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Genetic Organization of the Hydrogen Uptake (*hup*) Cluster from *Rhizobium leguminosarum*

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In symbiosis with peas, *Rhizobium leguminosarum* UPM791 induces the synthesis of a hydrogen uptake (Hup) system that recycles hydrogen generated in nodules by nitrogenase. A cosmid (pAL618) containing *hup* genes from this strain on a 20-kilobase-pair (kb) DNA insert has previously been isolated in our laboratory (A. Leyva, J. M. Palacios, T. Mozo, and T. Ruiz-Argüeso, J. Bacteriol. 169:4929–4934, 1987). Here we show that cosmid pAL618 contains all of the genetic information required to confer high levels of hydrogenase activity on the naturally Hup$^-$ strains *R. leguminosarum* UML2 and *Rhizobium phaseoli* CFN42, and we also describe in detail the organization of *hup* genes on pAL618. To study *hup* gene organization, site-directed transposon mutagenesis and complementation analysis were carried out. According to the Hup phenotype associated with the transposon insertions, *hup* genes were found to span a 15-kilobase-pair region within pAL618 insert DNA. Complementation analysis revealed that Hup$^-$ mutants fell into six distinct complementation groups that define six transcriptional units, designated regions *hup*I to *hup*VI. Region *hup*I was subcloned and expressed in *Escherichia coli* cells under the control of a bacteriophage T7 promoter. A polypeptide of ca. 65 kilodaltons that was cross-reactive with antiserum against the large subunit of *Bradyrhizobium japonicum* hydrogenase was detected both in *E. coli* cells carrying the cloned *hup*I region and in pea bacteroids from strain UPM791, indicating that region *hup*I codes for structural genes of *R. leguminosarum* hydrogenase.

Many aerobic N_2-fixing bacteria, including cyanobacteria, azotobacter, and rhizobia, are capable of synthesizing a hydrogenase system that oxidizes H_2 generated by the nitrogenase complex during the N_2 fixation process. Among the root legume-infecting bacteria, H_2 uptake hydrogenase-positive (Hup$^+$) strains have been found in species of the three genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* (4, 36). However, most of our present knowledge on the biochemistry and genetics of the H_2-oxidizing system comes from studies with soybean bacteroids and vegetative cells from Hup$^+$ strains of *Bradyrhizobium japonicum*. In free-living cultures, these strains are able to induce hydrogenase activity and to grow autotrophically with H_2 and CO_2 (13). In symbiosis with soybeans, the recycling of H_2 by the bacteroids inside the nodules has been reported to increase plant productivity (12). The H_2 uptake hydrogenase from *B. japonicum* has been purified from vegetative (14, 37) and symbiotic (1) cells and shown to contain two polypeptide subunits of approximately 65 and 35 kilodaltons (kDa). Besides FeS clusters, nickel (1, 14) and probably selenium (3) are also components of the active hydrogenase. Mutants defective in hydrogenase activity have been generated and used in the isolation of H_2 uptake (*hup*) genes (6, 17). The genes coding for the two polypeptides of the hydrogenase from *B. japonicum* 122DES have recently been sequenced (32). Several other genes are also likely to be involved in H_2 uptake in *B. japonicum*, since Tn*5* Hup$^-$ mutants mapping outside of the structural genes have been obtained (16, 21). This view is also supported by the demonstration that certain mutants unable to oxidize H_2 with oxygen exhibit hydrogenase activity with artificial electron acceptors (27). However, despite the considerable knowledge accumulated on the physiology and biochemistry of H_2 oxidation in *B. japonicum*, the number and organization of *hup* genes in *B. japonicum* and in any other endosymbiotic bacteria are unknown.

The DNA involved in H_2 uptake seems to be conserved in *B. japonicum* and *Rhizobium leguminosarum* Hup$^+$ strains (25). However, unlike the case with *B. japonicum*, hydrogenase activity is not expressed in free-living cultures of *R. leguminosarum*, and the screening for Hup$^-$ mutants requires individual plant testing (19, 30). The H_2 uptake determinants are located in the symbiotic plasmid in all the Hup$^+$ strains of *R. leguminosarum* examined (5, 25). *hup* genes were isolated in this laboratory from *R. leguminosarum* UPM791 by screening a gene library with *B. japonicum* *hup*-specific DNA probes (24). A cosmid, pAL618, containing a 20-kilobase (kb) DNA insert was able to complement a collection of Hup$^-$ mutants generated by random insertion of Tn*5* into the symbiotic plasmid of strain UPM791 (30).

In this study, we investigated the organization of *hup* genes in pAL618. Evidence is presented which demonstrates that pAL618 contains the entire *hup* DNA region of *R. leguminosarum* and that *hup* genes within this region are organized in six transcriptional units, designated *hup*I to *hup*VI. The large subunit of hydrogenase has been specifically localized to region *hup*I.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *R. leguminosarum*, *B. japonicum*, and *Escherichia coli* strains and the plasmids used are listed in Table 1. The pAL cosmids were constructed by inserting Tn*5* transposons into DNA cloned in pAL618 as described by Ditta (8). Similarly, the pHL series of cosmids was generated by mutagenesis of pAL618 insert DNA with transposon Tn*3*-HoHo1 as described by Stachel et al. (35). *R. leguminosarum* AL series strains were obtained by transferring Tn*5* insertions from the corresponding pAL cosmid into the genome of strain

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TABLE 1. Bacterial strains and plasmids

