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Rhizobium leguminosarum hupE Encodes a Nickel Transporter Required for Hydrogenase Activity▽

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Synthesis of the hydrogen uptake (Hup) system in *Rhizobium leguminosarum* bv. *viciae* requires the function of an 18-gene cluster (*hupSLCDEFGHIJK-hypABFCDEX*). Among them, the *hupE* gene encodes a protein showing six transmembrane domains for which a potential role as a nickel permease has been proposed. In this paper, we further characterize the nickel transport capacity of HupE and that of the translated product of *hupE2*, a hydrogenase-unlinked gene identified in the *R. leguminosarum* genome. HupE2 is a potential membrane protein that shows 48% amino acid sequence identity with HupE. Expression of both genes in the *Escherichia coli nikABCDE* mutant strain HYD723 restored hydrogenase activity and nickel transport. However, nickel transport assays revealed that HupE and HupE2 displayed different levels of nickel uptake. Site-directed mutagenesis of histidine residues in HupE revealed two motifs (HX_5DH and FHGX[AV]HGXE) that are required for HupE functionality. An *R. leguminosarum* double mutant, SPF22A (*hupE hupE2*), exhibited reduced levels of hydrogenase activity in free-living cells, and this phenotype was complemented by nickel supplementation. Low levels of symbiotic hydrogenase activity were also observed in SPF22A bacteroid cells from lentil (*Lens culinaris* L.) root nodules but not in pea (*Pisum sativum* L.) bacteroids. Moreover, heterologous expression of the *R. leguminosarum hup* system in bacteroid cells of *Rhizobium tropici* and *Mesorhizobium loti* displayed reduced levels of hydrogen uptake in the absence of *hupE*. These data support the role of *R. leguminosarum* HupE as a nickel permease required for hydrogen uptake under both free-living and symbiotic conditions.

Nickel is an essential microelement required for the synthesis of at least nine metalloenzymes, including hydrogenases and ureases (34). Most natural habitats contain very small amounts of available nickel, and bacteria require transport systems designed to take up and concentrate this element. However, an excess of free nickel can be toxic to cells by displacing essential metals from their native binding sites or by generating reactive oxygen species that cause oxidative DNA damage (14). For this reason, nickel uptake mechanisms are strictly regulated and balanced with efflux systems to avoid nickel overload in the cell.

Proteins involved in nickel transport have been described for different bacteria, in many cases in studies performed in connection to the enzymatic system requiring this element (hydrogenase, urease, or others). Nickel uptake systems are classified in two broad groups, single-component Ni^{2+} permeases and ATP-dependent binding cassette (ABC) transporters (39). The single-component permeases include three different groups, namely, the NiCoT, HupE/UreJ, and UreH families, whereas the multicomponent transporters include the NikMQO/CbiMQO and NikABCDE families.

Two nickel-dependent enzyme systems, hydrogenase and urease, have been described for *Rhizobium leguminosarum* (38, 48). However, no nickel transporter has been identified in this bacterial species, although one of the components of the hydrogenase gene cluster, *hupE*, encodes a candidate protein showing six putative transmembrane domains and a predicted signal peptide. Besides these features as a membrane protein, HupE does not show significant sequence similarities with NiCoT permeases such as HoxN from *Ralstonia eutropha*, HupN from *Bradyrhizobium japonicum*, or NixA from *Helicobacter pylori* (22, 23, 50). A homologous protein, UreJ, is encoded in several urease gene clusters, such as that from *Bordetella bronchiseptica* (33). The association of HupE/UreJ proteins with Ni-dependent enzymes has led to the assignment of a potential role as a nickel permease for these proteins (33). This possibility was further substantiated by experiments with heterologous expression of HupE from *Rhodopseudomonas palustris* and UreJ from *Cupriavidus necator* H16 in *Escherichia coli* (18). However, there is no previous evidence for a role for any of these proteins in the provision of nickel for nickel-dependent systems.

[NiFe] hydrogenases from diazotrophic bacteria catalyze the oxidation of molecular hydrogen evolved from nitrogenase, thus improving the energy efficiency of the nitrogen fixation

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process (43). In the case of *R. leguminosarum*, synthesis of the hydrogen-uptake (Hup) [NiFe] hydrogenase requires 18 genes (*hupSLCDEFGHIJK-hypABFCDEX*) (38). As in other hydrogenase systems, *R. leguminosarum* [NiFe] hydrogenase is a heterodimeric enzyme with two structural subunits encoded by the *hupSL* genes. HupL, the hydrogenase large subunit, contains a NiFe heterobimetallic center with a unique structure (21). Assembly of this NiFe center requires the concerted action of many of the *hup* and *hyp* gene products in a process that has been elucidated for *E. coli* (6). Incorporation of nickel into the active center requires the interaction of HypA and HypB, two nickel-binding proteins whose function can be substituted for by large amounts of nickel in the medium (5). An additional protein, SlyD, interacts with HypB to facilitate Ni release for hydrogenase synthesis in *Escherichia coli* (29). The final step in HupL maturation is the proteolytic processing of the C terminus of the large subunit precursor, carried out by the nickel-dependent protease HupD (40).

A particular feature of *Rhizobium leguminosarum* bv. *viciae* hydrogenase activity is its dependence on the plant host. In this bacterium, hydrogenase expression is induced during the nitrogen fixation process in bacteroid cells differentiating in pea root nodules. This symbiotic expression is driven by a NifA-dependent promoter controlling expression of structural and downstream genes (7). The obligate symbiotic expression of hydrogenase has hampered analysis of the hydrogenase system in *R. leguminosarum*. In order to allow hydrogenase expression in cultured vegetative cells, the NifA-dependent promoter of the *R. leguminosarum hup* operon was replaced with the FnrN-dependent promoter of the *fixN* gene, thus generating strain SPF25, where hydrogenase synthesis is induced when cells are exposed to microaerobic conditions (10).

Hydrogen uptake in pea bacteroids is limited by the availability of nickel to the plant, since addition of nickel chloride to the plant nutrient solution strongly increases levels of hydrogenase activity (9). Nickel limitation might be responsible for an additional level of plant-dependent control of *hup* expression. Legume hosts, such as lentil, which are nonpermissive for hydrogenase activity, have been described (32). This host effect, exerted at both transcriptional and posttranscriptional levels, is linked to a strong limitation of supply of nickel to the bacteroid in lentil nodules, suggesting the involvement of an unidentified metalloregulator (11).

Mechanisms for metal provision in vegetative and symbiotic forms of bacterial cells are relevant for the synthesis of metalloenzymes. In this work, we describe the role of *R. leguminosarum* HupE and HupE2, two proteins involved in nickel transport for hydrogenase synthesis under both free-living and symbiotic conditions.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. For induction of hydrogenase activity in *E. coli* cells, strains MC4100 and HYD723 were grown at 28°C under anaerobic conditions in TGYEP medium (41) modified to contain glycerol (0.4%) and fumarate (20 mM). Antibiotics were added at the following concentrations (in $\mu g \cdot ml^{-1}$): for tetracycline, 10; for kanamycin, 50; and for ampicillin, 100. *Rhizobium leguminosarum* bv. *viciae*, *Mesorhizobium loti*, *Rhizobium tropici*, and *Rhizobium etli* strains were routinely grown at 28°C in tryptone-yeast extract (TY) (4) or yeast mannitol broth (YMB) medium (49). Strain SPF25 derives from *R. leguminosarum* UPM791 by replacement of the native NifA-dependent *hupSL* promoter with the *fixN* gene promoter controlled by FnrN (10). Cosmid pALPF1 is a pAL618 derivative carrying the same promoter modification as that in SPF25. Cosmid pALPFE is a pALPF1 derivative carrying a *hupE* gene deletion (see below). Plasmids pALPF1 and pALPFE were introduced into *Rhizobium* and *Mesorhizobium* strains by conjugation, and transconjugants were selected in *Rhizobium* minimal medium (Rm) (37) supplemented with tetracycline ($5 \mu g \cdot ml^{-1}$).

