APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1987, p. 2539–2543
0099-2240/87/102539-05$02.00/0

Vol. 53, No. 10

Conserved Plasmid Hydrogen-Uptake (*hup*)-Specific Sequences within Hup$^+$ *Rhizobium leguminosarum* Strains

ANTONIO LEYVA, JOSÉ M. PALACIOS, AND TOMÁS RUIZ-ARGÜESO*

Departamento de Microbiología, Escuela Tecnica Superior de Ingenieros Agrónomos, Universidad Politécnica, 28040-Madrid, Spain

Received 20 April 1987/Accepted 31 July 1987

Thirteen *Rhizobium leguminosarum* strains previously reported as H_2-uptake hydrogenase positive (Hup$^+$) or negative (Hup$^-$) were analyzed for the presence and conservation of DNA sequences homologous to cloned *Bradyrhizobium japonicum hup*-specific DNA from cosmid pHU1 (M. A. Cantrell, R. A. Haugland, and H. J. Evans, Proc. Natl. Acad. Sci. USA 80:181–185, 1983). The Hup phenotype of these strains was reexamined by determining hydrogenase activity induced in bacteroids from pea nodules. Five strains, including H_2 oxidation-ATP synthesis-coupled and -uncoupled strains, induced significant rates of H_2-uptake hydrogenase activity and contained DNA sequences homologous to three probe DNA fragments (5.9-kilobase [kb] *Hin*dIII, 2.9-kb *Eco*RI, and 5.0-kb *Eco*RI) from pHU1. The pattern of genomic DNA *Hin*dIII and *Eco*RI fragments with significant homology to each of the three probes was identical in all five strains regardless of the H_2-dependent ATP generation trait. The restriction fragments containing the homology totalled about 22 kb of DNA common to the five strains. In all instances the putative *hup* sequences were located on a plasmid that also contained *nif* genes. The molecular sizes of the identified *hup-sym* plasmids ranged between 184 and 212 megadaltons. No common DNA sequences homologous to *B. japonicum hup* DNA were found in genomic DNA from any of the eight remaining strains showing no significant hydrogenase activity in pea bacteroids. These results suggest that the identified DNA region contains genes essential for hydrogenase activity in *R. leguminosarum* and that its organization is highly conserved within Hup$^+$ strains in this symbiotic species.

The generation of hydrogen by legume nodules as a consequence of the N_2 reduction catalyzed by nitrogenase is a potential source of inefficiency of the *Rhizobium*-legume symbiosis. In nodules produced by a limited number of *Bradyrhizobium* and *Rhizobium* strains, H_2 is recycled by means of an H_2 uptake system and oxidized to water (3, 12, 14). The utilization of the nitrogenase-generated H_2 in these nodules has been shown to provide ATP for nitrogen fixation, to protect nitrogenase against O_2, and to use up H_2, preventing nitrogenase inhibition by H_2 (3, 12). Some of these putative advantages may be responsible for the beneficial effects of the H_2-uptake system on whole-plant growth and nitrogen fixation that have been observed in soybeans nodulated by Hup$^+$ (H_2-uptake-hydrogenase-positive) strains of *Bradyrhizobium japonicum* (14, 16). However, the actual contribution of the H_2-uptake system to increased productivity has been difficult to assess in a rigorous manner (16).

Only certain strains of *Rhizobium leguminosarum* possess an H_2-uptake hydrogenase (23, 27, 29), and the H_2-oxidizing activity of most of them is insufficient to recycle all the H_2 produced during nitrogen fixation in pea nodules (23, 27). Nelson and Salminen (25) demonstrated that H_2 oxidation is coupled to ATP generation in certain Hup$^+$ strains (ATP-coupled strains) of *R. leguminosarum* but not in others.

Genetic evidence has been presented that determinants involved in H_2 oxidation in the ATP-uncoupled strain 128C53 of *R. leguminosarum* are linked in a plasmid (pRL6JI) to other determinants for symbiotic functions (4). When pRL6JI was transferred by recombination with a self-transmissible plasmid into other *R. leguminosarum* genetic backgrounds, an improvement in the symbiotic performance of the new host was demonstrated (11). However, by carrying out plant growth experiments using Hup$^-$ mutants generated by Tn*5* insertions into pRL6JI as inocula, it was later concluded that the observed improvement produced by pRL6JI may be associated with some trait other than the Hup phenotype (10).