Strain	Description[a]	Source or reference
Strains		
R. leguminosarum		
128C53	Wild type; Hup$^+$ on peas	31
UPM791	128C53; Str^r Hup$^+$ on peas	24
AL series	UMP791 derivatives with genomic Tn*5* insertions	This study; 24
UML2	Wild type; Hup$^-$ on peas	31
R. phaseoli CFN42	Wild type	R. Palacios
B. japonicum 122DES	Wild type; Hup$^+$ on soybeans	11
E. coli		
HB101	*proA2 leu thi lacY endA recA13 hsdR hsdM rpsL20*	G. Ditta
C600	*supE44 thi-1 leuB6 lacY1 tonA21*	G. Ditta
Plasmids		
pAL618	pLAFR1 cosmid containing *hup* genes from UPM791 in a 20-kb insert DNA; Tc^r	24
pAL series	pAL618::Tn*5*; Tc^r Km^r	This study; 24
pHL series	pAL618::Tn*3*-HoHo1; Tc^r Ap^r	This laboratory (Palacios et al., submitted)
pGP1-2	pBR322 encoding *c*I857 and T7 RNA polymerase from promoter p_L; Km^r	38
pT7-5	pACYC177 containing promoter ϕ10 from phage T7; Ap^r	38
pRLH391	pT7-5 carrying a 6.3-kb *Bgl*II fragment from pAL618	This study
pRLH398	Same as pRLH391 but with opposite transcription orientation	This study
pRK2073	Km^s Spc^r derivative of pRK2013	G. Ditta

[a] Abbreviations: Str, streptomycin; Tc, tetracycline; Km, kanamycin; Ap, ampicillin; Spc, spectinomycin.

UPM791 by a marker exchange procedure (8), with modifications previously described (24).

Conditions for routinely growing *E. coli*, *Rhizobium*, and *Bradyrhizobium* strains were as given elsewhere (24) except that the composition of TY medium (in grams per liter) was as follows: tryptone, 6; yeast extract, 3; and $CaCl_2$, 0.5. For induction of hydrogenase activity, *R. leguminosarum* UPM791 and *B. japonicum* 122DES were grown for 5 days at 28°C in 150-ml Erlenmeyer flasks containing 10 ml of the mineral medium of Hanus et al. (13) supplemented with glutamate (5 g/liter) and 50 mM potassium phosphate (pH 5.8) and provided with an atmosphere of 0.7% O_2–5% H_2–5% CO_2 in N_2.

Chemicals. All restriction endonucleases, DNA-modifying enzymes, and nucleotide triphosphates were purchased from Boehringer Mannheim GmbH (Mannheim, Federal Republic of Germany). Radioactive isotopes were obtained from Amersham International (Amersham, United Kingdom).

Medium constituents were from Oxoid Ltd. (Basingstoke, United Kingdom). Antibiotics used in growth media were purchased from Boehringer Mannheim and Serva Feinbiochemica (Heidelberg, Federal Republic of Germany). All other chemicals were of reagent grade.

Preparation and analysis of DNA. Plasmid DNA isolation, restriction enzyme digestions, agarose gel electrophoresis, and DNA cloning and labeling by nick translation with [α-^{32}P]dATP were performed by standard procedures (28). Total DNA for hybridization experiments was isolated as previously described (24). Hybridization of Southern blots was carried out for 24 h at 42°C in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1× Denhardt solution–0.2% sodium dodecyl sulfate (SDS)–200 μg of herring sperm DNA per ml. After hybridization, blots were washed three times (30 min each) in 200 ml of 2× SSC–0.1% SDS at 42°C and then dried and exposed at −70°C to Kodak-S X-ray film with Cronex intensifying screens.

Bacterial genetic techniques. Transfer of plasmids from *E. coli* into *Rhizobium* strains was carried out by the triparental mating system of Ditta et al. (9), using pRK2073 as the helper plasmid. *Rhizobium* transconjugants carrying pAL or pHL cosmids were selected in *Rhizobium* minimal medium (29) containing tetracycline (5 μg/ml). Transformation of *E. coli* strains was performed as described by Maniatis et al. (28).

To determine the stability of cosmid pAL618 in nodules, bacteroids were prepared by crushing random samples of 10 surface-sterilized nodules in a buffer containing 0.25 M sorbitol, 0.25 M mannitol, 2 mM $CaCl_2$, 2.5 mM $MgCl_2$, 30 mM KH_2PO_4, and 20 mM K_2HPO_4 at pH 5.8 (16). Appropriate dilutions of the resulting bacteroid suspensions were plated on TY medium to obtain single colonies. One hundred colonies were then streaked onto TY plates with and without tetracycline (5 μg/ml), and the frequency of tetracycline-resistant colonies was determined.

Plant tests and hydrogenase activities. Wild-type or Tn*5*-mutated *Rhizobium* strains and derivative transconjugant strains containing pAL or pHL cosmids were checked for hydrogenase activity in peas (*Pisum sativum* cv. Frisson) or in beans (*Phaseolus vulgaris* cv. Negro Jamapa). Seedlings were inoculated and plants were grown as previously described (24). Bacteroids were prepared from nodules of 3-week-old plants by a simplification of the procedure described by Ruiz-Argüeso et al. (31). Nodule samples (0.2 to 0.5 g) were crushed in 5-ml plastic tubes with 1 to 2 ml of Mg-phosphate buffer (2.5 mM $MgCl_2$, 50 mM potassium phosphate [pH 6.8]) and 0.1 to 0.2 g of polyvinylpolypyrrolidone. The crushed paste was diluted by adding 2 ml of Mg-phosphate buffer. Plant cell debris and polyvinylpolypyrrolidone were removed by centrifugation at low speed (121 × *g*) for 1 min. Bacteroids were recovered from the supernatant by centrifugation at 4,500 × *g* for 5 min and were finally suspended in Mg-phosphate buffer and used for determinations of hydrogenase activity and protein content.

Hydrogenase activities of bacteroids and vegetative cell suspensions were estimated by measuring consumption of externally supplied H_2 by the amperometric method as previously described (31), using O_2 or methylene blue (MB) as the terminal electron acceptor. The protein contents of cell suspensions were determined by the procedure of

Lowry et al. (26) after alkaline digestion of cells at 90°C in 1 N NaOH for 10 min.

