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Isolation of Genes (*nif/hup* Cosmids) Involved in Hydrogenase and Nitrogenase Activities in *Rhizobium japonicum*†

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Recombinant cosmids containing a *Rhizobium japonicum* gene involved in both hydrogenase (Hup) and nitrogenase (Nif) activities were isolated. An *R. japonicum* gene bank utilizing broad-host-range cosmid pLAFR1 was conjugated into Hup$^-$ Nif$^-$ *R. japonicum* strain SR139. Transconjugants containing the *nif/hup* cosmid were identified by their resistance to tetracycline (Tc^r) and ability to grow chemoautotrophically (Aut$^+$) with hydrogen. All Tc^r Aut$^+$ transconjugants possessed high levels of H_2 uptake activity, as determined amperometrically. Moreover, all Hup$^+$ transconjugants tested possessed the ability to reduce acetylene (Nif$^+$) in soybean nodules. Cosmid DNAs from 19 Hup$^+$ transconjugants were transferred to *Escherichia coli* by transformation. When the cosmids were restricted with *Eco*RI, 15 of the 19 cosmids had a restriction pattern with 13.2-, 4.0-, 3.0-, and 2.5-kilobase DNA fragments. Six *E. coli* transformants containing the *nif/hup* cosmids were conjugated with strain SR139. All strain SR139 transconjugants were Hup$^+$ Nif$^+$. Moreover, one *nif/hup* cosmid was transferred to 15 other *R. japonicum* Hup$^-$ mutants. Hup$^+$ transconjugants of six of the Hup$^-$ mutants appeared at a frequency of 1.0, whereas the transconjugants of the other nine mutants remained Hup$^-$. These results indicate that the *nif/hup* gene cosmids contain a gene involved in both nitrogenase and hydrogenase activities and at least one and perhaps other *hup* genes which are exclusively involved in H_2 uptake activity.

Rhizobium japonicum is the microorganism which forms an effective nitrogen-fixing symbiosis with soybeans (*Glycine max*). Nitrogenase catalyzes both the ATP-dependent reduction of N_2 to NH_4^+ and the reduction of protons to H_2. The evolution of H_2 by the nitrogenase reaction results in a substantial energy loss during nitrogen fixation (27). Some strains of *R. japonicum* express a membrane-bound H_2 uptake hydrogenase system as bacteroids in soybean nodules (5). In 1972, Dixon proposed that the H_2 uptake system may be used to (i) reoxidize the H_2 evolved from nitrogenase for additional ATP and reductant for nitrogen fixation, (ii) utilize the excess O_2 to protect the oxygen-labile nitrogenase, and (iii) prevent H_2 inhibition of nitrogenase (5). In both greenhouse and field studies, soybean plants inoculated with *R. japonicum* hydrogen uptake-positive (Hup$^+$) strains have been shown to possess increased plant dry matter and increased amounts of nitrogen in seeds compared with plants inoculated with *R. japonicum* Hup$^-$ strains (8, 31).

The *R. japonicum* strains which possess H_2 uptake capability in bacteroids can also utilize H_2 in free-living cultures when they are grown chemoautotrophically under microaerophilic conditions (9, 14). Chemoautotrophic growth conditions have been used to isolate numerous *R. japonicum* Hup$^-$ mutants (15, 18). Biochemical and physiological characterizations of some of these Hup$^-$ mutants have helped to elucidate the components involved in H_2 uptake and also how the system is regulated. Hydrogen oxidation activity is reconstituted when cell-free extracts from two different *R. japonicum* Hup$^-$ mutants are combined (22). Other mutants have H_2 uptake systems that are hypersensitive to repression by carbon substrates and O_2 (21). One of the most interesting classes of mutants is the class of mutants which are defective in both nitrogenase (Nif$^-$) and hydrogenase (Hup$^-$) activities (26). Nif$^-$ Hup$^-$ mutant strain SR139 lacks activity for hydrogenase and both of the nitrogenase component proteins. However, reversion studies have indicated that strain SR139 has only a single lesion which affects both nitrogenase and hydrogenase (26). Furthermore, this mutant strain produces nodules containing normal levels of leghemoglobin and has a growth rate in free-living cultures like that of the parent strain. These results indicate that the lesion is specific for the symbiotic properties Nif and Hup. The isolation of Nif$^-$ Hup$^-$ mutants indicates that common biochemical components or genetic components or both are involved in both nitrogen fixation and hydrogen oxidation.

