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Construction of a *Rhizobium japonicum* gene bank and use in isolation of a hydrogen uptake gene

(nitrogen fixation/hydrogenase/cloning/cosmid pLAFR1/soybean)

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ABSTRACT **A gene bank of *Rhizobium japonicum* DNA was constructed by using the broad host range conjugative cosmid pLAFR1. Eighty-three percent of the clones in the bank contained cosmids with insert DNA averaging 22.6 kilobase pairs in length. A series of cosmids containing a hydrogen uptake (*hup*) gene was identified by transferring the gene bank into a H_2 uptake-negative (Hup^-) *R. japonicum* point mutant (PJ17nal) and screening tetracycline-resistant colonies for the ability to grow chemolithotrophically and to reduce methylene blue in a recently devised colony assay. Hup^+ transconjugants arose at a frequency of approximately 6×10^{-3}. Plasmid DNAs from 11 of the Hup^+ transconjugants were isolated and used to transform *Escherichia coli*. *Eco*RI digests of all plasmids isolated from Hup^+ transconjugants had three DNA fragments in common. Eight of the *E. coli* transformants containing *hup* gene cosmids were conjugated with PJ17nal and another Hup^- point mutant, PJ18nal. All PJ17nal transconjugants were Hup^+. The frequency of Hup^+ transconjugants with PJ18nal was approximately 10^{-3}. The results indicate that the *hup* gene cosmids may contain one gene and a portion of another.**

Nitrogenases from all known sources catalyze an ATP-dependent reduction of not only N_2 to NH_4 but also protons to H_2. This H_2 loss results in an inefficient use of the energy provided by the organism to the N_2-fixing process. Most N_2 fixing, free-living microorganisms, but a minority of strains of *Rhizobium*, possess the capacity for synthesis of an H_2-recycling system that oxidizes the H_2 produced during N_2 fixation, thus recapturing some of the energy expended during H_2 evolution (1, 2). Dixon (3) pointed out several potential advantages of H_2-oxidizing capability to N_2-fixing organisms. Subsequent investigations have shown that an active H_2-uptake system in *R. japonicum* can support ATP synthesis for utilization in N_2 fixation and other processes and also provide a mechanism for additional protection of bacteroid nitrogenase from O_2 inactivation (4). In greenhouse and field experiments, soybean plants inoculated with groups of hydrogenase-positive (H_2 uptake-positive; Hup^+) strains of *R. japonicum* were reported to contain higher percentages of N in their tissues and seeds than plants inoculated with groups of hydrogenase-negative (Hup^-) strains (5, 6). Recent work by DeJong *et al.* has indicated that the Hup determinants in *R. leguminosarum* may increase the yield of pea plants (7). Highly active H_2 uptake systems have been found in only about 20% of *R. japonicum* strains and in no strain of *R. trifolii* or *R. meliloti* (1). Most strains of *R. leguminosarum* are Hup^- and, with few exceptions (8), the Hup^+ strains recycle only a small proportion of the H_2 produced by the nitrogenase reaction. It may be beneficial, therefore, to transfer the genes for the Hup phenotype from highly active Hup^+ strains to Hup^- strains of several species of *Rhizobium*.

At least some of the determinants for the Hup phenotype have been shown to be plasmid-borne and transmissible in *Alcaligenes eutrophus* (9) and *R. leguminosarum* (7, 10). There is much less information about Hup determinants in *R. japonicum*. If *R. japonicum* Hup determinants are plasmid encoded, they must be located on a plasmid with a molecular weight greater than 280×10^6. Efforts to resolve such large plasmids have not been successful (11). As a result, we have utilized recombinant DNA techniques to isolate and characterize genes specific for the Hup phenotype in *R. japonicum*.

In this paper, we describe the construction of a gene bank by utilizing the conjugative cloning vector pLAFR1 and DNA from the Hup^+ *R. japonicum* strain 122 DES. We have screened this gene bank for *hup*-specific DNA sequences by analysis for complementation of *R. japonicum* Hup^- point mutants. We report here the isolation of several recombinant cosmids containing at least one gene specific for the Hup phenotype.

