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Rhizobium japonicum Mutants Defective in Symbiotic Nitrogen Fixation

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Rhizobium japonicum strains 3I1b110 and 61A76 were mutagenized to obtain 25 independently derived mutants that produced soybean nodules defective in nitrogen fixation, as assayed by acetylene reduction. The proteins of both the bacterial and the plant portions of the nodules were analyzed by two-dimensional polyacrylamide gel electrophoresis. All of the mutants had lower-than-normal levels of the nitrogenase components, and all but four contained a prominent bacteroid protein not observed in wild-type bacteroids. Experiments with bacteria grown ex planta suggested that this protein was derepressed by the absence of ammonia. Nitrogenase component II of one mutant was altered in isoelectric point. The soluble plant fraction of the nodules of seven mutants had very low levels of heme, yet the nodules of five of these seven mutants contained the polypeptide of leghemoglobin. Thus, the synthesis of the globin may not be coupled to the content of available heme in soybean nodules. The nodules of the other two of these seven mutants lacked not only leghemoglobin but most of the other normal plant and bacteroid proteins. Ultrastructural examination of nodules formed by these two mutants indicated normal ramification of infection threads but suggested a problem in subsequent survival of the bacteria and their release from the infection threads.

The symbiosis between Rhizobium and leguminous plants requires the appropriate expression of many plant and bacterial genes. The result is a plant organ, the root nodule, in which physiology is coordinated so that the resident bacteria, called bacteroids, are able to reduce molecular nitrogen to ammonia, which the plant assimilates further (23). As a tool for understanding the symbiosis between soybean and Rhizobium japonicum, we have sought R. japonicum mutants whose root nodules did not fix nitrogen. Previous studies of soybean nodule physiology have suffered because only one or two R. japonicum mutants of the desired phenotype were available and, in cases where a defective field isolate was used, there was no isogenic control (15, 28). Our goal was to isolate a large number of mutants independently derived from two wild-type strains. The approach (4, 7, 18, 19, 24) was to screen directly for mutants defective only in symbiosis.

Using this approach, we isolated mutants which did not nodulate, Nod⁻ (3), and mutants which formed inactive nodules, Fix⁻ (3). The Nod mutants are the subject of a separate report (G. Stacey et al., Arch. Microbiol., in press). Here we present the Fix mutants, with emphasis on the nitrogenase components, leghemoglobin synthesis, and the overall protein content of the nodule as analyzed by two-dimensional gel electrophoresis (21). Two Fix mutants with severely altered nodule protein levels were studied further by electron microscopic examination of the defective nodules.

MATERIALS AND METHODS

Bacterial strains and plant variety. R. japonicum colony type SM, of the wild-type strain 61A76, has been described previously (18). R. japonicum 110 (HS) is strain 3I1b110, colony type I-110, originally obtained from G. H. Elkan, North Carolina State University, Raleigh. The soybean (Glycine max (L) Merrill) cultivar was Corsoy, obtained from the Agronomy Department of the University of Wisconsin, Madison.

Media. AMA is a yeast extract-mannitol broth medium (27). RM is a minimal medium (5) for culturing R. japonicum. PBS buffer contains 6.8 g of KH_2PO_4, 8.7 g of K_2HPO_4, and 8.7 g of NaCl per liter of water, giving a pH of 6.8. RBN is a plant nutrient solution without nitrogen (27).

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Mutagenesis and screening for mutants. Mutants SM4 and SM5 (18) and HS11, HS102, and HS104 (this study) were isolated after treatment of four cultures with N-methyl-N'-nitro-N-nitrosoguanidine (18). The remainder of the mutants were isolated after mutagenesis of nine cultures with UV radiation. Cells from 4-day AMA cultures were pelleted and washed with and resuspended in sterile PBS to a final concentration of approximately 10^8 cells per ml. This cell suspension was irradiated as a thin layer, continuously mixed, in the bottom of a sterile glass petri dish. The UV dose was determined to give 90 to 99% killing of the cells irradiated. After irradiation, the cells were diluted into AMA and incubated in the dark at 30°C. The mutagenized culture was then cycled through minimal medium to exclude auxotrophs and diluted to give individual colonies.

After mutagenesis, surviving clones were tested separately on soybean plants, using the effectiveness assay of Wacek and Brill (27) in which bacteria are incubated with plants in vials containing sterile vermiculite and covered with small plastic bags for 2 weeks in a plant growth room. Those clones that repeatedly lacked acetylene-reducing activity were kept as mutants. When subsequent analysis indicated that two or more clones from the same mutagenized culture had the same phenotype, only one was kept.

Mutants producing nodules that were defective in nitrogen fixation by the above assay were tested further under conditions that led to more vigorous plant growth and higher acetylene-reducing activity of plants inoculated with the wild type as follows. Two-day-old surface-sterilized seedlings were planted in growth pouches (Scientific Products) containing 25 ml of sterile RBN and 25 ml of sterile water. Two plants were grown per pouch. Each was inoculated with 0.5 ml of an AMA culture of *R. japonicum*. Sterile water was added as needed to maintain the water level between 20 and 50 ml. Nodules appeared after 11 days. Acetylene-reducing activity was measured after 25 to 30 days by exposing freshly detached roots to 5% (vol/vol) acetylene in air. Nodules were stripped and weighed to determine a specific activity per nodule (fresh weight).