Expression of *hup*I-encoded proteins in *E. coli*. Genes from region *hup*I were expressed in *E. coli* cells by using the bacteriophage T7 RNA polymerase-promoter system of Tabor and Richardson (38). The 6.3-kb *Bgl*II fragment from pAL618 was cloned in both orientations in the *Bam*HI site of the pT7-5 polylinker, and the resulting plasmids (pRLH391 and pRLH398) were introduced into *E. coli* C600, which has been shown to produce no detectable cross-reactive material with antisera against polypeptide subunits of the hydrogenase from *B. japonicum* (15). Cultures from *E. coli* C600 containing both pRLH391, pRLH398, or pT7-5 and pGP1-2 were induced for specific plasmid-encoded protein synthesis as suggested by S. Tabor (personal communication). Cells were grown in LB with kanamycin and ampicillin (40 μg/ml each) at 30°C with aeration (300 rpm). When cultures reached an optical density at 590 nm of 0.5, 0.8 ml was centrifuged, and cells were washed with 5 ml of M9 medium (28), suspended in 4 ml of M9 supplemented with 20 μg of thiamine per ml and 0.01% of a mixture of 20 amino acids, and incubated with shaking (300 rpm) at 30°C in Falcon 2059 tubes for 150 min. The temperature was then shifted to 42°C, and after 15 min of incubation, rifampin was added to a final concentration of 200 μg/ml. Cultures were left at 42°C for 10 min and then incubated at 30°C for 20 min. Finally, cells from 1-ml culture samples were harvested and suspended in SDS lysis buffer (62.5 mM Tris [pH 6.8], 2% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue, 10% glycerol), heated at 100°C for 3 min, and centrifuged in an Eppendorf centrifuge at 12,000 × *g* for 3 min to pellet insoluble material. The supernatant, referred to as the cell lysate, was used for immunoblot analysis.

Preparation of cell extracts. Crude extracts from vegetative cells of *R. leguminosarum* UPM791 and *B. japonicum* 122DES grown under hydrogenase induction conditions and crude extracts from pea bacteroids were prepared essentially as described by Harker et al. (15). Vegetative cells and bacteroids (approximately 2.5 mg of cell protein) were suspended in 0.5 ml of SDS lysis buffer without bromophenol blue. Cell suspensions were heated at 60°C for 20 min and centrifuged at 30,000 × *g* for 1 h at 4°C. The supernatants were retained, stored at 4°C, and used as crude extracts in the Western (immunoblot) assay.

Western blot analysis. *E. coli* cell lysates and crude extracts from *R. leguminosarum*, *B. japonicum*, and pea bacteroids were subjected to SDS-polyacrylamide gel electrophoresis (20). The resolving gel was 10% acrylamide. After electrophoresis, proteins were electrophoretically transferred onto nitrocellulose filters (type HA; 0.45-μm pore size; Millipore, Molsheim, France) essentially as described by Towbin et al. (39). The transfer was performed in a blotting apparatus (Transphor TE52; Hoefer Scientific Instruments, San Francisco, Calif.) for 8 h at 45 V. The nonspecific protein-binding sites were blocked by incubating the Western blots with saturant (5% nonfat dry milk in Tris-buffered saline, which contained 20 mM Tris [pH 7.5] and 0.5 M NaCl) for 1 h. The blots were probed overnight with whole rabbit antiserum against the 65-kilodalton (kDa) subunit of the *B. japonicum* SR hydrogenase (37; obtained from Robert J. Maier, The Johns Hopkins University, Baltimore, Md.) at a dilution of 1:1,000 in saturant. The blots were washed twice with Tris-buffered saline for 10 min each time and treated with a 1:1,000 dilution of *Staphylococcus aureus* protein A-horseradish peroxidase conjugate (Materiales y Reactivos S.A., Madrid, Spain) in saturant for 6 h, followed by two 10-min washes in Tris-buffered saline and staining with 4-chloro-1-naphthol.

TABLE 2. Symbiotic expression of *R. leguminosarum* UPM791 *hup* genes in different rhizobial backgrounds

Strain (legume host)	Bacteroid hydrogenase activity[a] with given electron acceptor		Cosmid stability in nodules[b] (10^{-1})
	O_2	MB	
R. leguminosarum (*Pisum sativum* cv. Frisson)			
UPM791	1,120	1,200	
UPM791(pAL618)	2,480	2,690	7.5
UML2	<50	<50	
UML2(pAL618)	1,022	1,220	8.2
R. phaseoli (*Phaseolus vulgaris* cv. Negro Jamapa)			
CFN42	<50	<50	
CFN42(pAL618)	5,502	13,090	6.5

[a] Expressed as nanomoles of hydrogen taken up per hour per milligram of protein.

[b] Frequency of tetracycline-resistant cells in bacteroid suspensions. Tetracycline resistance is encoded in cosmid pAL618.

RESULTS

Cosmid pAL618 contains the complete *hup* region of *R. leguminosarum* UPM791. To investigate whether the *hup* DNA region cloned in cosmid pAL618 contained all *R. leguminosarum* genetic determinants required for H_2 uptake during symbiosis, we examined the ability of pAL618 to confer H_2-utilizing capacity on Hup^- *Rhizobium* strains. Accordingly, pAL618 was transferred by conjugation into the naturally Hup^- strains *R. leguminosarum* UML2 and *Rhizobium phaseoli* CFN42, as well as into the Hup^+ parental strain *R. leguminosarum* UPM791. Transconjugants were selected by tetracycline resistance and tested for Hup phenotype in symbiosis with their respective legume hosts (Table 2). As expected, no hydrogen-oxidizing capacity was detected in pea and bean bacteroids from recipient strains UML2 and CFN42, respectively, either with O_2 or with methylene blue (MB) as the terminal electron acceptor. However, both strains UML2 and CFN42 became Hup^+ after receiving pAL618. Correspondingly, no homology to *R. leguminosarum hup* DNA was found when total DNA from UML2 or CFN42 was examined in hybridization experiments using pAL618 DNA as a probe (Fig. 1B, lanes 3 and 5), whereas DNA bands corresponding to *Eco*RI fragments of pAL618 were clearly seen in total DNA from the transconjugants (Fig. 1B, lanes 4 and 6). From comparison of the relative intensities of hybridizing bands in the Hup^+ parental strain UPM791 and in transconjugants UPM791 (pAL618), UML2(pAL618), and CFN42(pAL618), we conclude that the cosmid is maintained at similar copy numbers in the three strains, estimated to be about 15 copies per genome. No differences were observed between the patterns of ethidium bromide-stained *Eco*RI restriction fragments obtained from the recipient and transconjugant strains, and the patterns of UML2 and CFN42 were clearly different from that of UPM791 (Fig. 1A).