DNA manipulation techniques. Plasmid DNA preparations, restriction enzyme digestions, agarose gel electrophoresis, DNA cloning, and transformation of DNA into *Escherichia coli* were carried out by standard protocols (44). *R. leguminosarum* genomic DNA was extracted as previously described (30). For Southern hybridizations, *hupE* and *hupE2* DNA probes were generated by PCR with primers UE1/LE1 and hupE2.3/hupE2.4, respectively (Table 2), and labeled with digoxigenin (DIG) by using DIG-11-dUTP (Roche Molecular Biochemicals, Mannheim, Germany) at a 40 μM final concentration. Hybridizing bands were visualized using a chemiluminescence DIG detection kit as described by the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany).

Cloning and sequencing the *hupE2* gene of *R. leguminosarum* UPM791. The DNA region containing the *R. leguminosarum* UPM791 *hupE2* gene was amplified by PCR from genomic DNA with oligonucleotides hupE2.1 and hupE2.2. These primers were designed from the *R. leguminosarum* bv. *viciae* 3841 genomic sequence (Table 2). The 1.6-kb DNA fragment amplified was cloned in plasmid pCR2.1TOPO (Invitrogen BV, Groningen, The Netherlands) and sequenced with the following synthetic oligonucleotides: T7, Reverse, hupE2.3, hupE2.4, hupE2.5, and hupE2.6. DNA sequencing was carried out using a BigDye Terminator cycle sequencing ready reaction kit and an ABI377 automatic sequencer (PE Biosystems, Foster City, CA). DNA sequence was analyzed with the Sequencher 4.1 and DNA Strider 1.2 programs. Searches for homologous proteins were carried out with BLAST software (1). The 1.6-kb nucleotide sequence containing the *R. leguminosarum* UPM791 *hupE2* gene has been deposited in GenBank (accession no. GQ420671). Transmembrane domains were predicted with the TMHMM2 (CBS, DTU, Denmark) and DAS (15) programs.

Mutant and plasmid constructions. To generate a *hupE* mutant in strain UPM791, an in-frame deletion of 144 bp was created by taking advantage of a unique NdeI restriction site in plasmid pRLH41, which contains a 5-kb EcoRI fragment of the *R. leguminosarum hup* gene cluster. The EcoRI fragment carrying the *hupE* deletion was cloned in suicide vector pK18*mobsac*, and the resulting plasmid, pKE1, was introduced into *R. leguminosarum* UPM791 by conjugation. Homologous recombination was selected using the *sacB* system (45). The *hupE* mutant generated was named UPM791.27. The PNIFA promoter of UPM791.27 hydrogenase structural genes was replaced with the PFIXN promoter cloned in plasmid pKPF1, giving strain SPF2714.

An in-frame deletion of *hupE* was also generated in plasmid pALPF1 by following the one-step method of Datsenko and Wanner (16) based on the phage λ Red recombinase. Briefly, a 1.6-kb DNA fragment containing a kanamycin resistance gene flanked by recombinase target sites was PCR amplified from template plasmid pKD4 and primers hupE1.1 and hupE1.2. The 5′ oligonucleotide sequences were homologous to DNA regions adjacent to the *hupE* gene and the 3′ sequences to plasmid pKD4. The 1.6-kb fragment was introduced by electroporation in strain BW25113(pALPF1) to replace the *hupE* gene with the kanamycin resistance cassette. The resistance gene was removed by introduction of plasmid pCP20 expressing the FLP recombinase. The *hupE* in-frame deletion was confirmed by PCR amplification of the corresponding region in plasmid pALPF1 and sequencing.

To generate a deletion in the *hupE2* gene, DNA regions corresponding to the 5′ and 3′ ends were amplified by PCR using plasmid pThupE2 as a template and oligonucleotides Reverse/hupE2.5 and T7/hupE2.6, respectively (Table 2). The resulting DNA fragments of ca. 900 and 600 bp were digested with BamHI/HindIII and EcoRI/HindIII, respectively, and cloned into pK18*mobsac*. The resulting plasmid (pKE2) was introduced into strains SPF25 and SPF2714Δ*hupE* by conjugation, and recombination of homologous DNA regions was selected by the *sac* system. The transconjugant strains carrying mutations in *hupE2* and *hupE hupE2* were named SPF228 and SPF22A, respectively. Deletions in the *hupE* and *hupE2* genes were confirmed by Southern blot hybridization using DNA probes of the corresponding genes and genomic DNA from single and double mutants.

For construction of plasmids pBADE1 and pBADE2, the *hupE* and *hupE2* genes were amplified from their ATG codons with primers EChupE1/E1BAD and E2BAD1/E2BAD2, using DNA from plasmid pRLH41 and from UPM791 genomic DNA, respectively, as templates. The amplified DNA fragments were cloned in plasmid pCR2.1TOPO, excised with NheI (a restriction site included in both upper primers) and XbaI, and cloned in vector pBAD18-Kan (26).

Site-directed mutagenesis of the *hupE* gene was carried out in plasmid pCR2.1TOPO containing the *hupE* gene. To do this, two 26- to 28-mer comple-

TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Genotype or relevant characteristic(s)	Source or reference
E. coli		
DH5α	*recA1 endA1 gyrA96 thi hsdRl7 supE44 relA1* Δ(*lacZYA-argF*)*U169* (ϕ80 *lacZ*ΔM15) *deoR phoA*	27; Bethesda Research Laboratories
S17.1	*thi pro hsdR hsdM*$^+$ *recA* RP4::2-Tc::Mu-Kan::T7; Sp^r Sm^r	46
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ Δ*hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	16
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 ptsF25 relA1 flb5301 rpsL150*	12
HYD723	MC4100; *nikA*::MudI (Ap^r *lac*)	51
R. leguminosarum bv. *viciae*		
UPM791	128C53 Str^r; Nod$^+$ Fix$^+$ Hup$^+$	30
SPF25	UPM791 with P*fixN*::*hupSL*	10
SPF2714	SPF25 Δ*hupE*	This work
SPF228	SPF25 Δ*hupE2*	This work
SPF22A	SPF25 Δ*hupE* Δ*hupE2*	This work
Rhizobium tropici CIAT899	Wild type; Nod$^+$ Fix$^+$ Hup$^-$	P. van Berkum (USDA, Beltsville, MD)
Rhizobium etli CE3	CFN42; Nod$^+$ Fix$^+$ Hup$^-$; Kan^r	36
Mesorhizobium loti MAFF303099	Wild type; Nod$^+$ Fix$^+$ Hup$^-$	NIAS (Japan)
Plasmids		
pCR2.1-TOPO	PCR product cloning vector; Ap^r Kan^r	Invitrogen[b]
pRLH41	pBluescriptSK containing a 5-kb DNA region of the UPM791 *hup* gene cluster	31
pAL618	Cosmid pLAFR1 containing the UPM791 *hup* cluster; Tc^r	30
pALPF1	pAL618 with P*fixN*::*hupSL*	10
pALPFE	pALPF1 Δ*hupE*	This work
pK18*mobsacB*	pK18 derivative *sacB*; Kan^r	45
pKPF1	pK18*mobsacB* with P*fixN*::*hupSL*	10
pKE1	pK18*mobsacB* with a deletion in *hupE*	This work
pKE2	pK18*mobsacB* with a deletion in *hupE2*	This work
pThupE2	pCR2.1-TOPO with a 1.6-kb DNA of the *hupE2* region	This work
pKD4	Template plasmid harboring an FLP-mediated excision sequence flanking the Kan^r gene	16
pCP20	Plasmid for thermal induction of FLP synthesis; Cm^r Ap^r	16
pBAD18-Kan	Expression vector carrying the arabinose PBAD promoter; Kan^r	26
pBADE1	pBAD18-Kan with the *hupE* gene	This work
pBADE2	pBAD18-Kan with the *hupE2* gene	This work
pBADE1.H series	pBADE1 with *hupE* mutated versions	This work

[a] Abbreviations: Ap, ampicillin; Kan, kanamycin; Str, streptomycin; Tc, tetracycline; Cm, chloramphenicol.

mentary oligonucleotides with centered nucleotide substitutions were designed to generate a His-to-Ile substitution for each His residue in HupE. PCR amplification with complementary primers and DNA from pCR2.1TOPO-*hupE* as a template generated complete plasmids that potentially harbored the corresponding mutation. The resulting plasmids were treated with DpnI and transformed into DH5α cells. Substitution of target nucleotides as well as absence of additional mutations was verified by sequencing. Mutated *hupE* versions were excised with the NheI/XbaI restriction enzymes and cloned into vector pBAD18-Kan for further analysis.