Polyacrylamide gel electrophoresis. Plants were grown from seedlings for 4 weeks in pouches with RBN altered such that the sulfate concentration was 2% of normal (27). The lowered sulfate did not affect the plants or nodules in any visible manner, nor was nitrogenase activity or nodule protein content changed. Four days before harvest, $Na_2^{35}SO_4$ (from New England Nuclear Corp.; 0.1 mCi per plant) was added to the liquid medium. Uninoculated plants developed infrequent, small nodules during week 4 on the extremities of lateral roots. Therefore, nodules from these regions of the root were not harvested for subsequent analysis.

In the following steps of fractionation, nodules or their contents were maintained at 0 to 4°C. To 200 mg of nodules freshly stripped from the plants was added 1.5 ml of PBS, and the nodules were crushed with a glass rod. After centrifugation for 2 min at $100 \times g$, the sedimented debris was crushed again in 1.0 ml of PBS and reextracted by centrifugation at $100 \times g$. The second supernatant extract was combined with the first, and the mixture was centrifuged for 15 min at $11,000 \times g$. The supernatant fluid was the soluble plant portion, called the "cytosol." The pellet was suspended in 1.0 ml of PBS and recentrifuged at $3,000 \times g$ for 10 min. The resulting pellet was the bacteroid fraction. Because this fraction was likely to contain plant-cell organelles as well, in one experiment it was further fractionated on a discontinuous sucrose gradient (8). Almost all of the material banded at the density corresponding to mature bacteroids (8). When this fraction was subjected to electrophoresis, it appeared identical in protein composition to the unfractionated bacteroid pellet. Polyvinylpyrrolidone (12) was not used in the above nodule extraction because it produced artifacts detected by silver staining of gels.

The method of O'Farrell (21) for two-dimensional separation of proteins was used. All bacterial cells, whether from cultures or nodules, were lysed in the same manner. The bacterial pellet was frozen on dry ice in 3-ml plastic tubes. Cold sonication buffer (21) containing 0.5 mg of DNase I (Sigma) per ml, 0.1 mg of RNase A (Sigma) per ml, and 5 mM $MgCl_2$ in 10 mM Tris-hydrochloride (pH 7.4) was added (0.15 ml of buffer per bacterial contents of 200 mg of nodules [fresh weight]). The suspension was treated without cooling for 30 s with the microtip probe of a Branson Sonifier W350 at 40 W output. After a further 5 min of incubation on ice, the extract was refrozen in dry ice. Per 0.15 ml of buffer, 100 mg of urea and 0.10 ml of lysis buffer (21) were added. After two more cycles of thawing and freezing, 30- to 100-μl samples were applied to the isoelectric focusing tube gels (21) or stored at −20°C. The soluble plant proteins of the nodules were prepared for gels as follows. Urea (100 mg) and 100 μl of lysis buffer were added to 150 μl of the cytosol portion of the nodule extract. This sample was then frozen at −20°C, or 100 μl was applied immediately to the isoelectric focusing gels.

The acrylamide concentration in the second (sodium dodecyl sulfate) dimension was 12%, with 0.32% bisacrylamide. Gels containing ^{35}S-proteins were treated with dimethyl sulfoxide and PPO (2,5-diphenyloxazole) (14), and the dried gels were placed against SR-1 film at −80°C for fluorography. Proteins were stained with Coomassie blue (21) or, more often, by the more sensitive silver staining technique (20).

Growth ex planta of *Rhizobium* for gels. A 0.3-ml portion of a fully grown broth culture in RM, which contains 6.5 mM glutamate as a nitrogen source, was subcultured into 5 ml of RM or RM containing 10 mM NH_4Cl. When the cultures had reached a density of 6×10^8 to 8×10^8/ml (24 to 31 Klett units), 0.2 mCi of $Na_2^{35}SO_4$ was added to 1 ml of the cultures. After 3 h of shaking at 30°C, the culture tubes were put in ice, 0.5 ml of an ice-cold nonradioactive culture of densely grown bacteria was added (to increase the size of the pellet), and the cultures were centrifuged. The pellet was washed once in PBS and then frozen on dry ice. The cells were disrupted by sonic oscillation in 0.1 ml of sonication buffer and incubated on ice, as described above. Urea (67 mg) and lysis buffer (67 μl) were then added, and the sample was frozen until applied to the gel. Cultures were also grown for 5 days to stationary phase, and 1.0-ml samples of these denser cultures (150 to 300 Klett units) were treated identically.

Identification of components I and II of nitrogenase. The position of nitrogenase components I and II in two-dimensional gels of bacteroid proteins was determined by using nitrogenase components purified from

nodules containing *R. japonicum* 110. These samples of pure component I and component II were kindly supplied by Daniel Arp, Biochemistry Department, University of Wisconsin, Madison. The pure components and bacteroid samples were analyzed separately, and they were analyzed together after being combined such that the concentration of added pure component I or component II was at least three times that contained in the bacteroid sample itself.