Although the cosmid was not fully stable in nodules (Table 2), the level of hydrogenase activity detected by the amperometric analysis in pea bacteroids from UML2(pAL618) or bean bacteroids from CFN42(pAL618) was of the same

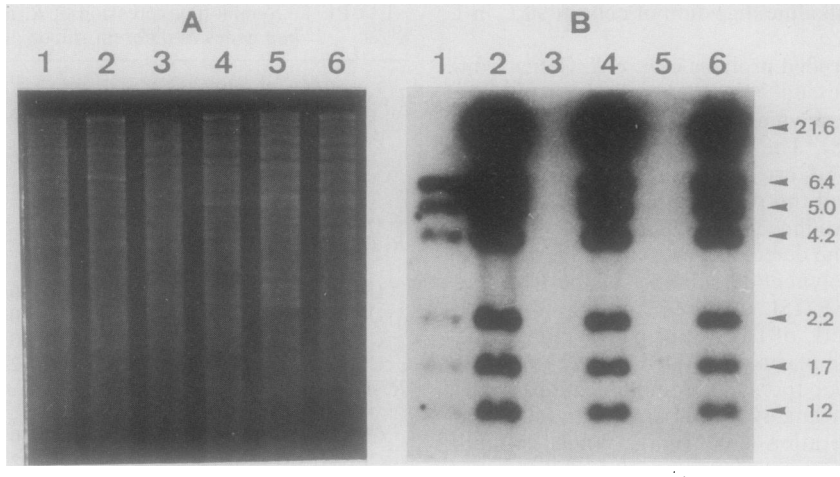

FIG. 1. Hybridization of *hup* DNA sequences cloned in pAL618 to total DNA from Hup$^-$ *R. leguminosarum* and *R. phaseoli* strains. (A) Ethidium bromide-stained agarose gel of *Eco*RI-digested total DNA from *R. leguminosarum* UPM791 (lane 1), UPM791(pAL618) (lane 2), UML2 (lane 3), and UML2(pAL618) (lane 4) and from *R. phaseoli* CFN42 (lane 5) and CFN42(pAL618) (lane 6). (B) Autoradiogram of panel A after Southern transfer and hybridization to pAL618 DNA labeled by nick translation. Lane designations are the same as in panel A. Sizes (in kilobases) of *Eco*RI fragments from pAL618 are shown on the right.

order as that observed in pea bacteroids from the parental strain UPM791. The hydrogenase activity values shown in Table 2 for pea bacteroids from strain UPM791 are significantly higher than those previously reported from this laboratory (25). The differences are attributable to the simpler procedure used here to prepare the bacteroid suspensions, which reduces the loss of hydrogenase activity.

Mutagenesis of the *hup* DNA cloned in pAL618. To identify the regions of pAL618 that are required for the ability to confer H_2 uptake capacity on *Rhizobium* spp., the 20-kb insert DNA of pAL618 was randomly mutagenized in *E. coli* HB101 with transposon Tn*5*. Twenty-nine Tn*5* insertions in the *R. leguminosarum* sequences were generated, and the location of each insertion was determined by restriction analysis using *Eco*RI and *Hin*dIII endonucleases (Fig. 2A). The resulting pAL618::Tn*5* constructs (pAL cosmids) were transferred by conjugation into *R. leguminosarum* UPM791, and the transposon was homogenotized into the genome by a marker exchange method exploiting plasmid incompatibility (8). The homogenotized Tn*5*-mutated strains were designated AL, followed by the number assigned to the corresponding Tn*5* insertion. Each mutagenized strain was used as an inoculum for peas, and the Hup phenotype was determined by measuring the O_2- and MB-dependent H_2 uptake of bacteroids. Twenty mutations spanning a central region of about 15 kb of pAL618 insert DNA (from insertions 11 to 91) resulted in an altered Hup phenotype (Fig. 2A). Transposon Tn*5* insertions throughout the DNA portions bordering this region had no effect on hydrogenase activity. All 20 Hup$^-$ mutants induced the formation of apparently normal nitrogen-fixing nodules on peas (data not shown).

Within the 15-kb DNA region essential for Hup activity, Hup$^-$ Tn*5* insertions could be divided into three distinct groups separated by Hup$^+$ Tn*5* insertions 48 and 71 (Fig. 2A). Seven insertions within the left group, 1 within the central group, and 12 within the right group abolished the H_2 uptake capacity of the bacteroids, both with O_2 and with MB as the final electron acceptor. Each of these mutant groups is likely to define at least one operon.

In addition, the pAL618 insert DNA was randomly mutagenized with transposon Tn*3*-HoHo1 (35) to create in vivo *hup-lacZ* fusions to be used in regulation studies (J. M. Palacios, J. Murillo, A. Leyva, G. Ditta, and T. Ruiz-Argüeso, submitted for publication). Twenty-nine Tn*3*-HoHo1 insertions were generated, and the position of each insertion in pAL618 was mapped (Fig. 2B). Since it was difficult to homogenotize the Tn*3*-HoHo1 insertions into the *R. leguminosarum* genome because of the poor expression of the β-lactamase gene (encoded in Tn*3*-HoHo1) in this bacterium, we decided to check the effect of the transposon insertions on the Hup phenotype in a Hup$^-$ *R. phaseoli* background. The resulting pAL618::Tn*3*-HoHo1 constructs (pHL cosmids) were introduced by conjugation into *R. phaseoli* CFN42. Each of the CFN42 transconjugant strains was used as an inoculum for beans, and the expression of hydrogenase activity in the bacteroids was examined. According to the phenotype associated with each Tn*3*-HoHo1 insertion (Fig. 2B), it clearly appeared that the distribution of DNA essential for Hup activity in pAL618 agreed with that found by the site-directed Tn*5* mutagenesis analysis. Tn*3*-HoHo1 insertions within any of the three DNA regions defined by this analysis suppressed the ability of pAL618 to confer H_2 uptake capacity to strain CFN42 in symbiosis with beans. The lack of Tn*3*-HoHo1 insertions near Tn*5* insertion 71 did not allow us to confirm the existence of nonessential DNA sequences around this location.

