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Gene Products of the *hupGHIJ* Operon Are Involved in Maturation of the Iron-Sulfur Subunit of the [NiFe] Hydrogenase from *Rhizobium leguminosarum* bv. viciae

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In the present study, we investigate the functions of the *hupGHIJ* operon in the synthesis of an active [NiFe] hydrogenase in the legume endosymbiont *Rhizobium leguminosarum* bv. viciae. These genes are clustered with 14 other genes including the hydrogenase structural genes *hupSL*. A set of isogenic mutants with in-frame deletions (Δ*hupG*, Δ*hupH*, Δ*hupI*, and Δ*hupJ*) was generated and tested for hydrogenase activity in cultures grown at different oxygen concentrations (0.2 to 2.0%) and in symbiosis with peas. In free-living cultures, deletions in these genes severely reduced hydrogenase activity. The Δ*hupH* mutant was totally devoid of hydrogenase activity at any of the O_2 concentration tested, whereas the requirement of *hupGIJ* for hydrogenase activity varied with the O_2 concentration, being more crucial at higher pO_2. Pea bacteroids from the mutant strains affected in *hupH*, *hupI*, and *hupJ* exhibited reduced (20 to 50%) rates of hydrogenase activity compared to the wild type, whereas rates were not affected in the Δ*hupG* mutant. Immunoblot experiments with HupL- and HupS-specific antisera showed that free-living cultures from Δ*hupH*, Δ*hupI*, and Δ*hupJ* mutants synthesized a fully processed mature HupL protein and accumulated an unprocessed form of HupS (pre-HupS). Both the mature HupL and the pre-HupS forms were located in the cytoplasmic fraction of cultures from the Δ*hupH* mutant. Affinity chromatography experiments revealed that cytoplasmic pre-HupS binds to the HupH protein before the pre-HupS-HupL complex is formed. From these results we propose that *hupGHIJ* gene products are involved in the maturation of the HupS hydrogenase subunit.

The protein core of [NiFe] hydrogenases is composed of two different subunits. The large subunit contains the catalytic site consisting of a heterobinuclear NiFe metallocenter, and the small subunit from most [NiFe] hydrogenases holds three Fe-S clusters (43). Multiple genes are required for the synthesis of [NiFe] hydrogenases, and most of them are conserved among different bacteria (40, 41). The role of proteins encoded by these genes in the synthesis process is only partially known. Analysis of *Escherichia coli* hydrogenase 3 revealed that Hyp proteins are involved in the biosynthesis of the Ni-Fe cofactor, a process which ends with the processing of the large subunit by an endopeptidase which removes a C-terminal tail from the protein (3). In contrast, no auxiliary proteins have been identified that are required for synthesis of a functional small subunit of the [NiFe] hydrogenases, although most of them contain three iron-sulfur clusters (two 4Fe-4S and one 3Fe-4S) that conduct electrons from the H_2-activating center in the large subunit to the physiological electron acceptor on the surface of the enzyme (11). Different proteins are known that facilitate the assembly of Fe-S clusters into other Fe-S proteins (29, 44).

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In symbiosis with peas, *Rhizobium leguminosarum* bv. viciae strain UPM791 induces an H_2 uptake [NiFe] hydrogenase whose genetic determinants are grouped in a cluster (*hupSLCDEFGHIJKhypABFCDEX*) required for the Hup^+ phenotype (33). The hydrogenase structural genes *hupSL* and most of the accessory genes show high sequence similarity with the corresponding genes from other bacteria (12, 40). Unlike the situation in *Bradyrhizobium japonicum* (21), *R. leguminosarum hupSL* gene expression is observed only in pea bacteroids, and it is controlled by the nitrogenase regulatory protein NifA (4). In contrast, *hyp* genes are induced in microaerobic as well as in symbiotic conditions by the transcriptional activator FnrN (13, 15). The entire *R. leguminosarum* hydrogenase gene cluster has been engineered for expression in free-living microaerobic cells by replacing the NifA-dependent *hupSL* promoter by the FnrN-dependent *fixN* promoter (6) in order to facilitate the analysis of gene functionality.

The *R. leguminosarum* hydrogenase gene cluster contains a subcluster of genes, *hupGHIJ* (30), whose specific role in hydrogenase synthesis is still unknown. This subcluster functions as an operon under the control of a promoter (P3) located upstream of *hupG* (23). Genes homologous to *hupGHIJ* are also present in other aerobic bacteria containing H_2 uptake [NiFe] hydrogenases such as *B. japonicum*, *Azotobacter vinelandii*, *Rhodobacter capsulatus*, and *Ralstonia eutropha* among others (40). In addition, HupG and HupH show homology to proteins from *E. coli* (HyaE and HyaF of hydrogenase 1), and a homologue to the gene encoding HupJ (HybE) is present in the gene cluster coding for hydrogenase 2 in the same bacte-

TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant genotype or phenotype	Reference or source
R. leguminosarum		
UPM791	128C53 wild type; Str^r Nod^+ Fix^+ Hup^+	32
UPM1155	UPM791 (Δ*hup*/*hyp* cluster) Hup^-	This lab
SM61	UPM791 *tatBC* mutant	25
Escherichia coli		
DH5α	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1*Δ(*lacZYA-argF*)*U169* ϕ80d*lacZ*Δ*M15*	14
S17.1	*thi pro hsdR*$^-$ *hsdM*$^+$ *recA* RP4 2-Tc::Mu-Km::Tn7 (Sp^r Sm^r)	36
Plasmids		
pALPF1	pAL618 with P1 promoter replaced by *fixN* promoter	6
pALPF15	pALPF1 Δ*hupS*	This work
pALPF2	pALPF1 Δ*hupL*	This work
pALPF4	pALPF1 Δ*hupD*	This work
pALPF6	pALPF1 Δ*hupG*	This work
pALPF7	pALPF1 Δ*hupH*	This work
pALPF8	pALPF1 Δ*hupI*	This work
pALPF9	pALPF1 Δ*hupJ*	This work
pKD3	Template plasmid harboring FLP-mediated excision sequence flanking Cm^r gene	9
pKD13	Template plasmid harboring FLP-mediated excision sequence flanking Km^r gene	9
pPM70	pKD3 derivative plasmid containing *Strep*-tag II sequence for N terminus end fusion	This work
pALPF34	pALPF1 derivative plasmid carrying $hupH_{strep}$ gene	This work
pBBR1MCS-2	Broad-host-range plasmid; Km^r mob^+	17
pPM1350	pBBR1MCS2 derivative plasmid containing a DNA fragment harboring P_{fixN} promoter from *R. leguminosarum*	This work
pPM125	pBBR1MCS2 derivative plasmid containing the *hupG*, $hupH_{strep}$, and *hupI* genes in an EcoRI DNA fragment	This work
pPM164	pPM1350 derivative plasmid containing an NdeI-XbaI fragment harboring *hupI* under the control of P_{fixN}	This work
pPM165	pPM1350 derivative plasmid containing an NdeI-XhoI fragment harboring *hupK* under the control of P_{fixN}	This work
pPM166	pPM1350 derivative plasmid containing an NdeI-XhoI fragment harboring $hupG_{strep}$, *hupH*, and *hupI* under the control of P_{fixN}	This work
PCR2.1-TOPO	PCR cloning vector	Invitrogen

