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Cloning and Characterization of Hydrogen Uptake Genes from *Rhizobium leguminosarum*

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A gene library of genomic DNA from the hydrogen uptake (Hup)-positive strain 128C53 of *Rhizobium leguminosarum* was constructed by using the broad-host-range mobilizable cosmid vector pLAFR1. The resulting recombinant cosmids contained insert DNA averaging 21 kilobase pairs (kb) in length. Two clones from the above gene library were identified by colony hybridization with DNA sequences from plasmid pHU1 containing *hup* genes of *Bradyrhizobium japonicum*. The corresponding recombinant cosmids, pAL618 and pAL704, were isolated, and a region of about 28 kb containing the sequences homologous to *B. japonicum hup*-specific DNA was physically mapped. Further hybridization analysis with three fragments from pHU1 (5.9-kb *Hin*dIII, 2.9-kb *Eco*RI, and 5.0-kb *Eco*RI) showed that the overall arrangement of the *R. leguminosarum hup*-specific region closely parallels that of *B. japonicum*. The presence of functional *hup* genes within the isolated cosmid DNA was demonstrated by site-directed Tn*5* mutagenesis of the 128C53 genome and analysis of the Hup phenotype of the Tn*5* insertion strains in symbiosis with peas. Transposon Tn*5* insertions at six different sites spanning 11 kb of pAL618 completely suppressed the hydrogenase activity of the pea bacteroids.

Certain strains of *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* (Hup^+ strains) induce, in symbiosis with soybeans and peas, respectively, the synthesis of an H_2 uptake system which catalyzes the recycling of H_2 generated by the nitrogenase complex as an obligate by-product of the nitrogen fixation process (11, 13). This H_2 uptake system has been studied in detail in free-living and symbiotic cells of Hup^+ strains of *B. japonicum* (13, 22, 26). The first component of the system is a membrane-bound hydrogenase which contains nickel and two polypeptide subunits. Hydrogen is activated by the hydrogenase and oxidized with O_2 to water through an electron transport chain, some of whose components may be specifically involved in the H_2 oxidation. The oxidation of H_2 in legume nodules has been shown to increase N_2 fixation and legume productivity (13, 14).

Hydrogen uptake (*hup*) genes are not expressed in normal cultures of rhizobia. However, derepression of *hup* genes in free-living *B. japonicum* has been shown under certain culture conditions including low-carbon medium and an atmosphere with H_2, CO_2, and low O_2 (27). Under these conditions the cells can use H_2 as the sole energy source for autotrophic growth (16). Mutants impaired in their H_2 uptake capacity have been isolated from mutagenized free-living cells of *B. japonicum* by screening for lack of H_2-dependent methylene blue reduction or lack of autotrophic growth with H_2 and CO_2 (24, 25, 33). Subsequently, recombinant cosmids containing *hup* genes were isolated from gene libraries of *B. japonicum* genomic DNA by complementing these Hup^- mutants (4, 18). Cosmid pHU1 was shown by site-directed Tn*5* insertion mutagenesis to contain at least 15 kilobase pairs (kb) of *hup*-specific DNA (17), but not all the *B. japonicum* Hup^- mutants were complemented by this cosmid. Additional *hup* genes are present on cosmid pHU52 which apparently encodes all essential *hup* determinants to confer hydrogenase activity to wild-type Hup^- strains of *B. japonicum* and *R. leguminosarum* (20). Both cosmids pHU1 and pHU52, however, contained the genes for the two polypeptide subunits of the hydrogenase (43).

The analysis of *hup* genes in *R. leguminosarum* has been hampered by the lack of mutants. Hydrogenase activity of these rhizobia is not induced under the culture conditions described for derepression of *B. japonicum hup* genes. Therefore, the isolation of Hup^- mutants necessitates plant-screening procedures. Recently, Kagan and Brewin (19) took advantage of the symbiotic plasmid location of *hup* genes in strain 128C53 (3) to isolate Hup^- mutants of this strain by Tn*5* mutagenesis and screening of mutants in plants. Homology between *B. japonicum hup*-specific DNA contained in pHU1 and symbiotic plasmid DNA from several Hup^+ strains of *R. leguminosarum* has been demonstrated (36, 37).

Here we report (i) the construction of a gene library from a Hup^+ strain of *R. leguminosarum*, (ii) the isolation of recombinant cosmids containing DNA sequences homologous to *B. japonicum hup*-specific DNA, and (iii) the demonstration of the presence in the isolated cosmids of genes essential for H_2 uptake in *R. leguminosarum*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* cells were grown in LB medium at 37°C, and when required, antibiotics were used at the following concentrations (micrograms per milliliter): tetracycline, 12; kanamycin, 50; nalidixic acid, 10; gentamicin, 25. *R. leguminosarum* cultures were grown at 28°C in either TY medium (1) or *Rhizobium* minimal medium (34); antibiotics were used in the selection media at the following concentrations (micrograms per milliliter): tetracycline, 5; kanamycin, 100; gentamicin, 10.