Complementation analysis of Hup$^-$ mutants. To more precisely define the organization of *hup* genes in the 20-kb insert DNA of pAL618, we carried out a complementation analysis of Hup$^-$ mutants from *R. leguminosarum* UPM791. Tn*5*- or Tn*3*-HoHo1-mutated pAL618 cosmids (pAL or pHL plasmids) were introduced into different Hup$^-$ mutants (AL strains) carrying genomic Tn*5* insertions. Eight different Hup$^-$ AL mutants with Tn*5* insertions along the *hup* DNA region were chosen as recipients for cosmids with Tn*5* or Tn*3*-HoHo1 insertions located, in each case, close to and to both sides of the corresponding Tn*5* genomic insertion (Fig. 2D). Also, cosmids carrying the transposon insertion far away from the genomic Tn*5* insertion in the recipient mutant were used as controls. Each of the resulting merodiploid strains was inoculated into pea plants, and the bacteroids were examined for the ability to oxidize H_2 with O_2 or MB as the final electron acceptor. On the basis of the ability of the

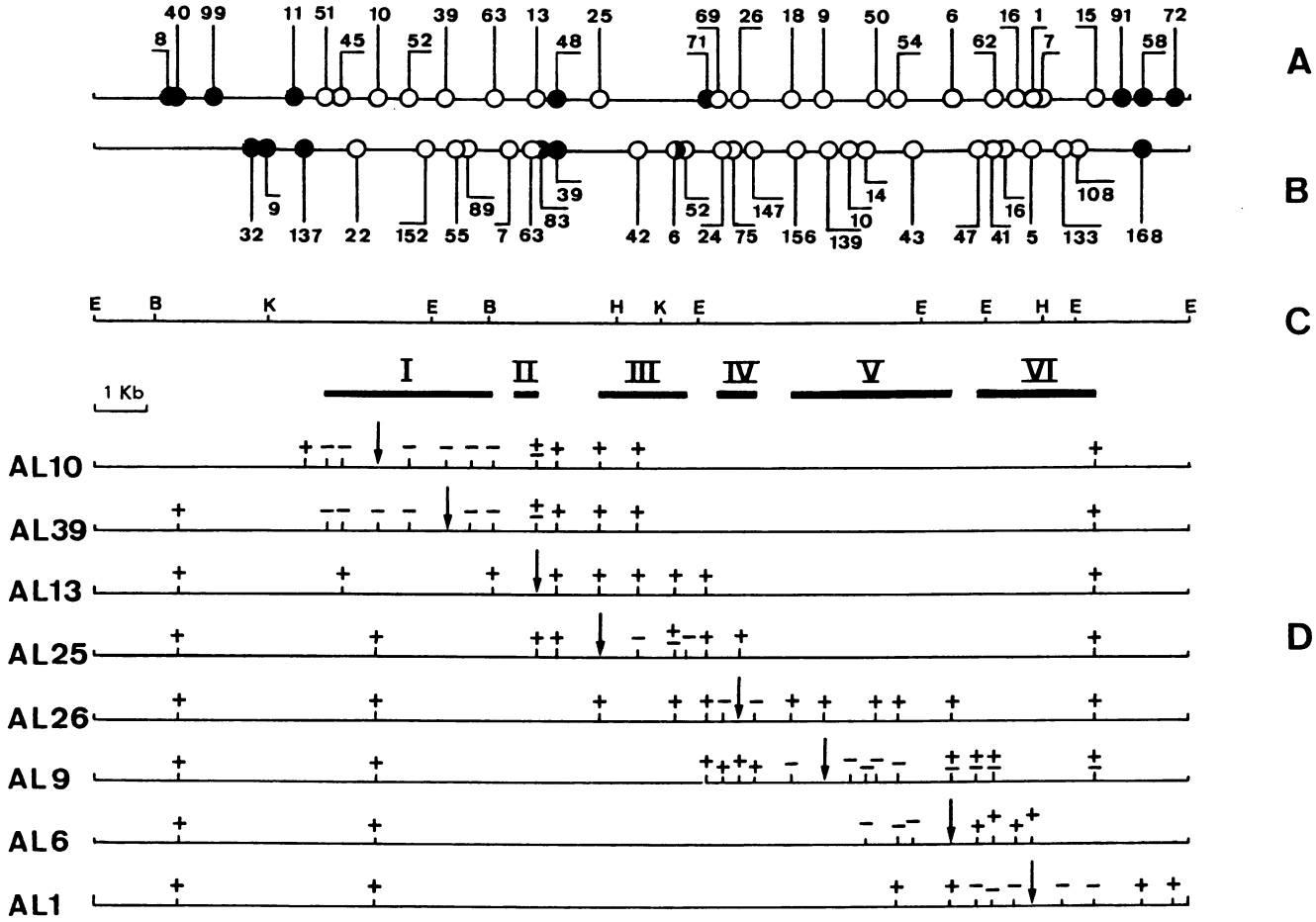

FIG. 2. Identification of *hup* DNA regions from *R. leguminosarum* UPM791 by Tn*5* and Tn*3*-HoHo1 transposon mutagenesis and complementation analysis. (A) Site-directed Tn*5* mutagenesis. Vertical lines indicate positions of transposon insertions, designated by numbers, in the genome of the UPM791::Tn*5* strains (AL strains). The open or solid circle below each vertical line indicates whether the insertion resulted in a Hup$^-$ or a Hup$^+$ phenotype, respectively, in symbiosis with peas (*Pisum sativum* cv. Frisson). (B) Tn*3*-HoHo1 mutagenesis. Vertical lines indicate positions of Tn*3*-HoHo1 insertions, designated by numbers, in the corresponding pAL618::Tn*3*-HoHo1 constructs (pHL cosmids). The open, solid, or half-solid circle above each vertical line indicates the Hup$^-$, Hup$^+$, or weak Hup$^+$ phenotype, respectively, of the corresponding *R. phaseoli* CFN42(pHL cosmid) transconjugant strain in symbiosis with beans (*Phaseolus vulgaris* cv. Negro Jamapa). (C) Restriction map of insert DNA of pAL618. Restriction sites: E, *Eco*RI; B, *Bgl*II; H, *Hin*dIII; K, *Kpn*I. (D) Complementation analysis. The Hup$^-$ AL mutants listed on the left and carrying genomic Tn*5* insertions in the positions shown by the vertical arrows were used as recipient strains for pAL618 derivative cosmids carrying Tn*5* or Tn*3*-HoHo1 insertions in the positions indicated by the short vertical lines. The resulting AL(pAL or pHL) merodiploid strains were used as inocula for peas and tested for Hup phenotype. The +, ±, or − above each vertical line indicates full, partial (<10%), or no complementation, respectively, of the Hup$^-$ phenotype of the corresponding AL mutant. The bars at the top represent the DNA regions *hup*I to *hup*VI, corresponding to each of the defined complementation groups.

pAL or pHL plasmids to restore a Hup$^+$ phenotype in the AL mutants, transposon insertions were found to fall into six distinct complementation groups (Fig. 2D). The DNA regions defined by these groups were termed, from left to right in pAL618, regions *hup*I to *hup*VI. The lengths of these regions shown in Fig. 2D are only approximate, since the limited number of insertions available does not allow a more precise definition. Since the mutants used in the complementation analysis were RecA$^+$ strains, the integrity of the transferred cosmids was checked in a random sample of 20 of the merodiploid strains by plasmid DNA restriction analysis. In all cases tested, the pattern of restriction fragments observed was identical to the restriction pattern expected for the corresponding pAL or pHL cosmid.