rium. Genes homologous to *hupGHIJ* have not been reported in anaerobic bacteria such as *Desulfovibrio* spp. (31). The *hupI* gene encodes a rubredoxin-type protein (7, 30), whereas no similarities, outside of equivalent hydrogenase-related proteins, have been reported for HupG, HupH, and HupJ proteins.

In this work we show that *hupGHIJ* gene products are required for hydrogenase activity in *R. leguminosarum* microaerobic cells but are not involved in the synthesis of a mature large subunit (HupL) of the hydrogenase. Based on these results, and on the evidence provided by the identification of a HupS-HupH complex in microaerobically induced hydrogenase-active cultures, a role for *hupGHIJ* gene products in the maturation of the hydrogenase small subunit in *R. leguminosarum* is proposed.

MATERIALS AND METHODS

Chemicals. All enzymes were purchased from Roche (Roche Applied Sciences, Mannheim, Germany) and were used according to manufacturer's indications. Media constituents were from Oxoid Ltd. (Basingstoke, United Kingdom). All other chemicals were of reagent or electrophoresis grade.

Bacterial strains, plasmids, media, and growth conditions. Strains and plasmids used in this study are listed in Table 1. *R. leguminosarum* strains were routinely grown at 28°C in YMB (42). *E. coli* DH5*a* was used for standard cloning procedures (14). *E. coli* S17.1 (36) was used for conjugative plasmid transfer between *E. coli* and *R. leguminosarum*. For cell extract preparations, cultures were grown on MM medium (39). Antibiotic concentrations used were as follows ($\mu g \cdot ml^{-1}$): ampicillin, 100; kanamycin, 50; tetracycline, 5 (for *R. leguminosarum*) or 10 (for *E. coli*). A stoppered-tube technique, adapted to 200-ml flasks with 45-ml cultures, was routinely used for hydrogenase induction assays with free-living microaerobic cells. To this end, cultures were previously grown aerobically in YMB medium to an optical density at 600 nm (OD_{600}) of 0.2. The flasks were then tightly capped, evacuated, and flushed three times with a mixture of 0.8% O_2 in N_2 and finally incubated for 16 h at 28°C. To study the effect of O_2 concentration on hydrogenase activity the stoppered-tube system was adapted to continuous flushing with different O_2-N_2 mixtures. To induce hydrogenase in larger cultures of cells, a fermentor (Microferm; New Brunswick) was used. Initially, *R. leguminosarum* cultures were aerobically grown to an OD_{600} of 0.35, and then hydrogenase was induced by a continuous flow of 0.2% O_2 in N_2 until an OD_{600} of ca. 2 was reached.

Plant tests and enzyme assays. Pea (*Pisum sativum* L. cv. Frisson) plants were used as host for *R. leguminosarum* bv. viciae. Conditions for plant inoculation, growth in nitrogen-free nutrient solution under bacteriologically controlled conditions, and bacteroid preparation were previously described (20). The nitrogen-free plant nutrient solution was supplemented with 20 mM $NiCl_2$ from day 10 after seedling inoculation. Hydrogenase activity was measured in bacteroid suspensions and in free-living cells by an amperometric method with oxygen as the terminal electron acceptor (32). Protein contents of cell extracts were determined by the bicinchoninic acid method (37) with bovine serum albumin as the standard. For whole cells, the same method was followed after alkaline digestion in 1 N NaOH at 90°C for 10 min.

Recombinant DNA techniques. DNA manipulations including purification, restriction, ligation, agarose gel electrophoresis, PCR amplification, and transformation into *E. coli* cells were carried out by standard methods (34). Oligonucleotides used for PCR and sequencing reactions were obtained from Sigma-Genosys (Haverhill, United Kingdom).

Generation of in-frame deletion mutants in *R. leguminosarum hup* genes. In-frame deletions of *hup* genes were generated in plasmid pALPF1 by the one-step procedure of Datsenko and Wanner (9) based on the phage λ Red recombinase. Primers used for deletions are presented in Table 2. These primers

TABLE 2. Primers used in this work

Primer	Sequence (5′–3′)	Use
HUPS5	ACTGCCGAGACTTTTTATGACGTCATTCGCCGCCAGGGGATTCCGGGGATCCGTCGACC	*hupS* deletion
HUPS3	GAATCGTCATGATGGTGTTTCCCGCCCAGAATGTTAAGGTGTAGGCTGGAGCTGCTTC	
HUPL5	AAGCGCTTGACCACCAAGCGCGAAAAAGCTGACGCTTAAGTGTAGGCTGGAGCTGCTTC	*hupL* deletion
HUPL3	TTATCGGACCTGGACCCTGGCCATTTCTTGCCCATCCGGCATATGAATATCCTCCTTAGT	
HUPD5	GATCTCGGGTGAACGGCTCTTCAAGGACAGGGAGGATTAGGTGTAGGCTGGAGCTGCTTC	*hupD* deletion
HUPD3	GCAGTTCAACGCGGCCGGTTCGGCACGGCGCTCGTAACGCATATGAATATCCTCCTTAGT	
HUPG5	TCGCAATGTTTTGCATCGTGATTTGTGGAGGAGACAATGATTCCGGGGATCCGTCGACC	*hupG* deletion
HUPG3	TTCATCGCGCGATCTCCTTGCCGGCATGGGTGATCTCGAGTGTAGGCTGGAGCTGCTTC	
HUPH5	CGAGATCACCCATGCCGGCAAGGAGATCGCGCGATGAGTGTAGGCTGGAGCTGCTTC	*hupH* deletion
HUPH3	CGCTCATTTGAAATAGGCCTCCCAGATGTCCCGCAGTCGATATGAATATCCTCCTTAGT	
HUPI5	CGACTGCGGGACATCTGGGAGGCCTATTTCAAATGAGCGATTCCGGGGATCCGTCGACC	*hupI* deletion
HUPI3	CCATCGCCAAGCCTCATGAACTTCGACTGCAGGGCATCGGTGTAGGCTGGAGCTGCTTC	
HUPJ5	CTGCAGCTCGAGATGCGCTACCGGGAGATTTATGCGACCATTCCGGGGATCCGTCGACC	*hupJ* deletion
HUPJ3	CCGAGAAGGAATGTCATTCCGATGCCTCCTCGCGCCCGCGTGTAGGCTGGAGCTGCTTC	
HUPH5	CGAGATCACCCATGCCGGCAAGGAGATCGCGCGATGAGTGTAGGCTGGAGCTGCTTC	pALPF34
TAGH	GGCGCCTTCGCCCTCGGGGGCAACCCAAAAACCAGCCTTCTTCTCGAACTGCGGGTGGC	
TAGI	CTCATATGTGGAGC CACCCGCAGTTCGAGAAGAGCGCCTTCGAGAATTT	pPM164
MAN2	GCGGTCGCATAAATCT	
KNDE	GCCACAGTCGTCATATGACATTCCTTCTCGGGGC	pPM165
MANK3	TGCCGCGCCGTCTCACAG	
TAGG	CTCATATGTGGAGCCACCCGCAGTTCGAGAAGCCATCTGCCCTGGTCCG	pPM166
MAN2	GCGGTCGCATAAATCT	
MANF5	GCGATGCTCGGCTTGCTG	pPM125
MAN2	GCGGTCGCATAAATCT	
STREP1	GTGTAGGCTGGAGCTGCTTC	pPM70
STREP2	CTTTTCGAACTGCGGGTGGCTCCAGTTCATATGAATATCCTCC	