Studies on the organization of the genes responsible for H_2 oxidation in *R. japonicum* have been initiated. Cantrell et al. (3) used an *R. japonicum* gene bank to isolate an H_2 uptake gene (*hup*) by complementation of an uncharacterized Hup$^-$ mutant. When several *hup* gene cosmids were transferred by conjugation into a different Hup$^-$ mutant, Hup$^+$ transconjugants appeared at a low frequency. These results showed that the cosmid may contain one *hup* gene and part of another. Recently, Haugland et al. (10) reported that some *hup* genes of *R. japonicum* DES122 are linked in one area of the chromosome and organized in a minimum of two transcriptional units. These authors showed that approximately 15 kilobases (kb) of cosmid pHU1 DNA code for *hup* genes.

In this study, we isolated a cosmid which contains an *R. japonicum* gene involved in both nitrogenase and hydrogenase activities (*nif/hup*) by complementation of Nif$^-$ Hup$^-$ *R. japonicum* mutant strain SR139. Moreover, one *nif/hup* recombinant cosmid also complemented several Hup$^-$ Nif$^+$ mutants. Our results indicate that this cosmid contains a *nif/hup* gene, as well as some other *hup* genes.

(A preliminary report of this study has been presented previously [Hom, Graham, and Maier, Abstr. 2nd Int. Symp. Mol. Genet. Plant-Bacteria Interaction, Ithaca, N.Y., Abstr. no. 50, 1984].)

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype[a]	Reference
R. japonicum		
SR	Sm^r Km^r Hup^+	23
SR139	Sm^r Km^r Hup^- Nif^-	26
SR114	Sm^r Km^r Hup^-	18
SR118	Sm^r Km^r Hup^-	18
SR119	Sm^r Km^r Hup^-	21
SR122	Sm^r Km^r Hup^+ Cfx^-	18
SR129	Sm^r Km^r Hup^-	21
SR140	Sm^r Km^r Hup^-	18
SR143	Sm^r Km^r Hup^- Nif^-	26
SR146	Sm^r Km^r Hup^-	22
SR152	Sm^r Km^r Hup^-	18
SR166	Sm^r Km^r Hup^-	18
SR174	Sm^r Km^r Hup^-	21
SR178	Sm^r Km^r Hup^-	21
SR180	Sm^r Km^r Hup^-	21
SR186	Sm^r Km^r Hup^-	21
SR192	Sm^r Km^r Hup^-	18
SU	Rif^r Hup^+ (derivative of USDA 110)	12
SU306-47	Rif^r (Tn*5* induced) Hup^-	This study
E. coli HB101	*pro leu thi lacY* Sm^r *endA*	2
Plasmids		
pLAFR1	Tc^r	6
pRK2013	Km^r	4

[a] Sm, streptomycin; Km, kanamycin; Rif, rifampin; Hup, oxygen-dependent hydrogen uptake activity; Nif, nitrogenase activity; Cfx, carbon dioxide fixation activity (via ribulose bisphosphate carboxylase).

MATERIALS AND METHODS

Chemicals. Streptomycin sulfate, kanamycin sulfate, rifampin, *d*-mannitol, Tween 40, sodium gluconate, α-ketoglutarate, and EDTA (sodium salt) were obtained from Sigma Chemical Co., St. Louis, Mo. Tetracycline hydrochloride was purchased from Calbiochem-Behring, La Jolla, Calif. Yeast extract, tryptone, and agar were supplied by Difco Laboratories, Detroit, Mich. N_2 and the gas mixture used for autotrophic growth were obtained from Arundel Sales and Service Co., Baltimore, Md. Tris, sodium dodecyl sulfate, phenol, agarose, chloroform, and restriction endonuclease *Eco*RI were purchased from International Biotechnologies Inc., New Haven, Conn. All other chemicals were reagent grade and were supplied by J. T. Baker Chemical Co., Phillipsburg, N.J.

Bacterial strains, plasmids, and gene bank. The bacterial strains and plasmids used in this study are listed in Table 1. *R. japonicum* strain SU306-47 was isolated as a Tn*5*-induced Hup^- mutant of strain USDA 110 Rif^r (characterization of strain SU306-47 will be described elsewhere). *R. japonicum* stock cultures were maintained on mannitol-yeast extract (MSY) agar plates supplemented with the following antibiotic(s), when necessary: streptomycin (250 μg/ml), kanamycin (100 μg/ml), rifampin (100 μg/ml) and tetracycline (60 μg/ml). *Escherichia coli* stock cultures were maintained on LB agar plates supplemented with 15 μg of tetracycline per ml and 50 μg of kanamycin per ml.