Genes essential for H_2 uptake (*hup* genes) have been isolated from libraries of *B. japonicum* DNA (6, 18), and at least two transcriptional units have been identified in cosmid pHU1 (17). By using DNA from pHU1 as a hybridization probe, Nelson et al. (24) found homology between *hup*-specific DNA of *B. japonicum* and genomic DNA from some Hup$^+$ *R. leguminosarum* strains but not from others. On the basis of these results, the investigators suggested the existence of two different types of *hup* sequences within Hup$^+$ strains of *R. leguminosarum*.

To further examine the contribution of the Hup phenotype of *R. leguminosarum* to plant nitrogen fixation and growth, additional information should be obtained from Hup$^+$ strains other than 128C53. Particularly attractive are strains in which H_2 oxidation is coupled to ATP generation, because the *hup* system of these strains may be associated with increased N_2 fixation. Since, in addition, the transfer of the H_2-uptake system into *Rhizobium* strains lacking H_2-recycling capacity would be facilitated by the location of *hup* genes on plasmids, it is of interest to identify and characterize *hup* plasmids which could be used as better sources of *hup* genes. We report the identification of plasmids containing *hup* determinants in ATP-coupled strains of *R. leguminosarum* and show that *hup*-specific DNA has a similar organization in both types of Hup$^+$ strains, ATP-coupled and -uncoupled.

MATERIALS AND METHODS

Bacterial strains and plasmids. *R. leguminosarum* UML2 and UML5 are native isolates from nodules of *Vicia ervilia*

* Corresponding author.

TABLE 1. Hydrogenase activity of pea bacteroids from *R. leguminosarum* strains and homology of plasmid and total DNA from these strains to *R. meliloti nif-* or *B. japonicum hup*-specific DNA sequences

Strain	Bacteroid hydrogenase activity[a]	Size (MDa) of plasmid with homology to *nif* DNA[b]	Homology to *hup* DNA[c]	
			Total DNA	Plasmid DNA
128C53	0.65 ± 0.15	186 ± 4	+	+
128C30	0.75 ± 0.18	212 ± 10	+	+
128C23	0.65 ± 0.12	184 ± 2	+	+
128C13	0.34 ± 0.08	203 ± 5	+	+
128C56	0.27 ± 0.06	200 ± 8	+	+
175G15	<0.05	160 ± 5	−	−
UML2	<0.05	>500	−	−
UML5	<0.05	174 ± 17	−	−
128C75	<0.05	139 ± 4	−	−
175G11	<0.05	160 ± 12	−	−
92A3	<0.05	ND[d]	−	ND
128C76	<0.05	ND	−	ND
128C78	<0.05	ND	−	ND

[a] Micromoles of O_2-dependent H_2 uptake · h^{-1} · mg of $protein^{-1}$. Values are the averages of three determinations ± standard errors.

[b] Values are averages ± standard errors of size determinations (megadaltons [MDa]) from three separate gels. DNA from plasmid pID1 containing *nifHD* genes from *R. meliloti* (1) was used as the hybridization probe.

[c] The 5.9-kb *Hin*dIII fragment from pHU1 (7) was used as the *hup*-specific hybridization probe.

[d] ND, Not determined.

and *Vicia faba* plants, respectively, grown in Spain. All other *R. leguminosarum* strains used (Table 1) were received from J. Burton, Nitragin Co., Milwaukee, Wis. Strains 128C53.5 and 128C30.2 are *sym* plasmid-cured derivatives of strains 128C53 and 128C30, respectively. They were obtained by screening single-colony isolates from stock cultures of the wild-type strains for their plasmid profiles. Some of these isolates apparently have spontaneously lost the *sym* plasmid and are not infective (data to be presented elsewhere). *B. japonicum* 122DES and plasmid pHU1 containing *hup*-specific sequences from this strain (6, 17) were provided by H. J. Evans, Oregon State University, Corvallis. Plasmid pID1 containing *nifHD* genes of *Rhizobium meliloti* (1) was received from J. Olivares, Consejo Superior de Investigaciones Científicas, Granada, Spain. Plasmid vectors pUC13 (30) and pACYC184 (8) were obtained from G. Ditta, University of California at San Diego, La Jolla.

