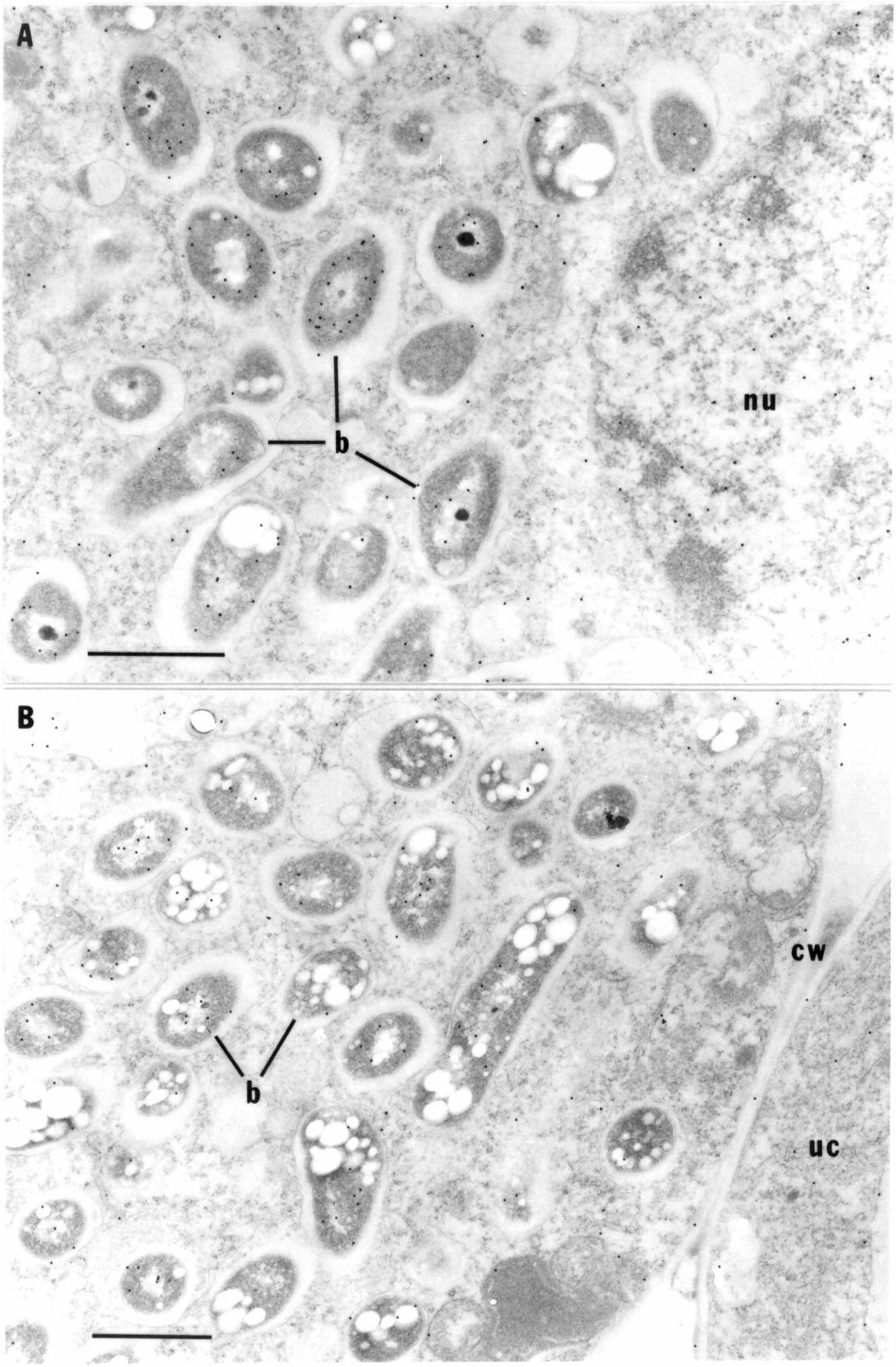
these findings and to probe more precisely for the presence of these proteins at the ultrastructural level in root nodules.

Detection of NodA and NodC in Soybean Nodules

The fact that the NodA and NodC proteins are highly conserved among different species of Rhizobium/Bradyrhizobium permits antibody raised against NodA or NodC from one particular species to be used to detect the homologous protein in induced cells of other species or in nodule tissue infected with other rhizobia. For example, John et al. (32) have successfully used anti-NodC antibody directed against the NodC protein of R. meliloti to detect NodC in mature nodules of Trifolium, Pisum, and Glycine, none of which is a host for R. meliloti. Similarly, a single preparation of anti-NodA or -NodC antibody can be used for immunogold localization studies with nodule tissue from different legume-Rhizobium/Bradyrhizobium symbioses.