Plant tests. Pea (*Pisum sativum* L. cv. Frisson), lentil (*Lens culinaris* cv. Magda), lotus (*Lotus corniculatus*), and bean (*Phaseolus vulgaris* cv. Torcaza INIA) seeds were surface sterilized, germinated on 1% water agar plates, and planted in Leonard jar-type assemblies under bacteriologically controlled conditions (42). Plants were grown on a nitrogen-free plant nutrient solution that was supplemented, when indicated, with 85 μM $NiCl_2$ on day 10 after seedling inoculation (9).

Hydrogenase activity assays. Hydrogenase activity in *R. leguminosarum*, *R. tropici*, *R. etli*, and *M. loti* bacteroid suspensions from 21-day-old plants was measured using an amperometric method with oxygen as an electron acceptor (42). Hydrogenase activity in *R. leguminosarum* vegetative cells was induced under stoppered-flask conditions with 50-ml cultures. Strains were aerobically grown in YMB medium to an optical density at 600 nm of 0.4. From these cultures, a 1:10 dilution was done in fresh YMB medium. When indicated, $NiCl_2$ was added to give a 1 μM final concentration. The 200-ml flasks were tightly capped, flushed five times with a 0.8% O_2 atmosphere, and incubated at 28°C for 16 h. Cell cultures were centrifuged and suspended in 5 ml Dixon buffer (32 mM K_2HPO_4, 24 mM KH_2PO_4, and 0.24 mM $MgCl_2$) before amperometric determination. For induction of hydrogenase activity in *E. coli* cells, strains MC4100 and HYD723 were grown in LB medium at 28°C for 16 h. A 1:20 dilution in a modified TGYEP medium was carried out, and arabinose was added to give a 200 μM final concentration. The 25-ml cultures were then capped, flushed five times with a N_2 atmosphere, and incubated at 28°C for 5 h. After incubation, cultures were centrifuged, resuspended in 5 ml Dixon buffer, and assayed for hydrogenase activity as described above. For quantification of hydrogenase activity, the protein content of bacteroids and vegetative cells was determined by the bicinchoninic acid method (47), with the modifications described by Brito et al. (9).

Nickel transport assays. *E. coli* strain HYD723 carrying plasmid pBAD18-Kan and derivatives were grown to mid-exponential growth phase under aerobic conditions in LB medium. Then, L-arabinose was added up to 200 μM and the culture incubated at 37°C for 1 h. Cells were harvested by centrifugation, washed, and resuspended in potassium phosphate buffer (65 mM KH_2PO_4-K_2HPO_4, pH 6.8) supplemented with 11 mM glucose and 10 mM $MgCl_2$ to give a final concentration of 0.2 to 0.4 mg of bacteria · ml^{-1} (dry weight). Nickel uptake was initiated by adding 150 nM $^{63}NiCl_2$ (7.52 mCi · ml^{-1} and 1.2 mg Ni · ml^{-1}; Amersham). Samples (100 μl) were taken at regular time intervals, vacuum filtered through cellulose nitrate membrane filters (0.45-μm pore size and 25-mm diameter; Millipore), and washed twice with 2.5 ml potassium phosphate buffer containing 10 mM nickel chelator EDTA. Filters were dried and placed in scintillation vials with 5 ml of scintillation fluid (OCS; Amersham) for counting with a Packard liquid scintillation counter. Similar growth and arabinose induction conditions were used for the analysis of membrane protein fractions. Cell cultures (250 ml) were centrifuged after induction, resuspended in 3 ml of buffer W (100 mM Tris-HCl, pH 8, 150 mM NaCl), mechanically disrupted by 3

TABLE 2. Oligonucleotides used in this work

Designation	Sequence (5′–3′)
hupE1.1	CATCCATGAAGTATAGTAAGATTACGAC GACGCTCGTGTAGGCTGGAGCTGC TTCG
hupE1.2	TGCCAGGAAGCCCGCCACGTAGCCCGTC GCGTTTGACATATGAATATCCTCC TTAG
UE1	CATATGAAGTATAGTAAGATTACG
LE1	TCAGCCGACCAGCAGTGCCGCGCC
hupE2.1	GGCGGCGACAACGAAGACGAA
hupE2.2	GACGTGGCTGGATTTGACTTG
hupE2.3	CGCCCGTTTGGTATTCGTCAT
hupE2.4	CGAAAACACCGACCATCACCA
hupE2.5	GGTGGTAAGCTTATGGTGATGGTCGGT GTTTT
hupE2.6	GGTGGTAAGCTTGCCGTCAACGAAAACT CCAT
E2BAD1	GCTAGCGAGGGAATCGAGCAATGAAA
E2BAD2	CTTGACGTGGCTGGATTTGACT
EChupE1	GCTAGCAGGAGGAATTCACCATGAAGT ATAGTAAGAT
E1BAD	CGGGAGCCACGACGGAGCAG
H36I.1	TGCGGGTCTCAACATTCCGTTCAGCG
H36I.2	CGCTGAACGGAATGTTGAGACCCGCA
H43I.1	GCGGCCTCGATATTATCCTGGCGATG
H43I.2	CATCGCCAGGATAATATCGAGGCCGCT
H126I.1	TGCGCGCTCTTCATCGGCCACGTCCAT
H126I.2	GGACGTGGCCGATGAAGAGCGCGCAT
H128I.1	CTCTTCCACGGCATCGTCCATGGCATC
H128I.2	ATGCCATGGACGATGCCGTGGAAGAG
H22I.1	CCCACGCCATTGTCGGGCTGCACGC
H22I.2	GCGTGCAGCCCGACAATGGCGTGGG
H130I.1	CACGGCCACGTCATCGGCATCGAGTTG
H130I.2	CAACTCGATGCCGATGACGTTGGCCGTG
H155I.1	ACGGTCATCCTGATCGTCCTCGGCATC
H155I.2	GATGCCGAGGACGATCAGGATGACCGT
H26I.1	GGGCTGATCGCCGATGGCACGCTTGC
H26I.2	AGCGTGCCATCGGCGATCAGCCCGAC
H22I/H26I.1	CCCACGCCATTGTCGGGCTGCACGCGGG CTGATCGCCGATGGCACGCTTGC
H22I/H26I.2	GCGTGCAGCCCGACAATGGCGTGGGAG CGTGCCATCGGCGATCAGCCCGAC

passages through a French press cell (SLM Aminco, Silver Spring, MD), centrifuged at 6,000 rpm to remove cell debris, and ultracentrifuged at 135,000 × *g* for 1 h. Membrane pellets were resuspended in 600 μl of buffer W and ultracentrifuged again. Finally, membrane pellets were resuspended in 200 μl of buffer W and quantified for protein content (47). SDS-PAGE analysis was performed with 60 μg per sample in 15% acrylamide gels run at 95 V for ca. 1 h and Coomassie blue stained after the run.