are homologous to DNA regions adjacent to the genes to be deleted and to template plasmids (pKD3 and pKD13) containing an antibiotic resistance gene that is flanked by recombinase target sites. In order to avoid polarity effects, specific precautions were taken at the primer design step to ensure that the deletions did not affect the ribosome binding sites of the overlapping genes. The desired deletions were confirmed by PCR amplification of the corresponding plasmid pALPF1 region containing the target genes followed by electrophoretic mobility assays.

Generation of *Strep*-tag II-HupH fusion. The *Strep*-tag II peptide, once fused to a protein, allows one-step protein purification because the tag interacts specifically with an immobilized variant of streptavidin called *Strep*-Tactin (IBA, Göttingen, Germany). To generate the *Strep*-tag II-HupH fusion protein, a modification of the Datsenko and Wanner deletion system (9) was used. The modification consisted in insertion of the sequence coding for the *Strep*-tag II peptide (WSHPQFEK) in the 5′ end of the antibiotic resistance gene of the pKD3 plasmid using primers STREP1 and STREP2, thus generating the new template plasmid pPM70. Subsequently, pPM70 was used as the template plasmid for in-frame fusing of the *Strep*-tag II sequence to the 5′ end of *hupH* from pALPF1 plasmid using primers HUPH5 and TAGH (Table 2). The resulting pALPF1 derivative plasmid (pALPF34) harbors a hydrogenase gene cluster encoding a *Strep*-tag II-HupH fusion protein.

Generation of plasmids expressing HupG, HupH, HupI, and HupK proteins in free-living *R. leguminosarum* cells. In order to express *hup* genes in microaerobically grown cultures of *R. leguminosarum*, the P_{fixN} promoter from pALPF1 was cloned in pBBR1MCS-2 vector plasmid (17) using PF1 and PF2 primers (6). P_{fixN} is expressed in microaerobic conditions under the control of the FnrN protein. The resulting plasmid (pPM1350) was later used to clone *hupI*, *hupK*, and *hupGHI* genes isolated by PCR amplification from the pALPF1 plasmid using specific primers (Table 2). The resulting pPM1350 derivative plasmids were designated pPM164, pPM165, and pPM166, respectively.

Cell fractionation for protein localization. Cells (1 g) from *R. leguminosarum* hydrogenase-induced cultures were suspended in 4 ml of buffer W (100 mM Tris-HCl, pH 8, 150 mM NaCl) containing a protease inhibitor mixture (Complete-mini; Roche Molecular Biochemicals, Mannheim, Germany). Cells were disrupted by three passages through a French pressure cell (SLM Aminco, Silver Spring, MD) at 100 MPa, and then the cell lysate was cleared for 20 min at 13,000 × *g*. The resulting supernatant was centrifuged at 135,000 × *g* for 1 h at 4°C in a TL-100 ultracentrifuge (Beckman Inc., Palo Alto, California.). The pellet, containing the cell membranes, was resuspended in the same volume of buffer W and precipitated again by an additional centrifugation at 135,000 × *g* for 1 h at 4°C.

Western immunoblot analysis. Protein portions (30 μg) from the soluble and the membrane fractions were resolved by either sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or native PAGE in 10% polyacrylamide and subsequently transferred to polyvinylidene difluoride membranes. HupL, HupS, and HypB proteins were identified immunologically as previously described (5) using antisera raised against *B. japonicum* HupL and *R. leguminosarum* HupS and HypB, respectively.

Purification of *Strep*-tag II-HupH fusion protein. Cell extracts from *R. leguminosarum* UPM1155 derivative strains containing plasmid pPM125 (Table 1) were obtained by French pressure cell disruption as above and subsequent centrifugation (12,000 × *g* for 30 min). The soluble fraction was applied to a 1-ml *Strep*-Tactin Superflow column (IBA, Göttingen, Germany) and developed by gravity flow. After the column was washed five times with 1 ml of buffer W to remove unbound proteins, the tagged protein was eluted six times with 0.5 ml of buffer W supplemented with 2.5 mM D-desthiobiotin. Eluted fractions were resolved by SDS-PAGE (12% polyacrylamide), and *Strep*-tagII-HupH, -HupL, and -HupS were identified by immunoblotting with *Strep*-Tactin conjugated to alkaline phosphatase (1:2,500; IBA, Göttingen, Germany) and antisera against HupL and HupS, respectively.