Experiments were performed with antibody preparations directed against the nodA and nodC gene products of R. meliloti to probe for proteins in mature nodules of soybean infected with B. japonicum. Immunogold labeling produced similar results for both NodA and NodC (Figure 10). Bacteroids appeared to

FIGURE 10. Immunogold detection of NodA and NodC using the appropriate antibody and PAG(15nm) on thin sections of Spurr's-embedded nodule tissue from soybean inoculated with B. japonicum. (A) Labeling obtained when sections were treated with anti-NodA antibody and the gold marker. Label is present primarily on bacteroids (b) and host cell nucleus (nu). x23,000; bar = 1 μm. (B) Immunolabeling of NodC using anti-NodC antibody and PAG. In the area pictured, bacteroids (b) have the heaviest label, while a few gold particles are present on plant cell walls (cw) and cytoplasm. Note low level of labeling in cytoplasm of an adjacent uninfected cell (uc). x19,800; bar = 1 μm.


A
nu
b
B
cw
b
uc

have the heaviest labeling, but gold particles were also found in the host cell cytoplasm and nucleus (Figure 10-A) and on cell walls and uninfected cell cytoplasm (Figure 10-B).

Quantitation of labeling is tabulated in Table 2. For NodA labeling the average density of gold particles is at least 4 times heavier on bacteroids than on any other structure. Similarly for NodC, labeling on bacteroids is heavier than on other structures by at least a factor of 4.4, except for the high density of gold particles on peroxisomes of uninfected cells. Density of gold particles on peroxisomes is 1.3 times greater than on bacteroids and at least 6 times greater than label on the remaining structures.

Labeling of peroxisomes is demonstrated in Figure 11-A; peroxisomes were identified by their characteristic crystalline arrays. Treatment of sections with preimmune serum produced a low level of background staining on peroxisomes and cytoplasm (Figure 11-B) and on nuclei and bacteroids (Figure 11-C).

While sufficient data were not available to quantify NodA labeling in peroxisomes, evidence from an experiment performed on isolated bacteroids indicates that heavy labeling of peroxisomes occurs in

TABLE 2. Density of immunolabeling of NodC and NodA in nodule tissue from soybean inoculated with *B. japonicum*

Structure[b]	Gold Particles per μm^2 (Mean ± SE)[a]		Total Surface Area Measured (μm^2)			
	NodC	NodA	NodC		NodA	
Bacteroids	18.42 ± 1.72	66.35 ± 9.31	19.72	(35)[c]	15.43	(35)
Mitochondria	4.16 ± 1.37	9.45 ± 1.48	5.20	(21)	6.71	(27)
Cytoplasm[d] of infected cells	3.29 ± 0.61	15.95 ± 4.88	27.08	(20)	34.13	(12)
Cytoplasm[d] of uninfected cells	2.72 ± 0.52	n. d.[e]	42.54	(8)	n. d.	
Nuclei	2.90 ± 0.78	4.70 ± 0.22	99.78	(5)	37.23	(3)
Plant cell walls	2.74 ± 0.70	6.49 ± 1.94	6.13	(10)	4.55	(7)
Vacuoles	1.12 ± 0.40	1.87 ± 1.15	33.61	(8)	10.80	(8)
Extracellular spaces	0.45 ± 0.17	3.48 ± 0.33	37.88	(4)	14.31	(3)
Peroxisomes in uninfected cells	24.74 ± 2.40	n. d.	18.24	(30)	n. d.	

[a] Average density of labeling for each protein was quantified from the combined data of two experiments.