Electron microscopy. Soybean nodules (3 to 4 weeks old) infected with the wild type or with mutant strain HS124 or HS146 were processed for transmission electron microscopy. Before harvest, plants infected with HS124 or HS146 showed no detectable acetylene reducing activity. Nodules of various sizes were stripped from the plant, sliced into approximately 1-mm sections, and fixed immediately at room temperature for 2 to 3 h in 3% glutaraldehyde in 0.05 M potassium phosphate buffer (pH 6.8). Nodule segments were then rinsed for 1.5 h in phosphate buffer and postfixed for 2 h in buffered 2% osmium tetroxide at room temperature. Samples were dehydrated in an acetone series (10 to 100%) and embedded in Spurr medium. Ultrathin sections were cut with a diamond knife of a Sorvall MT-2 Ultramicrotome, mounted on copper grids, and stained in aqueous 2% uranyl acetate for 20 min followed by lead citrate for 10 min. The sections were observed with a Hitachi 11A electron microscope at an accelerating voltage of 75 kV.

Heme determination. Heme concentrations in plant nodule cytosol preparations were determined by the hemochrome method (10) as described by Bisseling et al. (6). Nodules from 20-day-old plants were used.

RESULTS

Nodulation and nitrogenase activity. Each mutant in Table 1 arose independently. The mutants derived from strain 3I1b110 were given strain numbers with the prefix HS. Likewise, strains from 61A76 were assigned numbers with the prefix SM, according to an earlier convention (18). The phenotypic designations were those suggested by Beringer (3) for nodulation (Nod) and nitrogenase activity (Fix).

The nitrogenase activity of nodulated roots was measured by the acetylene reduction assay (13). The roots inoculated with 9 of the 25 mutants exhibited partial activity after 25 days of growth, ranging from 0.3 to 10% of the specific activity of roots with wild-type *R. japonicum* (Table 1).

Sixteen strains formed as many nodules per plant as the wild type and in some cases consistently more (e.g., strain HS11). Seven were partially defective in macroscopic nodulation (Stacey et al., in press). The two remaining strains, HS124 and HS146, were defective in nodulation at the microscopic level, as will be detailed below, but visible nodulation was not delayed, nor were there fewer nodules than normal.

Analysis of nodule proteins. Two-dimensional polyacrylamide gel electrophoresis was used to analyze the nodule proteins of the mutants. Stained gels of proteins from the bacteroid and the plant portion of a nodule are shown in Fig. 1A and Fig. 2, respectively. Autoradiographs of bacteroid proteins labeled during growth with

TABLE 1. Phenotypes of the *R. japonicum* mutants defective in symbiotic nitrogen fixation

Strain	Phenotype[a]
SM4	Fix$^-$ Nod[b]
SM5	Fix$^-$; cII altered (18)
SM100	Fix$^-$
SM105	Fix$^-$ Heme$^-$ cII$^-$ Nod[c]
SM113	Fix$^-$
HS11	Fix$^-$
HS101	Fix$^-$ Nod[c]
HS102	Fix$^-$
HS104	Fix$^-$ Nod[b]; cII barely detectable
HS105	Fix (10%), Heme$^-$
HS108	Fix$^-$
HS113	Fix (2%)
HS116	Fix$^-$ cII$^-$ Nod[c]
HS118	Fix (0.3%)
HS119	Fix$^-$
HS122	Fix (1%), cII altered
HS124	Fix$^-$ leghemoglobin$^-$ cI$^-$ cII$^-$ protein 3$^-$; overall content of bacteroid and plant proteins greatly altered and decreased, defective in survival or release from infection threads
HS126	Fix (1%)
HS129	Fix (2%), Heme$^-$ Nod[c]
HS131	Fix (10%), Nod[c]
HS137	Fix$^-$
HS141	Fix (5%)
HS145	Fix (2%), Heme$^-$
HS146	Fix$^-$ leghemoglobin$^-$ cI$^-$ cII$^-$ protein 3$^-$; overall content of bacteroid and plant proteins greatly altered and decreased, defective in survival or release from infection threads
HS152	Fix$^-$ cI$^-$ cII$^-$ protein 3$^-$ Heme$^-$

[a] Ability to fix nitrogen (Fix) was assayed by measuring acetylene reduction of pouch-grown plants; a number in parentheses following "Fix" indicates the percentage of the wild-type activity per milligram (fresh weight) of nodules (35 to 55 μmol g^{-1} h^{-1}); Fix$^-$ indicates a specific activity less than 0.1% of the wild type. The absence (superscript minus) or altered structure of nitrogenase component I (cI), component II (cII), bacteroid protein 3, and leghemoglobin was assayed by two-dimensional polyacrylamide gel electrophoresis. Heme$^-$ indicates that less than 10% of the normal concentration was found in the soluble plant fraction of the nodule. The nodules of all mutants were decreased in plant proteins f and l (Fig. 2), and in all but those of strains HS105, HS124, HS146, and HS152, bacteroid protein 4 (Fig. 1C) was derepressed.

[b] Nodules were not visible until at least 7 days later than with the wild type.