RESULTS

Contribution of *hupGHIJ* genes to free-living and symbiotic hydrogenase activity. Since *hup* genes from *R. leguminosarum* bv. viciae are not expressed in free-living cells, in-frame deletion mutations in each of the *hupG*, *hupH*, *hupI*, and *hupJ* genes were generated in plasmid pALPF1 (Fig. 1). This plasmid contains the whole *hup* cluster from strain UPM791 under the control of the promoter of the *fixN* gene from the same strain, thus allowing microaerobic expression of hydrogenase activity in free-living cells and in bacteroids of Hup^- strains of *R. leguminosarum* bv. viciae and other rhizobial species (6). The resulting pALPF1 mutant plasmid derivatives were transferred by conjugation into the Hup^- *R. leguminosarum*

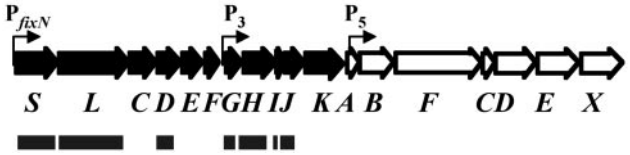

FIG. 1. *R. leguminosarum* UPM791 hydrogenase gene cluster cloned in plasmid pALPF1. *hup* and *hyp* genes are shown by full and empty horizontal arrows, respectively, and designated by capital letters. The locations of characterized promoters in the hydrogenase gene cluster are shown by bent horizontal arrows. DNA fragments deleted in pALPF1 derivative plasmids containing single-gene deletions are shown by horizontal bars.

UPM1155 strain. This strain is a UPM791 derivative strain with the entire *hup*/*hyp* gene cluster deleted that was constructed in our laboratory (our unpublished results). The hydrogenase activity of the corresponding transconjugant strains was tested in vegetative cells grown in microaerobic conditions (0.8% oxygen) as specified in Materials and Methods. pALPF1 mutant plasmid derivatives containing in-frame deletions in *hupS* or *hupL* hydrogenase structural genes, and in *hupD*, encoding HupL endopeptidase, were also constructed and used as controls (Fig. 1).

Hydrogenase activities associated with mutant plasmids affected in each of the *hupG*, *hupH*, *hupI*, and *hupJ* genes were severely reduced in vegetative cells as regards the hydrogenase activity associated with wild-type plasmid pALPF1 (Table 3). Particularly drastic for hydrogenase activity in free-living conditions was the mutation in the *hupH* gene, since strain UPM1155(pALPF7) (Δ*hupH*) exhibited no hydrogenase activity whereas mutation of the *hupG* gene caused only a 50% reduction of hydrogenase activity under the same conditions.

The UPM1155(pALPF1) derivative strains were also used to inoculate pea plants, and the hydrogenase activity was similarly measured in bacteroids. Remarkably, levels of hydrogenase activity exhibited by pea bacteroids from strains UPM1155 (pALPF6) (Δ*hupG*), UPM1155(pALPF7) (Δ*hupH*), UPM1155 (pALPF8) (Δ*hupI*), and UPM1155(pALPF9) (Δ*hupJ*) were much higher than those observed in free-living cells. In particular, wild-type levels of hydrogenase activity were detected in bacteroids of the strain containing the *hupG* deletion, and only a 50% reduction in activity was associated with the *hupH* deletion mutant. Strains carrying deletions in *hupI* and *hupJ* showed activity levels between 30 and 40% of those in the wild-type strain (Table 3).

TABLE 3. Relative free-living and symbiotic hydrogenase activities associated with pALPF1 derivative plasmids containing deletions in *hup* genes

Plasmid	Hydrogenase activity[a]	
	Free-living microaerobic cells	Pea bacteroids
pALPF15 (Δ*hupS*)	<1	<1
pALPF2 (Δ*hupL*)	<1	<1
pALPF4 (Δ*hupD*)	<1	<1
pALPF6 (Δ*hupG*)	45	100
pALPF7 (Δ*hupH*)	<1	50
pALPF8 (Δ*hupI*)	10	30
pALPF9 (Δ*hupJ*)	5	40

[a] Hydrogenase activities associated with pALPF1 derivative plasmids were measured in UPM1155 transconjugant strains and expressed as percentages of hydrogenase activity associated with the wild-type pALPF1 plasmid. The absolute values (100%) of hydrogenase activity for the UPM1155 (pALPF1) strain were (nmol of $H_2 \cdot h^{-1} \cdot$ mg protein^{-1}) 6,520 ± 730 in microaerobic cultures and 4,490 ± 100 in pea bacteroids. Free-living cultures were bubbled with 0.8% O_2. Values are averages of three replicates.

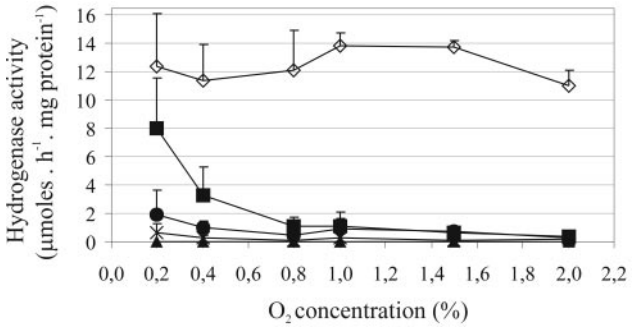

FIG. 2. Effect of *hupGHIJ* genes on the O_2 tolerance of hydrogenase induction in free-living cultures of *R. leguminosarum*. Hydrogenase activity of UPM1155 transconjugant strains containing pALPF1 or pALPF1 derivative plasmids with in-frame deletions in *hupG*, *hupH*, *hupI*, *or hupJ* genes were determined in cell cultures bubbled with gas mixtures containing different O_2 concentrations. The values correspond to the average of three replicate cultures, and error bars represent standard deviations. Symbols: ◇, UPM1155(pALPF1); ■, UPM1155(pALPF6) (Δ*hup*G); ▲, UPM1155(pALPF7) (Δ*hupH*); ●, UPM1155(pALPF8) (Δ*hupI*); ×, UPM1155(pALPF9) (Δ*hupJ*).

Effect of oxygen concentration on the contribution of *hupGHIJ* genes to hydrogenase activity. Since *hupGHIJ* genes appeared less relevant for hydrogenase activity in symbiosis than in free-living conditions and since the concentration of free oxygen inside the nodule is extremely low and precisely regulated (19), we decided to investigate the requirement of *hupGHIJ* genes for hydrogenase activity at different O_2 concentrations in free-living conditions. The assay was performed with bacterial cultures continuously bubbled with a gas mixture containing O_2 concentrations ranging from 0.2% to 2%. We first investigated the effect of O_2 on hydrogenase activity in the wild-type strain UPM1155(pALPF1). Maximum levels of hydrogenase activity were observed in 1.0 to 1.5% O_2 cultures, whereas small variations were observed at the remaining O_2 concentrations assayed (Fig. 2).