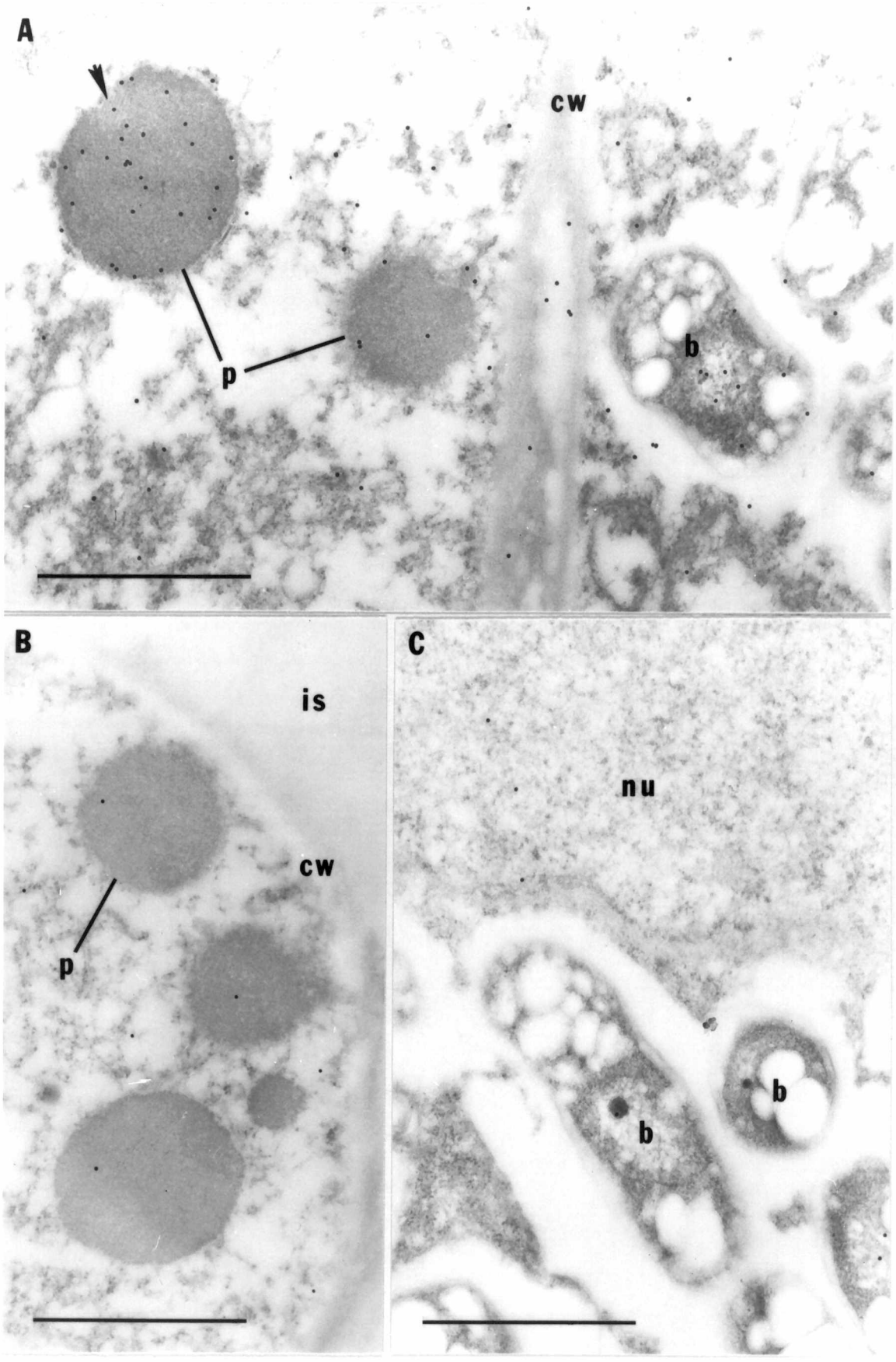
[b] Unless otherwise noted, quantitation was performed on structures within infected cells.

[c] Number in parentheses is number of separate measurements made for each type of structure.

[d] "Cytoplasm" refers to cytoplasmic areas without large membrane-bound organelles, such as mitochondria, vacuoles, or lysosomes.

[e] n. d. = not determined

FIGURE 11. Gold labeling of NodC in soybean nodule tissue fixed and embedded in LR White resin 28 days after inoculation with B. japonicum. All bars = 1 µm. (A) Large peroxisomes (p) in uninfected cells are heavily labeled in sections incubated on antibody to NodC followed with PAG(15nm). Note crystalline array characteristic of peroxisomes (arrow). A bacteroid (b) in an adjacent cell is labeled, and a few gold particles are scattered over the cytoplasm of both cells. cw = cell wall. x35,700. (B) Preimmune control showing nonspecific labeling of peroxisomes in an uninfected cell. cw = cell wall; is = intercellular space. x35,700. (C) Background labeling obtained by treating sections with preimmune serum and the gold marker. A very low level of labeling occurs on bacteroids and cell nucleus (nu) in this region of an infected cell. x36,000.