[c] Less than 30% of the number of nodules per plant formed by the wild type, but of normal size and time of appearance.
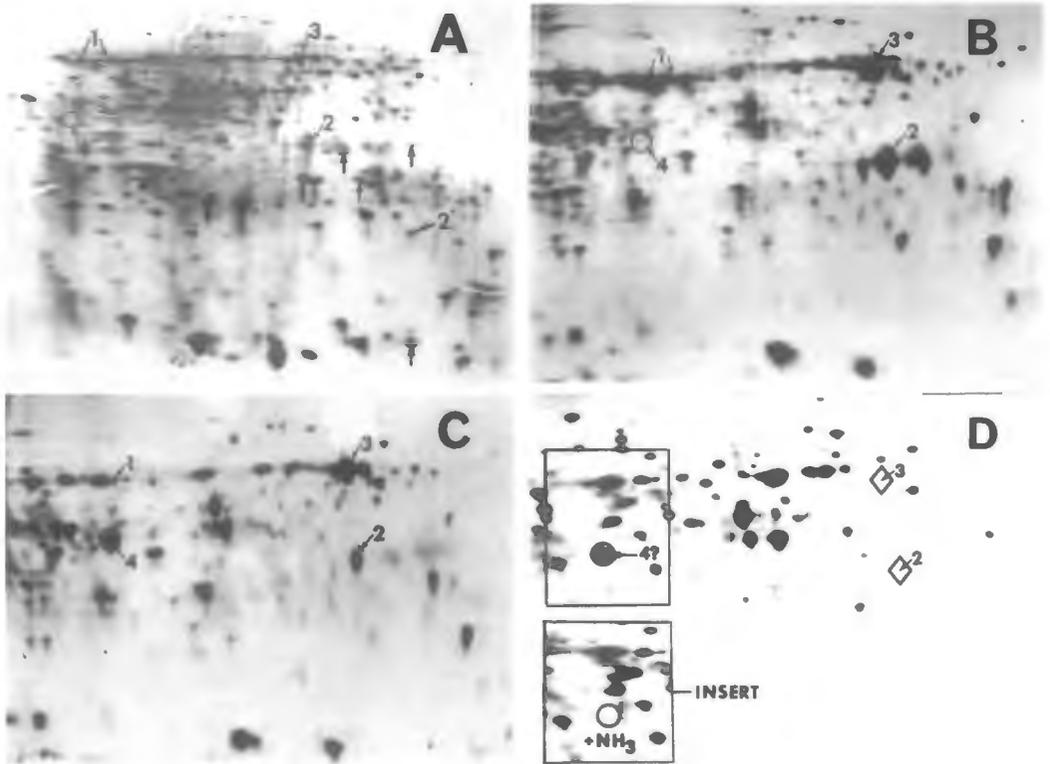
FIG. 1. Two-dimensional polyacrylamide gel of the bacteroid proteins of wild-type strain 110 (HS) stained with silver (A); autoradiographs of the ^{35}S-proteins of bacteroids of strain 110 (B) and mutant strain HS11 (C), and the ^{35}S-proteins of strain 110 in exponential growth ex planta in minimal medium RM (D). Proteins 1 and 2 are components I and II, respectively, of nitrogenase. The other three labeled proteins are explained in the text. Arrows without numerals in (A) point to proteins that were relatively enhanced in the nodule bacteria of mutant strains HS124 and HS146. In (D) are indicated the positions that would be occupied by bacteroid proteins 2, 3, and 4. The insert immediately below the boxed area shows that the protein marked "4?" was absent when strain 110 was grown in the same medium but with 10 mM ammonium added. In this figure and Fig. 2 the acidic end of the isoelectric focusing is on the right, and migration in the sodium dodecyl sulfate dimension is from top to bottom.

$^{35}SO_4^{2-}$ are shown in Fig. 1B and C. Such gels can be used as an indication of the bacteroid condition since bacteroids and free-living bacteria (Fig. 1D) each have distinct signature proteins. (Compare Fig. 1B with Fig. 1D.) Approximately half of the total protein of the plant portion was a series of proteins with the same low molecular weight (proteins a, b, c, d, and e, Fig. 2). Three of these proteins (b, c, and d) comigrated with purified leghemoglobins (Stacey, unpublished data). Since at least five soybean leghemoglobins of differing isoelectric points have been observed (11), it is likely that the other two major proteins were leghemoglobin as well. Cross-contamination of the plant proteins with bacteroid proteins, and vice versa, did not seem to be a problem. Leghemoglobin was not detected in the bacteroid gels, nor were the major bacteroid proteins apparent among the plant proteins. With the exceptions to be noted below (and summarized in Table 1), nodules of the mutants yielded the same two-dimensional gel patterns of plant and bacteroid proteins that the wild-type strain gave.