MATERIALS AND METHODS

Bacterial Strains and Growth Media. Strains of *Escherichia coli* and *R. japonicum* used in this study are listed in Table 1. *E. coli*, unless otherwise indicated, was cultured on LB medium at 32°C (19). Cells used as recipients for cosmid transduction were grown in LB medium supplemented with 0.4% maltose (15). *R. japonicum* was routinely cultured in broth or on agar plates containing the yeast extract/mannitol (YEM) medium described by Vincent (20). *R. japonicum* was cultured chemolithotrophically on plates containing Repaske's medium (21, 22) with 1.5% Noble agar, using the procedures described by Lepo *et al.* (18). Prior to derepression of *R. japonicum* for hydrogenase activity, cells were grown either on plates of YEM medium or on plates containing the H_2 uptake medium (HUM) of Maier *et al.* (23) adjusted to pH 7.0 but modified by adding 0.2 g of $KH_2PO_4 \cdot H_2O$ and 0.03 g of $NaH_2PO_4 \cdot H_2O$ per liter instead of 0.15 g of $NaH_2PO_4 \cdot H_2O$.

DNA Isolations. Large-scale preparations of cosmid DNA from *E. coli* and *R. japonicum* were obtained by modification of the procedure of Currier and Nester (24) as described by Cantrell *et al.* (11). For use in preparation of the clone bank, pLAFR1 DNA from *E. coli* was further purified by two CsCl/ethidium bromide gradient centrifugations in 49% (wt/wt) CsCl and ethidium bromide at 0.35 mg/ml in a buffer solution at pH 8.0 containing 50 mM Tris·HCl and 20 mM EDTA (TE buffer). The initial refractive index of the gradient solution was 1.3910. Samples were centrifuged in a Beckman VTi65 rotor (12–16 hr) at 55,000 rpm. Covalently closed circular DNA recovered from the second gradient centrifugation was diluted with TE buffer

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Abbreviations: Hup, hydrogen uptake; kb, kilobase pair(s); YEM, yeast extract/mannitol; HUM, H_2 uptake medium.

Table 1. Bacterial strains

Strain	Relevant characteristics	Ref.
E. coli		
HB101	*recA*$^-$, *hsdR*, *hsdM*, *pro*, *leu*, Str^r	12
HB101(pLAFR1)	Contains pLAFR1	13
HB101(pRK2013)	Contains pRK2013	14
BHB2688	N205, *recA*$^-$, (λ*imm434*, *cIts*, *b2*, *red3*, *Eam4*, *Sam7*)/λ	15
BHB2690	N205, *recA*$^-$, (λ*imm434*, *cIts*, *b2*, *red3*, *Dam15*, *Sam7*)/λ	15
R. japonicum		
122 DES	Hup$^+$	16
SR	Hup$^+$, Str^r, Kan^r, derivative of 122 DES	17
SRnal	Hup$^+$, Str^r, Kan^r, Nal^r, derivative of SR	This paper
PJ17	Hup$^-$, Str^r, Kan^r, derivative of SR	18
PJ17nal	Hup$^-$, Str^r, Kan^r, Nal^r, derivative of PJ17	This paper
PJ18	Hup$^-$, Str^r, Kan^r, derivative of SR	18
PJ18nal	Hup$^-$, Str^r, Kan^r, Nal^r, derivative of PJ18	This paper

Str, streptomycin; Kan, kanamycin; Nal, nalidixic acid; ^{r}, resistant.

and pelleted by centrifugation in a Beckman SW 60 rotor at 50,000 rpm. The pelleted DNA was resuspended in 0.37 ml of a buffer containing 6 mM Tris·HCl (pH 7.4), 10 mM NaCl, and 0.1 mM EDTA (DNA storage buffer), extracted repeatedly with isoamyl alcohol, and precipitated with ethanol. After the precipitate had been dried by evacuation at room temperature, it was redissolved and stored in 0.25 ml of DNA storage buffer. Small-scale isolations of cosmid DNA from *E. coli* for use in restriction analyses were performed by use of a cleared lysate procedure (19). Total genomic DNA from *R. japonicum* was isolated by a previously described procedure (25) with the modification that an RNA digestion step (RNase A at 50 μg/ml in 150 mM NaCl/15 mM sodium citrate at pH 7.0 for 20 min at 37°C) and a second ethanol precipitation were carried out prior to the CsCl/ethidium bromide gradient centrifugation.