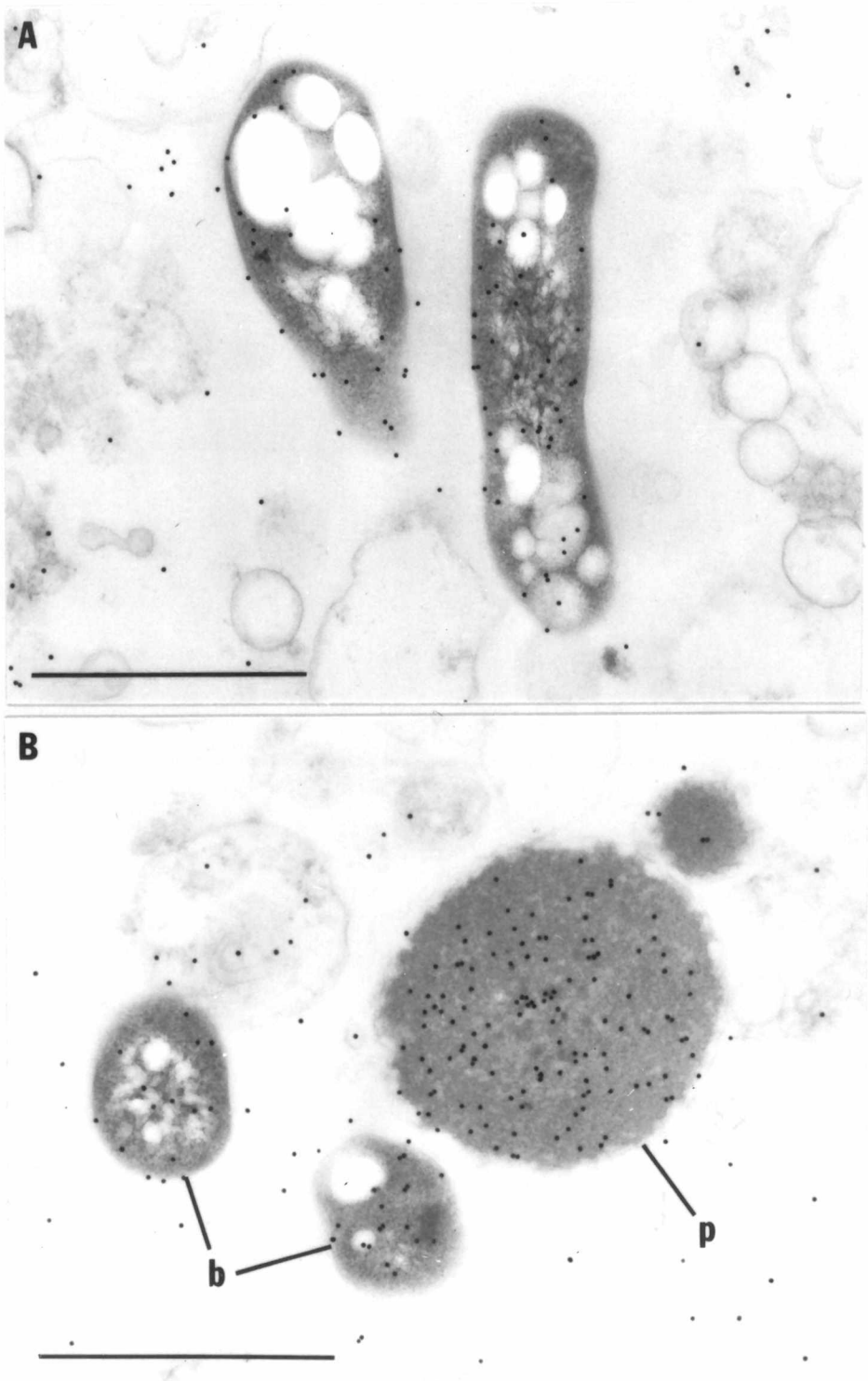

A
cw
p
b
B
is
cw
p
C
nu
b
b

nodule tissue probed for NodA as well as NodC. Immunogold labeling of NodA on thin sections of B. japonicum bacteroids isolated from mature soybean nodules (Figure 12-A) produced the same distribution of label seen in immunolabeled bacteroids of intact nodule tissue and in free-living R. meliloti probed for NodA and NodC in thin section. Figure 12-B shows labeling on two bacteroids in the vicinity of a heavily labeled structure that appears to be a peroxisome. This suggests that, similar to the NodC labeling, a high density of gold particles occurs on large peroxisomes in uninfected cells of soybean nodules probed for NodA.

Detection of NodA and NodC in Infection Threads of Alfalfa Nodules

While it was of interest to perform experiments localizing NodA and NodC in infected regions of alfalfa nodules in parallel with experiments carried out on soybean nodules, no data are available for the former because of technical problems related to the quality of thin sections used for the experiments and the limited amount of antibody available for performing and repeating the experiments. However, data were generated from experiments performed on thin sections cut from the invasion zone of alfalfa nodules, where

FIGURE 12. Immunogold localization of NodA on thin sections of Spurr's-embedded B. japonicum bacteroids isolated from mature soybean nodules. (A) Detection of NodA in bacteroids after thin sections were incubated with anti-NodA antibody and PAG(15nm). x39,000; bar = 1 μm. (B) Heavy NodA labeling appears on two bacteroids (b) and a large peroxisome-like body (p) that was isolated with the bacteroid fraction. x42,000; bar = 1 μm.