DNA techniques. Large- and small-scale plasmid DNA preparations were made by the alkaline lysis procedure described by Maniatis et al. (30). Total DNA for hybridization experiments was isolated essentially as described by

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli		
HB101	*pro leu thi lacY endA recA hsdR hsdM* Str^r	G. Ditta
NS428	Su$^-$ *recA* (λ *A*am11 *b2 red3 c*I857 *S*am7)	38
NS433	Su$^-$ *recA* (λ *E*am4 *b2 red3 c*I857 *S*am7)	38
HB101::Tn*5*	Km^r	G. Ditta
C2110	*polA* Nal^r	G. Ditta
R. leguminosarum		
128C53	Nod$^+$ Fix$^+$ Hup$^+$	35
UPM791	128C53 Str^r	This study
AL8	Nod$^+$ Fix$^+$ Hup$^+$ UPM791::Tn*5*	This study
AL6, AL10, AL13, AL18, AL25, AL26	Nod$^+$ Fix$^+$ Hup$^-$ UPM791::Tn*5*	This study
Plasmids		
pLAFR1	pRK290 (Tc^r *mob* IncP) containing *cos*	15
pRK2073	Km^s derivative of pRK2013, helper plasmid	10
pPH1JI	Str^r Gm^r IncP	2
pUC13	Ap^r	32
pACYC184	Cm^r Tc^r	5
pHU1	pLAFR1 cosmid containing *hup* DNA from *B. japonicum*	4
pHU52	pLAFR1 cosmid containing *hup* DNA from *B. japonicum*	20
pAL618, pAL704	pLAFR1 cosmids containing *hup* DNA from *R. leguminosarum*	This study

[a] Abbreviations: Str^r, streptomycin resistant; km^r, kanamycin resistant; Nal^r, nalidixic acid resistant; Tc^r, tetracycline resistant; Gm^r, gentamicin resistant; Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant.

Corbin et al. (8) with the following modifications. (i) Cells from 1 ml of a 48-h-old culture in TY medium were washed first with 1 M NaCl and then with 0.1% Sarkosyl in TE buffer (50 mM Tris, 20 mM disodium EDTA, pH 8.0). (ii) The dialysis step was omitted, and the final DNA pellet was washed with 70% ethanol. Total DNA for construction of the gene library was isolated by the method of Clewell and Helinski (6) as modified by Takeda et al. (39). DNA restriction fragments from recombinant cosmids were subcloned into plasmid vector pUC13 or pACYC184, and the inserted DNAs were isolated from low-melting-point agarose gels by the method of Langridge et al. (23). Restriction enzyme digestions, agarose gel electrophoresis, and enzymatic cloning techniques were standard (30).

Construction of *R. leguminosarum* gene library. Total DNA from *R. leguminosarum* UPM791 was partially digested with *Eco*RI and size fractionated with a preparative vertical gel electrophoresis apparatus from Bethesda Research Laboratories, Inc., Gaithersburg, Md. (model 1100PG) with 0.6% agarose in Tris-acetate (40 mM Tris, 20 mM acetic acid, 2 mM disodium EDTA, pH 8.1). The size of the DNA present in each fraction was determined by electrophoresis in 0.5% agarose gels. Fractions containing DNA in the size range of 10 to 35 kb were pooled and used for construction of the gene library.

*Eco*RI-digested DNA from cosmid pLAFR1 was ligated with size-fractionated *R. leguminosarum* DNA at concentrations of 66 and 462 μg/ml, respectively, in the presence of T4 DNA ligase (72 U/ml). The ligation product was packaged in vitro into λ bacteriophage heads by the method of Friedman et al. (15). Packaging extracts were prepared from *E. coli* lysogenic strains NS428 and NS433 as described previously (30) except that cultures of strains NS428 and NS433 were used in the ratio 1:2 and putrescine was omitted from the cell suspension buffer. The resulting phage particles containing the recombinant cosmids were used to infect *E. coli* HB101. After 1 h of incubation at 37°C, the transducted cells were plated on LB medium supplemented with tetracycline.

Hybridization procedures. In vitro α-^{32}P labeling of plasmid DNA or purified DNA fragments was accomplished by the nick translation procedure of Rigby et al. (34a). After digestion and electrophoresis, DNA fragments were transferred to nitrocellulose filters (type HAHY; Millipore Corp., Molsheim, France) by the method of Southern (37a). DNAs from bacterial colonies were transferred to Whatman no. 540 filter disks by the procedure of Grunstein and Hogness (15a). Hybridization of nick-translated DNA to blots and filter disks was done in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0)–1× Denhardt solution–0.2% sodium dodecyl sulfate–0.02% denatured herring sperm DNA at 42°C for 48 h. After hybridization, filter disks and blots were rinsed three times with 0.1× SSC–0.1% sodium dodecyl sulfate and then washed three times with 2× SSC–0.1% sodium dodecyl sulfate at 42°C for 30 min. RP-X7 (Manufacturas Fotográficas Españolas, Madrid, Spain) X-ray film was exposed to the filters and blots with a Cronex intensifying screen at −70°C.

Site-directed Tn*5* mutagenesis. Tn*5* mutagenesis of cloned DNA in *E. coli* and recombination of the cosmids carrying Tn*5* with the corresponding region of the UPM791 genome were done as described by Ditta (9). pPH1JI was used as the incoming incompatible plasmid, and selection was made on *Rhizobium* minimal medium containing kanamycin and gentamicin. The loss of the pLAFR1 recombinant cosmid was confirmed by checking the tetracycline sensitivity of the putative marker-exchanged mutants. The fidelity of the Tn*5* recombinations in the genome was verified by hybridization analysis as described by Haugland et al. (17).