Size Fractionation of *R. japonicum* DNA. *R. japonicum* total DNA (200 μg) was incubated at 37°C with 10 μl of *Eco*RI (Bethesda Research Laboratories, 10 units/μl) for periods between 45 and 115 min in 100 mM Tris·HCl, pH 7.2/50 mM NaCl/5 mM $MgCl_2$/2 mM 2-mercaptoethanol. Partial digests were pooled and subjected to sucrose gradient centrifugation as described by Ditta *et al.* (14). Agarose gel electrophoresis was performed by the method of Meyers *et al.* (26) with modifications of Haugland and Verma (25) to identify fractions from the gradient containing DNA in the size range of 12–30 kilobase pairs (kb).

Construction of *R. japonicum* DNA/pLAFR1 Recombinants. *Eco*RI-digested pLAFR1 DNA was ligated with pooled, partially digested 12- to 30-kb fractions of *R. japonicum* DNA at concentrations of 0.05 and 1.0 mg/ml, respectively, in the presence of bacteriophage T4 ligase (Bethesda Research Laboratories) at approximately 700 units/ml. The ligation mixture was incubated for 18 hr at 12°C in a buffer containing 66 mM Tris·HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol, and 66 μM ATP.

Cosmid Packaging and Transductions. Packaging extracts were prepared and DNA was packaged essentially as described by Blattner *et al.* (27), using the λ lysogenic strains of *E. coli*, BHB2688 and BHB2690 (15). Protein A was not purified, but the sonic extract prepared from BHB2690 was centrifuged as described (27) and aliquots of the supernatant were stored at −70°C. DNA packaging was initiated by addition of the following in the order indicated: 7 μl of buffer A of Blattner *et al.* (27), 3 μl of ligated DNA, 1 μl of M1 buffer of Blattner *et al.*, 5 μl of BHB2690 extract, and 5 μl of BHB2688 extract. After incubation for 1 hr at 23°C, a drop of $CHCl_3$ was added, the mixture was serially diluted, and transduction with strain HB101 was performed as described by Hohn (15).

Bacterial Matings and Transformations. The triparental mating system of Ditta *et al.* (14) was used for conjugative transfer of pLAFR1 recombinants to *R. japonicum*. *E. coli* donor cells (approximately 5×10^7) and *R. japonicum* recipient cells (approximately 5×10^8) from early and late logarithmic growth phase cultures, respectively, were mixed and spread on YEM plates at pH 7.0 and incubated at 29°C for 3–4 days. Cells from the matings were then suspended in the mineral salts solution used for HUM to which was added 0.01% Tween 80. Dilutions between 10^{-1} and 10^{-8} were plated on either YEM medium or HUM with tetracycline at 100 μg/ml and nalidixic acid at 100 μg/ml.

Transformation of cryogenically preserved competent HB101 cells (28) with pLAFR1 recombinants was performed according to the procedure of Davis *et al.* (29) and selection was made on LB medium with tetracycline at 20 μg/ml.

Methylene Blue Colony Screen for Hup. *R. japonicum* transconjugant colonies grown on selective HUM or YEM plates were replicated onto sterile filter paper discs (Whatman 541), which were then placed on plates of Repaske's medium. Cells on the filters were then derepressed by incubation for 4–5 days in an atmosphere that initially contained 5% H_2, 5% CO_2, approximately 0.7% O_2, and the remainder N_2. The levels of O_2 gradually decreased to less than 0.1% of the gas volume during this time and were subsequently maintained at about 0.2%. The discs with colonies attached were removed from the Repaske plates and soaked for 1 hr in 0.8 ml of a solution containing 200 mM iodoacetic acid, 200 mM malonic acid (30), 10 mM methylene blue, 50 mM KH_2PO_4, and 2.5 mM $MgCl_2$ adjusted to pH 5.6 with KOH. The treated discs with colonies were placed in Petri dishes, sealed inside a Plexiglas container (29 × 39 × 3 cm), and flushed with a continuous stream of H_2 during the assay. Colonies with H_2 uptake activity reduced methylene blue to its leuko form within 0.5–3 hr. Further details of the procedure will be presented elsewhere.

Hydrogen Uptake Assay by the Amperometric Method. *R. japonicum* cells were grown to an optical density (540 nm) of 0.25 in 8 ml of YEM medium containing tetracycline at 50 μg/ml. Cells were harvested by centrifugation and cell pellets were resuspended in 0.2 ml of HUM. The suspensions were spread on plates of Repaske's medium and derepressed for 4 days under the gas mixture described for the methylene blue colony screen. Cells were removed from plates and suspended in 5 ml of 50 mM KH_2PO_4 and 2.5 mM $MgCl_2$ buffer adjusted to pH 7.0. The suspensions were diluted 1:10 in the above buffer and assayed for rates of H_2 uptake by the method of Hanus *et al.* (31). After digestion of cells (32) protein contents were determined by the method of Goa (33) with bovine serum albumin as the standard.