A
B
b
p

bacteria in infection threads are invading newly differentiated nodule tissue even as older cells filled with bacteroids are actively fixing nitrogen in other regions of the nodule.

Immunogold localization of NodA resulted in heavy labeling on bacteria in infection threads, with gold particles distributed over the cytoplasm and occasionally located on or near membranes (Figure 13-A). Gold particles were also numerous throughout the host cytoplasm, while infection thread matrix, infection thread wall, and plant cell wall possessed a low amount of label, and vacuoles were virtually free of label. Sections treated with preimmune serum had reduced labeling on bacteria, host cytoplasm, and infection threads (Figure 13-B).

Immunogold labeling of NodC (Figure 14-A) produced results similar to those for NodA. Bacteria in infection threads were well labeled, and gold particles appeared throughout the host cytoplasm. Lower amounts of label occurred on infection thread wall and matrix and plant cell wall. Treatment of sections with preimmune serum produced a low level of background labeling on nearly all structures (Figure 14-B). Sufficient data were not available to quantitate the results of NodA and NodC labeling in these experiments.

FIGURE 13. Immunogold labeling of the NodA protein in a Spurr's-embedded alfalfa nodule. (A) Sections from the invasion of a nodule excised 28 days post inoculation were labeled with anti-NodA antibody and PAG(15nm). Gold particles are numerous on bacteria (b) in an infection thread and are also present on the infection thread wall (itw) and matrix (itm). The plant cell cytoplasm and cell wall (cw) contain some label, and gold particles appear on the periphery of mitochondria (m). Vacuoles (v) are essentially free of label. x17,600; bar = 1 µm. (B) Preimmune control shows a low level of nonspecific staining after sections were treated with preimmune serum and PAG. x17,600; bar = 1 µm.