The same analysis was performed with strains containing the *hup*-deleted mutant plasmids (Fig. 2). The hydrogenase activity of cultures from Δ*hupG*, Δ*hupI*, and Δ*hupJ* mutant strains increased as the O_2 concentration decreased. This gradual increase was particularly evident in cultured cells from the Δ*hupG* strain and less so for the strains containing the *hupI* and *hupJ* deletions. No hydrogenase activity was detected in cells from the Δ*hupH* strain at any of the O_2 concentrations assayed. These results indicate that HupH is essential for hydrogenase activity in free-living *R. leguminosarum* cells and that the requirement of *hupGIJ* genes for the hydrogenase activity of free-living cultures increases at higher free-O_2 concentrations in the medium.

Effect of *hupGHIJ* genes on the maturation of hydrogenase structural proteins. The potential role of *hupGHIJ* genes in the maturation of hydrogenase subunits was investigated immunochemically for HupL (Fig. 3A) and HupS (Fig. 3B) in

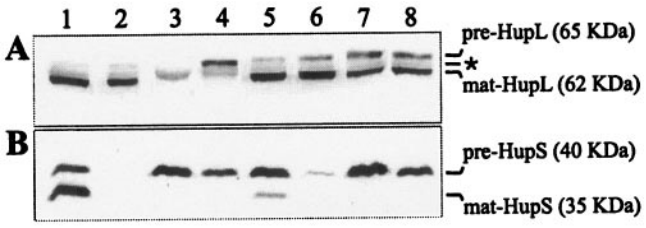

FIG. 3. Effect of *hupGHIJ* genes in the processing of hydrogenase subunits. Shown is the immunodetection of hydrogenase subunits (HupL and HupS) in cell extracts from microaerobically induced cultures of *R. leguminosarum* UPM1155 transconjugant strains containing pALPF1 or pALPF1 derivative plasmids with in-frame deletions in *hup* genes. Each lane was loaded with crude cell extracts containing 30 μg of total proteins from cell cultures bubbled with 0.8% O_2. Antibodies generated against HupL (A) and HupS (B) were used. Lines on the right side indicate the positions and molecular sizes of the unprocessed (pre) and the mature (mat) forms of the structural hydrogenase proteins and the presence of an unspecific anti-HupL reactive band (*). Lanes: 1, UPM1155(pALPF1); 2, UPM1155(pALPF15) (Δ*hupS*); 3, UPM1155(pALPF2) (Δ*hupL*); 4, UPM791(pALPF4) (Δ*hupD*); 5, UPM791(pALPF6) (Δ*hupG*); 6, UPM791(pALPF7) (Δ*hupH*); 7, UPM791(pALPF8) (Δ*hupI*); 8, UPM791(pALPF9) (Δ*hupJ*).

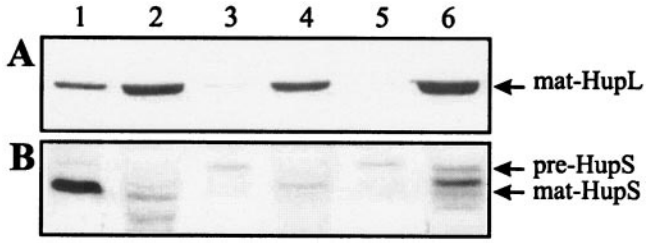

FIG. 4. Subcellular location of HupS and HupL in *R. leguminosarum* containing a deletion in the *hupH* gene. Hydrogenase subunits were immunologically detected using antisera generated against HupL (A) and HupS (B). SDS-PAGE gels were loaded with cell membrane (lanes 1, 3, and 5) or soluble (lanes 2, 4, and 6) fractions from microaerobically grown free-living culture cells. Lanes 1 and 2, UPM1155(pALPF1); lanes 3 and 4, UPM1155(pALPF7) (Δ*hupH*); lanes 5 and 6, SM61(pALPF1). pre and mat are as defined for Fig. 3.

total cell extracts from 0.8% O_2 microaerobic cultures of strains containing pALPF1 or pALPF1 derivative plasmids with deletions in each of the *hupGHIJ* genes. The unprocessed and processed forms of both HupS and HupL subunits were identified in free-living cells of the wild-type strain (Fig. 3A and B, lane 1). The processed form of HupL (mature HupL) was present in free-living cells from the *hupG*, *hupH*, *hupI*, and *hupJ* mutants (Fig. 3A, lanes 5 to 8), including the strain containing the *hupH* mutation, which exhibited no hydrogenase activity (Fig. 3A, lane 6). These results suggest that the *hupGHIJ* genes are not required for any biosynthetic step previous to full processing of HupL.

In contrast, the band corresponding to the processed form of HupS (mature HupS) was not detected in strains containing mutations in *hupH*, *hupI*, or *hupJ* (Fig. 3B, lanes 6 to 8), which exhibited low or no hydrogenase activity. In these mutant strains most of the HupS protein was in the unprocessed form (pre-HupS). A weak band corresponding to the processed form was observed associated with the *hupG* deletion, which showed the highest hydrogenase activity (50%) among mutants (Fig. 3B, lane 5). The processed form of HupL (Fig. 3A, lane 2) and the unprocessed form of HupS (Fig. 3B, lane 3) were observed in cell extracts from *hupS* and *hupL* mutants, respectively. As expected, the unprocessed forms of both subunits were detected in the *hupD* mutant (Fig. 3A and B, lane 4). These results support the idea that the *hupGHIJ* genes are required either for maturation of pre-HupS or for the formation or stability of the pre-HupS-HupL complex previous to its translocation to the membrane or for both processes. This was further investigated with the *hupH* mutant, the only one that did not have residual hydrogenase activity.

HupH is required for translocation of hydrogenase structural proteins to the membrane. The subcellular localization of structural subunits was investigated in the *hupH* mutant with UPM1155(pALPF1) and SM61(pALPF1) as controls. SM61 is a *tatBC* mutant affected in hydrogenase translocation to the cytoplasmic membrane (25). In the mutant strain containing the *hupH* deletion, HupL was located exclusively in the soluble fraction (Fig. 4A, lane 4). As expected, the large subunit in the wild type was located in the membrane fraction (Fig. 4A, lane 1), whereas in the *tatBC* mutant HupL was detected only in the soluble fraction (Fig. 4A, lane 6). The presence of HupL in the soluble fraction of the wild-type strain (Fig. 4A, lane 2) may be due to residual levels of HupL that has not been translocated.