The *R. japonicum* DNA gene bank used in this study was supplied by G. Stacey, University of Tennessee. *R. japonicum* I-110 DNA was partially digested with restriction endonuclease *Eco*RI and ligated into cosmid pLAFR1. The average insertion size was approximately 20 kb (P. Russell et al., manuscript in preparation).

Media and growth conditions. *R. japonicum* was routinely grown in either the MSY medium described by Lim and Shanmugam (16) or the yeast extract medium described by Hom et al. (12). LB medium was used as described by Miller to cultivate *E. coli* (25). For autotrophic growth, BM medium containing no carbon was used as described by Maier (18). Since Stults and co-workers have recently shown that nickel is an integral component of hydrogenase in *R. japonicum* SR (28), BM medium containing no carbon was supplemented with 5 μM $NiCl_2$. After inoculation, agar plates (1.5% Noble agar) were placed in gas-tight 20-liter polyethylene buckets. The containers were sparged first with N_2 and then with a gas mixture consisting of 10% H_2, 5% CO_2, 1% O_2, and 84% N_2. The buckets were incubated at 30°C for 7 to 14 days and resparged with the gas mixture every 2 days.

Bacterial filter mating technique. Late log phase *E. coli* donor cells and mid-log phase *R. japonicum* recipient cells were grown as previously described (12). Donor and recipient cells (10^9 cells each) were mixed, collected onto a metrical membrane filter (diameter, 25 mm; pore size, 0.45 μm; Gelman Sciences, Inc., Ann Arbor, Mich.), and incubated on yeast extract agar plates at 30°C for 5 days. The cells were then suspended in 5 ml of 0.01% (vol/vol) Tween 40 and serially diluted, and portions were spread onto the appropriate selection plates. The plates were incubated at 30°C for 7 to 14 days. The titer of recipients at the time of plating onto the selective medium was determined by spreading samples onto mannitol-glutamate agar plates and incubating the plates at 30°C for 7 days.

Determination of the MIC. The MIC was defined as the lowest antibiotic concentration at which no colonies appeared. Mid-log phase *R. japonicum* cells were cultivated, and samples containing 100 to 300 cells were spread onto MSY agar plates containing different levels of tetracycline, as described previously (12).

Hydrogen uptake induction and assay. The H_2 uptake activity of free-living cultures of *R. japonicum* was measured by the induction procedure of Maier (18), with minor modifications. After growth in yeast extract medium (supplemented with 50 μg of tetracycline per ml when transconjugants were cultured) to an optical density at 540 nm of 0.5 to 0.7, the bacteria were harvested by centrifugation at 1,500 × *g* for 10 min at room temperature and suspended in 0.05 M potassium phosphate buffer (pH 7.0) containing 2.5 mM $MgCl_2$. Then 10-ml portions of cells (optical density at 540 nm, 0.5) were dispensed into 160-ml serum bottles (catalog no. 223748; Wheaton, Millerville, N.J.). The serum bottles were sealed with rubber stoppers (13 by 20 mm) fitted with aluminum collars and then sparged with 10% H_2–5% CO_2–1% O_2–84% N_2. The bottles were incubated at 30°C for 48 h on a rotary shaker (100 rpm; model G-24; New Brunswick Scientific Co., Inc., Edison, N.J.).

H_2 uptake specific activities were measured amperometrically as described previously (20, 30), except that the amperometric chamber size was 4.5 ml. Cell numbers were estimated by utilizing a standard curve of colony-forming units versus optical density at 540 nm.

Soybean nodulation and nitrogenase assays. *R. japonicum* strains were checked for effectiveness (nitrogen fixation) with soybeans (*G. Max* L. Merr. cv. Essex). Seeds were surface sterilized by immersion first in 95% ethanol and then in 0.2% $HgCl_2$, rinsed 10 times with sterile distilled water, and incubated in the dark for 2 days at 30°C on the surfaces of 1% water agar plates, as described by Vincent (29). *R. japonicum* cultures for seed inoculation were grown in MSY medium as described previously (12). The MSY medium used for growing transconjugants was supplemented with 50 μg of tetracycline per ml. The germinated soybean seeds

were inoculated with 10^9 *R. japonicum* cells. Soybean plants were grown for 6 weeks by the pouch method, as described previously (19). Uninoculated control plants did not form nodules.

Nitrogenase activities were measured by placing root sections containing nodules into 160-ml serum bottles. After the bottles were sealed with rubber stoppers fitted with aluminum collars, acetylene was added to a final concentration of 10% (vol/vol). After incubation for 30 min at room temperature, acetylene reduction (ethylene formation) was determined by gas chromatography as described previously (12). Acetylene reduction activity was linear through this time period.