Nodulation and N_2 Effectiveness of Strains. Soybean seeds [*Glycine max* (L.) Merr., cultivar Wilkin] were surface disinfected, germinated on agar plates (20), and immersed in YEM broth cultures (with tetracycline at 45 μg/ml for transconjugants) of the desired *R. japonicum* strains. Plants were provided with a half-strength nitrogen-free nutrient solution (34) and grown for 30 days in a growth cabinet (20). Nodules were as-

sayed for acetylene reduction and bacteroids for hydrogenase activity by methods used by Lepo *et al.* (18).

RESULTS

Preliminary Experiments with pRK290. Initial studies were designed to identify a suitable cloning vector that could be transferred by conjugation into *R. japonicum*, would be maintained within that host, and would confer increased antibiotic resistance to that host. The concentration of antibiotics necessary to inhibit background growth of *R. japonicum* was found to be decreased when cells were suspended in a solution containing 0.01% Tween 80 prior to plating. The conjugative cloning vehicle pRK290 constructed by Ditta *et al.* (14) from the naturally occurring antibiotic resistance plasmid RK2 was tested. Tetracycline resistance was conferred to derivatives of *R. japonicum* strain 122 DES at frequencies of 10^{-3} to 10^{-4} per recipient cell when the helper plasmid pRK2013 was present. Conjugation did not occur (frequency less than 10^{-8}) in the absence of pRK2013. Autonomous existence of the plasmid in *R. japonicum* was verified by plasmid isolation and gel electrophoresis.

A derivative of pRK290 containing the *cos* sites of the bacteriophage λ was subsequently developed by Friedman *et al.* (13). Because use of the cosmid vector pLAFR1 had several advantages, it was used instead of the plasmid vector in all further experiments.

Gene Bank of *R. japonicum* DNA. *R. japonicum* strain 122 DES total DNA that had been partially digested with *Eco*RI and fractionated by size was ligated with *Eco*RI-digested pLAFR1. Ligation was performed at a ratio of 20 μg of *R. japonicum* 122 DES DNA per μg of vector DNA to minimize vector–vector ligations. The ligation mixture was then packaged with an extract containing phage λ head and tail components and the resultant packaged cosmids were allowed to adsorb onto *E. coli*. Tetracycline-resistant transductants were obtained at a frequency of 7.8×10^5 per μg of vector DNA. The resultant gene bank contains more than 40,000 clones.

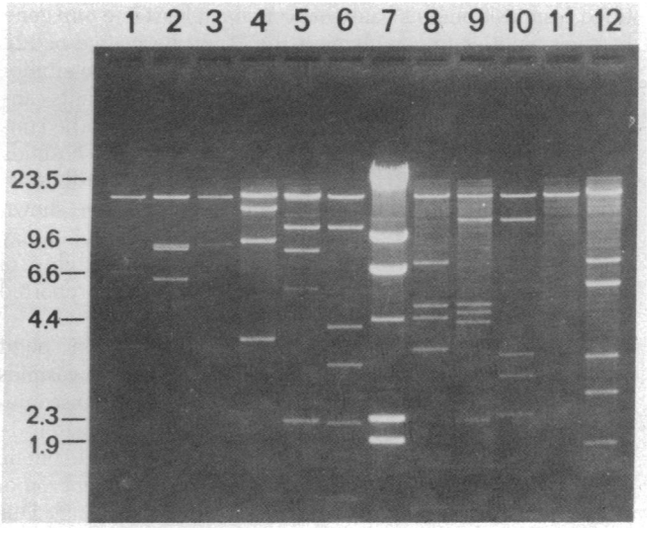

FIG. 1. *Eco*RI-digested cosmid DNAs from a *R. japonicum* gene bank. Partially purified cosmid DNAs from 11 randomly chosen HB101 transductants were digested with *Eco*RI restriction endonuclease and fragments were separated by gel electrophoresis in 0.7% agarose. Lane 7 contains *Hin*dIII-cut bacteriophage λ DNA and fragment sizes in kb are indicated at the left. Lane 11 shows DNA from a clone whose cosmid had no inserts. The other lanes show DNAs from clones containing cosmids with *R. japonicum* DNA inserts of various sizes.