On the other hand, the analysis of the subcellular localization of HupS subunit was consistent with the results observed for HupL. As expected, the small subunit was associated with the membrane cell fraction in the wild-type strain and with the soluble cell fraction in the *tatBC* mutant strain (Fig. 4B, lanes 1 and 6, respectively). In the strain containing the *hupH* deletion, HupS was not detected at all in the membrane fraction and was hardly detected in the soluble fraction (Fig. 4B, lanes 3 and 4). The weak bands corresponding to HupS in the Δ*hupH* and *tatBC* mutant strains could be due to the high lability of the HupS protein caused by the action of nonspecific cytoplasmic proteases (see below). These results are consistent with those obtained with the large subunit and indicate that HupH is required for translocation of hydrogenase to the membrane.

Formation of HupS-HupL complex requires HupH. The effect of HupH on HupS-HupL complex formation was first investigated by immunological analyses of native polyacrylamide gels loaded with membrane fractions of the Δ*hupH* mutant, using UPM1155(pALPF1) and UPM1155(pALPF15) (Δ*hupS*) as controls. Strains carrying *hupG* and *hupI* deletions were also included. HupS-HupL complexes were detected by using both HupL (Fig. 5A) and HupS (Fig. 5B) antisera.

A strongly labeled band was detected by both antisera in the membrane fraction from the wild-type strain (Fig. 5A and B, lane 1). This band, likely corresponding to the HupS-HupL complex, was not detected in membrane fractions from the mutant strain containing the *hupH* deletion (Fig. 5A and B, lane 2) or in the control Δ*hupL* strain (Fig. 5A and B, lane 3). This result suggests that the HupH protein is required for heterodimeric complex formation. This observation might also apply to HupG and HupI since only weak bands corresponding to the potential HupS-HupL complex were detected in mutant strains containing the *hupG* or *hupI* deletions (Fig. 5A and B, lanes 4 and 5). The presence of these faint bands likely ac-

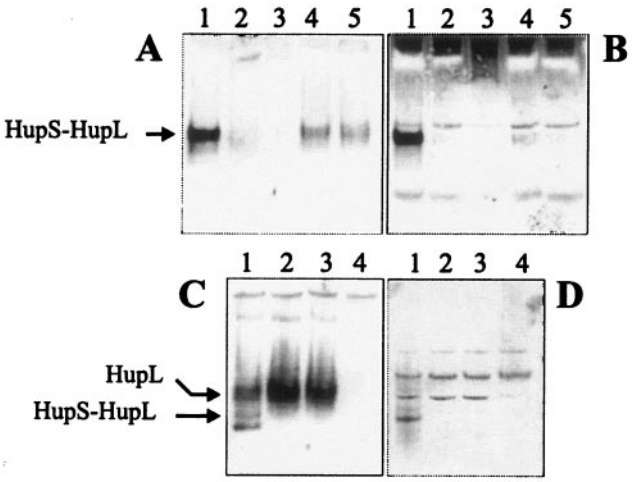

FIG. 5. Effect of HupH protein on the assembly of the HupS-HupL complex in free-living cultures of *R. leguminosarum*. The analysis was performed on native gels loaded with membrane (A and B) or soluble (C and D) fractions from cultures microaerobically induced for hydrogenase activity. The heterodimer complex was immunologically identified with HupL (A and C) or HupS (B and D) antisera. Lanes for panels A and B: 1, UPM1155(pALPF1); 2, UPM1155(pALPF7) (Δ*hupH*); 3, UPM1155(pALPF2) (Δ*hupL*); 4, UPM1155(pALPF6) (Δ*hupG*); 5, UPM1155(pALPF8) (Δ*hupI*); lanes for panels C and D: 1, UPM1155(pALPF1); 2, UPM1155(pALPF7) (Δ*hupH*); 3, UPM1155 (pALPF15) (Δ*hupS*); 4, UPM1155(pALPF2) (Δ*hupL*).

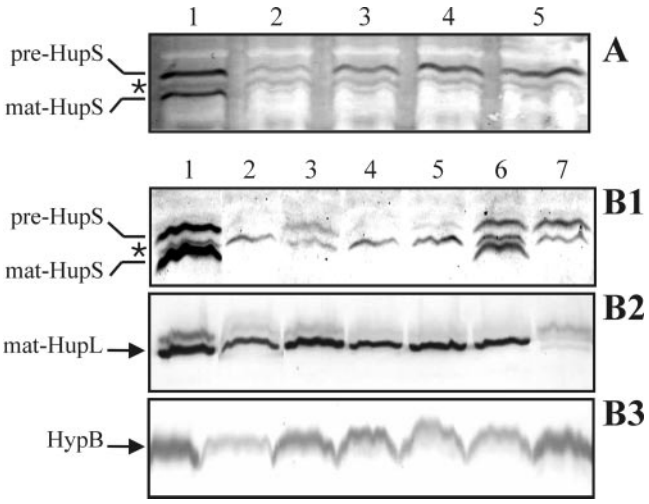

FIG. 6. Effect of HupH on the accumulation of HupS. Cell extracts from microaerobically grown (0.8% oxygen) cultures of *R. leguminosarum* UPM1155 transconjugant strains were subjected to Western immunoblotting analysis after PAGE separation. Samples of cell extracts containing 30 μg of total proteins were applied to each lane. HupS (A and B1), HupL (B2), and HypB (B3) proteins were detected by using the corresponding antisera. Arrows on the left side of panels indicate the position of the unprocessed (pre) and the mature (mat) forms of the structural hydrogenase proteins; the presence of an unspecific band resulting from cross-reactivity with HupS-antiserum is indicated by an asterisk. (A) Analysis of mutants. Lanes: 1, UPM1155 (pALPF6) (Δ*hupG*); 2, UPM1155(pALPF7) (Δ*hupH*); 3, UPM1155 (pALPF8) (Δ*hupI*); 4, UPM1155(pALPF9) (Δ*hupJ*); 5, UPM1155 (pALPF4) (Δ*hupD*). (B1 to B3) Complementation analysis of mutants. Lanes: 1, UPM1155(pALPF1); 2, UPM1155(pALPF15) (Δ*hupS*); 3, UPM1155(pALPF7) (Δ*hupH*); 4, UPM1155(pALPF7/pPM164) (Δ*hupH*/*hupG*); 5, UPM1155(pALPF7/pPM165) (Δ*hupH*/*hupK*); 6, UPM1155 (pALPF7/pPM166) (Δ*hupH*/*hupGHI*); 7, UPM1155(pALPF4) (Δ*hupD*).

counts for the low hydrogenase activity induced by Δ*hupG* and Δ*hupI* mutant strains (Table 3). The absence of the HupS-HupL complex in the membrane fraction of the *hupH* mutant is consistent with the observed subcellular localization of hydrogenase structural proteins (Fig. 4A and B).