Region *hup*I was defined by using Hup$^-$ mutants AL10 and AL39. Both mutants were not complemented by cosmids pAL51, pAL45, pAL10, pAL52, pAL39, pAL63, and pHL89 but were restored to a normal Hup$^+$ phenotype by pAL48 or by cosmids with insertions located to the right of Tn*5* insertion 48. Mutant AL13, which exhibited a clear Hup$^-$ phenotype, seemed to fall in a complementation group different from that of region *hup*I, since it was fully complemented by cosmids pAL51 and pAL63. We designated *hup*II the Hup essential DNA region identified by mutant AL13. However, it is not clear why mutants AL10 and AL39 within region *hup*I were only partially complemented by pAL13.

Region *hup*III, defined by using Hup$^-$ mutant AL25, is located between Tn*5* insertions 48 and 71. Tn*3*-HoHo1 insertions 42 and 52 are located within this region, since mutant AL25 was not complemented by cosmids pHL42 and pHL52. The weak hydrogenase activity induced by pHL6 in mutant AL25 could probably be attributed to the generally leaky Hup phenotype displayed by this insertion when examined in *R. phaseoli* (Fig. 2B). Tn*5* insertion 26 is located

in a region of DNA essential for Hup, region *hup*IV, limited on the left side by Tn*5* insertion 71 and on the right side by Tn*5* insertion 18, whose corresponding pAL18 cosmid complemented mutant AL26. Tn*3*-HoHo1 insertions 24 and 147 are also located within this *hup* region, since no hydrogenase activity was detected in the merodiploid strains AL26 (pHL24) and AL26(pHL147).

Regions *hup*V and *hup*VI were identified by using mutants AL9 and AL1, respectively. Tn*5* insertions 18, 50, and 54 and Tn*3*-HoHo1 insertions 10 and 14 are located within region *hup*V, since their corresponding cosmids did not complement mutant AL9. Likewise, Tn*5* insertions 16 and 15 and Tn*3*-HoHo1 insertions 47, 41, and 133 are likely within region *hup*VI. Mutant AL9 was clearly complemented by cosmids carrying transposon insertions within region *hup*IV, indicating that regions *hup*IV and *hup*V are different. In general, the data are consistent with the interpretation of regions *hup*V and *hup*VI as distinct complementation groups, as shown by the full complementation of mutant AL1 by cosmids pAL54 and pAL6 and by results obtained when mutant AL6 was used as a recipient strain for cosmids with insertions to the left and right of Tn*5* insertion 6. However, it is not clear why wild-type levels of complementation were not observed for AL9(pHL47), AL9 (pHL41), and AL9(pAL15) and why pAL6 partially complemented mutant AL9.

Location of hydrogenase structural genes. We had previously shown that a DNA probe containing structural genes for the *B. japonicum* hydrogenase hybridized to the 6.6-kb *Kpn*I-*Hin*dIII fragment of pAL618 (24). This fragment contains regions *hup*I and *hup*II and part of *hup*III (Fig. 2). To more specifically localize hydrogenase structural genes within this region, we first expressed in *E. coli* the genes encoded in a 6.3-kb *Bgl*II fragment containing region *hup*I by using an expression vector based on a T7 RNA polymerase-promoter system (38) and then examined the translational products for cross-reactivity with antiserum raised against the large (65-kDa) subunit of the *B. japonicum* hydrogenase (37). The existence of immunological cross-reactivity between hydrogenases from these two bacteria was expected in light of the close immunological relationship found among different H_2 uptake hydrogenases (2).