Plant portion of the nodules. The interior of normal soybean nodules is red due to the presence of leghemoglobin (11). Nodules of mutant strains HS124, HS146, HS152, HS105, HS129, HS145, and SM105 lacked this red color, and no heme (less than 4 μmol/g of fresh weight) was detected by the hemochrome assay (6) in the cytosol fraction of these nodules. Between 30 and 55 μmol of heme per g (fresh weight) of nodules (depending on plant age) was routinely found for nodules formed by the wild type. On the other hand, only the nodules of strains HS124 (Fig. 2) and HS146 completely lacked the polypeptide portion of leghemoglobin as ana-
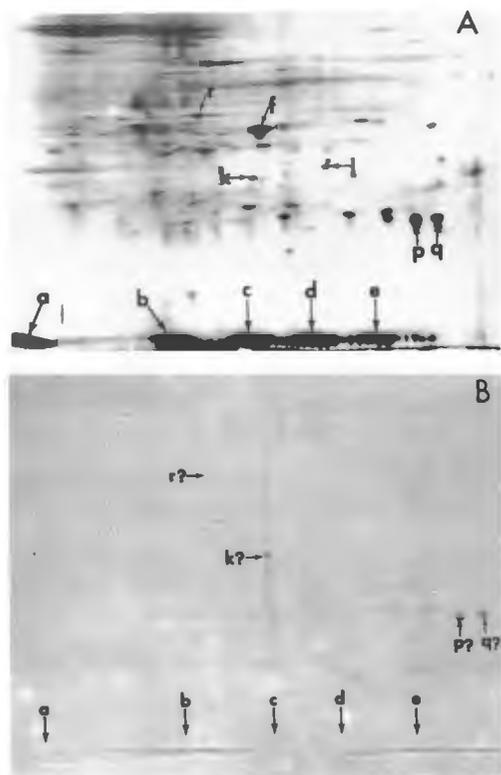
FIG. 2. Two-dimensional polyacrylamide gels of the soluble plant proteins of nodules infected by wild-type strain 110 (HS) (A) or by mutant strain HS124 (B). They have been stained with silver. Symbols are described in the text. Proteins a, b, c, d, and e, among others, are absent from the lower gel.

lyzed on two-dimensional polyacrylamide gels. The nodules of mutants HS105, HS152, and SM105 appeared to contain somewhat less of the leghemoglobin polypeptides relative to other proteins, but strains HS129 and HS145 seemed to allow the normal production of globin.

Only two plant proteins (f and l, Fig. 2) were consistently decreased in the nodules of all the Fix mutants. Protein f is a major protein of the nodules formed by wild-type *R. japonicum*. Protein l was a relatively minor constituent in nodules of the wild type and was generally undetectable in nodules of the mutants.

Proteins of mutant bacteroids. The position of nitrogenase component II in the gels of strain 3I1b110 (HS) was determined by co-electrophoresis with purified component II. It was a doublet of heterogeneous molecular weight (protein 2 of Fig. 1). A major protein with the same molecular weights and similar charge was likewise termed component II of strain SM. During storage of the purified component II a protein contaminant (2', Fig. 1) appeared to increase in

relative concentration and may be a product of component II.

The component II of strain HS122 was altered in isoelectric point. Strain SM5 also had an altered component II (18), but its component II migrated on gels identically to that of the wild type. No component II (or protein 2') was detectable from nodules of strains SM105, HS116, HS124, HS146, or HS152.

Analysis by two-dimensional gels of nitrogenase component I of strains derived from wild-type 3I1b110 was difficult because of nearby proteins (Fig. 1). We suggest tentatively that only nodules of strains HS124, HS146, and HS152 lacked this nitrogenase component. Component I of strain SM has not yet been identified.

Protein 3 was a major protein of normal bacteroids. It was not observed in *R. japonicum* grown ex planta (Fig. 1D) but was especially prominent relative to other bacteroid proteins in young nodules sampled just 3 days after they became visible, when nitrogenase component II was barely detectable. Nodules of strains HS124, HS146, and HS152 appeared not to contain this protein.

A feature common to all mutants was a visibly decreased amount of both nitrogenase component I and component II (Fig. 1C). The relative extent of this decrease varied from mutant to mutant. Another common aspect was that bacteroids of all mutants except strains HS105, HS124, HS146, and HS152 produced a major protein (protein 4, Fig. 1C) not present in wild-type bacteroids. The relative amount of this protein varied in roughly an inverse fashion with the specific acetylene-reducing activity of the nodules. In the wild-type bacteria grown ex planta on glutamate (Fig. 1D) a protein of identical electrophoretic migration was present, but when 10 mM ammonium was added, this protein was not observed. Purified glutamine synthetase II (22) of strain 61A76 (SM) (obtained from R. Darrow, Charles F. Kettering Research Laboratory, Yellow Springs, Ohio) migrated identically with this protein in the sodium dodecyl sulfate dimension but existed as two species of different charge, neither exactly matching that of protein 4 of strain SM.

Strains HS124 and HS146. In the cataloging of specific proteins to this point, the nodules of strains HS124 and HS146 have been described as lacking every one (Table 1). The nodules of these mutants were smaller than those formed by most of the mutant strains, approximately 70% of the normal fresh weight, and occasionally (perhaps 10%) were strangely amorphous. Unlike the nodules of other mutants, there was practically no bacteroid pellet after differential centrifugation. When this small pellet was ana-

lyzed by two-dimensional polyacrylamide gel electrophoresis, only about 40 proteins could be detected, most of which were found also in the wild-type bacteroids, and a few of which were in relatively high levels (Fig. 1A). Among others, proteins 1, 2, and 3 of Fig. 1 were missing. Likewise, most of the nodule plant proteins, including leghemoglobin, were missing. The ones that appeared to be present, proteins p, q, r, k, and perhaps 10 others in Fig. 2, could be the result of coincident migration of different proteins. Obviously, the symbiosis with these mutants was severely affected.