The possibility that a HupS-HupL complex is formed in the soluble fraction but not transported to the periplasm was also investigated in native gels by using HupL and HupS antisera. The results obtained indicate the presence of a band identified with both antisera in the soluble cell fraction from the wild-type strain (Fig. 5C and D, lanes 1) but not from the Δ*hupS* and Δ*hupL* strains used as controls (Fig. 5C and D, lanes 3 and 4). This band can be attributed to a HupS-HupL complex and was not detected in the soluble cell fraction from the mutant strain containing the *hupH* deletion (Fig. 5C and D, lane 2). In contrast to the HupL protein, clearly identified in these native gels (Fig. 5C, lane 2), a band corresponding to the HupS protein was not detected in the *hupH* mutant (Fig. 5D, lane 2). Taken together, these results with membrane and soluble cell fractions suggest that HupH is required for pre-HupS-HupL complex formation.

The level of HupS decreases in the absence of a functional HupH protein. The fact that HupS frequently appeared as a weak band in extracts from mutant strains containing the *hupH* deletion (Fig. 3B, lane 6) compared, for instance, with the band appearing in cell extracts from the Δ*hupL* mutant (Fig. 3B, lane 3) prompted us to investigate the effect of HupH on HupS protein accumulation.

First, immunoblot analyses of HupS were performed on extracts from Δ*hupG*, Δ*hupH*, Δ*hupI*, and Δ*hupJ* mutants using extracts from the Δ*hupD* mutant as a control (Fig. 6A). Cell extracts from the strain containing the Δ*hupH* mutation clearly exhibited a band corresponding to the unprocessed form of HupS (Fig. 6A, lane 2), but much weaker than the corresponding band in cell extracts from strains containing the Δ*hupG*, Δ*hupI*, Δ*hupJ*, or Δ*hupD* mutations (Fig. 6A, lanes 1, 3, 4, and 5).

Second, wild-type HupS levels could be restored by introduction of the *hupH* gene (Fig. 6B1). The decrease of HupS levels in the *hupH* mutant background was corrected by complementation with plasmid pPM166 containing *hupGHI* genes (Fig. 6B1, lane 6), but not by plasmid pPM164 or pPM165, containing the *hupI* and *hupK* genes, respectively (Fig. 6B1, lanes 4 and 5). The effect on HupS accumulation associated with plasmid pPM166 could not be due to the *hupG* gene since the unprocessed form of HupS was stable in the Δ*hupG* strain (Fig. 3, lane 5). Similarly, HupH-dependent accumulation of immature HupS was not related to formation of the HupS-HupL complex, since pre-HupS levels were also high in a Δ*hupD* background (Fig. 6B1, lane 7) where no HupL processing took place. Other nonspecific reasons for the low intensity of the pre-HupS band in the Δ*hupH* background were ruled out by assaying HupL and HypB levels in the same extracts (Fig. 6B2 and B3, respectively). Since there are no reasons to postulate a change on the level of *hupS* translation, our interpretation of the above results is that the presence of HupH is required for HupS stability.

HupH forms a complex with HupS. Since HupH is essential for hydrogenase activity in free-living conditions but seems not to be needed for HupL maturation, we decided to analyze its involvement in HupS maturation. To investigate the potential formation of a HupS-HupH complex, we used an affinity chromatography-based approach. To this end, a *Strep*-tag II sequence was fused to the N terminus of the HupH protein. We expected that the resulting construct, designated $HupH_{strep}$ and encoded by plasmid pALPF34, would allow us a one-step affinity purification of proteins interacting with HupH. Since foreign protein overexpression in *E. coli* frequently has limitations, we decided to work with the original host, *R. leguminosarum*. First, we checked for functionality of the $HupH_{strep}$ modified protein. The pALPF34 plasmid induced in free-living cells of *R. leguminosarum* UPM1155 the same levels of hydrogenase activity associated with the wild-type plasmid, pALPF1, and the $HupH_{strep}$ protein encoded by pALPF34 could be detected using a *Strep*-Tactin-alkaline phosphatase conjugate (data not shown). Next, the EcoRI fragment containing the $hupGH_{strep}I$ genes and the upstream P3 promoter was isolated from pALPF34 and cloned in a pBBR1MCS-2 broad-host-range plasmid. The resulting plasmid, pPM125, complemented the Δ*hupH* mutation for hydrogenase activity (data not shown). This indicates both that $hupGH_{strep}I$ genes are transcribed in microaerobic cultures, likely from a functional P3 promoter (23), and that the tagged HupH protein complements the *hupH* mutation. Finally, the resulting pPM125 plasmid was introduced into UPM1155 transconjugant strains carrying plasmids with the Δ*hupS* mutation (pALPF15), as a control, and Δ*hupL* (pALPF2) or Δ*hupD* (pALPF4) mutations to favor the accumulation of a potential HupS-$HupH_{strep}$ complex. Cell extracts were loaded into a *Strep*-Tactin column, and, after standard washes, proteins bound to the column were eluted with desthiobiotin (2.5 mM) and separated in SDS gels. $HupH_{Strep}$, HupS, and HupL proteins were identified by immunoblotting with the corresponding antisera. The eluted fractions from all strains harboring the pPM125 plasmid contained large amounts of the tagged $HupH_{strep}$ protein (Fig. 7A, lanes 2, 3, and 4).

A band of an apparent molecular mass of 35 kDa and reactive with the anti-HupS antiserum was present in the eluate from cell extracts from Δ*hupL* and Δ*hupD* strains (Fig. 7B, lanes 3 and 4, respectively), but not from the control Δ*hupS* strain (Fig. 7B, lane 2). This result is consistent with the presence of a cytoplasmic unprocessed form of HupS (pre-HupS) protein that has been retained by the $HupH_{strep}$ fusion protein. The amount of the HupS protein in the purified HupS-$HupH_{strep}$ complex may represent only a portion of the total pre-HupS pool, since the wild-type copy of *hupH* is still present in the strain and its product may compete with the tagged HupH for available pre-HupS. Copurification of HupL with $HupH_{strep}$ was not observed in any case (Fig. 7C, lanes 2, 3, and 4). These results indicate the formation of a complex between pre-HupS and $HupH_{strep}$ proteins, prior to HupS-HupL complex assembly.