RESULTS

Two *hupE* genes are involved in hydrogen oxidation in *R. leguminosarum* bv. *viciae*. The role of *hupE*, a gene located into the *R. leguminosarum* hydrogenase gene cluster, was investigated for SPF25, a strain that expresses hydrogenase activity in microaerobic (1% O_2) free-living cells (10). For this purpose, SPF2714, a nonpolar *hupE* mutant with a 144-bp deletion, was generated (see Materials and Methods). Analysis of hydrogen oxidation in microaerobic free-living cells showed that SPF2714 expressed hydrogenase activity to a level similar to that observed in the wild-type strain (see Table 4), suggesting that either HupE was not necessary for hydrogen uptake or another protein was replacing its role. Inspection of the genome sequence of *R. leguminosarum* bv. *viciae* strain 3841 revealed the presence of an open reading frame, pRL90274, whose predicted product is described as a putative transmembrane hydrogenase-related protein. This protein showed 48% sequence identity with *R. leguminosarum* UPM791 HupE (Table 3). The pRL90274 gene appears as a monocistronic operon flanked by a potential transcriptional regulator adjacent to the *cydDCAB* operon and a gene encoding a putative dehydrogenase (Fig. 1) (52). We investigated the presence of a pRL90274 orthologous gene in UPM791. As described in Materials and Methods, a 1.6-kb DNA fragment was amplified by PCR using genomic DNA of *R. leguminosarum* UPM791 as a template. Sequence analysis of this region revealed the presence of an open reading frame, named *hupE2*, which appeared as a monocistronic operon (Fig. 1). Since previous work in our laboratory had led to the identification of the *cydDCAB* operon in plasmid pRLUPM791b of *R. leguminosarum* UPM791 (unpublished data), we assume this location for the newly identified *hupE2* gene. *hupE2* encodes a 199-amino-acid-residue protein that showed 92% identity with the predicted product of pRL90274 and 48% with the *R. leguminosarum* UPM791 HupE protein. Comparison of the HupE and HupE2 amino acid sequences with the HupE/UreJ proteins from other organisms (Table 3) showed higher percentages of identity for HupE2 (up to 92%) than for HupE (up to 48%). Protein sequence analysis of HupE2 suggested a cleavage site for the signal peptidase and

TABLE 3. Sequence conservation between HupE-like proteins from different bacterial groups and *R. leguminosarum* bv. *viciae* HupE and HupE2

Microorganism	Gene index designation	Value[a] for *R. leguminosarum* UPM791	
		HupE	HupE2
R. leguminosarum bv. *viciae* 3841	116249722	48.0 (61.0)	92.0 (96.0)
Rhizobium etli CFN42	86360024	42.3 (56.7)	79.9 (86.4)
A. caulinodans ORS571	46392545	43.3 (61.6)	54.4 (73.5)
Mesorhizobium loti MAFF303099	13474132	41.1 (54.3)	47.0 (60.4)
Rhodopseudomonas palustris CGA009	39934041	48.3 (62.7)	47.3 (62.7)
Deinococcus radiodurans R1	15807977	50.3 (62.3)	46.7 (59.8)
Methylococcus capsulatus strain Bath	53802532	42.8 (57.7)	40.9 (58.1)
Agrobacterium tumefaciens C58	159185133	42.7 (56.0)	40.1 (55.0)
Ralstonia solanacearum GMI1000	17546753	43.8 (55.8)	39.3 (54.5)
Ralstonia eutropha H16	113867102	41.5 (58.0)	38.6 (55.3)

[a] Values represent the percentages of identity (similarity) between amino acid sequences of HupE-like proteins from each bacterium and HupE/HupE2 from *R. leguminosarum* UPM791.

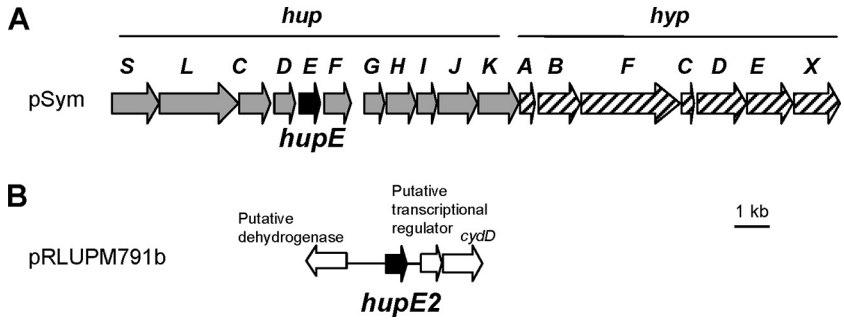

FIG. 1. Genomic context of *hupE* and *hupE2* genes in *R. leguminosarum* bv. *viciae* UPM791. (A) The *hupE* gene is part of the *hupSLCDEF* operon located in the symbiotic plasmid. Expression of *hupE* is controlled by the hydrogenase structural gene promoter. (B) The *hupE2* gene is found in plasmid pRLUPM791b and bears an independent promoter. Black arrows highlight the positions of the *hupE* and *hupE2* genes. Gray and hatched arrows show the *hup* and *hyp* genes, respectively. White arrows indicate genes adjacent to *hupE2* unlinked to hydrogenase biosynthesis.

six potential transmembrane domains in the mature protein, with both N- and C-terminal ends exposed to the periplasm (Fig. 2).

To investigate the role of HupE2 in hydrogenase activity, single *hupE2* (SPF228) and double *hupE hupE2* (SPF22A) mutant strains were generated as described in Materials and Methods. Levels of hydrogenase activity in microaerobic cells of the mutant SPF228 were similar to those in the wild-type strain SPF25 (Table 4). In contrast, the double mutant SPF22A showed a drastic reduction of hydrogenase activity. Supplementation of medium with 1 μM $NiCl_2$ resulted in complete reversion of the hydrogenase-defective phenotype of SPF22A (Table 4). We also analyzed the effect of nitrilotriacetate (NTA) on hydrogenase activity in the *hupE* and *hupE2* mutants. NTA is a metal chelator that shows a higher binding affinity for Ni^{2+} than for other metal ions, such as Co^{2+}, Mn^{2+}, and Zn^{2+} (25). The presence of 50 μM NTA did not have an effect on hydrogen oxidation in the wild-type or *hupE2* mutant strains. However, cells from the *hupE* mutant SPF2714 reached only 50% of the hydrogenase activity (Table 4). These results indicate that HupE and HupE2 have similar functions, likely related to nickel transport, that are required for hydrogenase activity in microaerobic free-living cells. However, HupE2 is apparently less efficient in providing nickel to hydrogenase when this metal is present at low concentrations, as is the case when the NTA chelator is present.

Since nickel is also a constituent of urease, a metalloenzyme present in *R. leguminosarum* UPM791 (48), we also checked the effect of the inactivation of both *hupE* genes on the ability of the cells to grow on minimal medium modified to contain urea as the only nitrogen source. In these experiments, we found no significant differences in growth between the *hupE hupE2* double mutant and the wild type, whereas a urease-deficient mutant (URE4) (48) showed a strong reduction in growth (data not shown). These data suggest that the residual levels of nickel uptake responsible for the very low hydrogenase activity observed in the double mutant are likely sufficient to sustain urease-based growth.

HupE and HupE2 restore hydrogenase activity and nickel transport in an *E. coli nik* mutant. To investigate the role of *R. leguminosarum* HupE and HupE2 proteins in hydrogenase biosynthesis, transport of $^{63}Ni^{2+}$ in strains SPF25 and SPF22A was assayed under aerobic conditions as well as under the microaerobic conditions that induce hydrogen oxidation. For these assays, cells were incubated with 100 nM or 1 μM $^{63}Ni^{2+}$ in a magnesium-containing buffer. However, no conclusive results could be obtained, since controls with disrupted cells of wild-type and mutant strains showed levels of nickel accumulation similar to those in the corresponding cell cultures (data not shown), suggesting a high level of nonspecific Ni^{2+} binding to the bacterial surface.