Bacteroid preparation and hydrogenase assays. Bacteroids were prepared from nodules of *Pisum sativum* cv. Frisson plants grown under bacteriologically controlled conditions as described by Ruiz-Argüeso et al. (27), except that a growth chamber provided with 25,000-lx illumination during a 16-h period was used instead of a greenhouse. Bacteroids from nodules were prepared under aerobic conditions as described previously (21). The hydrogenase activities of bacteroid suspensions were determined by measuring the rate of H_2 uptake with O_2 or methylene blue as terminal electron acceptors by an amperometric technique (15, 27).

Plasmid identification. *Rhizobium* plasmids were identified by using the in-well cell lysis and electrophoresis procedure of Eckhardt (13) with the modification previously described (5). The sizes of the plasmids were calculated from their relative mobilities in agarose gels by using plasmids of known molecular weights as standards (5).

DNA techniques. Total *Rhizobium* DNA was isolated by the method of Corbin et al. (9). Plasmid DNA from *Escherichia coli* was prepared by the alkaline lysis procedure described by Maniatis et al. (22). DNA fragments from pHU1 (6) to be used as *hup*-specific probes were subcloned into plasmid vector pUC13 (5.9-kilobase [kb] *Hin*dIII and 5.0-kb *Eco*RI fragments) or pACYC184 (2.9-kb *Eco*RI fragment). The resulting recombinant plasmids (pCM5, pCM7, and pCM3, respectively) were digested with *Hin*dIII or *Eco*RI, and the inserted DNA was isolated from gels by the low-melting-agarose procedure of Langridge et al. (20). Restriction enzyme digestions, agarose gel electrophoresis, Southern blot transfer, and enzymatic cloning techniques were standard (22).

Hybridization procedures. DNA from plasmid pID1 and DNA fragments from pHU1 were labeled with [α-^{32}P]dATP (410 Ci/mmol; Amersham International) by nick translation (22) and used as *nif*- and *hup*-specific probes, respectively. The Eckhardt agarose gels used for visualization of plasmids were dried as previously described (5). Genomic DNA restriction fragments were separated by electrophoresis and transferred to nitrocellulose filters (Millipore, Molsheim, France) by the method of Southern (22). DNA hybridizations of labeled probes to dried gels and Southern blot transfers were done essentially as described previously (5). Hybridization was carried out for 48 h with 50% formamide at 42°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). After hybridization, the nitrocellulose filters and dried gels 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. Autoradiography was conducted at −70°C with RP-X7 (MAFE) X-ray film and Cronex (Du Pont Co.) intensifying screens.

RESULTS

Hup phenotype. The hydrogenase activities of bacteroids from nodules produced by 13 strains of *R. leguminosarum* in peas were determined (Table 1). Strains 128C53, 128C30, 128C23, 128C13, and 128C56, which have been previously reported as Hup^+ strains (23, 27), exhibited rates of H_2 uptake ranging from 0.27 to 0.65 $\mu mol \cdot h^{-1} \cdot mg$ of $protein^{-1}$. No significant O_2- or methylene blue-dependent H_2-uptake activities were detected in bacteroids from the remaining strains. These strains have been shown (27, 29) to produce nodules in peas with no or low capacity to recycle H_2.

***hup* sequence conservation in *R. leguminosarum*.** To investigate the sequence conservation and organization of H_2-uptake genes in *R. leguminosarum*, we used *B. japonicum hup*-specific DNA sequences contained on the 5.9-kb *Hin*dIII, 2.9-kb *Eco*RI, and 5.0-kb *Eco*RI fragments from cosmid pHU1 (6, 17) as hybridization probes. The DNA from the 13 strains of *R. leguminosarum* examined for Hup phenotype was purified and digested with *Eco*RI or *Hin*dIII, and the resulting restriction fragments were separated by electrophoresis, transferred to nitrocellulose paper, and hybridized to the *B. japonicum* probes. Total DNA from all strains showing a Hup^+ phenotype contained identical patterns of *Eco*RI fragments with homology to each of the three *hup* probe DNAs (Fig. 1, lanes A, C, D, E, and F). The 5.0-kb *Eco*RI probe showed three hybridizing bands, the 5.9-kb *Hin*dIII probe showed two bands, and the 2.9-kb *Eco*RI probe showed one band. None of these bands was present in *Eco*RI-digested genomic DNA from each of the *R. leguminosarum* strains exhibiting a Hup^- phenotype (Fig. 1, lanes G to L) or from strain 128C30.2 (*sym* plasmid-cured derivative of Hup^+ strain 128C30; Fig. 1, lane B). Low-

intensity hybridization bands were also observed with the 5.0-kb *Eco*RI probe to *Eco*RI-digested DNA from some Hup^+ and Hup^- strains.