From approximately 2,500 individually isolated and stored clones, 24 were chosen at random for plasmid analysis. Gel electrophoresis of *Eco*RI digests of these plasmids showed that 20 of the colonies (83%) contained insert DNA. Fig. 1 shows the patterns obtained after gel electrophoresis of the digests of 11 of the cosmids. The length of insert DNA in the clones was between 9 and 32.4 kb, with an average DNA insert size of 22.6 kb. On the basis of theoretical calculations, 1,200 clones randomly selected from the above bank should have a probability greater than 99% of containing any gene with a size of 1 kb if the bank is completely random (35).

Complementation Analysis of *R. japonicum* Hup⁻ Mutants by Using the Gene Bank. The two revertible Hup⁻ mutants PJ17nal and PJ18nal were conjugated with the clone bank *en masse* and tetracycline-resistant, nalidixic acid-resistant transconjugants were selected. Tetracycline-resistant transconjugant colonies appeared at frequencies of 10^{-3} to 10^{-4} per recipient cell (see Table 2, crosses 1 and 2). Tetracycline-resistant colonies were then screened for H_2 uptake activity by the methylene blue assay. Approximately 5×10^3 colonies derived from PJ18nal were tested for H_2 uptake activity by this procedure, but no complementation was observed. Hup⁺ colonies did arise, however, amongst the PJ17nal transconjugants as indicated in Table 2. Some Hup⁺ transconjugants identified in the above manner were independently identified by chemolithotrophic growth on replica plates (18). The O_2-dependent H_2 uptake activities of 11 of the isolated Hup⁺ PJ17nal transconjugants were tested amperometrically and the results are presented in Table 3. All of these transconjugants showed high H_2 uptake activities. The variability seen in specific activities is characteristic of single determinations on heterotrophically grown derepressed cells.

Table 2. Conjugal transfer frequencies of pLAFR1 recombinant cosmids into Hup⁻ *R. japonicum* strains and frequency of Hup⁺ transconjugants

Cross	Donor*	Recipient	Frequency of Tet^r colonies per recipient	Frequency of Hup⁺ colonies per transconjugant†
1	HB101(pLAFR1-bank)	PJ17nal	3.1×10^{-4}‡	6.4×10^{-3}
2	HB101(pLAFR1-bank)	PJ18nal	4.5×10^{-4}‡	$<2.1 \times 10^{-4}$
3	HB101(pHU1)	PJ17nal	1.4×10^{-3}	1.0
4	HB101(pHU2)	PJ17nal	1.2×10^{-3}	1.0
5	HB101(pHU5)	PJ17nal	1.4×10^{-3}	1.0
6	HB101(pHU6)	PJ17nal	1.0×10^{-3}	1.0
7	HB101(pHU7)	PJ17nal	8.1×10^{-4}	1.0
8	HB101(pHU11)	PJ17nal	1.8×10^{-3}	1.0
9	HB101(pHU14)	PJ17nal	1.5×10^{-3}	1.0
10	HB101(pHU18)	PJ17nal	2.3×10^{-3}	1.0
11	HB101(pHU1)	PJ18nal	1.4×10^{-3}	2.3×10^{-3}
12	HB101(pHU2)	PJ18nal	1.7×10^{-3}	1.6×10^{-3}
13	HB101(pHU5)	PJ18nal	9.8×10^{-4}	5.8×10^{-3}
14	HB101(pHU6)	PJ18nal	1.5×10^{-3}	4.7×10^{-3}
15	HB101(pHU7)	PJ18nal	7.0×10^{-4}	3.9×10^{-3}
16	HB101(pHU11)	PJ18nal	3.1×10^{-3}	1.4×10^{-3}
17	HB101(pHU14)	PJ18nal	5.1×10^{-3}	2.9×10^{-3}
18	HB101(pHU18)	PJ18nal	3.3×10^{-3}	7.5×10^{-3}

* All crosses contained HB101(pRK2013) to mobilize pLAFR1 derivatives.

† Frequency of Hup⁺ colonies per transconjugant was calculated by dividing the number of Hup⁺ tetracycline-resistant (Tet^r) colonies by the total number of tetracycline-resistant colonies.

‡ Frequency values for crosses with the clone bank are averages of more than one cross.