Plant tests and nitrogenase and hydrogenase assays. *R. leguminosarum* UPM791 derivative strains containing Tn*5* insertions were checked for nodulation and symbiotic nitrogenase and hydrogenase activities in peas (*Pisum sativum* L. cv. Frisson). Pea seeds were surface sterilized, germinated on 1% water agar plates, and planted in vermiculite in Leonard jar-type assemblies (42) at a density of four germinated seeds per jar. *Rhizobium* cells grown for 48 h on yeast-mannitol broth (42) were used as the inoculum. Plants were provided with a nitrogen-free solution and grown for 30 days in a growth chamber (day/night temperature, 24°C/15°C; light intensity, 25 klx, 16-h photoperiod). Acetylene reduction and hydrogen evolution by nodules and hydrogenase activity of bacteroids were determined as previously described (35).

RESULTS

Gene library of *R. leguminosarum* UPM791. Cosmid pLAFR1 was chosen as the cloning vector to construct the gene bank because it can be mobilized into and stably replicates in *Rhizobium* species. Tetracycline-resistant (Tc^r) transductants were obtained at a frequency of 4×10^4/μg of vector DNA. The resulting gene bank contained more than 16,000 independent clones and was maintained in *E. coli*

HB101. Twenty-four clones were chosen at random for cosmid DNA analysis. Gel electrophoresis of *Eco*RI digests of these cosmids showed that all contained insert DNA ranging in size between 9.5 and 34.6 kb and averaging 21 kb. All cosmids analyzed contained a fragment that comigrated with *Eco*RI-digested pLAFR1. Assuming that the molecular weight of the *Rhizobium* genome is about the same as that of the *E. coli* genome, i.e., 4,200 kb, and on the basis of theoretical calculations (7), 990 clones of the above gene bank should have a probability of greater than 99% of containing a given 1-kb sequence of DNA.

Isolation of recombinant cosmids containing presumptive *hup*-specific DNA. We took advantage of the existence of homology between *hup*-specific DNA from *B. japonicum* and genomic DNA of Hup^+ strains of *R. leguminosarum* (36, 37) to identify recombinant cosmids containing *hup*-specific sequences from a gene bank of strain UPM791. The 5.9-kb *Hin*dIII and 5.0-kb *Eco*RI DNA fragments from pHU1 (see Fig. 2A) were used as hybridization probes to screen 1,500 clones of the gene bank by the colony hybridization method. The 5.9-kb *Hin*dIII fragment contains the 60-kilodalton subunit gene for the *B. japonicum* hydrogenase (43), and the 5.0-kb *Eco*RI fragment also contains essential genes for hydrogen uptake in *B. japonicum* (17). Two clones were identified, and the corresponding recombinant cosmids (pAL618 and pAL704) were isolated. After gel electrophoresis and blotting, *Eco*RI digests from these cosmids and from total DNA of strain UPM791 were hybridized to probe DNAs from pHU1 to confirm that the cosmids did contain sequences homologous to *B. japonicum hup* DNA and that the hybridizing fragments were present in the *R. leguminosarum* genome. In addition to the DNA probes mentioned above, the 2.9-kb *Eco*RI fragment from pHU1, containing the gene coding for the 30-kilodalton subunit of the *B. japonicum* hydrogenase (43), was also used as a hybridization probe (Fig. 1).

Cosmids pAL618 and pAL704 contained approximately 21 and 30 kb of insert DNA, respectively, and had a 1.7-kb and a 2.2-kb *Eco*RI fragment in common. These two fragments strongly hybridized to the 5.0-kb *Eco*RI probe (Fig. 1, panel 2). Cosmid pAL704 also contained an additional *Eco*RI fragment of 7.2 kb with weak homology to the same probe. The 5.9-kb *Hin*dIII probe strongly hybridized to two *Eco*RI fragments of 6.4 and 5.0 kb from pAL618, but not to pAL704 (Fig. 1, panel 3). The 2.9-kb *Eco*RI probe showed hybridization to only the 5.0-kb *Eco*RI fragment from pAL618 (Fig. 1, panel 4). The hybridization to pLAFR1 DNA observed in lanes 4b and 4c of Fig. 1 is probably due to contamination of the probe DNA with vector pACYC184 used for subcloning. All the hybridizing fragments were present in the UPM791 genome (Fig. 1, lanes 2a, 3a, and 4a).

Physical organization of presumptive *hup* genes from *R. leguminosarum*. To define the DNA region of *R. leguminosarum* containing the homology to *hup*-specific DNA of *B. japonicum* and to determine its physical organization, we analyzed DNA from cosmids pAL618 and pAL704 by restriction endonuclease mapping techniques. Combinations of *Eco*RI, *Hin*dIII, *Bam*HI, *Xho*I, *Sal*I, and *Kpn*I endonucleases were used in the mapping analysis. The order of the *Eco*RI fragments determined for cosmid pAL618 and part of cosmid pAL704 is shown in Fig. 2C. By hybridizing filter blots of *Hin*dIII-, *Bam*HI-, *Sal*I-, and *Xho*I-digested total DNA from UPM791 to isolated and α-^{32}P-labeled *Eco*RI fragments from pAL618, it was demonstrated that the order of *Eco*RI fragments in this cosmid corresponds to that in the UPM791 genome. Similar hybridization analysis with