When total DNA from Hup^+ strains of *R. leguminosarum* was digested with *Hin*dIII restriction enzyme, a common pattern of fragments hybridizing to each of the *B. japonicum* DNA probes was observed (data not shown). The molecular sizes of the *Hin*dIII DNA fragments containing the *hup* homology were 10.5 kb (5.9-kb *Hin*dIII probe), 8.2 kb (2.9-kb *Eco*RI probe), and 8.2 and 4.0 kb (5.0-kb *Eco*RI probe). The weakest hybridization signals were observed in all the Hup^+ *R. leguminosarum* strains examined with the 2.9-kb *Eco*RI probe DNA.

Plasmid location of *hup* sequences. By using a modified Eckhardt agarose gel procedure, three to six plasmid bands were visualized in each of the 13 strains of *R. leguminosarum* examined. Representative examples are shown in Fig. 2. The sizes of the plasmids, estimated by their relative mobilities in agarose gels with plasmids of known molecular weight as standards, ranged from 60 to >500 megadaltons.

To investigate whether *hup* sequences were located on plasmids, the Eckhardt gels containing the plasmid DNA bands were dried and directly hybridized to the 5.9-kb *Hin*dIII *B. japonicum hup*-specific DNA probe. In each of the strains exhibiting H_2-uptake capability a single plasmid was found to hybridize to the *hup* probe (Fig. 2, lanes A, a, C, c, E, e, F, f, G, and g). In experiments in which *nifHD* DNA from *R. meliloti* was used as the hybridization probe,

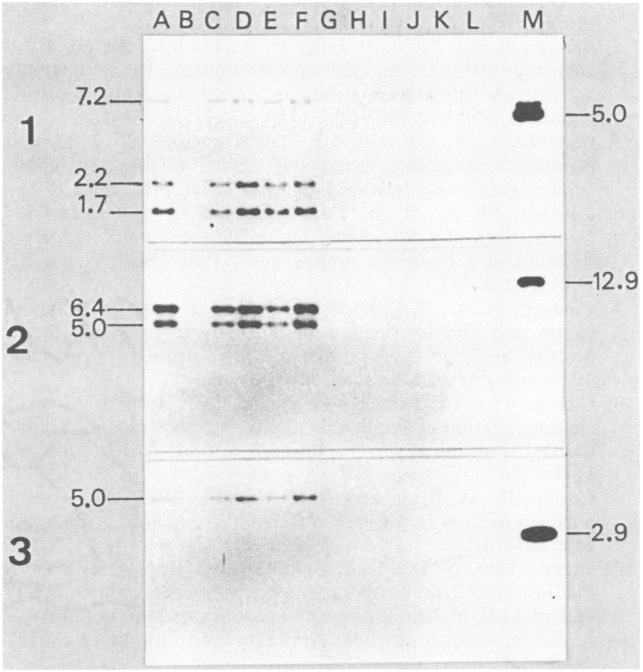

FIG. 1. Autoradiograms of Southern blots of *Eco*RI-digested total DNA from *R. leguminosarum* strains hybridized to *hup*-specific DNA from *B. japonicum* 122DES. Hybridization probes were DNA fragments from pHU1 (17): 5.0-kb *Eco*RI (panel 1), 5.9-kb *Hin*dIII (panel 2), and 2.9-kb *Eco*RI (panel 3). The *R. leguminosarum* strains used are as follows: lane A, 128C30; lane B, 128C30.2; lane C, 128C53; lane D, 128C23; lane E, 128C13; lane F, 128C56; lane G, 175G15; lane H, 92A3; lane I, 128C76; lane J, 128C78; lane K, 128C75; lane L, UML2. Lane M, *B. japonicum* 122DES. The numbers in the margins are the molecular sizes (kilobases) of the hybridizing DNA bands.