Table 3. Comparison of hydrogenase activities of representative Hup$^+$ SRnal and PJ17nal transconjugants with the Hup$^-$ PJ17nal recipient

Rhizobium strains*	Hydrogenase assays†	
	Methylene blue reduction	H_2 uptake, nmol·min^{-1} per mg protein
PJ17nal(pHU11)	+	147
PJ17nal(pHU12)	+	166
PJ17nal(pHU13)	+	146
PJ17nal(pHU14)	+	179
PJ17nal(pHU16)	+	142
PJ17nal(pHU17)	+	103
PJ17nal(pHU18)	+	162
PJ17nal(pHU19)	+	126
PJ17nal(pHU20)	+	249
PJ17nal(pHU21)	+	170
PJ17nal(pHU22)	+	236
SRnal(pLAFR1)‡	+	158
PJ17nal	−	<5

* All strains are transconjugants except the PJ17nal recipient.
† Cells were derepressed and assayed for hydrogenase activity.
‡ A transconjugant from a cross with the pLAFR1-122 DES gene bank.

Analysis of Potential *hup* Gene Cosmids. Eleven of the above Hup$^+$ PJ17nal transconjugants were isolated for further study. Cosmid DNA isolated from each of these transconjugants was used to transform *E. coli* HB101. Subsequent isolation of cosmids from these HB101 transformants and gel electrophoresis of *Eco*RI restriction fragments resulted in the patterns presented in Fig. 2. The DNA fragments with sizes of 13, 2.9, and 2.3 kb were present in the cosmids isolated from all of the Hup$^+$ transconjugants. It would appear that some portion of the DNA fragments in common may be necessary for complementation leading to Hup activity in PJ17nal.

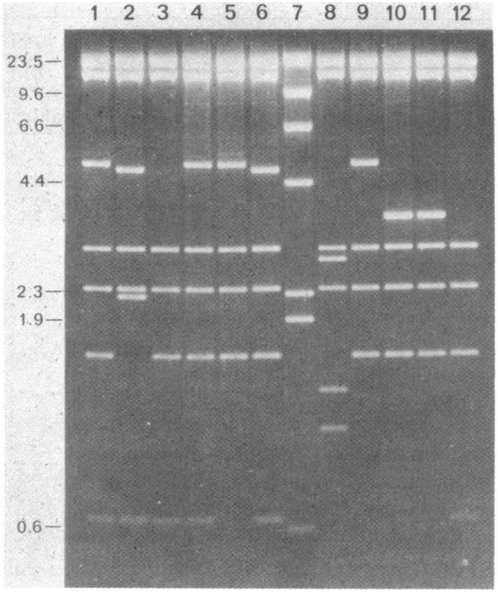

FIG. 2. *Eco*RI-restriction digests of *R. japonicum hup* gene-containing cosmids. Cosmid DNAs isolated from 11 independent Hup$^+$ PJ17nal transconjugants were used to transform *E. coli* strain HB101. Cosmid DNAs were then reisolated from the transformants and digested with *Eco*RI restriction endonuclease, and the resulting DNA fragments were separated by gel electrophoresis in 0.7% agarose. Lane 1, pHU1; lane 2, pHU2; lane 3, pHU4; lane 4, pHU5; lane 5, pHU6; lane 6, pHU7; lane 7, *Hin*dIII-digested bacteriophage λ DNA (fragment sizes in kb indicated at left); lane 8, pHU11; lane 9, pHU12; lane 10, pHU14; lane 11, pHU17; lane 12, pHU18.

In order to demonstrate that the above cosmids contain a gene specific for the Hup phenotype, eight were transferred by conjugation from HB101 back into PJ17nal. In each case, all of the resultant PJ17nal transconjugants were Hup$^+$ as determined by the methylene blue colony screen (see Table 2, crosses 3–10). These results indicate that the isolated cosmids contain at least one *hup*-specific gene.

Nodulation Tests of Hup$^+$ PJ17nal Transconjugants. Selected Hup$^+$ PJ17nal transconjugants were tested for effective nodulation on soybean plants. Soybean plants were inoculated with PJ17nal isolates containing pHU1, pHU2, pHU4, pHU5, pHU6, and pHU7. All test plants formed nodules and showed N_2 fixation as measured by acetylene reduction, whereas all uninoculated control plants remained nodule free. Bacteroids were prepared from nodules and assayed amperometrically for H_2 uptake. PJ17nal(pHU4) bacteroids had no H_2 uptake activity, but all other bacteroids had specific activities of 5–25% of the H_2 uptake activity observed for 122 DES bacteroids. The hydrogenase activities of nodules formed by PJ17nal(pHU1), PJ17nal(pHU2), PJ17nal(pHU5), and PJ17nal(pHU7), however, were sufficient to recycle all the H_2 produced by nitrogenase, whereas nodules produced by PJ17nal(pHU6) failed to recycle all H_2 and, as a consequence, showed H_2 evolution. Preliminary evidence indicates that a significant percentage of the bacteroids have become tetracycline sensitive and thus may have lost the cosmids.