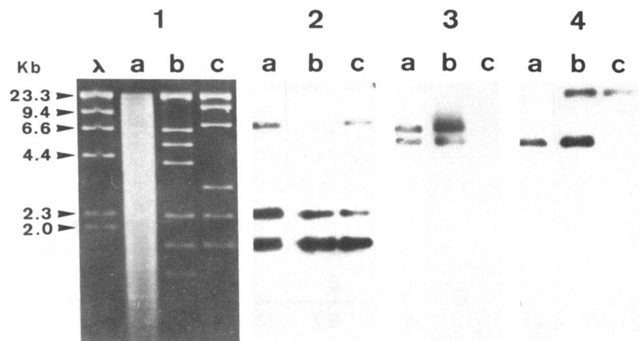

FIG. 1. Hybridization of *hup*-specific DNA from *B. japonicum* to DNA from *R. leguminosarum*. Lanes a, b, and c show *Eco*RI digests of total DNA from UPM791, cosmid pAL618, and cosmid pAL704, respectively. Panel 1 shows agarose gel electrophoresis of restriction fragments, and panels 2, 3, and 4 show the autoradiographs of Southern blots of replicates of gels in panel 1 after hybridization with the 5.0-kb *Eco*RI (panel 2), 5.9-kb *Hin*dIII (panel 3), and 2.9-kb *Eco*RI (panel 4) probe DNAs. The autoradiographs of total DNA (lanes a) were exposed twice as long as the autoradiographs of cosmid DNAs (lanes b and c). Numbers in the margin indicate fragment sizes of *Hin*dIII-digested λ phage DNA.

subcloned *Eco*RI fragments from pAL704 indicated that in the genome of UPM791 the 7.2-kb *Eco*RI fragment from pAL704 was contiguous with the pAL618 DNA at the 2.2-kb *Eco*RI border fragment. The DNA of pAL704 to the left of the 1.7-kb *Eco*RI fragment in common with pAL618 is not contiguous to the rest of the region. The resulting physical map of the DNA region defined by both cosmids is shown in Fig. 2B.

Based on hybridization and restriction mapping analysis of cosmids and genomic DNAs, the relative positioning of DNA regions homologous to the pHU1 DNA probes in the *R. leguminosarum* genome is shown in Fig. 2B by the solid bars above it. The bars show maximum limits of hybridization; the actual regions of DNA homology may be smaller. As can be seen, the relative order of the *R. leguminosarum* presumptive *hup* genes is the same as that of *B. japonicum hup* genes. When the 7.2-kb *Eco*RI DNA fragment from pAL704 was used as a probe to hybridize *Eco*RI-restricted DNA from pHU1 and pHU52, no hybridization signals were observed to the unique 5.5-kb *Eco*RI fragment of pHU52, reported to be essential for autotrophic growth in *B. japonicum* (20).

Evidence for functional *hup* genes in cosmid pAL618. To determine whether the DNA region of UPM791 showing homology to *hup*-specific DNA of *B. japonicum* contains in fact genes essential for H_2 uptake in *R. leguminosarum*, we used pAL618 to produce site-directed Tn*5* insertions into the UPM791 genome by a marker exchange technique. The physical location of Tn*5* insertions into pAL618 was determined by restriction enzyme analysis of isolated DNA from each pAL618::Tn*5* derivative, using *Eco*RI and *Hin*dIII restriction endonucleases. After the exchange by homologous recombination in *R. leguminosarum* UPM791, each UPM791::Tn*5* insertion strain was used to inoculate peas. The Hup phenotypes of insertion strains with Tn*5* located at seven different sites are shown in Fig. 2B. All seven strains formed effective nitrogen-fixing nodules (Table 2). Six insertions resulted in essentially the complete loss of O_2- and methylene blue-dependent H_2 uptake activity in bacteroids; three of them (AL10, AL13, AL25) were in the DNA region with homology to the 5.9-kb *Hin*dIII probe, and the other