The *R. japonicum* strains responsible for infection were isolated from nodules by the nodule surface sterilization procedure of Vincent (29), which utilized 95% ethanol and 3% hydrogen peroxide. Nodules were crushed onto MSY agar plates containing 60 μg of tetracycline per ml. These agar plates were incubated at 30°C for 10 days.

Nitrogenase induction and assay. Nitrogenase activity in free-living cultures of *R. japonicum* was induced by the procedure of Agarwal and Keister (1). Tetracycline (50 μg/ml) was included in the induction cultures of *R. japonicum* transconjugants. Prescription bottles (volume, 250 ml) were used instead of 500-ml reagent bottles. Acetylene (10%, vol/vol) was added at the beginning of the induction period. Nitrogenase activities and estimates of cell numbers were determined as described above.

Cosmid DNA isolation and bacterial transformations. Cosmid DNAs from strain SR139 transconjugants were isolated as described by Kado and Liu (13), with modifications. *R. japonicum* transconjugants were grown in yeast extract medium containing 50 μg of tetracycline per ml to late log phase, washed twice with 40 mM Tris-acetate–2 mM EDTA (pH 7.9) (E buffer) containing 3% (wt/vol) NaCl and once with E buffer to remove the exopolysaccharides. The final cell pellet was suspended in 0.5 ml of E buffer, and the cells were lysed with 1.0 ml of 3% (wt/vol) sodium dodecyl sulfate in E buffer (pH 12.6). After incubation at 65°C for 1 h, the preparation was extracted with 1.5 ml of unbuffered phenol-chloroform (1:1) and centrifuged at 8,000 × *g* for 15 min. The DNA in the aqueous phase was precipitated by adding 0.1 volume of 2.5 M sodium acetate (pH 5.2) and 2 volumes of cold ethanol. After incubation at −20°C overnight, the DNA was harvested by centrifugation for 15 min in a microfuge at 4°C. After the DNA pellet was dried, the DNA was suspended in 6 mM Tris–0.1 mM EDTA–10 mM NaCl (pH 7.4).

Competent *E. coli* HB101 cells were prepared by the procedure of Maniatis et al. (24). Freshly prepared competent strain HB101 cells were transformed with cosmid recombinant DNA as described previously (24). Transformants were selected on LB agar plates containing 15 μg of tetracycline per ml.

RESULTS

Complementation of an *R. japonicum* Nif⁻ Hup⁻ mutant with an *R. japonicum* gene bank. Nif⁻ Hup⁻ mutant strain SR139 was conjugated with the pLAFR1 cosmid clone library. *R. japonicum* colonies able to grow autotrophically (Aut⁺) with hydrogen and carbon dioxide as sole energy and carbon sources were selected. In filter matings between *E. coli* donors containing the *R. japonicum* I-110 clone bank and strain SR139 recipients, the frequency of Aut⁺ colonies per recipient (3.2×10^{-6}) was two times greater than the frequency of spontaneous strain SR139 Aut⁺ revertants (1.6×10^{-6}). The Aut⁺ strains were isolated and then screened for growth on MSY agar plates containing tetracycline (60 μg/ml). Of 156 Aut⁺ colonies tested, 27 tetracycline-resistant (Tc^r) strains were isolated. Three randomly chosen Aut⁺ Tc^r strains [strains SR139(pSH7), SR139(pSH14), and SR139(pSH20)] possessed substantially increased tetracycline resistance compared with strain SR139. The single-cell MIC for tetracycline for these three strains was >150 μg/ml, which was at least three times greater than that of parental strain SR139 (45 μg/ml). Moreover, all of the Tc^r Aut⁺ isolates possessed the streptomycin resistance and kanamycin resistance of the strain SR139 parent. These results suggest that the Tc^r Aut⁺ strains were strain SR139 transconjugants that harbored a clone containing a gene which complemented the Hup⁻ phenotype.

TABLE 2. Hydrogen oxidation activities of strain SR139 transconjugants

Strain	Hydrogen uptake activity (nmol of H_2 oxidized per h per 10^8 cells)[a]
SR	136
SR139	0
SR139(pLAFR1)	0
SR139(pSH7)	201
SR139(pSH14)	156
SR139(pSH15)	102
SR139(pSH16)	45
SR139(pSH17)	78
SR139(pSH18)	53
SR139(pSH19)	84
SR139(pSH20)	158
SR139(pSH21)	76
SR139(pSH22)	82
SR139(pSH23)	83
SR139(pSH24)	62
SR139(pSH25)	112
SR139(pSH26)	93
SR139(pSH27)	153
SR139(pSH28)	45
SR139(pSH29)	68
SR139(pSH30)	126
SR139(pSH31)	76
SR139(pSH32)	84
SR139(pSH33)	110
SR139(pSH34)	79
SR139(pSH35)	91
SR139(pSH37)	69
SR139(pSH38)	131

[a] Cells were grown, harvested, suspended in 0.05 M potassium phosphate buffer, and derepressed for 48 h in an atmosphere containing 84% N_2, 10% H_2, 5% CO_2, and 1% O_2, as described in the text. Oxygen-dependent hydrogen uptake specific activities were determined amperometrically as described in the text.