For *E. coli*, the ABC-type nickel transport system *nikABCDE* and the phenotype of the HYD723 Nik^- mutant have been well characterized (35). In the mutant, nickel transport is impaired, and hence, no hydrogenase activity is detected in a nickel-depleted culture medium. We first investigated complementation of hydrogen oxidation in HYD723 through expression of the *hupE* and *hupE2* genes. Plasmids pBADE1 and pBADE2 containing the *hupE* and *hupE2* gene coding sequences, respectively, under the arabinose PBAD promoter system were introduced into HYD723. To induce hydrogen oxidation, strains were grown under anaerobic conditions in a TGYEP modified medium containing glycerol and fumarate. Under these conditions, high levels of hydrogenase activity were observed in wild-type strain MC4100, whereas *nik* mutant HYD723 carrying control plasmid pABD18-Kan showed very low levels of activity (Fig. 3). This defective phenotype was reverted by supplementation of Ni into the medium (data not shown). High levels of hydrogen oxidation were also detected in cultures of strains HYD723(pBADE1) and HYD723 (pBADE2) under low-Ni conditions, but only when arabinose was added as an inducer to the culture medium (Fig. 3). These results indicate that HupE and HupE2 can individually complement the function of the NikABCDE nickel transport system, which is lacking in strain HYD723, thus restoring hydrogenase activity in *E. coli*.

We then investigated the nickel transport capability of *R. leguminosarum* HupE and HupE2 in *E. coli* cells by measuring $^{63}Ni^{2+}$ uptake in the complemented strains. Strain HYD723 derivatives carrying plasmids pBADE1, pBADE2, and pBAD18-Kan were incubated aerobically with 150 nM $^{63}Ni^{2+}$ and arabinose as an inducer of PBAD promoter expression. In these experiments, expression of HupE led to much higher levels of nickel accumulation than those observed with the *E. coli* mutant strain carrying control plasmid pBAD18-Kan (Fig. 4). Surprisingly, expression of HupE2 (HYD723 carrying plas-

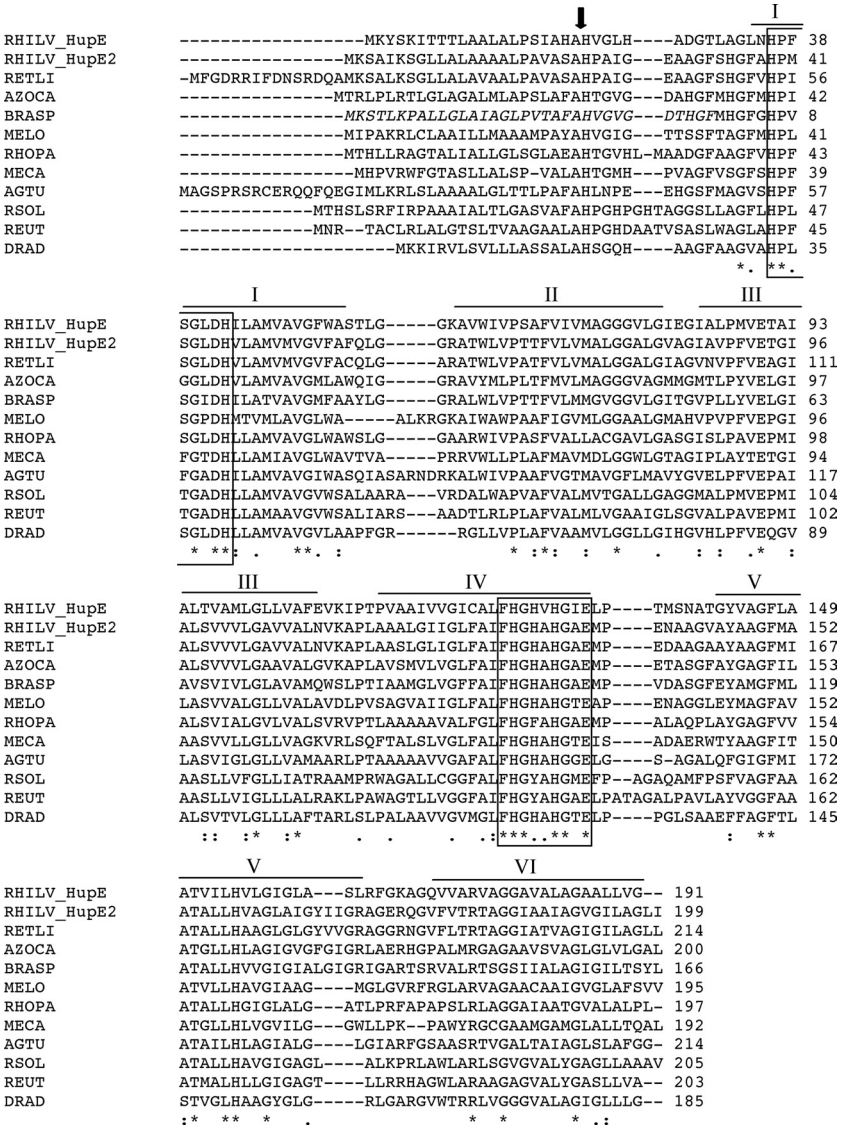

FIG. 2. Sequence alignment of HupE/UreJ proteins. Asterisks indicate identical amino acids. Horizontal lines correspond to transmembrane domains, designated by roman numerals, as predicted using TMHMM2 software. The vertical arrow shows the predicted cleavage site for a signal peptidase. Boxed residues correspond to conserved regions in HupE/UreJ proteins. RHILV, *R. leguminosarum* bv. *viciae* 3841 (GI|116249722); RETLI, *R. etli* CFN42 (GI|86360024); AZOCA, *Azorhizobium caulinodans* ORS571 (GI|46392545); BRASP, *Bradyrhizobium* sp. BTAi (GI|148252085); MELO, *Mesorhizobium loti* MAFF303099 (GI|13474132); RHOPA, *Rhodopseudomonas palustris* CGA009 (GI|39934041); DRAD, *Deinococcus radiodurans* R1 (GI|15807977); MECA, *Methylococcus capsulatus* strain Bath (GI|53802532); AGTU, *Agrobacterium tumefaciens* C58 (GI|159185133); RSOL, *Ralstonia solanacearum* GMI1000 (GI|17546753); REUT, *Ralstonia eutropha* H16 (GI|113867102). Inspection of the sequence corresponding to BRASP HupE revealed an annotation mistake in the database. The corrected sequence includes 33 additional residues (shown in italics) at the N terminus.

TABLE 4. Hydrogenase activity in microaerobic free-living cells from *R. leguminosarum hupE*- and *hupE2*-deficient mutants

Strain	Hydrogenase activity[a] obtained with:		
	No metal added	NTA[b]	$NiCl_2$[b]
SPF25	820 ± 160	920 ± 70	1,000 ± 260
SPF2714 Δ*hupE*	890 ± 170	520 ± 50	1,140 ± 100
SPF228 Δ*hupE2*	930 ± 240	1,120 ± 20	1,110 ± 270
SPF22A Δ*hupE* Δ*hupE2*	140 ± 70	40 ± 20	1,300 ± 100

[a] Values are expressed in nmol H_2 h^{-1} mg $prot^{-1}$. Hydrogenase activities were obtained using oxygen as the electron acceptor. Values are averages of results from four experiments ± SE.