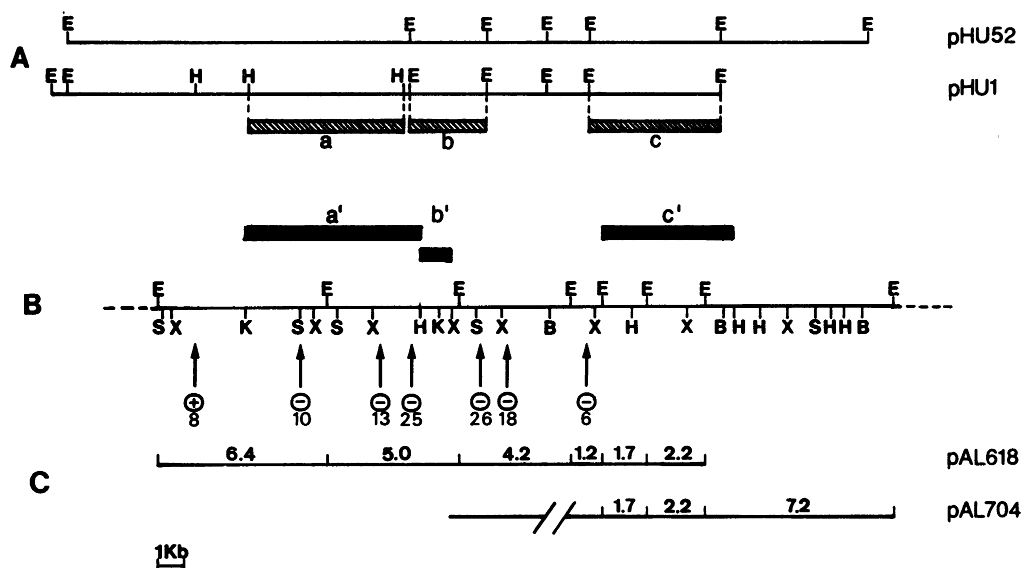

FIG. 2. Organization of *hup* region of *R. leguminosarum* genome, relative to the *hup* region of *B. japonicum*. (A) Restriction maps of pHU1 and pHU52 cosmids containing *hup*-specific DNA from *B. japonicum* 122DES (17, 20). The hatched bars show the DNA fragments used as hybridization probes: a, 5.9-kb *Hin*dIII; b, 2.9-kb *Eco*RI; and c, 5.0-kb *Eco*RI. (B) Restriction map of the *hup* region of *R. leguminosarum* UPM791. Restriction site abbreviations: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; S, *Sal*I; X, *Xho*I; K, *Kpn*I. The solid bars denote regions within which the *hup* DNAs hybridize. The arrows indicate positions of transposon Tn*5* insertions that have been incorporated into the UPM791 genome by marker exchange. Insertion positions are numbered according to the corresponding AL insertion strains, and the + or − beneath each arrow indicates whether the insertion resulted in a Hup^+ or Hup^- phenotype. (C) Cosmid DNAs isolated from a gene bank of *R. leguminosarum* UPM791. The sizes of the *Eco*RI fragments are shown. The broken segments indicate additional DNA that is not contiguous with the rest of the region shown.

three (AL6, AL18, AL26) were between the homology regions defined by the 2.9-kb *Eco*RI and 5.0-kb *Eco*RI probes.

To confirm that the sites of Tn*5* insertions in the genome of the marker-exchanged strains were the same as that determined in pAL618, filter blots containing *Eco*RI digests of total DNA from each *R. leguminosarum* Tn*5* insertion strain were hybridized to pAL618::Tn*5* DNA. The results of the hybridization experiment are shown in Fig. 3. The pattern of fragments hybridizing to pAL618::Tn*5* in each UPM791::Tn*5* insertion strain was identical to the corresponding pattern in strain UPM791, except for an *Eco*RI fragment that is larger by the size of Tn*5*. The two upper bands in each of the lanes corresponding to UPM791(pPH1JI) and all the UPM791::Tn*5* derivatives but not UPM791 are due to plasmid pPH1JI used to remove pAL618 in the marker exchange procedure. DNA from pPH1JI has homology to pLAFR1 vector DNA.

TABLE 2. Nitrogenase and hydrogenase activities of nodules and bacteroids produced by *R. leguminosarum* UPM791 and various Tn*5* insertion derivative strains in symbiosis with peas

Strain	Nodule activity[a]			Bacteroid hydrogenase activities[c] (electron acceptors)	
	C_2H_4 production	Hydrogen evolution	Relative efficiency[b]	O_2	MB
UPM791	19.2 ± 0.2	4.1 ± 0.6	0.78	0.28	0.54
UPM791::Tn*5* derivatives					
AL6	17.8 ± 1.4	8.2 ± 1.3	0.54	<0.05	<0.05
AL8	16.9 ± 1.7	3.8 ± 0.9	0.77	0.29	0.64
AL10	18.7 ± 2.5	5.9 ± 1.5	0.68	<0.05	<0.05
AL13	18.1 ± 0.8	5.8 ± 1.2	0.68	<0.05	<0.05
AL18	18.0 ± 1.1	5.6 ± 0.5	0.68	<0.05	<0.05
AL25	21.9 ± 0.7	7.4 ± 2.2	0.66	<0.05	<0.05
AL26	18.3 ± 0.5	7.5 ± 0.8	0.58	<0.05	<0.05

[a] Micromoles per hour per gram of fresh nodule weight. Values are means of four replicate plant cultures ± standard error of the mean.

[b] Relative efficiency was calculated as 1 − (H_2 evolved in air/C_2H_2 reduced).

[c] Micromoles of O_2- or methylene blue (MB)-dependent H_2 uptake per hour per milligram of protein. Values are the average of at least two determinations.

DISCUSSION

A major advancement in the genetics of H_2 oxidation was the isolation of *hup* genes from *B. japonicum* (4, 17) and the demonstration that the complete *hup* system is clustered in a DNA region of about 20 kb (20). In *R. leguminosarum* the need of plant growth experiments to select for Hup^- mutants made the study of *hup* genes more difficult. The conservation of *hup* DNA observed between *B. japonicum* and *R. leguminosarum* (36, 37) enabled us to isolate recombinant cosmids (pAL618 and pAL704) containing DNA sequences homologous to *hup*-specific DNA of *B. japonicum* from a gene bank of *R. leguminosarum* UPM791 constructed in the broad-host-range cloning vector pLAFR1.