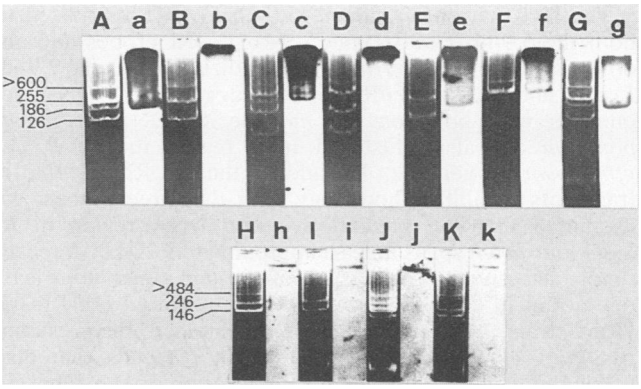

FIG. 2. Composite photograph showing hybridization of *hup*-specific DNA from *B. japonicum* to plasmid DNA from *R. leguminosarum* strains. Uppercase letters indicate photographs of ethidium bromide-stained agarose gels after electrophoresis of in-well cell lysates from *R. leguminosarum* 128C53 (lane A), 128C53.5 (lane B), 128C30 (lane C), 128C30.2 (lane D), 128C13 (lane E), 128C56 (lane F), 128C23 (lane G), UML2 (lane H), 128C75 (lane I), UML5 (lane J), and 175G15 (lane K). The sizes (megadaltons) of the plasmids from strains 128C53 and UML2 are shown in the margins. The migration distances are comparable from strain to strain within the top or bottom panel. Lowercase letters indicate the corresponding autoradiograms of the dry gels (lanes A to K) after hybridization to the α-^{32}P-labeled 5.9-kb *Hin*dIII fragment from pHU1 (7).

plasmids exhibiting *hup* homology were shown to also contain DNA sequences homologous to the *R. meliloti nif* genes (data not shown). The molecular sizes of plasmids hybridizing to *hup* and *nif* probes are indicated in Table 1. No hybridization signal with the *hup* probe was observed in any of the plasmids harbored in Hup^- strains (Fig. 2, lanes H to K and h to k) or in *sym* plasmid-cured derivatives of strains 128C53 and 128C30 (Fig. 2, lanes B, b, D, and d). These results clearly demonstrate that *hup* sequences in all Hup^+ strains of *R. leguminosarum* tested are linked to symbiotic genes in a plasmid (*sym-hup*).

DISCUSSION

The results presented here show that all the strains of *R. leguminosarum* that induced significant hydrogenase activity in pea bacteroids contained DNA sequences homologous to *B. japonicum* 122DES. The pattern of *Eco*RI and *Hin*dIII genomic DNA restriction fragments hybridizing to each of three *hup*-specific probe DNAs from pHU1 was identical in all the Hup^+ strains of *R. leguminosarum* examined (Fig. 1). Since these DNA sequences were not present in any of the strains exhibiting a Hup^- phenotype, they may be specifically involved in H_2 recycling in *R. leguminosarum*. Of particular interest are the findings by Zuber et al. (31) that the 5.9-kb *Hin*dIII and 2.9-kb *Eco*RI fragments from pHU1 contain the structural genes for the two *B. japonicum* hydrogenase subunits. This suggests that the corresponding homologous DNA sequences in *R. leguminosarum* contain the structural genes for the hydrogenase. Obviously, besides the hydrogenase structural genes other genes involved in the synthesis or functioning of the H_2-uptake system may also be present in the 5.9-kb *Hin*dIII and 2.9-kb *Eco*RI probe DNA. The significance of the hybridization to the 5.0-kb *Eco*RI fragment from pHU1 is unknown since no functions have been assigned to this DNA. However, it was demonstrated by Haugland et al. (17) that this fragment contains essential genes for hydrogen uptake in *B. japonicum*.

The fact that one fragment (5.0-kb *Eco*RI) hybridized to both the 5.9-kb *Hin*dIII and 2.9-kb *Eco*RI probes and another fragment (8.2-kb *Hin*dIII) hybridized to both the 2.9-kb *Eco*RI and 5.0-kb *Eco*RI probes suggests that DNA sequences with homology to *hup*-specific DNA of *B. japonicum* are also clustered in a region in the *R. leguminosarum* genome. By adding the *Eco*RI or *Hin*dIII fragments exhibiting homology with the three probes, we estimated that the presumptive *hup* DNA region in *R. leguminosarum* spans at least approximately 22-kb. A recent report also suggests that some of the *hup* genes in at least one strain of *R. leguminosarum* are clustered (19). In all Hup^+ strains of *R. leguminosarum* examined, the presumptive *hup* sequences were located on plasmids that also carried *nif* genes. Evidence for the location of *hup* genes on *sym* plasmids had previously been shown for strain 128C53 (4) and for some native isolates of *R. leguminosarum* (28). Therefore, unlike the situation in *B. japonicum* (7) and *Azotobacter chroococcum* (26), the plasmid location of *hup* genes seems to be a general trait in *R. leguminosarum*. The maintenance of *sym* and *hup* determinants in the same plasmid (*sym-hup*) during evolution suggests that the Hup phenotype is beneficial for the *R. leguminosarum*-plant interaction.