Conjugation of *hup* Gene Cosmids into PJ18nal. The *hup* gene cosmids listed in Table 2 were transferred by conjugation into PJ18nal and the resultant tetracycline-resistant recipients were tested for H_2 uptake by the methylene blue colony assay. The results shown in Table 2 (crosses 11–18) indicate that Hup$^+$ colonies surprisingly appeared at frequencies of approximately $1–8 \times 10^{-3}$

DISCUSSION

We have described the construction of a gene bank from the Hup$^+$ *R. japonicum* strain 122 DES in *E. coli*. We have identified cosmids from this bank that contain at least one *hup* gene by their abilities to complement the Hup$^-$ derivative of 122 DES, PJ17nal. Because the cells used were *rec*$^+$, the possibility of a low level of recombination within the transconjugants cannot be eliminated. However, Hup activity appears to be conferred by complementation, because isolation of *hup* cosmids and transfer back into PJ17nal results in conversion of all transconjugants to a Hup$^+$ phenotype. These cosmids were shown to confer high levels of H_2 uptake activity upon PJ17nal but may be unstable in nodule bacteroids. A similar instability of pLAFR1 derivatives in *R. meliloti* bacteroids has been reported by Long *et al.* (36).

Although screening of the clone bank with the Hup$^-$ point mutant PJ18nal revealed no Hup$^+$ clones, the *hup* cosmids isolated by the use of PJ17nal converted PJ18nal transconjugants to a Hup$^+$ phenotype at frequencies of approximately $1–8 \times 10^{-3}$. It is possible that the *hup* cosmids isolated contain only a portion of another *hup* determinant (another operon or cistron) that is necessary for complementation in PJ18nal. Due to lack of a complete operon, restoration of H_2 uptake would be seen only after homologous recombination between the mutated sequences in the genomic DNA of PJ18nal and the corresponding wild-type sequences in the cosmids. The cosmids isolated may, therefore, contain all of one *hup* gene and a portion of another.

There are a number of possible reasons for our inability to identify a cosmid that will complement the PJ18nal *hup* mu-

tation. This lesion may be *trans*-dominant. If this is the case, H_2 uptake would be seen only when reciprocal recombination was followed by loss of the vector containing the mutated sequence. Alternatively, the *hup* gene in question may be lethal or near a gene that is lethal in *E. coli*, or the gene may be part of an extremely large operon that is not present in its entirety in any single clone from the gene bank. Another possibility is that the gene may be on a fragment that is very rare in the population of *Eco*RI-digested DNA fragments used for production of the gene bank due to the relative accessibility or number of *Eco*RI restriction sites in that region. It may be necessary, therefore, to screen the clone bank further (the bank contains more than 40,000 clones, of which approximately 5,000 have been screened) or it may be necessary to produce another bank from 122 DES DNA.

Until recently, very little was known about the genetic organization of the Hup system in *R. japonicum*. Maier has isolated mutants that show no O_2-dependent H_2 uptake but exhibit methylene blue-dependent H_2 uptake (37). Further studies have shown *in vitro* biochemical complementation for methylene blue-dependent H_2 uptake by mixing extracts from groups of Hup^- mutants (38). These results indicate that more than one gene may be specifically involved in the Hup system. Our results are consistent with this possibility. Transfer of H_2 uptake activity to wild-type Hup^- strains of *Rhizobium* may, therefore, require transfer of two or more genes. Alternatively, there is no reason at this point to rule out the possibility that wild-type Hup^- strains of *Rhizobium* may already contain some *hup* determinants, particularly if these determinants are pleiotropic for other functions. In this respect, it will be of interest to determine the effect of introduction of the *hup* cosmids described here into various wild-type Hup^- strains of *Rhizobium*. It will also be of interest to determine the effect of introduction of these cosmids into *Rhizobium* strains with low H_2 uptake activity.

Examination of the gel patterns obtained after *Eco*RI digestion of the *hup* cosmids allows only a tentative listing of the arrangement and contiguity of the fragments.

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