[b] Nickel chloride and nitrilotriacetate were added at 1 μM and 50 μM, respectively.

mid pBADE2), which was shown to fully complement the *nik* mutation for hydrogenase activity, resulted in Ni accumulation levels that were ca. 20-fold lower than those mediated by HupE although still significantly higher than those detected in cells carrying control plasmid pBAD18-Kan. These results indicate that both HupE and HupE2 are nickel permeases. Both proteins, however, appear to differ in their affinities or efficiencies in transporting this metal. Analysis of the expression levels of both HupE and HupE2 in arabinose-induced *E. coli* cells was attempted by SDS-PAGE of membrane fractions followed by Coomassie blue staining. In these experiments, we were not

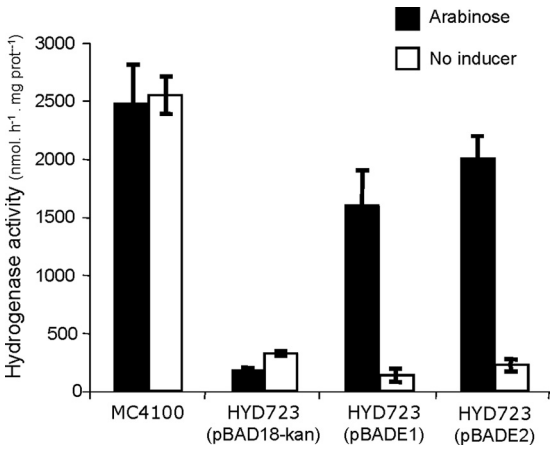

FIG. 3. Effect of HupE and HupE2 on the hydrogenase activity of the *E. coli* HYD723 *nik* mutant. Wild-type strain MC4100 and *nik* mutant HYD723 expressing pBAD-derivative plasmid pBADE1 (*hupE*) or pBADE2 (*hupE2*) or control plasmid pBAD18-Kan were assayed for hydrogenase activity in anaerobic cultures grown in a glycerol-fumarate medium for 5 h with 200 μM arabinose or without inducer. Values for hydrogenase activities are averages of results from four independent assays, with error bars corresponding to standard errors (SE). prot, protein.

able to detect any protein band associated with arabinose-inducible HupE expression (data not shown), suggesting that very low levels of HupE protein are required for full hydrogenase activity in *E. coli*. Since we could not detect HupE, we cannot exclude the possibility that HupE2 is produced at lower levels.

Identification of amino acid residues required for HupE activity. Expression of HupE in the *E. coli* heterologous system was used to investigate HupE amino acid residues required for nickel transport. Analysis of amino acid sequences of *R. leguminosarum* HupE and HupE-like proteins from other

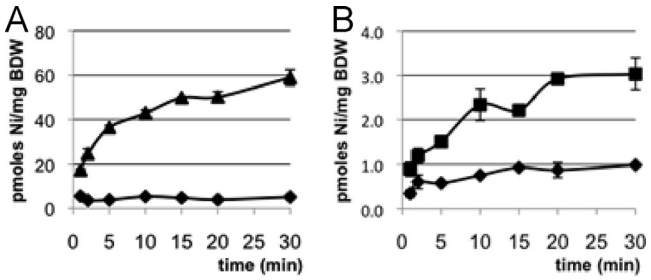

FIG. 4. Nickel transport of the *E. coli* HYD723 *nik* mutant as a function of HupE and HupE2 expression. Strain HYD723 derivatives harboring plasmid pBADE1 (*hupE*; ▲ in panel A) or pBADE2 (*hupE2*; ■ in panel B) or control plasmid pBAD18-Kan (◆ in both panels) were grown in Luria-Bertani medium under aerobic conditions at mid-log phase (OD_{600}, ≈0.5). Then, arabinose (final concentration, 200 μM) was added to induce PBAD expression. The uptake assay was performed in the presence of 150 nM $^{63}Ni^{2+}$ by using a cell density equal to that in the original culture for pBADE1 (A) or 10-fold higher for pBADE2 (B). Aliquots (100 μl) were filtered at the indicated times. The intracellular concentration of ^{63}Ni per milligram of bacteria (dry weight) (BDW) was determined. The values shown represent a typical experiment and are averages of results from two replicates, with error bars indicating standard deviations. Error bars smaller than the symbols are not shown.

TABLE 5. Hydrogenase activity in *E. coli* HYD723 expressing pBADE1 or derivative plasmids carrying mutations in histidine residues of HupE

Plasmid	Hydrogenase activity[a] obtained with:	
	200 μM arabinose	No inducer
pBADE1	1,700 ± 420	20 ± 20
pBAD18-Kan	100 ± 80	40 ± 30
pBADE1.H22I	2,450 ± 670	70 ± 60
pBADE1.H26I	1,390 ± 200	100 ± 30
pBADE1.H22I/H26I	720 ± 240	40 ± 10
pBADE1.H36I	70 ± 20	210 ± 10
pBADE1.H43I	260 ± 130	210 ± 100
pBADE1.H126I	610 ± 190	30 ± 30
pBADE1.H128I	1,960 ± 510	60 ± 40
pBADE1.H130I	190 ± 100	140 ± 40
pBADE1.H155I	1,190 ± 60	40 ± 10

[a] Hydrogenase activities (expressed in nmol H_2 h^{-1} mg $prot^{-1}$) in anaerobic cells were measured, using oxygen as an electron acceptor. Values are means of results from three independent assays ± SE.

microorganisms revealed the presence of a histidine domain (HX_5DH [motif I]) located in transmembrane domain I (Fig. 2). This domain is highly similar to a domain (HX_4DH) that has been reported as essential for nickel transport in permeases of the NiCoT family, such as HoxN of *Cupriavidus necator* (formerly *Ralstonia eutropha*) and NixA of *Helicobacter pylori* (17, 23, 24). An additional histidine-rich sequence (FHGX[AV]HGXE [motif II]), located in transmembrane domain IV, was highly conserved in HupE/UreJ proteins from different microorganisms (Fig. 2). To analyze the importance of these sequences in HupE functionality, conserved histidine residues H36 and H43, located in motif I; H126 and H130, present in motif II; and H22 and H155, located at the N-terminal and C-terminal parts of the protein, respectively, were replaced by isoleucine through site-directed mutagenesis. In addition, single mutants were generated in nonconserved histidine residues H26 and H128 (see Materials and Methods for details). Expression of mutated variants of HupE under the PBAD promoter system was induced in the *E. coli nik* mutant HYD723, and hydrogenase activity was measured under the anaerobic conditions previously established. Expression of the different mutant forms of HupE protein led to three different phenotypes (Table 5). Variants of HupE mutated in residues H22, H26, H128, and H155 showed wild-type levels of hydrogen oxidation. In contrast, hydrogenase activity was drastically reduced in strain HYD723 expressing versions H36I, H43I, and H130I of HupE, whereas an intermediate phenotype was associated with the H126I HupE variant, which retained 35% of the hydrogenase activity. We also generated a double mutant involving residues H22 and H26, located in the N-terminal part of HupE and predicted to be exposed to the periplasm. Strain HYD723 expressing this variant protein showed reduced (42%) hydrogenase activity in comparison to the strain complemented with wild-type plasmid pBADE1. These data indicate that conserved histidine residues located in motifs I and II are required for the function or stability of the HupE protein.

HupE is required for symbiotic hydrogen oxidation. Symbiotic hydrogen uptake is a particular trait present only in certain strains of the *Rhizobium* group (2). We investigated the role of

TABLE 6. Hydrogenase activity in bacteroid cells as a function of *R. leguminosarum hupE* and *hupE2* genes

Host	Strain	Hydrogenase activity[a] obtained with:	
		No $NiCl_2$ added	85 μM $NiCl_2$
Pisum sativum	SPF25	2,010 ± 500	5,580 ± 650
	SPF2714 Δ*hupE*	2,820 ± 160	5,740 ± 440
	SPF228 Δ*hupE2*	3,460 ± 140	4,380 ± 680
	SPF22A Δ*hupE* Δ*hupE2*	2,200 ± 350	5,430 ± 910
	SPF25(pALPF1)	2,480 ± 210	11,120 ± 1,150
	SPF22A(pALPFE)	2,710 ± 530	10,250 ± 700
Lens culinaris	SPF25(pALPF1)	1,100 ± 350	2,690 ± 450
	SPF22A(pALPFE)	360 ± 120	2,080 ± 650
Lotus corniculatus	MAFF303099(pALPF1)	1,910 ± 320	3,930 ± 200
	MAFF303099(pALPFE)	1,100 ± 60	3,570 ± 560
Phaseolus vulgaris	CIAT899(pALPF1)	1,210 ± 290	1,350 ± 180
	CIAT899(pALPFE)	360 ± 140	940 ± 240
	CE3(pALPF1)	6,730 ± 1,540	5,410 ± 320
	CE3(pALPFE)	5,600 ± 1,610	4,890 ± 920

[a] Values are expressed in nmol H_2 h^{-1} mg $protein^{-1}$. Hydrogenase activities were measured using oxygen as an electron acceptor. Values were determined for bacteroids from 21-day-old plants and represent averages of results from at least two experiments ± SE.