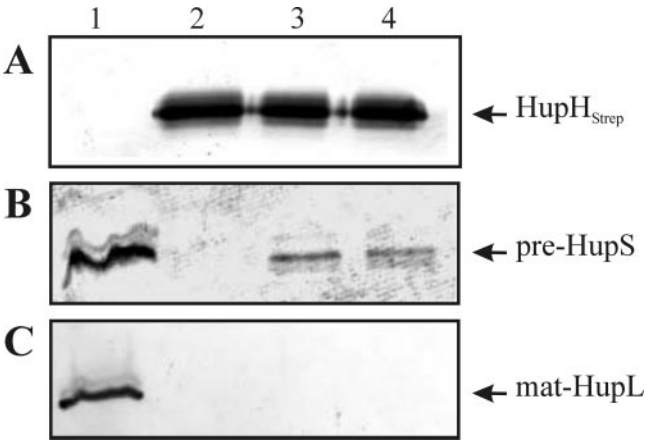

FIG. 7. Analysis of the formation of a HupS-$HupH_{Strep}$ complex. Proteins eluted from a *Strep*-Tactin column loaded with cell extracts from different strains were separated in a 15% SDS-PAGE gel (lanes 2, 3, and 4). Lane 1 contains crude cell extracts from microaerobically grown cells. $HupH_{Strep}$ (A), HupS (B), and HupL (C) proteins were identified using a streptavidin-alkaline phosphatase conjugate and antisera against HupS and HupL, respectively. Plasmid pPM125 encodes the HupH-*Strep*-tag fusion. Lanes: 1, UPM1155(pALPF1); 2, UPM1155 (pALPF15/pPM125) (Δ*hupS*/*hupGHI*); 3, UPM1155(pALPF2/pPM125) (Δ*hupL*/*hupGHI*); 4, UPM1155(pALPF4/pPM125) (Δ*hupD*/*hupGHI*).

DISCUSSION

The results reported in this work suggest a role for the *hupGHIJ* gene products in the maturation of HupS, the hydrogenase small iron-sulfur subunit, and show the formation of a HupS-HupH complex in *R. leguminosarum*. These results are specially relevant since, apart from the structural component and the Tat system (25), no other proteins with a role in synthesis of a functional iron-sulfur subunit of [NiFe] hydrogenase have been identified.

Immunoblot analyses of in-frame deletion mutants revealed that the *R. leguminosarum* HupGHIJ proteins are not required for synthesis of a processed large hydrogenase subunit (HupL) but that instead they are involved in the maturation of the hydrogenase small subunit of this bacterium. Consistent with these results is the fact that an *E. coli* mutant lacking the HyaE protein, a homologue of *R. leguminosarum* HupG, was unable to process HyaA, the small subunit of Hyd1 hydrogenase (26). Also, a mutant with a deletion in *hybE*, a *hupJ*-homologous gene in the *E. coli* hydrogenase 2 gene cluster, was able to C-terminally process the large subunit HybC (16). Similarly, HoxO and HoxQ, the *Ralstonia eutropha* homologues of HupG and HupH, respectively, have also been shown to be required for hydrogenase activity in this aerobic bacterium (2) and to interact with HoxK, the hydrogenase small subunit (T. Schubert, M. Bernhard, O. Lenz, and B. Friedrich, Abstr. 7th Int. Hydrogenase Conf., abstr. P3-5, 2004).

The requirement of the HupGHIJ proteins for hydrogenase synthesis seems to be related to the O_2 concentration in the medium during hydrogenase induction and appears crucial when hydrogenase is induced under high O_2. These proteins might be fully or partially replaced by housekeeping Fe-S cluster biosynthetic proteins at low free-O_2 concentrations in the media or in symbiosis. In this regard, it is known that some Fe-S clusters are labile under oxidative conditions and require repair or resynthesis by specific proteins, as it is the case for *Erwinia chrysanthemi* SufC (27) and *A. vinelandii* IscA (18). Circumstantial evidence supporting a connection between the

oxygen level in the hydrogenase-inducing environment and the role of HupGHIJ proteins in hydrogenase synthesis is the absence of these proteins in anaerobic, [NiFe] hydrogenase-containing bacteria such as *Desulfovibrio* species (31).

Regarding the potential participation of HupGHIJ proteins in reduction chemistry leading to the assembly or maintenance of the Fe-S clusters into pre-HupS, it should be noted that HupI shows homology to rubredoxins (7, 30). Although the precise physiological function of rubredoxins remains elusive, especially in aerobic bacteria, they have been repeatedly associated with electron transfer reactions to diverse substrates with a wide range of reduction potentials (35, 38). Also, HupG and homologous proteins contain a structural domain related to thioredoxins and thiol-disulfide isomerases, proteins that participate in redox reactions, via the reversible oxidation of an active center disulfide bond (COG0526) (22). There is also some evidence indicating that HupH, HupI, and HupJ have related roles. First, HyaF2, homologous to HupH in the hydrogenase I gene cluster of *Salmonella enterica*, is likely a HupH-I fusion protein that contains the functional domain characteristic of rubredoxins at the C terminus (24). Second *hupJ* from some bacteria encodes a combined protein with the N terminus homologous to HupI and the remainder of the protein homologous to HupJ (1, 8).

The involvement of HupH on the maturation of a pre-HupS subunit able to form a periplasm-translocatable complex with the HupL subunit is evident from the experiments with the Δ*hupH* mutant. Besides the requirement of HupH for the translocation of the HupS-HupL complex to the membrane, we have also found evidence indicating that HupH binds the pre-HupS subunit. This binding may be required in the process of Fe-S cluster incorporation to the pre-HupS protein, with the likely participation of HupG, HupI, and HupJ proteins. In addition, the binding of HupH to HupS may be needed either to mediate the interaction between the pre-HupS and HupL modules or to prevent the formation of a defective complex before the completion of both the HupL and pre-HupS moieties and the subsequent wasteful export of incompletely folded or immature enzyme. Such a role has been proposed for *E. coli* HyaE and HybE chaperone-like proteins based on their interactions with the Tat signal peptide-bearing hydrogenase precursors HyaA of hydrogenase 1 and HybO of hydrogenase 2, respectively (10, 16). This role would be also similar to that of the DmsD chaperone, which binds the Tat signal peptides of the dimethyl sulfoxide and trimethylamine *N*-oxide reductases (28).

In conclusion, in this work we demonstrate that the *R. leguminosarum* HupGHIJ proteins are involved in the maturation of the hydrogenase small subunit. We propose that they play a common role related to the incorporation or maintenance of the iron-sulfur clusters in the pre-HupS form and that the relevance of this role is dependent on the oxygen levels of the hydrogenase induction conditions. It is also clear from this work that HupH forms a complex with pre-HupS. Unraveling the precise role of HupGHIJ proteins in HupS maturation will require additional experimentation that will shed light on the biosynthetic process of this complex but fascinating metalloenzyme.

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