The 6.3-kb *Bgl*II fragment was cloned in both orientations in the *Bam*HI site of vector pT7-5, downstream of the T7 RNA polymerase promoter ϕ10 (Fig. 3A). Assuming the orientation of the pAL618 DNA restriction fragments to be shown in Fig. 2C, the resulting recombinant plasmids, pRLH391 and pRLH398, contain the *Bgl*II fragment oriented left to right and right to left, respectively, with respect to the direction of transcription from the ϕ10 promoter. pRLH391 and pRLH398 were transformed into *E. coli* C600, and exclusive transcription from the ϕ10 promoter was accomplished by introducing a second plasmid, pGP1-2, which provides the T7 RNA polymerase in *trans*, and by shutting off activity of the host RNA polymerase with rifampin. Proteins from the *E. coli* culture lysates were transferred by Western blotting onto nitrocellulose filters, and the immunoassay was performed. Cell extracts from *B. japonicum* 122DES and *R. leguminosarum* UPM791 cultures grown under hydrogenase-inducing conditions and from pea bacteroids induced by strain UPM791 were also included in the immunoblot as controls. A polypeptide of approximately 65 kDa, which showed a clear immunological cross-reaction with the 65-kDa antiserum, was present in crude extracts from pea bacteroids and in culture lysates from *E. coli* C600(pRLH391) (Fig. 3B, lanes 3 and 4). This polypeptide

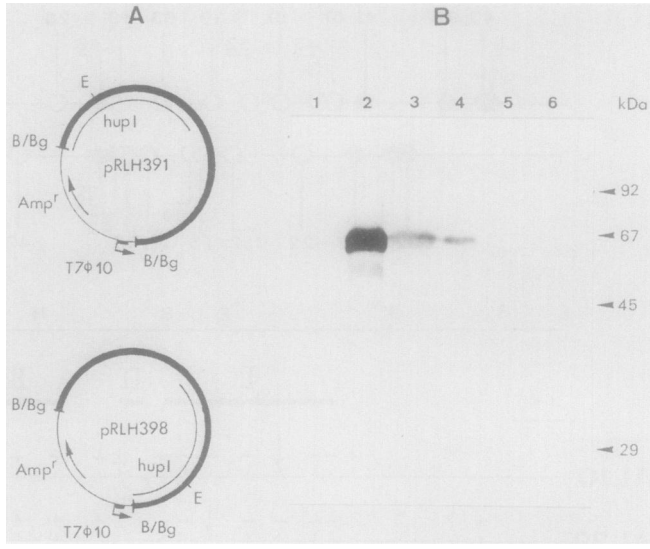

FIG. 3. Expression of *hup*I genes from *R. leguminosarum* UPM791 in *E. coli* cells. (A) Diagram showing plasmids pRLH391 and pRLH398 containing a 6.3-kb *Bgl*II fragment from pAL618 cloned in both orientations in the *Bam*HI site of the expression vector pT7-5. (B) Immunological detection of polypeptides cross-reactive with antiserum against the large subunit of the *B. japonicum* hydrogenase in crude extracts from *R. leguminosarum* UPM791 (lane 1) and *B. japonicum* 122DES (lane 2) cells grown under hydrogenase induction conditions, UPM791 pea bacteroids (lane 3), and *E. coli* C600(pRLH391) (lane 4), C600(pRLH398) (lane 5), and C600(pT7-5) (lane 6). Positions of molecular size markers are indicated on the right.

comigrated in the gel with a strongly reacting protein present in crude extracts from hydrogenase-induced cells of *B. japonicum* 122DES (Fig. 3B, lane 2). The absence of any detectable protein band cross-reactive with the 65-kDa antiserum in cell lysates from *E. coli* C600(pRLH398) (Fig. 3B, lane 5) indicated that transcription in *E. coli* was only occurring from the T7 ϕ10 promoter of the expression vector and not from internal promoters in the insert DNA. This result also confirmed the polarity of transcription for region *hup*I as being that of pRLH391. From these results, we conclude that the 65-kDa polypeptide is a component of the *R. leguminosarum* hydrogenase and that the structural gene coding for this component is contained within region *hup*I and transcribed from left to right according to the map in Fig. 2C. This direction of transcription was confirmed by analysis of the expression of β-galactosidase activity of *hup*I-*lacZ* fusions created by the Tn*3*-HoHo1 insertions (Palacios et al., submitted). Moreover, the fact that cross-reacting polypeptides of identical molecular weight were detected in both pea bacteroids and *E. coli* C600(pRLH391) extracts indicates that the translational initiation and stop signals in the *hup*I mRNA can be recognized by *E. coli* ribosomes.

B. japonicum 122DES grown ex planta under hydrogenase induction conditions exhibited an average hydrogenase activity of 11.3 μmol of H_2 uptake h^{-1} (mg of protein)$^{-1}$. Under the same growing conditions, *R. leguminosarum* UPM791 cells produced no detectable H_2 uptake activity with either O_2 or MB as the terminal electron acceptor. When crude extracts from UPM791 cells were analyzed by the immunoblot assay, no band cross-reacting with antibodies against the large subunit of the *B. japonicum* hydrogenase was detected (Fig. 3B, lane 1), indicating that vegetative cells from *R. leguminosarum* do not induce the

synthesis of hydrogenase proteins in the same cultural conditions that derepress *hup* genes in *B. japonicum*. The possibility that this lack of protein was due to mRNA instability or proteolysis is unlikely, since no activation of region *hup*I was observed in microaerobically grown vegetative cells containing *hup*I-*lacZ* fusions (Palacios et al., submitted).

DISCUSSION

We have recently isolated *R. leguminosarum* genes essential for H_2 uptake by pea bacteroids in a 20-kb DNA fragment cloned in cosmid pAL618 (24). From the cosmid transfer and *hup* gene expression experiments reported here, it is evident that pAL618 has the capacity to confer H_2 uptake ability to Hup^- strains of *R. leguminosarum* and *R. phaseoli*, both with O_2 and with MB as the terminal electron acceptor. Since the Hup^- recipient strains contained no DNA sequences with detectable homology to *hup* DNA from pAL618 under hybridization conditions in which *hup* DNA from *B. japonicum* was clearly detected (Fig. 1B), we conclude that all genetic determinants specifically required for H_2 uptake in *R. leguminosarum* are likely to be contained within the insert DNA of pAL618. Similarly, it has also been shown that all *hup* genes from *B. japonicum* are clustered in a DNA region cloned in cosmid pHU52 (21). However, the possibility that rhizobial genes others than those present in pAL618 and pHU52 were also needed for H_2 uptake or *hup* gene expression cannot be excluded. These putative additional genes should be present in both Hup^+ and Hup^- strains and might be common to different endosymbiotic bacteria.