HupE and HupE2 in hydrogen oxidation in bacteroid cells of strains SPF25, SPF2714 (*hupE*), SPF228 (*hupE2*), and SPF22A (*hupE hupE2*) obtained from 21-day-old pea nodules. In contrast to what was observed for the vegetative cells, no differences in hydrogenase activity were observed among bacteroids of wild-type and mutant strains from plants grown without nickel supplementation (Table 6). In order to increase the nickel demand inside the bacteroid cell, extra copies of the hydrogenase gene cluster were introduced. To this effect, plasmids pALPF1, containing the *R. leguminosarum hup* cluster, and pALPFE, a pALPF1 derivative containing a nonpolar *hupE* mutation (see Materials and Methods), were introduced into strains SPF25 and SPF22A, respectively. Pea bacteroids from SPF25(pALPF1) and SPF22A(pALPFE) were assayed for hydrogenase activity (Table 6). Despite the extra copies of the *hup* gene cluster, no reduction in hydrogen oxidation was observed under any plant growth condition tested (i.e., nutrient solution supplemented or not supplemented with nickel). We then analyzed symbiotic hydrogenase activity in the *R. leguminosarum* SPF25-*Lens culinaris* association. This symbiosis requires larger amounts of nickel in the plant nutrient solution for hydrogenase activity (11). As shown in Table 6, bacteroids of strain SPF22A(pALPFE) obtained from lentil plants grown on a standard nutrient solution were strongly impaired in hydrogen oxidation in comparison to those of strain SPF25(pALPF1). Hydrogenase activity in SPF22A(pALPFE) bacteroids was restored by cultivation of lentil plants on a nutrient solution containing 85 μM $NiCl_2$.

The relevance of HupE in symbiotic hydrogen oxidation was further investigated for other rhizobium-legume symbioses (Table 6). Plasmids pALPF1 and pALPFE were introduced into *Mesorhizobium loti* MAFF303099, an endosymbiont of different *Lotus* species, and *Rhizobium tropici* CIAT899 and *Rhizobium etli* CE3, which establish symbiosis with *Phaseolus vulgaris*. Bacteroids of MAFF303099(pALPFE) extracted from 21-day-old *Lotus corniculatus* plants grown without nickel addition showed a 42% reduction in hydrogenase activity in comparison to the level for the wild-type strain MAFF303099 (pALPF1). Addition of nickel to the nutrient solution restored hydrogen oxidation to wild-type levels. In the case of *Rhizobium*-bean symbioses, hydrogen uptake of CIAT899(pALPFE) bacteroid cells decreased more than 70% compared to that of CIAT899(pALPF1), and again, this phenotype was reverted by incorporation of nickel in the plant nutrient solution. In contrast, no difference in hydrogenase activity was observed between CE3(pALPF1) and CE3(pALPFE) bacteroids obtained from bean plants grown in plant nutrient solutions supplemented or not supplemented with nickel. The latter result can be explained by the presence in *R. etli* CE3 of a *hupE* orthologous gene, showing 42% sequence identity with *R. leguminosarum* HupE, that might complement its function in bean bacteroids (Table 3). In conclusion, these results indicate that HupE and HupE2 are involved in symbiotic hydrogenase activity providing nickel to the bacteroid cell inside the nodule. The relevance of their role depends on the particular legume-rhizobium symbiosis, suggesting the occurrence of alternative mechanisms in the host plant that replace the function of HupE and HupE2.

DISCUSSION

In this paper, we characterize the hydrogenase-related protein HupE as a nickel transporter required for hydrogenase activity in microaerobic free-living cells and bacteroids of *R. leguminosarum* bv. *viciae* UPM791. We have also identified HupE2, a HupE-homolog that displays a similar role in this strain. HupE and HupE2 belong to the HupE/UreJ family of single component metal permeases. An increasing number of *hupE*-like genes have been identified as a result of bacterial genome sequencing efforts. Some of them are linked to hydrogenase or urease gene clusters, but many appear as isolated genes in the bacterial genome. A recent database search identified 103 transporters of this family in a wide diversity of bacteria, including members of several proteobacterial subdi-

visions and cyanobacteria (53). Members of the HupE/UreJ family of transporters have been postulated as nickel transporters on the basis of their features as membrane proteins, the presence of amino acid sequence motifs involved in nickel transport in other proteins, and their association with nickel-containing systems such as hydrogenase or urease (3, 33). Functional evidence for a direct involvement of HupE in nickel transport had been obtained by measuring nickel accumulation in *E. coli* cells that express the *R. palustris* HupE protein (18). However, no reports had demonstrated the physiological role of a HupE-based transport system in the activity of the associated metalloenzyme. In this work, we identified two *hupE*-like genes whose simultaneous disruption led to a drastic reduction of hydrogenase activity in *R. leguminosarum* vegetative cells. Hydrogenase activity was recovered upon the addition of nickel to the culture medium, indicating that the defect in the double mutant was at the level of provision of nickel to the enzyme. The presence of residual activity in the double mutant indicates that nickel can enter the cell via some other mechanisms, such as nonspecific magnesium transporters (50). Nickel-dependent complementation of metalloenzyme activity has previously been described. Nickel transport mediated by the *Ralstonia eutropha* HoxN nickel permease, a member of the NiCoT family, could be demonstrated in an *E. coli* heterologous system involving urease activity (50). In *R. palustris*, a HupE-deficient mutant was impaired in hydrogenase activity, but this phenotype was not recovered by an excess of nickel (18). In contrast, a HupE-deficient mutant from the cyanobacterium *Synechocystis* sp. PCC6803 was not affected in hydrogenase activity (28).

Hydrogenase activity and nickel uptake assays with *R. leguminosarum* HupE overexpressed in *E. coli* further confirmed that this protein mediates Ni transport and promotes hydrogenase activity, indicating a physiological role for this transport. A technical problem, likely related to nonspecific binding of nickel to *Rhizobium* cells, prevented direct transport experiments in the original genetic background. This nonspecific binding of nickel to cell structures has previously been reported as a major problem interfering with the analysis of nickel transport in different bacteria and in liposome systems (50). Nickel transport assays performed with *E. coli* cells indicate that HupE is able to mediate a highly efficient nickel uptake system. More interestingly, expression of HupE and HupE2 proteins in the *E. coli* mutant HYD723 showed different levels of nickel transport. These differences in Ni accumulation rates mediated by HupE and HupE2 were not due to the different growth conditions used for both sets of experiments, since cells grown anaerobically showed a similar pattern of results (data not shown). The different uptake activities might indicate either different levels of active protein or different affinities and/or capacities of these transport proteins.