Restriction fragments covering the *hup* DNA region of cosmid pHU1 (17) hybridized to cloned DNA in cosmids pAL618 and pAL704 in the same relative order as they appear in pHU1 (Fig. 2). Besides, the size of the homology region in pAL cosmids (about 19 kb) corresponds with the size of the *hup*-specific region of pHU1. These observations demonstrate that the overall arrangement of *R. leguminosarum* UPM791 presumptive *hup* DNA closely parallels

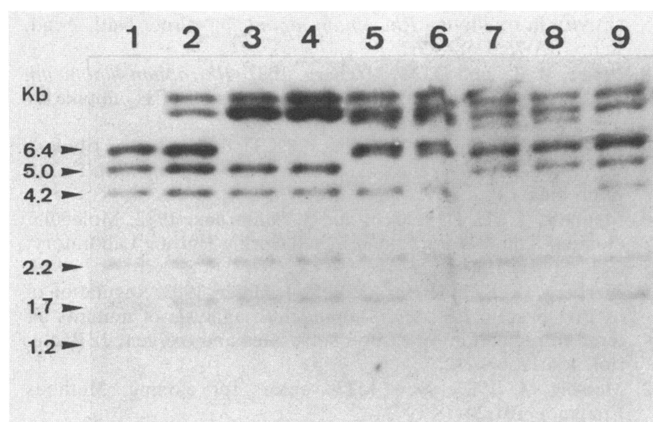

FIG. 3. Southern blot analysis of *R. leguminosarum* Tn*5* insertion strains. *Eco*RI-digested total DNA samples were hybridized with α-^{32}P-labeled pAL618::Tn*5* DNA. Lanes: 1, UPM791; 2, UMP791(pPHIJI); 3, AL8; 4, AL10; 5, AL13; 6, AL25; 7, AL18; 8, AL26; 9, AL6. Numbers in margin indicate the sizes of *Eco*RI DNA fragments from cosmid pAL618.

that of *B. japonicum*. Preliminary results presented by Tichy et al. (41) also suggest a similar organization of *hup* sequences in the *R. leguminosarum* B10 genome and in plasmid pHU1. Since, in addition, a recent report (40) also demonstrates similarities in organization and functionality between *Azotobacter chroococcum* and *B. japonicum*, it is likely that the type of *hup* DNA organization observed in *B. japonicum* 122DES (17, 20) is widely distributed among aerobic nitrogen-fixing bacteria.

The presence of functional *hup* genes in the isolated cosmids was demonstrated by generating UPM791::Tn*5* derivatives by site-directed transposon mutagenesis and examining their Hup phenotypes in symbiosis with peas. Tn*5* insertions at six different sites of pAL618 insert DNA abolished the pea bacteroid hydrogenase activity (Table 2). Three of these Hup$^-$ Tn*5* mutants are located within a pAL618 DNA region of about 8 kb with strong and weak homology to the 5.9-kb *Hin*dIII and 2.9-kb *Eco*RI fragments, respectively, from pHU1 (4). These two DNA fragments have been shown to direct the synthesis of the 60- and 30-kilodalton polypeptide subunits of the *B. japonicum* hydrogenase (43). Although the *R. leguminosarum* hydrogenase has not been purified yet and the number and the sizes of its components are unknown, on the basis of the DNA-DNA homology observed, we can also presume a two-subunit polypeptide structure for the *R. leguminosarum* hydrogenase. The genes coding for these subunits would then be within that 8-kb DNA region from pAL618. Since some evidence for functional equivalence between the *hup* genes of *R. leguminosarum* and *B. japonicum* has been reported by Kagan and Brewin (19), it can be expected that cosmid pAL618 complements *B. japonicum* Hup$^-$ mutants known to be affected in hydrogenase structural genes (12, 24).

Other genes essential for H_2 uptake in *R. leguminosarum* are also located in pAL618 to the right of the putative structural region, as suggested by the Hup$^-$ phenotype shown by three Tn*5* insertions (AL6, AL18, AL26) and by the existence of a 5-kb DNA region of homology to the *hup*-specific 5.0-kb *Eco*RI fragment from pHU1 (Fig. 2). Candidate genes are genes involved in regulation of the synthesis and activity of hydrogenase. Little or nothing is known about the genetics of *hup* regulation in either *B. japonicum* or *R. leguminosarum*. Mutants altered in regulation of hydrogenase activity or mutants defective in both nitrogenase (Nif$^-$) and hydrogenase (Hup$^-$) activities have been isolated in *B. japonicum* (28, 31). We are currently examining the symbiotic complementation of these mutants by cosmids pAL618 and pAL704 to compare the *hup* gene constituents of *B. japonicum* and *R. leguminosarum*.

Apparently, cosmid pAL618 contains all the DNA homology to pHU1 except for about 1 kb present in the adjacent 7.2-kb *Eco*RI fragment of pAL704 (Fig. 2). According to Lambert et al. (21), pHU1 may contain all the genetic determinants required for symbiotic *hup* activity. Therefore, it would be interesting to know whether pAL618 also contains all the information needed for hydrogenase expression in symbiosis with peas. In *B. japonicum*, genetic information present in cosmid pHU52 in a 5.5-kb *Eco*RI fragment contiguous to pHU1 insert DNA seems to be required for autotrophic growth with H_2 and CO_2 (20). We found no DNA sequences homologous to this fragment in the 7.2-kb *Eco*RI fragment from pAL704 which is adjacent to the pAL618 insert DNA in the *R. leguminosarum* UPM791 genome. This observation is consistent with the lack of chemolithotrophic growth with H_2 (20) and the absence of ribulose bisphosphate carboxylase activity (29) in strain 128C53 and suggests probable differences in regulatory control and integration into cellular metabolism of the H_2 uptake system of *B. japonicum* and *R. leguminosarum*.