The existence of DNA sequences with homology to *hup*-specific DNA of *B. japonicum* was previously shown in some Hup^+ strains of *R. leguminosarum* but not in others (24). Nelson et al. (24) demonstrated that eight Hup^+ strains of *R. leguminosarum* contained a common *Hin*dIII DNA fragment of about 10.0 kb which hybridized to the 5.9-kb *Hin*dIII DNA fragment from pHU1. However, these investigators found no hybridization of DNA from pHU1 to plasmid or genomic DNA from strains 128C30 and 128C23, and only weak hybridization was observed to strains 128C13 and 128C53. On the basis of these results, they suggested the existence of two different types of *hup* DNA sequences in *R. leguminosarum* (24). Interestingly, H_2 oxidation is coupled to ATP generation in strains 128C30, 128C23, and 128C13 (24). In our hands both plasmid and genomic DNA from the four strains mentioned above hybridized to DNA from pHU1 regardless of the H_2-dependent ATP generation trait. In addition, the organization of the presumptive *hup* sequences was identical in the genomes of the four strains, all of which contain a *Hin*dIII fragment of approximately 10.5 kb homologous to the 5.9-kb *Hin*dIII probe. These results indicate that the organization of the *hup* DNA is highly conserved within Hup^+ strains of *R. leguminosarum*. The most likely explanation for the lack of hybridization to *B. japonicum hup*-specific DNA observed by Nelson et al. (24) in clearly Hup^+ strains of *R. leguminosarum* could be the absence of *sym* plasmids in all or most rhizobial cells of the stock cultures used in their experiments. This is supported by the observation that the plasmid profiles reported by them for strains 128C30, 128C23, 128C13, and 128C53 are identical to the plasmid profiles shown in Fig. 2 for the same strains, except that the DNA band corresponding to the *sym* plasmid is missing. We have observed (data to be presented elsewhere) that plasmids are spontaneously lost in stock cultures of *R. leguminosarum* strains, resulting in most cells of the culture being cured of the *sym* plasmid.

Strains 175G15, 92A3, 175G11, and UML5, which showed no significant O_2-dependent H_2-uptake activity in pea bacteroid preparations (Table 1), were reported by Truelsen and Wyndaele (29) as Hup^+ on the basis of their capacity to take up H_2 in excised, halved pea nodules. In the experiments carried out by these investigators, the H_2-uptake rate was very low, except for strain 175G15. Since we used a different pea cultivar and plant growing conditions, it is possible that host and environmental effects could have been responsible for the differences observed in the Hup phenotype (2, 21). However, under our hybridization conditions we found no homology of plasmid or genomic DNA from any of these strains to *B. japonicum hup*-specific DNA of pHU1. This suggests a Hup^- phenotype for these strains. Nevertheless, the existence of Hup^+ strains of *R. leguminosarum* with *hup* DNA sequences not homologous to *hup*-specific DNA of *B. japonicum* cannot be completely ruled out.

ACKNOWLEDGMENTS

We are grateful to Harold J. Evans for cosmid pHU1 and *B. japonicum* strains, Joe Burton for *R. leguminosarum* strains, José Olivares for pID1, and Gary Ditta for pUC13 and pACYC184. We also thank Teresa Mozo for valuable discussions.

This work was supported by grants from the Comisión Asesora de Investigación Científica y Técnica (project 2462/83) and from the Ministerio de Agricultura, Pesca y Alimentación (project 0243/84).