The presence of pAL618 in *R. leguminosarum* or *R. phaseoli* transconjugants was always associated with high levels of hydrogenase activity and cosmid stabilities in bean and pea nodules (Table 2). In contrast, the transfer of pHU52 to Hup^- strains of *B. japonicum*, *Rhizobium meliloti*, and *Rhizobium trifolii* resulted in low levels of Hup activity (22). This low expression was attributed to the high instability of cosmid pHU52 observed in the nodules when plants inoculated with transconjugants were grown in the absence of tetracycline (pHU52 codes for tetracycline resistance). Since both pHU52 and pAL618 are pLAFR1 derivative cosmids, it is likely that the bacterial host has a strong effect on the stability of the cosmids, being more stable in *R. leguminosarum* and *R. phaseoli* than in other rhizobial species. Alternatively, rhizobial DNA of pHU52 might be responsible for the high instability of this cosmid.

It seems likely that the defined *hup*I to *hup*VI regions represent operons whose transcription is interrupted in the Hup^- mutants by the Tn*5* or Tn*3*-HoHo1 transposon insertions, and complementation of the Hup^- phenotype is obtained only by providing in *trans* the corresponding intact operon. However, since the complementation analysis was conducted in a $RecA^+$ genetic background, the possibility that some of the Hup^+ phenotypes observed in the merodiploid strains were due to recombination events rather than to true complementations cannot be completely ruled out. Nevertheless, the lack of selection pressure for a Hup^+ phenotype after plant inoculation makes this explanation unlikely, particularly since full recovery of wild-type levels of hydrogenase activity was observed in merodiploids. Such a selection pressure does exist, however, in complementation analysis of nodulation deficient (Nod^-) mutants (18).

Region *hup*III clearly represents an independent transcriptional unit, since it is separated from regions *hup*IV and *hup*II by DNA not essential for Hup both in beans and in peas (Fig. 2A and B). Also, the complementation data indicate that region *hup*IV is an operon distinct from that defined by region *hup*V. Thus, at least four distinct transcriptional units are clearly identified in pAL618: regions *hup*I/*hup*II, *hup*III, *hup*IV, and *hup*V/*hup*VI. Although most data are consistent with the idea that regions *hup*V and *hup*VI represent independent transcriptional units, the partial complementation of mutant AL9 (in region *hup*V) by cosmids pHL47, pHL41, and pAL15 (in the putative region *hup*VI) (Fig. 2D) is puzzling. It should be noted that despite the clear complementation of AL1 and AL6 by appropriate flanking Tn*5* insertions, the possibility cannot be excluded that regions *hup*V and *hup*VI actually constitute a single transcription unit and that this fact is relevant to the partial complementation seen with AL9. This would require, however, that the Tn*5* insertions in AL9, AL43, and AL6 be substantially nonpolar. This seems unlikely. The same interpretation can also be applied to regions *hup*I and *hup*II, whose partial complementation is observed between AL10 and AL39. Since fewer data are available in this case, however, it is correspondingly more plausible that regions *hup*I and *hup*II constitute a single operon.

The general organization of *hup* genes in other nitrogen-fixing bacteria has barely been studied. By using Tn*5* mutagenesis analysis, Haugland et al. (16) demonstrated the existence of at least two distinct transcriptional units in the *B. japonicum* 122DES *hup* cluster. Similarly, Yates et al. (41) identified in *Azotobacter chroococcum* two independent complementation regions by complementing Hup^- mutants with recombinant cosmids carrying DNA fragments homologous to *B. japonicum hup* DNA. Also, in *Rhodobacter capsulatus*, the isolation of three unrelated recombinant plasmids complementing distinct Hup^- mutants suggests that several operons are involved in the synthesis of hydrogenase (23). In each case, one of the transcriptional units has been shown by sequence analysis to code for the structural genes of the hydrogenase (23, 32, 41). Although the H_2 uptake hydrogenase from *R. leguminosarum* has not yet been purified, mainly because of difficulties in obtaining working amounts of hydrogenase-induced cells, a dimeric ($\alpha\beta$) polypeptide structure similar to that of hydrogenases from other N_2-fixing bacteria such as *B. japonicum* (1, 14, 37), *A. vinelandii* (33), or *Rhodobacter capsulatus* (34) is expected. Several lines of evidence indicate that the structural genes for the *R. leguminosarum* hydrogenase are located in the operon defined by region *hup*I. First, the immunological data reported here (Fig. 3) demonstrate that region *hup*I codes for a 65-kDa polypeptide immunologically cross-reactive with antiserum against the 65-kDa subunit of the *B. japonicum* hydrogenase. Second, region *hup*I is within the pAL618 DNA fragment that hybridizes with the structural genes of *B. japonicum* hydrogenase (24). Third, the hydrogenase structural genes that have been sequenced from nitrogen-fixing bacteria (23, 32, 41) are contiguous and constitute a single operon in which the gene for the small subunit precedes that for the large subunit. Nucleotide sequence analysis of region *hup*I is currently being carried out by our group, and it is hoped that the results will allow us to determine the gene organization of this operon.

The number of genes encoded in each of the other *hup* regions identified in *R. leguminosarum*, as well as the functions of their products, are unknown at present. However, the fact that all of the Hup^- mutants with mutations in these regions were unable to oxidize H_2 with MB as the final electron acceptor suggests that the products of all *hup* genes

are required to synthesize an active hydrogenase protein. Assuming that the hydrogenase from *R. leguminosarum* is of the Ni-Fe or Se-Ni-Fe type, as has been shown for other nitrogen-fixing bacteria (1, 3, 14, 33), we can expect that proteins involved in metal transport or processing are candidate products for genes encoded in the *hup* regions of pAL618. Genes for hydrogenase Ni transport and processing have been identified in *Alcaligenes eutrophus* (10) and *E. coli* (7, 40). Also, regulatory proteins or proteins needed for proper insertion or coupling to electron acceptors in the membrane may also be encoded in the pAL618 *hup* cluster. Obviously, further characterization of *hup* regions in this cluster, at the nucleotide sequence and biochemical levels, will be required to identify their internal gene organization and function.

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