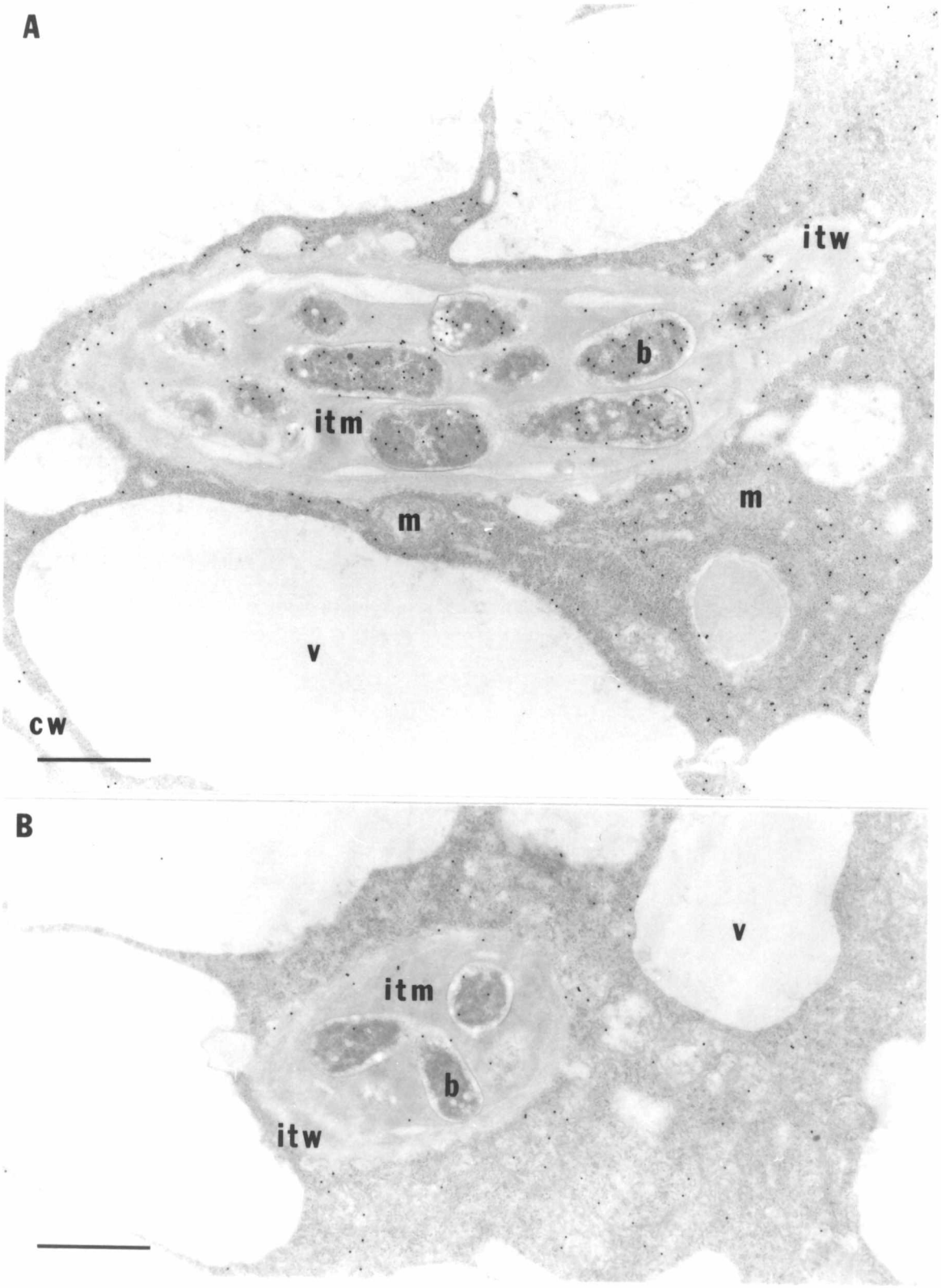
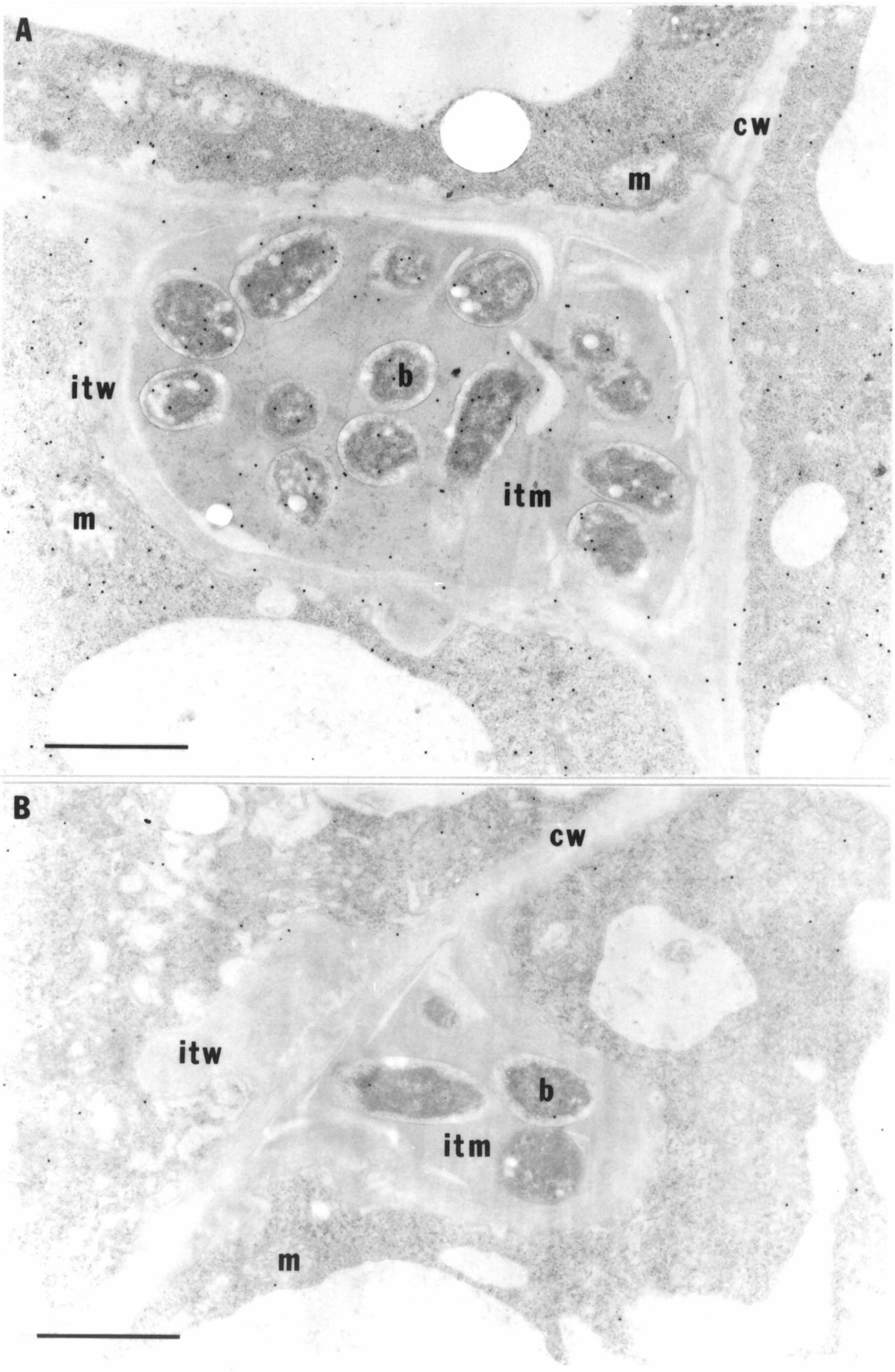