Hydrogen uptake and nitrogenase activities in putative strain SR139 transconjugants. A reversion analysis of strain SR139 had previously shown that the genetic defect which resulted in the Hup⁻ Nif⁻ phenotype was a single mutation (26). Strain SR139 revertants able to grow autotrophically were shown to possess both hydrogen uptake and symbiotic nitrogenase activities. To further establish positive complementation of both defects of strain SR139, oxygen-dependent H_2 uptake activities were measured amperometrically with whole cells of strain SR139 Tc^r Aut⁺ transconjugants (Table 2). All transconjugants possessed high levels of H_2 uptake specific activities. That the various transconjugants showed H_2 uptake activities that ranged from 30 to 150% of the wild-type activity should not be stressed, because vari-

TABLE 3. Symbiotic nitrogenase activities of strain SR139 transconjugants

Strain	Nitrogenase sp act (μmol of C_2H_2 reduced per h per g of fresh nodule wt)[a]
SR	17.8
SR139	0.00
SR139(pLAFR1)	0.00
SR139(pSH15)	0.59
SR139(pSH16)	1.02
SR139(pSH17)	0.52
SR139(pSH19)	1.06
SR139(pSH22)	0.50
SR139(psH26)	0.22

[a] Six-week-old soybean plants were harvested, and nodules on root sections were assayed for acetylene reduction as described in the text.

ability in H_2 oxidation rates even in a single strain after derepression has been observed previously (3).

Second, nitrogenase activities (as determined by acetylene reduction assays) were measured by using root sections containing nodules from soybean plants that had been inoculated with six transconjugants [Table 3, strains SR139(pSH15), SR139(pSH16), SR139(pSH17), SR139(pSH19), SR139(pSH22), and SR139(pSH26).]. These six transconjugants were chosen for analysis because the cosmids that were isolated from them complemented strain SR139 at a frequency of 1.0 (see below). Nodules from plants inoculated with the transconjugants possessed nitrogenase specific activities that were approximately 1 to 6% of the value obtained for wild-type strain SR. On the other hand, nodules from plants inoculated with strain SR139 or SR139(pLAFR1) did not have nitrogenase activity. Tc^r Hup^+ strains were reisolated from nodules from soybean plants that had been inoculated with these *R. japonicum* transconjugants.

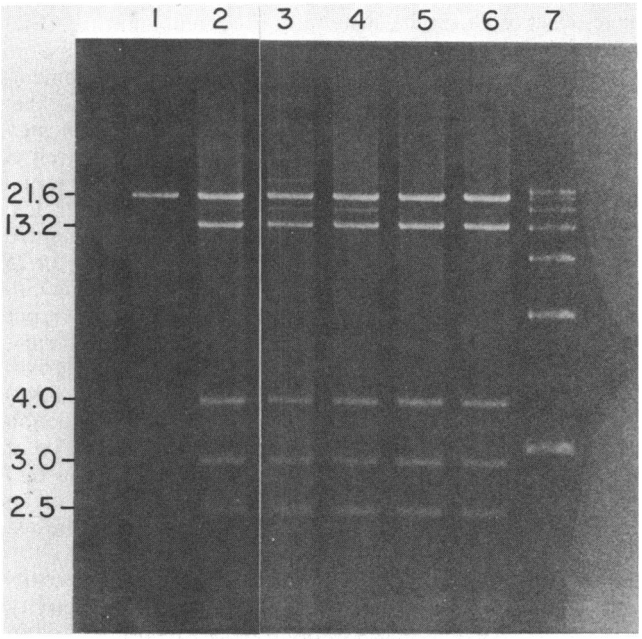

FIG. 1. *Eco*RI digests of cosmids containing the *nif/hup* gene. Cosmid DNAs partially purified from five independent Hup^+ strain SR139 transconjugants were used to transform *E. coli* HB101. Cosmid DNAs were isolated from the transformants and digested with restriction endonuclease *Eco*RI, and the DNA fragments were separated by gel electrophoresis in 0.6% agarose. Lane 1, pLAFR1; lane 2, pSH7; lane 3, pSH19; lane 4, pSH20; lane 5, pSH22; lane 6, pSH26; lane 7, linear oligomers of λdv21 DNA. The sizes of the λdv21 DNA oligomers were 22.1, 18.9, 15.8, 12.6, 9.4, 6.3, and 3.1 kb.

