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Nif⁻ Hup⁻ Mutants of *Rhizobium japonicum*†

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Two H_2 uptake-negative (Hup^-) *Rhizobium japonicum* mutants were obtained that also lacked symbiotic N_2 fixation (acetylene reduction) activity. One of the mutants formed green nodules and was deficient in heme. Hydrogen oxidation activity in this mutant could be restored by the addition of heme plus ATP to crude extracts. Bacteroid extracts from the other mutant strain lacked hydrogenase activity and activity for both of the nitrogenase component proteins. Hup^+ revertants of the mutant strains regained both H_2 uptake ability and nitrogenase activity.

Some strains of *Rhizobium japonicum* contain a membrane-bound H_2 uptake hydrogenase system. Hydrogen oxidation by bacteroids from soybean root nodules increases intracellular steady-state ATP levels which support nitrogenase activity (9). Free-living cells of *R. jeponicum* can also express hydrogenase activity (16, 19); H_2 oxidation in free-living culture can support chemoautotrophic growth (14).

The isolation and characterization of *Rhizobium* sp. mutant strains altered in H_2 oxidation activity has recently received a good deal of research attention (15, 17, 20–22, 27). Some of these mutants can reconstitute H_2 oxidation activity when cell-free extracts from two different H_2 uptake-negative (Hup^-) mutants are mixed together (21), and some are altered in regulation of H_2 oxidation by oxygen (20, 22). We now report mutants that lack N_2 fixation activity as well as H_2 uptake activity. The isolation of such mutants indicates that there are common biochemical or genetic factors involved in both symbiotic N_2 fixation and H_2 oxidation.

Properties of the wild-type Hup^+ *R. japonicum* strain SR have been described previously (17). The isolation and characterization of Hup^- mutants from strain SR (17) and methods for screening of the mutants for symbiotic properties on soybean plants (cultivar Essex) were described previously (21). The mutants retain the antibiotic resistance characteristics of the parent strain (17).

Growth conditions of *R. japonicum* SR and its mutants have previously been reported (17). Mutant strain SR143 grew poorly on minimal medium (see below); therefore it was routinely cultured on complex medium containing yeast extract (20). Derepression of H_2 oxidation activity in free-living cultures involved suspending cells to a concentration of approximately 4×10^8 viable cells per ml in 0.05 M potassium phosphate buffer (pH 7.0) containing 2.5 mM $MgCl_2$. A sample was then derepressed by incubation in an atmosphere composed of 84% N_2, 10% H_2, 5% CO_2, and 1% O_2 for 24 h as previously described (17, 20). H_2 uptake was monitored amperometrically (33) as previously described (17, 20, 26). Oxygen concentration was also monitored and varied between 50 and 120 μM during H_2 uptake assays. When only the H_2-activating hydrogenase was assayed, methylene blue was injected into the amperometric chamber to a concentration of 1 mM (for whole cells) or 100 μM (for membranes), and H_2 uptake was assayed in the absence of O_2 (24).

Soybean plants (cultivar Essex) were inoculated with approximately 5×10^8 cells and grown in sterile Leonard jar assemblies as described (32). Assays for nitrogenase activity (acetylene reduction) were performed on intact nodules on root sections (0.24- to 0.37-g nodule samples), and ethylene was quantitated by gas chromatography. Hydrogen uptake activity was determined on whole bacteroids (approximately 0.1 mg of bacteroid protein per assay) harvested from nodules, as described (9). Leghemoglobin heme concentration was determined by the pyridine hemochrome assay (8) on the nodule cytosol fraction obtained from crushing 4 to 5 g of nodules as described (3, 5). Crude extracts were prepared with butylated hydroxytoluene (4 mg/ml of buffer) in the 0.05 M potassium phosphate buffer used for rupturing cells, as described (24). The cell macerate was centrifuged in a stoppered tube (in a 100% H_2 atmosphere) at $12,000 \times g$ for 10 min, and the supernatant was removed with a syringe to an Ar-flushed stop-

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pered vial. For the heme addition experiments (see Table 2), 25 μl of hematin solution (13) and 0.25 ml of 100 mM ATP were added to 5.0 ml of the crude extracts (13). The extracts were then incubated in stoppered vials in 100% Ar for 2 h at 30°C, and 0.5-ml samples were then assayed. The addition of ATP alone (without heme) did not affect activity. Cell-free nitrogenase reactions were carried out in 12-ml vials for 15 min at 30°C as described (29, 30). Reactions were terminated by the addition of 0.1 ml of 30% (wt/vol) trichloroacetic acid, and ethylene was quantitated by gas chromatography. *Azotobacter vinelandii* strains (obtained from V. K. Shah of the University of Wisconsin, Madison) were grown and extracts were prepared as described (29); the extracts contained 12.0 to 17.1 mg of protein per ml of extract. Bacteroid crude extracts contained 3.5 to 7.2 mg of protein per ml of extract. Protein concentration was determined by the dye-binding method of Bradford (6) after hydrolysis of samples with 0.75 M NaOH at 60°C for 30 min.