LITERATURE CITED

1. **Banfalvi, S. V., C. Koncz, A. Kiss, I. Dusha, and A. Kondorosi.** 1981. Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *Rhizobium meliloti*. Mol. Gen. Genet. **184**:318–325.
2. **Bedmar, E. J., S. A. Edie, and D. A. Phillips.** 1983. Host plant cultivar effects on hydrogen evolution by *Rhizobium leguminosarum*. Plant Physiol. **72**:1011–1015.
3. **Brewin, N. J.** 1984. Hydrogenase and energy efficiency in nitrogen fixing symbionts, p. 179–203. *In* D. P. S. Verma and T. H. Hohn (ed.), Genes involved in microbe-plant interactions. Springer-Verlag, New York.
4. **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.
5. **Cadahia, E., A. Leyva, and T. Ruiz-Argüeso.** 1986. Indigenous plasmids and cultural characteristics of rhizobia nodulating chickpeas (*Cicer arietinum* L.). Arch. Microbiol. **146**:239–244.
6. **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.
7. **Cantrell, M. A., R. E. Hickok, and H. J. Evans.** 1982. Identification and characterization of plasmids in hydrogen uptake positive and hydrogen uptake negative strains of *Rhizobium japonicum*. Arch. Microbiol. **131**:102–106.
8. **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.
9. **Corbin, D., G. Ditta, and D. R. Helinski.** 1982. Clustering of nitrogen fixation (*nif*) genes in *Rhizobium meliloti*. J. Bacteriol. **149**:221–228.
10. **Cunningham, S. D., Y. Kapulnik, N. J. Brewin, and D. A. Phillips.** 1985. Uptake hydrogenase activity determined by plasmid pRL6JI in *Rhizobium leguminosarum* does not increase symbiotic nitrogen fixation. Appl. Environ. Microbiol. **50**:791–794.
11. **DeJong, T. M., N. J. Brewin, A. W. B. Johnston, and D. A. Phillips.** 1982. Improvement of symbiotic properties in *Rhizobium leguminosarum* by plasmid transfer. J. Gen. Microbiol. **128**:1829–1838.
12. **Dixon, R. O. D.** 1972. Hydrogenase in legume root nodule bacteroids: occurrence and properties. Arch. Microbiol. **85**: 193–201.
13. **Eckhardt, T.** 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid **1**:584–588.
14. **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.
15. **Emerich, D. W., T. Ruiz-Argüeso, T. M. Ching, and H. J. Evans.** 1979. Hydrogen-dependent nitrogenase activity and ATP formation in *Rhizobium japonicum* bacteroids. J. Bacteriol. **137**:153–160.
16. **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.
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. **Langridge, J., P. Langridge, and P. L. Bergquist.** 1980. Extraction of nucleic acids from agarose gels. Anal. Biochem. **103**: 264–271.
21. **Lopez, M., V. Carbonero, E. Cabrera, and T. Ruiz-Argüeso.** 1983. Effects of host on the expression of the H_2-uptake hydrogenase of *Rhizobium* in legume nodules. Plant Sci. Lett. **29**:191–199.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Nelson, L. M., and J. J. Child.** 1981. Nitrogen fixation and hydrogen metabolism by *Rhizobium leguminosarum* isolates in pea root nodules. Can. J. Microbiol. **27**:1028–1034.
24. **Nelson, L. M., E. Grosskopf, H. V. Tichy, and W. Lotz.** 1985. Characterization of *hup*-specific DNA in *Rhizobium leguminosrum* strains of different origin. FEMS Microbiol. Lett. **30**:53–58.
25. **Nelson, L. M., and S. O. Salminen.** 1982. Uptake hydrogenase activity and ATP formation in *Rhizobium leguminosarum* bacteroids. J. Bacteriol. **151**:989–995.
26. **Robson, R. L., J. A. Chesshyre, C. Wheeler, R. Jones, P. R. Woodley, and J. R. Postgate.** 1984. Genome size and complexity in *Azotobacter chroococcum*. J. Gen. Microbiol. **130**:1603–1612.
27. **Ruiz-Argüeso, T., J. Hanus, and H. J. Evans.** 1978. Hydrogen production and uptake by pea nodules as affected by strains of *Rhizobium japonicum*. Arch. Microbiol. **116**:113–118.
28. **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, The Netherlands.
29. **Truelsen, T. A., and R. Wyndaele.** 1984. Recycling efficiency in hydrogenase uptake positive strains of *Rhizobium leguminosarum*. Physiol. Plant. **62**:45–50.
30. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
31. **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.