TABLE 4. Complementation of strain SR139 with recombinant cosmids containing the *nif/hup* gene[a]

E. coli donor[b]	Tc^r/recipient[c]	Aut^+/Tc^r[d]
HB101(pSH15)	1.0×10^{-3}	1.0
HB101(pSH16)	8.1×10^{-4}	1.0
HB101(pSH17)	7.2×10^{-4}	1.0
HB101(pSH19)	1.0×10^{-3}	1.0
HB101(pSH22)	1.8×10^{-4}	1.0
HB101(pSH26)	9.8×10^{-4}	1.0
HB101(pSH25)	5.8×10^{-4}	$<2.0 \times 10^{-2}$
HB101(pLAFR1)	1.0×10^{-4}	$<1.0 \times 10^{-2}$

[a] The recipient for all matings was Nif^- Hup^- strain SR139.
[b] Strain HB101(pRK2013) was included in all matings to mobilize pLAFR1 and recombinant cosmids.
[c] Frequency of plasmid transfer as expressed by the number of tetracycline-resistant colonies per recipient.
[d] Frequency of *nif/hup* gene transfer as expressed by the number of colonies able to grow under autotrophic conditions per tetracycline-resistant colony.

Analysis of cosmids containing the *nif/hup* gene. Cosmid DNAs from 19 strain SR139 transconjugants were isolated and transferred to *E. coli* HB101 by transformation. Cosmid DNAs from the strain HB101 transformants were digested with restriction endonuclease *Eco*RI, and DNA restriction fragments were separated by agarose gel electrophoresis (Fig. 1). All of the cosmids possessed a 21.6-kb DNA fragment which comigrated with the linearized pLAFR1 vector (Fig. 1, lane 1). Three distinct *Eco*RI restriction patterns were detected among the 19 cosmid clones. The predominant pattern (15 of 19 cosmids restricted) (Fig. 1, lanes 2 through 6) contained 21.6-, 13.2-, 4.0-, 3.0-, and 2.5-kb DNA fragments. The second type pattern (3 of 19 cosmids) showed a single DNA fragment which comigrated with the pLAFR1 band. Since the strain SR139 recipients were *rec*$^+$, the *R. japonicum* DNA insertion could have recombined into the genome. The three strain SR139 Tc^r Hup^+ transconjugants which showed this pattern were not studied further. Finally, the third type of restriction pattern, which was found only in pSH25, had 21.2-, 11.7-, and 9.7-kb DNA fragments (data not shown).

To show the presence of the *nif/hup* gene in cosmid clones, representatives from the two types of cosmids with

TABLE 5. Ex planta nitrogenase activity of strain SR139 transconjugants obtained from 1:1 complementation studies

Strain	Nitrogenase sp act (nmol of C_2H_2 reduced per h per 10^9 cells)[a]
SR	3.70
SR139	0.00
SR139(pLAFR1)	0.00
SR139(pSH15)	1.19
SR139(pSH16)	1.21
SR139(pSH17)	1.18
SR139(pSH19)	1.69
SR139(pSH22)	1.71
SR139(pSH26)	1.36

[a] Cultures were induced and assayed for nitrogenase activity as described in the text.

different *Eco*RI restriction patterns were transferred via conjugation from *E. coli* HB101 to strain SR139. When six randomly chosen recombinant cosmids which possessed the predominant *Eco*RI restriction pattern (21.6-, 13.2-, 4-, 3-, and 2.5-kb DNA fragments) were transferred from strain HB101 to strain SR139 (Hup^- Nif^-), all Tc^r strain SR139 transconjugants were found to be Hup^+ by their ability to grow under autotrophic conditions (Table 4). Moreover, randomly selected Tc^r Hup^+ strain SR139 transconjugants from each mating possessed whole-cell (ex planta) nitrogenase activities (Table 5). The transconjugant nitrogenase activities were 30 to 50% of the value obtained for the parent strain, strain SR. Strains SR139 and SR139(pLAFR1) did not express nitrogenase activity. On the other hand, the recombinant cosmid with the other restriction pattern (represented by pSH25) did not complement strain SR139, since strain SR139 transconjugants containing pSH25 were not able to grow autotrophically with H_2 (Table 4).