Ultrathin sections of nodules infected with wild type or mutant strains HS124 or HS146 were examined by transmission electron microscope (Fig. 3 and 4). Unlike nodules formed by the wild type, nodules formed by the mutant strains contained very few infected plant cells in their central symbiotic region. When the rare infected cells were observed, they appeared to be collapsed, with the peribacteroid vesicles and bacteroids in various stages of disintegration (Fig. 3B and 4C). The number of infection threads traversing the root nodule cells infected with mutant strain HS124 or HS146 appeared similar to those formed by the wild type; however, rarely were the bacteria released from these infection threads (Fig. 3A and 4A and B). The bacteria within the infection threads were also observed to be in various stages of disintegration. These ultrastructural results suggested that these mutants were perhaps unable to survive the entire length of the infection thread. They perhaps also were defective in release from the infection thread or in an early stage of bacteroid establishment. Unlike similarly described mutants of *R. meliloti* (25), the defect probably was not auxotrophy, for strains HS124 and HS146 grew as well as the wild type in minimal *R. japonicum* medium.

DISCUSSION

Biochemical studies have suggested that *Rhizobium* is the source of the heme in leghemoglobin (1, 9) and have predicted that there would be bacterial mutants whose nodules lacked cytosol heme. Indeed, one of the most common phenotypes of *Rhizobium* symbiotic mutants is the formation of white nodules (2, 4, 17, 18, 25, 26). However, rarely are these nodules examined for heme content (17, 18) or the presence of bacteroids (2, 4, 25, 26), and in none of the cases just cited was the presence of the polypeptide of leghemoglobin assayed. We have assayed all three. Although more sensitive analysis of heme and better quantitation of leghemoglobin is warranted, three tentative suggestions about leghemoglobin synthesis can be made. First, the five

strains that formed bacteroids, as indicated by bacteroid proteins, but whose nodules did not have detectable cytosol heme (SM105, HS105, HS129, HS145, and HS152) are the ones to assay for defective heme synthesis or export, in support of the above hypothesis that *Rhizobium* supplies leghemoglobin-heme. Second, the synthesis of the polypeptide of leghemoglobin may not be regulated by the available heme content, for almost normal globin concentrations were observed in some of the cases in which cytosol heme content was less than 10% of normal (e.g., the nodules of strain HS145). Finally, since leghemoglobin was present in nodules which contained nitrogenase mutants, such as HS122 and SM5, but absent from nodules of strains HS124 and HS146, it is synthesized independently of nitrogenase activity but perhaps only after the *Rhizobium* is released into plant cells from infection threads.

Lacking nitrogenase activity had little effect on either the overall extent or the pattern of synthesis of the 100 to 200 easily observed bacteroid proteins (Fig. 1C). In other words, the regulatory factors controlling these proteins were independent of whether the bacteroids could fix nitrogen or not. The common exception to this lack of change, the derepression of protein 4 in the mutant bacteroids (Fig. 1C), may be a response to lower internal ammonia concentrations since a protein of identical migration on two-dimensional gels was repressed by adding ammonium to the wild-type strain grown ex planta. This protein may be glutamine synthetase II (22), but the evidence with purified glutamine synthetase II was weak.

For only two mutants, SM5 (18) and HS122 (Table 1), could the defect tentatively be ascribed to a particular protein, in this case nitrogenase component II. The problem of strains HS105 and HS145 might be confined to heme production. Although specific biochemical defects were observed in other mutants, in every case there was also a problem in nodulation, or more than one biochemical parameter was altered (Table 1). The mutants designated merely as Fix⁻ (Table 1) are presented in the expectation that they will be assayed for specific biochemical functions believed essential for nitrogen fixation. They are reasonable candidates for searches of single biochemical defects, because they are unaffected in the synthesis of the major bacterial and plant proteins of the nodule (aside from the five changes common to all, mentioned in Table 1).