A
itw
b
itm
m
m
v
cw
B
v
itm
b
itw

FIGURE 14. Gold labeling of NodC in the invasion zone of Spurr's-embedded nodule tissue from alfalfa. (A) Numerous gold particles are present in the cytosol of bacteria (b) within an infection thread following treatment of sections with anti-NodC antibody and PAG(15nm). The host cell cytoplasm also contains many gold particles. Other structures, such as mitochondria (m), plant cell wall (cw), infection thread wall (itw), and infection thread matrix (itm), contain a small amount of label. x21,600; bar = 1 µm. (B) Preimmune control shows a low level of background staining after sections were treated with preimmune serum and the gold marker. x22,000; bar = 1 µm.


A
cw
m
itw
b
itm
m
B
cw
itw
b
itm
m

V. DISCUSSION

Elucidation of the function of nodulation gene products will be advanced by the knowledge of their precise intracellular location. For this reason immunogold labeling was employed to localize the protein products of two common nodulation genes, *nodA* and *nodC*. Previous studies using biochemical and physiological approaches have localized the 46.8-kDa NodC protein on the surface of induced *R. meliloti* (31, 32). In the work reported here, whole-mount immunogold labeling localized NodC on the surface of induced cells (Figure 9), while immunolabeling of cells in thin-section detected NodC both in the cytoplasm and cell envelope (Figure 5). In view of the strong evidence for NodC as an outer membrane protein, these results suggest that NodC is translated on free cytosolic ribosomes prior to assembly in the membrane and that immunogold labeling detects nascent and precursor forms in the cytosol as well as the native protein in the outer membrane.

The relatively low level of detection of NodC as a surface antigen may reflect the true distribution of this protein or may be due to the use of antibody directed against the denatured (solubilized) form of the protein. Tommassen *et al.* (74) have shown that

the mature trimeric form of PhoE, an inducible outer membrane protein of E. coli, is not detectable with immunocytochemistry using anti-denatured PhoE serum. Antibody against mature PhoE trimer is required to localize the protein in the membrane. Biochemical cross-linking of surface components of induced R. meliloti has shown that NodC exists in a multimeric form in the outer membrane (32). Thus the use of anti-denatured NodC antibody to probe for NodC with immunogold cytochemistry may at best permit only partial detection of the native multimeric protein.

In contrast to NodC, the 21.8-kDa NodA protein was not localized on cell surfaces when whole-mount immunogold labeling was performed (Figure 9). This is consistent with biochemical and physiological studies reported by Schmidt et al. (63) in which Western blot analysis detected NodA in the soluble fraction of induced R. meliloti. Similar to NodC, immunogold labeling of NodA on thin sections of cells localized the majority of antigenic sites in the cytoplasm with a smaller number on or near membranes (Figure 6). Because antibody to denatured NodA was used for these experiments, it is not clear how much of the mature form of the protein was detected. While these results do not conclusively demonstrate the subcellular

location of mature NodA in induced R. meliloti, detection of NodA determinants in the cytoplasm and cell envelope raises the possibility that NodA is synthesized on free ribosomes in the cytoplasm and associates with the inner membrane after it achieves its mature conformation.

Immunolabeling on thin sections of R. meliloti showed a 3.5- to 4-fold increase in the level of detection of both NodA and NodC in induced compared to uninduced cells (Table 1). This compares favorably with results from induction studies utilizing lacZ fusions to nod genes. For example, Mulligan and Long (43) recombined a cloned nodC-lacZ fusion into its genomic location on the symbiotic megaplasmid of R. meliloti and found a 2- to 3-fold increase in expression of nodC after exposure of cells to plant exudate. A similar study using R. trifolii showed a 5- to 10-fold induction of all nod genes except nodD when cells were grown in the presence of clover (29). Immunogold detection of NodA and NodC in uninduced cells is very likely a reflection of low-level constitutive expression of these proteins and is thus a demonstration of the sensitivity of the technique.