Transfer of a cosmid containing the *nif/hup* gene into other *R. japonicum* Hup^- mutants. Since the *nif/hup* gene cosmid possessed an insertion of approximately 23 kb, it was possible that other genes involved in the expression of hydrogenase or nitrogenase or both were encoded on this recombinant cosmid. Cosmid pSH22, which complemented Nif^- Hup^- strain SR139, was transferred by conjugation from *E. coli* HB101 to 15 other Hup^- mutants (Table 6). In previous studies, the nature of the defect causing a Hup^- phenotype was shown to differ among various Hup^- mutants (Table 1). Tc^r transconjugants were isolated and then screened for H_2 uptake capability by measuring their growth responses under autotrophic conditions. Hup^+ Tc^r transconjugants of *R. japonicum* SR114, SR118, SR119, SR140, SR146, and SU 306-47 appeared at a frequency of 1.0 per cosmid transfer (Table 6). All of these mutants were Hup^- but Nif^+. Therefore, the *nif/hup* cosmid also contained a gene(s) involved only in H_2 oxidation. Although the Hup^- Nif^+ mutants have not been completely characterized, these complemented mutants can be placed into different Hup^- classes (18, 21, 22). On the other hand, no complementation was detected for Hup^- Nif^+ mutant strains SR152, SR166, SR174, SR178, SR180, SR186, and SR192, a different Nif^- Hup^- strain (strain SR143), and Cfx^- strain SR122 (Table 6). Therefore, not all of the genes involved in hydrogenase expression are on cosmid pSH22.

TABLE 6. Complementation of *R. japonicum* Hup^- mutants with a cosmid containing the *nif/hup* gene[a]

Recipient	Phenotype	Tc^r/recipient[d]	$Aut^+/Tc^{r e}$
SR114	Hup^-	2.4×10^{-5}	1.0
SR118	Hup^-	6.0×10^{-5}	1.0
SR119	Hup^-	1.0×10^{-4}	1.0
SR140	Hup^-	2.2×10^{-5}	1.0
SR146	Hup^-	4.2×10^{-5}	1.0
SU306-47	Hup^-	8.5×10^{-6}	1.0
SR122[b]	Cfx^-	3.1×10^{-5}	$<1.0 \times 10^{-2}$
SR129	Hup^-	1.9×10^{-4}	$<1.0 \times 10^{-2}$
SR143	Nif^- Hup^-	ND[c]	$<1.0 \times 10^{-2}$
SR152	Hup^-	1.8×10^{-5}	$<1.0 \times 10^{-2}$
SR166	Hup^-	3.0×10^{-4}	$<1.0 \times 10^{-2}$
SR174	Hup^-	2.6×10^{-3}	$<1.0 \times 10^{-2}$
SR178	Hup^-	2.9×10^{-4}	$<1.0 \times 10^{-2}$
SR180	Hup^-	1.3×10^{-4}	$<1.0 \times 10^{-2}$
SR186	Hup^-	7.5×10^{-3}	$<1.0 \times 10^{-2}$
SR192	Hup^-	4.8×10^{-5}	$<1.0 \times 10^{-2}$

[a] The recombinant cosmid used in all matings was pSH22 harbored in strain HB101. Moreover, HB101(pRK2013) was used to mobilize pSH22.
[b] Strain SR122 is a derivative of strain SR that lacks ribulose bisphosphate carboxylase activity (18).
[c] ND, Not determined.
[d,e] See Table 4, footnotes *c* and *d*.

DISCUSSION

Recombinant cosmids which contain a *nif/hup* gene that is involved in both nitrogenase and hydrogenase activities were isolated from an *R. japonicum* I-110(pLAFR1) clone library. When the gene bank was transferred from *E. coli* to *R. japonicum* SR139 (Nif^- Hup^-), strain SR139 recipients containing the *nif/hup* cosmid were identified as Tc^r transconjugants that were able to grow chemoautotrophically with H_2 as the sole energy source (Aut^+). Positive complementation (but not recombination) was indicated when the *nif/hup* cosmids were transformed into *E. coli* and retransferred back into strain SR139 (Nif^- Hup^-). All of the resulting Tc^r transconjugants possessed a Nif^+ Hup^+ phenotype. The *Eco*RI endonuclease restriction pattern of these *nif/hup* cosmids (i.e., pSH22) was similar to the pattern of *R. japonicum* SR *hup* gene-containing cosmids isolated by Cantrell and co-workers (3). These workers showed that all *hup* cosmids possessed 13-, 2.9-, and 2.3-kb DNA fragments. The restriction patterns of the *nif/hup* cosmids have 13.2-, 4-, 3-, and 2.5-kb DNA fragments.