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LITERATURE CITED

1. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84:**188–198.
2. **Beringer, J. E., J. L. Beynon, A. V. Buchanan-Wollaston, and A. W. B. Johnston.** 1978. Transfer of the drug-resistance transposon Tn*5* to *Rhizobium*. Nature (London) **276:**633–634.
3. **Brewin, N. J., T. M. DeJong, D. A. Phillips, and A. W. B. Johnston.** 1980. Co-transfer of determinants for hydrogenase activity and nodulation ability in *Rhizobium leguminosarum*. Nature (London) **288:**77–79.
4. **Cantrell, M. A., R. A. Haugland, and H. J. Evans.** 1983. Construction of a *Rhizobium japonicum* gene bank and use in isolation of a hydrogen uptake gene. Proc. Natl. Acad. Sci. USA **80:**181–185.
5. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
6. **Clewell, D. B., and D. R. Helinski.** 1972. Effect of growth conditions on the formation of the relaxation complex of supercoiled ColE1 deoxyribonucleic acid and protein in *Escherichia coli*. J. Bacteriol. **110:**1135–1146.
7. **Collins, J.** 1979. *Escherichia coli* plasmids packageable *in vitro* in λ bacteriophage particles. Methods Enzymol. **68:**309–326.
8. **Corbin, D., G. Ditta, and D. R. Helinski.** 1982. Clustering of nitrogen fixation (*nif*) genes in *Rhizobium meliloti*. J. Bacteriol. **149:**221–228.
9. **Ditta, G.** 1986. Tn5 mapping of *Rhizobium* nitrogen fixation genes. Methods Enzymol. **118:**519–528.
10. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
11. **Dixon, R. O. D.** 1978. Nitrogenase-hydrogenase interrelation-

ships in rhizobia. Biochimie **60**:233–236.

12. **Drevon, J. J., L. Frazier, S. A. Russell, and H. J. Evans.** 1982. Respiratory and nitrogenase activities of soybean nodules formed by hydrogen uptake negative (Hup^-) mutant and revertant strains of *Rhizobium japonicum* characterized by protein patterns. Plant Physiol. **70**:1341–1346.
13. **Eisbrenner, G., and H. J. Evans.** 1983. Aspects of hydrogen metabolism in nitrogen fixing legumes and other plant-microbe associations. Annu. Rev. Plant Physiol. **34**:105–136.
14. **Evans, H. J., F. J. Hanus, R. A. Haugland, M. A. Cantrell, L.-S. Xu, S. A. Russell, G. R. Lambert, and A. R. Harker.** 1985. Hydrogen recycling in nodules affects nitrogen fixation and growth of soybeans, p. 935–942. *In* R. Shibles (ed.), World Soybean Research Conference III: Proceedings. Westview Press, London.
15. **Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene **18**:289–296.

15a. **Grunstein, M., and D. S. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA **72**:3961–3965.