Inoculation of 78 Hup^- mutants (17, 20) individually onto a group of soybean plants resulted in two strains which gave obviously poor plant growth (Fig. 1). The two Hup^- mutants SR139 and SR143, however, still nodulated the plants well (Fig. 1). We were interested in determining other symbiotic properties of the mutants, and nitrogenase activity, H_2 uptake activity, nodule color, and leghemoglobin heme concentration were measured (Table 1). Both mutants produced nodules deficient in nitrogenase and H_2 uptake activity. Strain SR143 nodules were green and deficient in heme in the leghemoglobin-containing nodule fraction. Strain SR139 nodules were pink in color and correspondingly contained almost wild-type levels of leghemoglobin heme.

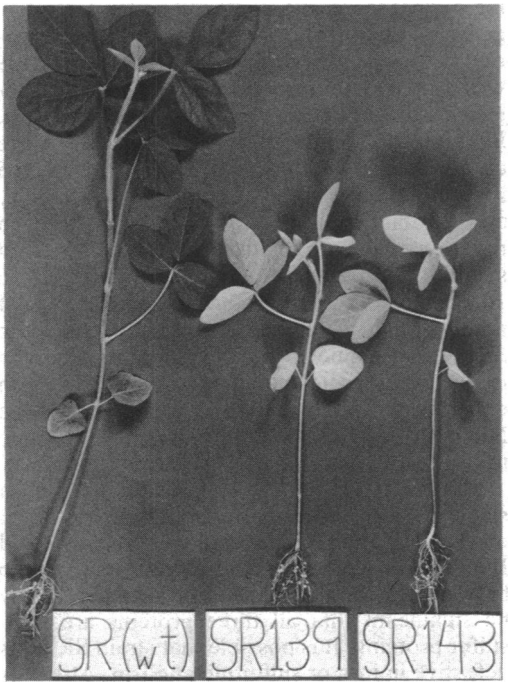

FIG. 1. Soybeans (cultivar Essex) in sterile Leonard jar assemblies (32) were inoculated with approximately 5×10^8 cells per seed, and the plants were grown in a greenhouse for 5 weeks as described (21).

We noticed that strain SR143 grew more slowly (doubling time of 34 h) than the parent strain SR (doubling time of 12 h) in minimal medium (4). After subjection of strain SR143 to the H_2 uptake derepression conditions, some H_2 uptake activity with methylene blue as the electron acceptor (2.1 nmol/h per 10^8 cells) was detected. This activity was approximately 20% of the wild-type activity. Therefore, this strain was still able to make active H_2-activating hydrogenase enzyme, but was deficient in some other component involved in H_2 oxidation.

We investigated the possibility that strain SR143 had a deficiency in heme synthesis. Electron transport activity can be restored in *Escherichia coli* heme mutants by the addition of heme plus ATP to crude extracts (12, 13). During an incubation period, the added heme is apparently inserted into apocytochromes in these mutants. By using this procedure (addition of heme to extracts) we were able to obtain H_2 oxidation activity (with O_2 as acceptor) in extracts of strain SR143 (Table 2). Addition of heme to strain SR143 extracts resulted in recovery of 18% of the wild-type activity. The added heme is not acting merely as an electron acceptor, since the added heme did not enhance wild-type activity (Table 2). Strain SR143 presumably makes all of the components (e.g., hydrogenase, quinone, apocytochromes) needed for H_2 oxidation (26), but is deficient in heme synthesis. Attempts to derepress H_2 oxidation activity by the addition

TABLE 1. Symbiotic characteristics of Nif^- Hup^- mutants[a]

Strain	Nitrogenase activity[b]	Hydrogen uptake activity[c]	Nodule color	Leghemoglobin concn[d]
SR	8.3	2.9	Red/pink	0.27
SR143	<0.2	<0.1	Green	0.05
SR139	<0.2	<0.1	Pink	0.22

[a] Nodules from 5- to 6-week-old greenhouse-grown plants (21) were used for all assays.

[b] Micromoles of C_2H_4 produced per hour per gram of nodule fresh weight.

[c] Micromoles of H_2 uptake per hour per milligram of bacteroid protein.

[d] Micromoles of heme per gram of nodule fresh weight.

TABLE 2. Effect of addition of heme to crude extracts of strain SR143 and the wild-type strain SR

Crude extract	Addition	H_2 uptake activity[a]
SR143	None	<0.05
SR143	Heme + ATP	0.7
SR	None	3.7
SR	Heme + ATP	3.8

[a] Nanomoles of H_2 consumed per minute per milligram of protein, with oxygen (50 to 80 μM) as the electron acceptor.

of δ-aminolevulinic acid to cultures of strain SR143 were unsuccessful.