Legocki and Verma (15) have also used two-dimensional gel electrophoresis to study the plant and bacteroid proteins of soybean nodules. The gel patterns that they observed were vastly different from ours, but so were their methods.
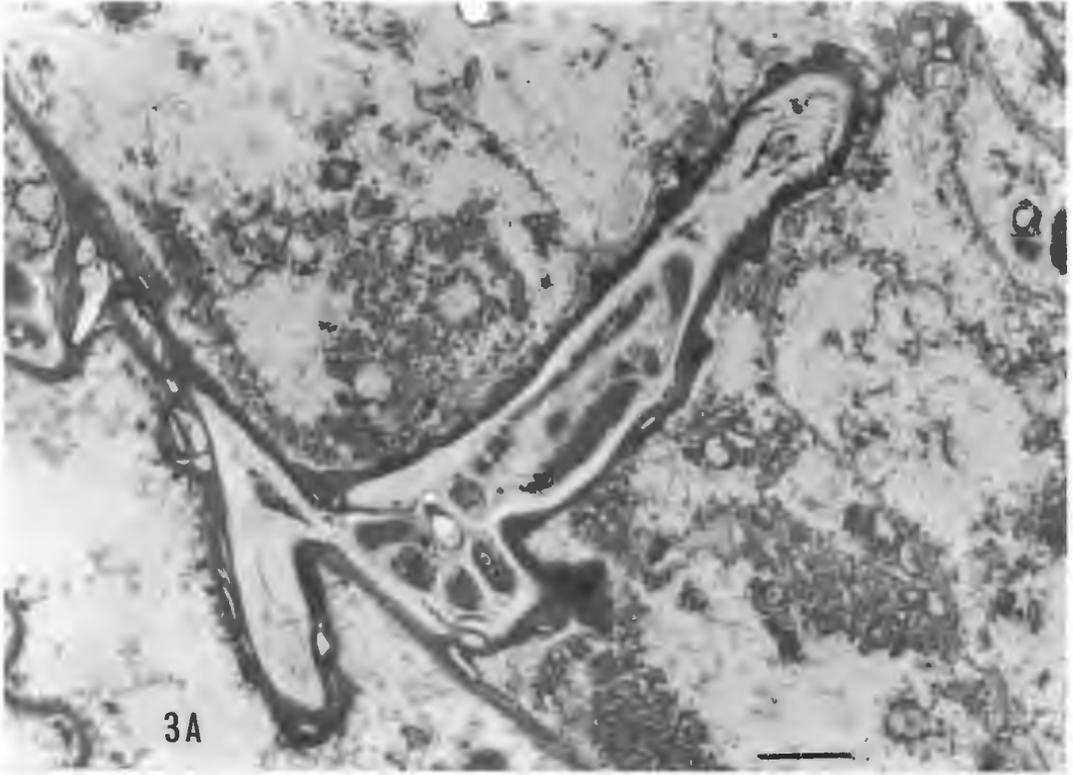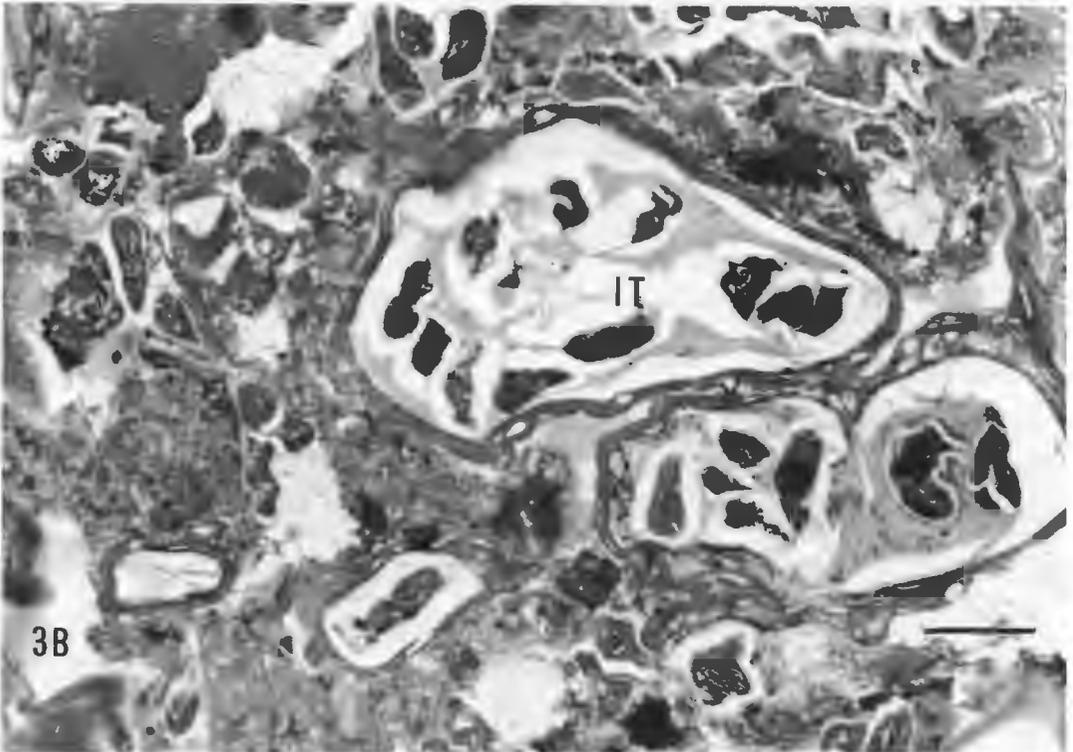
FIG. 3. Ultrathin sections of soybean root nodules infected with mutant HS124. (A) Collapsed infected cells with infection thread containing degenerated bacteria. (B) An infected cell with peribacteroid vesicles and bacteroids in various stages of disintegration. IT, Cross section of an infection thread with degenerated bacteria. Bar, 1.0 μm.
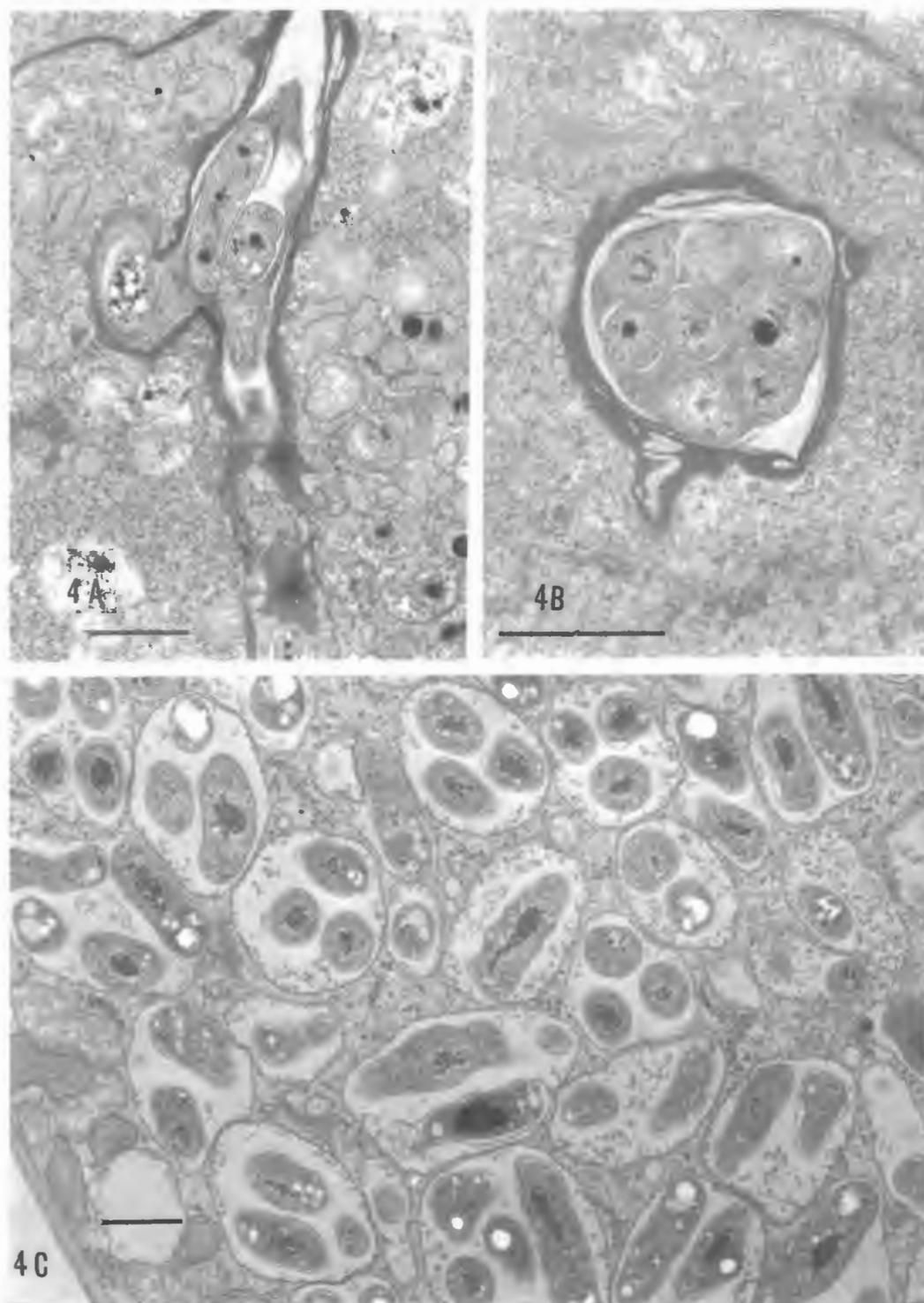
FIG. 4. Ultrathin sections of soybean root nodules infected with wild type (*R. japonicum* 3I1b110). (A) Infection thread traversing an infected cell. (B) Cross-section of an infection thread packed with healthy bacteria. (C) Peribacteroid vesicles with bacteroids. Bar, 1.0 μm.

It might be of interest to compare our in situ labeling method with their in vitro methods, using the same radioactive amino acid, for their gels indicate a greater preponderance of low-molecular-weight polypeptides.

Strains HS124 and HS146 are examples of *Rhizobium* mutants that traditionally have been classified as "ineffective" or "fixation" mutants, but which are in reality defective in nodulation. Electron microscopy of nodules formed by strains HS124 and HS146 showed that bacteria were rarely released from infection threads and, when released, were not maintained in the plant cell. Similar observations have been made after infecting alfalfa with a leucine auxotroph of *R. meliloti* (26) and clover with a mutant strain of *R. trifolii* (2). These findings with bacterial mutants are consistent with the view that the inner cortex root cells are induced to proliferate at a distance from the bacteria in the infection thread (16). In the case of strains HS124 and HS146, this proliferation was extensive enough to produce a nearly normal-size nodule, apparently without plant cells being successfully infected. On the other hand, although the plant cells multiplied extensively, they did not differentiate completely. The aforementioned alfalfa nodule plant cells did not become polyploid, as normally occurs (26). Likewise, soybean nodules formed by HS124 or HS146 did not synthesize most of the normal plant proteins, including leghemoglobin. These events may not occur until populations of bacteria are established within the plant cells.

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