Strain SR139 transconjugants containing the *nif/hup* cosmid possessed high levels of H_2 uptake activity, as well as low but significant levels of symbiotic nitrogenase activity. The low nitrogenase levels in soybean nodules inoculated with transconjugants suggest that a large number of bacteroids may have lost the *nif/hup* cosmid. Other workers have observed a similar instability in recombinant pLAFR1 cosmids during growth under nonselective conditions (no tetracycline) (i.e, during infection and nodule development) (3, 10, 17). For example, Long et al. (17) showed that pLAFR1 recombinant cosmids containing *Rhizobium meliloti* DNA were unstable in bacteroids, whereas Cantrell et al. (3) and Haughland et al. (10) reported similar observations of *hup* cosmid instability in *R. japonicum* bacteroids. Furthermore, when *R. japonicum* SR cells which contained the parent plasmid of pLAFR1 (pRK290) were grown for 25 generations under nonselective conditions in free-living cultures, the cells lost plasmid pRK290 at a rate of 3.3% per generation (Hom, Ph.D. thesis, University of California, Davis, 1983). It has been reported (4) that deletion derivatives (e.g., pRK290) of wild-type plasmids (e.g., RK2) possess reduced stability in the absence of positive selection (medium containing the appropriate antibiotic). It will be of interest to test new generations of broad-host-range plasmid derivatives for enhanced stability so that the effect of an introduced gene on biological nitrogen fixation can be assessed more adequately.

The *nif/hup* cosmid also contains at least one and perhaps other *hup* genes which are involved in H_2 uptake activity exclusively. When *nif/hup* cosmid pSH22 was transferred to 15 *R. japonicum* Hup^- Nif^+ mutants, Hup^+ transconjugants were detected at a frequency of 1.0 per cosmid transfer for mutant strains SR114, SR118, SR119, SR140, SR146, and SU306-47. These six Hup^- Nif^+ mutants do not possess any whole-cell H_2 uptake activity when oxygen or methylene blue is used as the electron acceptor (18, 22). Moreover, the hydrogenase polypeptides which were detected in bacteroid extracts of the wild type after two-dimensional protein polyacrylamide gel analysis were not detected in any of

these six mutants (E. B. O'Hara, Ph.D. thesis, Johns Hopkins University, Baltimore, Md., 1984).

On the other hand, some of the *R. japonicum* mutants that were not complemented by *nif/hup* cosmid pSH22 possessed different characteristics. For example, unlike strain SR139, strain SR143 was a Nif$^-$ Hup$^-$ mutant that was deficient in the production of heme (26). Therefore, the lesion in this mutant is not directly related to *nif* or *hup*. Another Hup$^-$ mutant that was not complemented by the *nif/hup* clone was strain SR166. Strain SR166 lacked whole-cell H_2 uptake activity when it was coupled to oxygen, and this Hup$^-$ mutant possessed Hup activity when methylene blue or phenazine methosulfate was used as an electron acceptor (18). Strain SR166 apparently retains a functional hydrogenase, but it may lack an electron transport component(s) operating between hydrogenase and the terminal electron acceptor, oxygen (18). Another mutant not complemented by the *nif/hup* cosmid was strain SR122. Strain SR122 is Hup$^+$, but it is Aut$^-$ due to a deficiency in ribulose bisphosphate carboxylase activity (18). Finally, some other, uncharacterized Hup$^-$ strains were not complemented. These results suggest that other distinct genetic determinants for the simultaneous expression of nitrogenase and hydrogenase activities, electron transport components for H_2 oxidation, carbon dioxide fixation, and H_2 uptake are not on *nif/hup* cosmid pSH22. These genetic determinants may be located on the DNA sequences adjacent to the ends of the *nif/hup* DNA insertion or may be unlinked.

The successful complementation of two types of *R. japonicum* mutants (i.e., Nif$^-$ Hup$^-$ and Hup$^-$) suggests that *nif/hup* recombinant cosmids such as pSH22 may contain a cluster of genes involved in H_2 uptake activity and at least one gene also involved with both of the nitrogenase component proteins. Although the *R. japonicum nif* HDK genes have been cloned (7, 11), the *nif/hup* recombinant cosmids that we have isolated must (due to the phenotype of strain SR139) contain another gene involved in nitrogen fixation in *R. japonicum*. Physical and genetic analysis of such clones should prove to be useful in elucidating the biochemical, genetic, and regulatory factors involved in the complex *R. japonicum* H_2 uptake and nitrogen fixation systems.

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