There is good evidence that *Rhizobium* sp. supplies heme for nodule leghemoglobin (2, 7). The inability of nodules formed by strain SR143 to express nitrogenase activity is probably a secondary consequence of low heme levels in the leghemoglobin cytosol portion of the nodules. Correlations between the ability of *Rhizobium* sp. to produce red-colored (leghemoglobin-containing) nodules and to fix N_2 are common (11, 32). Recently, mutants of *R. japonicum* that are very poor in symbiotic nitrogen fixation ability and also lack heme in the cytosol nodule fraction have been described (25). Mutant strain SR143 may be useful for studies on heme production in nodules, but it does not seem to have a defect directly related to hydrogenase and nitrogenase.

Bacteroid extracts of *R. japonicum* can complement with the two nitrogenase component proteins of *A. vinelandii* to give an active nitrogen-fixing preparation (23). We tested bacteroid extracts of strain SR139 for the presence of the two active nitrogenase component proteins by complementation with *A. vinelandii* Nif$^-$ mutants. *A. vinelandii* mutant strain UW38 makes active component II but not component I, whereas *A. vinelandii* mutant strain UW91 makes active component I but not component II (30). Cell-free extracts from bacteroids of strain SR139 had no acetylene-reducing activity, whereas extracts from the parent strain SR were able to reduce acetylene (Table 3). The addition of component I (UW91 extract) or component II (UW38 extract) did not complement strain SR139 extracts. However, an extract from bacteroids of a component II-negative *R. japonicum* mutant (strain SM5) (18, 25) could be complemented by *A. vinelandii* component II (UW38 extract). We can conclude that strain SR139 bacteroids seem to be missing activity for both of the nitrogenase component proteins.

Methylene blue is an excellent electron acceptor for purified *R. japonicum* hydrogenase (1). We tested whole cells and membranes of strain SR139 (from both bacteroids and derepressed free-living cells) for H_2 uptake with methylene blue as electron acceptor (24). Neither membranes nor whole cells of strain SR139 took up H_2 with methylene blue; therefore, this mutant lacks activity for the H_2-activating hydrogenase enzyme.

TABLE 3. Complementation of nitrogenase in bacteroid extracts of *R. japonicum* with extracts from Nif$^-$ *A. vinelandii* mutants[a]

Source of extract (mg of protein per assay)	Nitrogenase activity (nmol of C_2H_4 formed per min per vial)
UW38 (0.4)	0.7
UW91 (0.4)	0.5
UW38 (0.4) + UW91 (0.4)	22.2
SR (0.5)	3.7
SR139 (0.5)	0.0
SR139 (0.5) + UW38 (0.4)	0.5
SR139 (0.5) + UW91 (0.4)	0.6
SM5 (0.5)	0.0
SM5 (0.5) + UW38 (0.4)	2.4

[a] Nodules were harvested from Essex soybeans grown in sterile Leonard jar assemblies (32) as described previously (21). Crude extracts were prepared from bacteroids by a modification (18) of the procedure described by Evans et al. (10).

It was possible that strain SR139 contained two or more independent mutations, one affecting hydrogenase and another one for the nitrogenase components. Reversion tests were carried out to examine this possibility. Strain SR139 was plated onto carbon-free medium and subjected to autotrophic growth conditions (17). Revertants able to grow autotrophically were obtained at a frequency of 7.4×10^{-7}. Seven revertants were tested for both H_2 uptake ability and symbiotic N_2 fixation activity. All seven revertants able to oxidize H_2 also expressed symbiotic nitrogenase activity (range of 3.4 to 10.1 μmol of C_2H_4 produced per h per g of nodule fresh weight). Therefore the lesion in SR139 that results in the Nif$^-$ Hup$^-$ phenotype is due to a single mutation. Similar reversion experiments on strain SR143 indicated that the Nif$^-$ Hup$^-$ phenotype of this strain was also due to a single mutation.

Mutant strain SR139 apparently contains a single genetic lesion that affects three proteins: hydrogenase and nitrogenase components I and II. Perhaps there is a structural relationship between the three proteins. For example, it may be significant that all three proteins are iron-sulfur proteins. It is possible that strain SR139 is defective in iron or sulfur metabolism. Attempts to derepress H_2 oxidation activity in this mutant by adding iron (up to 5 mM ferrous sulfate) to the derepression medium have been unsuccessful.

Alternatively, there may be a genetic or regulatory relationship between the hydrogenase enzyme and nitrogenase components in *R. japonicum*. Nif$^-$ Hup$^-$ mutants of *Rhodopseudomonas acidophila* have been described by Siefert and Pfennig, but the mutants were not characterized further (31). However, Nif$^+$ revertants of these mutants also were Hup$^+$. These authors suggested that a regulatory relationship exists between nitrogenase and hydrogenase. Further analysis of strain SR139 for such properties as reaction to antiserum prepared against purified hydrogenase and nitrogenase should aid in determining nitrogenase-hydrogenase relationships.

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