16. **Hanus, F. J., R. J. Maier, and H. J. Evans.** 1979. Autotrophic growth of H_2-uptake positive strains of *Rhizobium japonicum* in an atmosphere supplied with hydrogen gas. Proc. Natl. Acad. Sci. USA **76**:1788–1792.
17. **Haugland, R. A., M. A. Cantrell, J. S. Beaty, F. J. Hanus, S. A. Russell, and H. J. Evans.** 1984. Characterization of *Rhizobium japonicum* hydrogen uptake genes. J. Bacteriol. **159**:1006–1012.
18. **Hom, S. S. M., L. A. Graham, and R. J. Maier.** 1985. Isolation of genes (*nif/hup* cosmids) involved in hydrogenase and nitrogenase activities in *Rhizobium japonicum*. J. Bacteriol. **161**:882–887.
19. **Kagan, S. A., and N. J. Brewin.** 1985. Mutagenesis of a *Rhizobium* plasmid carrying hydrogenase determinants. J. Gen. Microbiol. **131**:1141–1147.
20. **Lambert, G. R., M. A. Cantrell, F. J. Hanus, S. A. Rusell, K. R. Haddad, and H. J. Evans.** 1985. Intra- and interspecies transfer and expression of *Rhizobium japonicum* hydrogen uptake genes and autotrophic growth capability. Proc. Natl. Acad. Sci. USA **82**:3232–3236.
21. **Lambert, G. R., F. J. Hanus, A. R. Harker, M. A. Cantrell, S. A. Rusell, and H. J. Evans.** 1985. Free-living and symbiotic expression of *Rhizobium japonicum* hydrogenase genes in slow and fast growing *Rhizobium* species, p. 228. *In* H. J. Evans, P. J. Bottomley, and W. E. Newton (ed.), Nitrogen fixation research progress. Martinus Nijhoff Publishers, Dordrecht.
22. **Lambert, G. R., A. R. Harker, M. Zuber, D. A. Dalton, F. J. Hanus, S. A. Russell, and H. J. Evans.** 1985. Characterization, significance and transfer of hydrogen uptake genes from *Rhizobium japonicum*, p. 209–215. *In* H. J. Evans, P. J. Bottomley, and W. E. Newton (ed.), Nitrogen fixation research progress. Martinus Nijhoff Publishers, Dordrecht.
23. **Langridge, J., P. Langridge, and P. L. Bergquist.** 1980. Extraction of nucleic acids from agarose gels. Anal. Biochem. **103**:264–271.
24. **Lepo, J. E., R. E. Hickok, M. A. Cantrell, S. A. Russell, and H. J. Evans.** 1981. Revertible hydrogen uptake-deficient mutants of *Rhizobium japonicum*. J. Bacteriol. **146**:614–620.
25. **Maier, R. J.** 1981. *Rhizobium japonicum* mutant strain unable to grow chemoautotrophically with H_2. J. Bacteriol. **145**:533–540.
26. **Maier, R. J.** 1986. Biochemistry, regulation and genetics of hydrogen oxidation in *Rhizobium*. Crit. Rev. Biotechnol. **3**:17–38.
27. **Maier, R. J., N. E. R. Campbell, F. J. Hanus, F. B. Simpson, S. A. Russell, and H. J. Evans.** 1978. Expression of hydrogenase activity in free-living *Rhizobium japonicum*. Proc. Natl. Acad. Sci. USA **75**:3258–3262.
28. **Maier, R. J., and D. M. Merberg.** 1982. *Rhizobium japonicum* mutants that are hypersensitive to repression of H_2 uptake by oxygen. J. Bacteriol. **150**:161–167.
29. **Manian, S. S., and F. O'Gara.** 1982. Derepression of ribulose bisphosphate carboxylase activity in *Rhizobium meliloti*. FEMS Microbiol. Lett. **14**:95–99.
30. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Merberg, D., E. B. O'Hara, and R. J. Maier.** 1983. Regulation of hydrogenase in *Rhizobium japonicum*: analysis of mutants altered in regulation by carbon substrates and oxygen. J. Bacteriol. **156**:1236–1242.
32. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
33. **Moshiri, F., L. Stults, P. Novak, and R. J. Maier.** 1983. Nif^- Hup^- mutants of *Rhizobium japonicum*. J. Bacteriol. **155**:926–929.
34. **O'Gara, F., and K. T. Shanmugam.** 1976. Regulation of nitrogen fixation by rhizobia: export of fixed nitrogen as NH_4^+. Biochim. Biophys. Acta **437**:313–321.

34a. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.

35. **Ruiz-Argüeso, T., F. J. Hanus, and H. J. Evans.** 1978. Hydrogen production and uptake by pea nodules as affected by strains of *Rhizobium leguminosarum*. Arch. Microbiol. **116**:113–118.
36. **Ruiz-Argüeso, T., A. Leyva, J. Palacios, T. Mozo, and B. Campos.** 1985. Plasmid location of *hup* genes in strains of *Rhizobium leguminosarum*, p. 230. *In* H. J. Evans, P. J. Bottomley, and W. E. Newton (ed.), Nitrogen fixation research progress. Martinus Nijhoff Publishers, Dordrecht.
37. **Seifert, B. L., H. V. Tichy, L. M. Nelson, M. A. Cantrell, R. A. Haugland, and W. Lotz.** 1984. Localization of *nif*- and *hup*-specific sequences on the plasmids of newly isolated strains of *Rhizobium leguminosarum*, p. 721. *In* C. Veeger and W. E. Newton (ed.), Advances in nitrogen fixation research. Martinus Nijhoff/Dr W. Junk Publishers, The Hague.

37a. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–507.

38. **Sternberg, N., D. Tiemeier, and L. Enquist.** 1977. In vitro packaging of λ Dam vector containing *Eco*RI DNA fragments of *Escherichia coli* and phage P1. Gene **1**:255–280.
39. **Takeda, Y., N. E. Harding, D. W. Smith, and J. W. Zyskind.** 1982. The chromosomal origin of replication (*oriC*) of *Erwinia carotovora*. Nucleic Acids Res. **10**:2639–2650.
40. **Tibelius, K. H., R. L. Robson, and M. G. Yates.** 1987. Cloning and characterization of hydrogenase genes from *Azotobacter chroococcum*. Mol. Gen. Genet. **206**:285–290.
41. **Tichy, H. V., C. Schild, H. M. Ripke, L. M. Nelson, H. Fees, and W. Lotz.** 1987. Analysis of *hup* DNA and Hup host range of *Rhizobium leguminosarum* B10, p. 279–281. *In* D. P. S. Verma and N. Brisson (ed.), Molecular genetics of plant-microbe interactions. Martinus Nijhoff Publishers, Dordrecht.
42. **Vincent, J. M.** 1970. A manual for the practical study of the root nodule bacteria. International Biological Programme Handbook 15. Blackwell Scientific Publications, Ltd., Oxford.
43. **Zuber, M., A. R. Harker, M. A. Sultana, and H. J. Evans.** 1986. Cloning and expression of *Bradyrhizobium japonicum* uptake hydrogenase structural genes in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **83**:7668–7672.