The site-directed mutagenesis analysis performed for the present report is the first attempt to study functional requirements in a member of the HupE/UreJ family of metal permeases. Data from mutagenesis of histidine residues suggest essential roles associated with two motifs, HX_5DH and FHGX[AV]HGXE, conserved in the HupE/UreJ family. Assuming that the identified signal peptide is actually cleaved, we have integrated our data into a hypothetical model for mature HupE topology (Fig. 5), based on previous models proposed

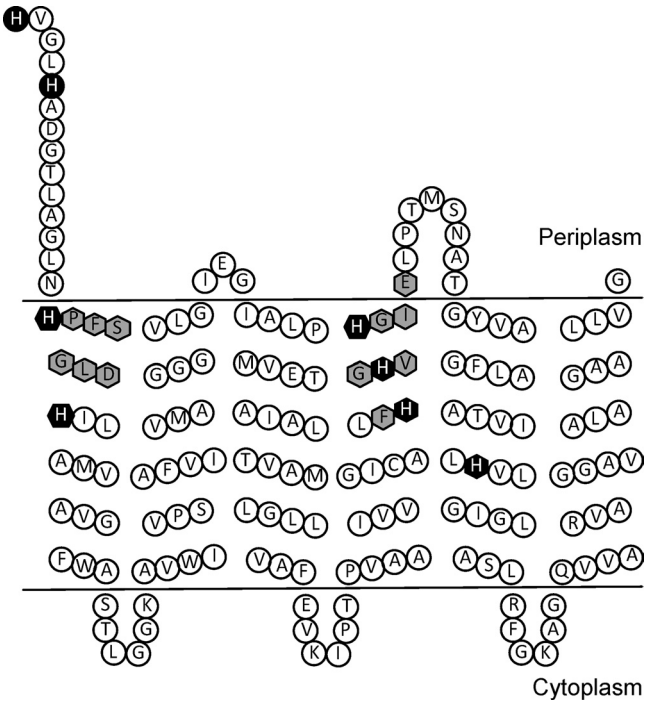

FIG. 5. Predicted topology of the *Rhizobium leguminosarum* HupE protein and localization of amino acid residues involved in activity. Proposed model of HupE topology based on hydropathy predictions and previous models for *C. necator* HoxN and *H. pylori* NixA (19, 24). Transmembrane domains are as predicted by DAS and HMMTOP 2.0 bioinformatic programs. Histidine residues are colored black. Residues highlighted in gray correspond to those participating in the conserved domains proposed as essential for nickel transport. Residues corresponding to a predicted cleavable signal peptide have been removed.

for other Ni permeases (19, 24). According to this model, the identified motifs I and II would be located within predicted transmembrane domains I and IV, respectively, and would participate in a membrane channel that allows the passage of nickel through the cytoplasmic membrane. Validation of this hypothesis requires additional experimentation to exclude the possibility that the histidine residues within the identified motifs are required for protein stability or membrane insertion.

The first motif is similar to the motif (HX_4DH) identified as essential for nickel uptake in permeases of the NiCoT and UreH families (50). Changes of the histidine residues in this motif suppressed the ability of HupE to provide nickel for hydrogenase synthesis. The second motif (FHGX[AV]HGXE) is likely specific for the HupE/UreJ family and had not previously been identified (Fig. 2). Mutagenesis data indicate that at least one of the histidine residues in the motif (H130) is essential for hydrogenase activity but that alteration of the other residue (H126) leads to reduced levels. A search of nonredundant protein database at NCBI for the sequence FHGX[AV]HGXE revealed that it is present in 142 proteins, all of them members of the HupE/UreJ family. These data suggest that this motif is a specific signature for permeases of this family. A different motif ($GX_5GHSSVV$) has been described for the NiCoT family of transporters; the histidine residue within this motif has been shown as essential for NixA func-

tionality (23). Such a motif is not present in HupE-like proteins. Finally, analysis of histidine residues in the N-terminal part of HupE suggests an interchangeable or additive effect of H22 and H26. Based on their predicted periplasm-exposed location and on the high nickel affinity of histidine residues, we hypothesize a role in ion scavenging and concentration around the transporter (Fig. 5). Then, histidine residues from motifs I and II might bind the ion with higher affinity and introduce it into the cell. Since these residues are well conserved in HupE/UreJ proteins from different microorganisms, an essential role for them in nickel transport in other bacterial backgrounds can be assumed.

The results reported here also indicate that HupE and HupE2 are involved in symbiotic hydrogenase activity, likely by providing nickel to the bacteroid cell inside the nodule. The presence of *hupE* homologs in *Rhizobium* genomes is not universal. For instance, this gene is absent in sequenced strains of *Sinorhizobium meliloti* and *B. japonicum.* In contrast, genomes from *R. etli* CE3 and *M. loti* MAFF303099 contain *hupE*-like genes (designated RHE_PB00145 and mll4945, respectively) whose predicted proteins show over 40% sequence identity with HupE and ca. 80% and 50%, respectively, with HupE2. These *hupE2*-like genes are probably responsible for the significant levels of hydrogenase activity induced by the *hupE*-deficient pALPFE *hup* cluster in these two strains. In the case of *R. tropici* CIAT899, the genome sequence is not available but the low levels of symbiotic hydrogenase activity associated with pALPFE suggest that strain CIAT899 does not contain the *hupE* gene.

The presence of two copies of a *hupE* gene in *R. leguminosarum* UPM791 might be related to the plasmid location of the corresponding genes. HupE is encoded by a gene within the hydrogenase gene cluster in the symbiotic plasmid, whereas *hupE2* is located in plasmid pRLUPM791b, unrelated to hydrogenase genes. While it is clear that the *hupE* gene, part of a highly conserved *hup* cluster (20), is evolutionarily related to hydrogenase genes, the rationale for a *hupE2* gene in *R. leguminosarum* is less clear. The low level of amino acid sequence identity between *hupE* and *hupE2* suggests that these two proteins may play different roles, either providing nickel with a different capacity/affinity or transporting a different ion. In fact, HupE from *Synechocystis* sp. PCC6803 is likely a cobalt transporter required for the activity of a cobalamin-dependent methionine synthase (28). In that system, as well as in *hupE* genes from other cyanobacteria, sequences encoding a B_{12}-dependent riboswitch have been identified upstream of *hupE*, as reported for other cobalt transporters (39). Such elements are not present upstream of the *R. leguminosarum hupE* and *hupE2* coding sequences (data not shown). Further analyses of HupE2 transport capacity and expression will help to elucidate this point.

Analysis of the role of HupE and HupE2 in symbiotic hydrogenase activity revealed differences for the *R. leguminosarum* symbioses with pea and lentil. The lack of a hydrogenase phenotype of the *hupE hupE2* double mutant in pea bacteroids suggests the existence of alternative mechanisms that replace the function of HupE and HupE2 in this symbiosis. Surprisingly, Ni provision by HupE/HupE2 was required in lentil bacteroids, suggesting that these potential alternative mechanisms do not operate in lentils. In symbiosis, bacteroid nickel ions are obtained from the plant cytosol surrounding the bacteroids. In this environment, nickel is likely to be present not as a free ion but rather as complexes with organic compounds (13). Although the possibility that different nickel transporters are induced in pea bacteroids but are absent in cultured cells and lentil bacteroids cannot be excluded, we favor the hypothesis that the observed differences are due to the presence of different nickel complexes in both symbioses (11). We have previously shown that bacteroid hydrogenase activity is reduced in lentil symbiosis due to both transcriptional and posttranscriptional factors (11), and similar limitations were observed when other symbiotic systems were analyzed for heterologous hydrogenase expression (8). One of these factors is apparently related to incorporation of nickel into the hydrogenase. Since the lentil nodule cytosol contains nickel levels similar to those observed in pea nodules (11), it is possible that the observed differences are due to differences in the natures of the nickel forms present. Confirmation of this hypothesis will require further analysis of the composition of nickel complexes in the cytoplasmic fractions of pea and lentil nodules. This analysis is currently under way in our laboratory.

The data presented here represent a first step toward the understanding of nickel provision by HupE, a member of a widespread bacterial nickel transporter family. Further work will allow us to get insight into the mechanism of metal provision in endosymbiotic bacteria in both free-living and symbiotic lifestyles.

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