Immunogold localization of NodA and NodC in bacteroids of mature soybean nodules (Figure 10) is

consistent with biochemical studies which have detected both proteins in alfalfa nodules (63) and have found NodC in bacteroids isolated from nodules of M. sativa and in nodule tissue from a variety of other legumes, including soybean (32). Quantitation of immunogold data established the specificity of NodA and NodC labeling on bacteroids in soybean nodules by showing that bacteroids contained at least 4 times more label than other structures, excluding NodC-labeled peroxisomes (discussed below). However, quantitation also confirmed the visual impression that many subcellular structures other than bacteroids were labeled. These stray gold particles may represent a true picture of the distribution of NodA and NodC in nodule tissue or they may be the result of nonspecific labeling caused by technical artifacts.

One possible source of nonspecific staining in these experiments was the use of antisera that may have been too concentrated. All polyclonal antibody preparations contain low-affinity cross-reactive immunoglobulins that are a source of nonspecific staining if the antiserum is insufficiently diluted (71). Determining the best dilution of antiserum to use depends on a trial-and-error approach. One attempts to maximize specific labeling on particular

structures while minimizing the amount of background labeling on other structures. For the experiments reported here several dilutions of antisera were tested in preliminary trials, and none of the dilutions gave heavier labeling than the undiluted antibodies obtained at a concentration of 0.81 mg/ml for anti-NodC antibody and 1.26 mg/ml for anti-NodA antibody. These concentrations were used in all of the experiments and were quite possibly high enough to produce nonspecific labeling in nodule tissue since it has been suggested that antiserum used for immunocytochemical procedures should be diluted no less than 1:1000 (71).

The phenomenon of heavily labeled peroxisomes in uninfected cells of soybean nodules does not appear to be attributable to nonspecific labeling because of the high density of gold particles on these structures. The labeling was 1.3 times heavier than on bacteroids and 6-22 times heavier than on other subcellular components in NodC-labeled sections (Table 2). Enlarged peroxisomes found in soybean nodules have the important physiological role of oxidizing purines to ureides, major nitrogen carriers in the flow of metabolites from root nodules to the shoots (28). Immunogold detection of NodC and NodA in these sites

of active metabolism suggests that the proteins accumulate and are metabolized there as a result of secretion from the bacteroids or because of a general process of degradation and turnover of bacteroids in nodule tissue. While there is no evidence for the latter possibility, there is biochemical evidence that NodC is processed to a shorter form in bacteroids (63, 32). The fate of the liberated peptide is not known, but it may be released from the bacteroids. Perhaps in the case of NodC this cleaved fragment accumulates in enlarged peroxisomes of uninfected cells and is thus detectable with immunogold labeling. NodA, however, does not appear to be processed to a smaller form in bacteroids (63).

Immunogold labeling experiments performed on alfalfa nodules provide the first direct evidence for NodA and NodC in infection threads (Figures 13 and 14). These results emphasize the value of the immunogold technique for localizing antigens in transient subcellular structures not amenable to biochemical analysis. Although the experiments did not provide sufficient data for quantitation, it is clear that structures other than bacteria were labeled. Again, it is likely that nonspecific staining occurred due to the use of relatively concentrated antisera.

While immunogold labeling is clearly a valuable tool for *in situ* localization of antigens, the results reported here indicate the pitfalls and limitations that must be appreciated in order to derive the maximum benefit from the technique. Antiserum specific for the antigen of interest is obviously the basis for generating accurate data. Futhermore, whether the antigen used to raise antibody was in its native or denatured state can affect the results of immunogold localization experiments. The work reported here also indicates that problems in interpretation of data arise when nonspecific labeling occurs. For this reason it is probably wise to perform extensive trial experiments for determining the dilution of antiserum that is optimal both for detecting the antigen on specific structures and for minimizing nonspecific labeling.

For future experiments immunogold labeling might be employed with antisera to NodA and NodC to investigate whether detection of these proteins in peroxisomes of soybean nodules is a generalized phenomenon occurring in alfalfa and other legumes. It would be especially interesting to perform immunogold experiments using domain-specific antibodies as suggested by John *et al.* (32) to follow the processing of NodC in

nodule tissue. With measures taken to reduce nonspecific labeling, experiments localizing NodA and NodC in infection threads of alfalfa could be repeated and expanded to other Rhizobium/ Bradyrhizobium-legume symbioses. As antisera to other nodulation proteins become available, immunogold localization studies will very likely have an important role in advancing our knowledge of their function by enabling detection of their precise intracellular location.

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