

Engineering the *Rhizobium leguminosarum* bv. *viciae* Hydrogenase System for Expression in Free-Living Microaerobic Cells and Increased Symbiotic Hydrogenase Activity

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***Rhizobium leguminosarum* bv. *viciae* UPM791 induces hydrogenase activity in pea (*Pisum sativum* L.) bacteroids but not in free-living cells. The symbiotic induction of hydrogenase structural genes (*hupSL*) is mediated by NifA, the general regulator of the nitrogen fixation process. So far, no culture conditions have been found to induce NifA-dependent promoters in vegetative cells of this bacterium. This hampers the study of the *R. leguminosarum* hydrogenase system. We have replaced the native NifA-dependent *hupSL* promoter with the FnrN-dependent *fixN* promoter, generating strain SPF25, which expresses the *hup* system in microaerobic free-living cells. SPF25 reaches levels of hydrogenase activity in microaerobiosis similar to those induced in UPM791 bacteroids. A sixfold increase in hydrogenase activity was detected in merodiploid strain SPF25(pALPF1). A time course induction of hydrogenase activity in microaerobic free-living cells of SPF25(pALPF1) shows that hydrogenase activity is detected after 3 h of microaerobic incubation. Maximal hydrogen uptake activity was observed after 10 h of microaerobiosis. Immunoblot analysis of microaerobically induced SPF25(pALPF1) cell fractions indicated that the HupL active form is located in the membrane, whereas the unprocessed protein remains in the soluble fraction. Symbiotic hydrogenase activity of strain SPF25 was not impaired by the promoter replacement. Moreover, bacteroids from pea plants grown in low-nickel concentrations induced higher levels of hydrogenase activity than the wild-type strain and were able to recycle all hydrogen evolved by nodules. This constitutes a new strategy to improve hydrogenase activity in symbiosis.**

In the nitrogen fixation process in legume root nodules, a large amount of hydrogen is released from nodules as an obligate by-product of the nitrogenase reaction. This hydrogen production is one of the major factors that affect the efficiency of symbiotic nitrogen fixation (39). In certain strains of *Bradyrhizobium japonicum* and *Rhizobium leguminosarum*, a hydrogen uptake (Hup) system oxidizes the hydrogen produced by the nitrogenase. This H₂ recycling reduces energy losses and has been shown to enhance legume productivity (11, 25).

The hydrogenase systems from *B. japonicum* and *R. leguminosarum* bv. *viciae* have been extensively characterized (for a review, see reference 34). In both cases, a membrane-bound heterodimeric [NiFe] hydrogenase is responsible for hydrogen uptake. The genetic determinants for hydrogen oxidation are clustered in large DNA regions spanning ca. 20 kb (21, 22); on the basis of sequence analysis, these regions have similar *hup* gene organizations. These hydrogenase clusters are composed by 17 common genes, *hupSLCDFGHIJK* and *hypABFCDEX*, which display a high sequence similarity (32 to 69% identity [34]). Most of these genes are also found in hydrogenase systems from other H₂-oxidizing bacteria, suggesting that they encode proteins with essential functions in hydrogenase biosynthesis. Among them, the *hupS* and *hupL* genes code for the

small and large hydrogenase structural polypeptides, respectively (16, 37). These polypeptides are synthesized as precursors in a soluble, immature form; after insertion of nickel, iron, and other metal groups, the polypeptides are processed into the active subunits and translocated into the membrane (for a review see reference 9). As has been shown for the hydrogenase-3 of *Escherichia coli*, the processing step implies the proteolytic cleavage of both an N-terminal signal peptide in HupS and a C-terminal peptide in HupL by a specific protease (32, 43). The recruitment and incorporation of metallic groups into the hydrogenase active site require the contribution of the remaining Hup and Hyp proteins in a complex and not completely elucidated process.

Despite the similarities, *B. japonicum* and *R. leguminosarum* hydrogenase systems show important differences, mainly in terms of gene composition and hydrogenase regulation (35). The *B. japonicum* *hup* gene cluster lacks the *hupE* gene present in *R. leguminosarum* but contains the *hupNOP*, *hupUV*, and *hoxAT* operons. It has been shown that HupNOP are involved in nickel metabolism (12) and that HoxA is a transcriptional regulator required for hydrogenase activation in microaerobic free-living cultures in the presence of hydrogen and nickel (10, 18, 44, 46). Functions of HupUV and HupT are not well established, but it has been proposed that HupUV might constitute a pseudohydrogenase that would act as a sensor for the adequate environmental conditions required for hydrogenase activity (4), while HupT could transduce the signal sensed by HupUV to HoxA by modifying its transcription-inducing ac-

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Source or reference
<i>R. leguminosarum</i>		
UPM791	128C53 Str ^t	24
SPF25	UPM791 with <i>PfixN::hupSL</i>	This work
DG2	UPM791 <i>furN1 furN2</i>	13
<i>E. coli</i>		
S17.1	<i>thi pro hsdR hsdM⁺ recA</i> RP4 2-Tc::Mu-Km::Tn7	41
C2110	<i>polA</i> Nal ^r	G. Ditta
Plasmids		
pAL618	Cosmid containing the UPM791 <i>hup</i> cluster; Tc ^r	23
pK18 <i>mobsac</i>	pK18 derivative <i>sacB</i> ; Km ^r	38
pSPV4	pMP220 derivative; Tc ^r	29
PCR2.1-TOPO	PCR product cloning vector; Ap ^r	Invitrogen
pALPF1	<i>PfixN::hupSL</i> in pAL618	This work
pSPF1	<i>PfixN::hupSL</i> in pSPV4	This work
pKPF1	<i>PfixN::hupSL</i> in pK18 <i>mobsac</i>	This work
pPF1	<i>PfixN</i> in PCR2.1-TOPO	This work
pPF2	<i>PfixN::hupSL</i> in PCR2.1-TOPO	This work
pDH1	<i>hupSL</i> in PCR2.1-TOPO	This work
pIM1	<i>'orf3orf4</i> in PCR2.1-TOPO	This work

tivity via phosphorylation (45). In contrast, *R. leguminosarum* UPM791 contains a truncated, functionally inactive HoxA, and no genes homologous to either *hupNOP*, *hupUV*, or *hupT* (7, 8). The absence of these genes may explain why hydrogenase expression in *R. leguminosarum* is observed only in symbiosis, while *B. japonicum* is able to induce hydrogenase activity in free-living as well as in symbiotic conditions. The analysis of *hupSL* promoter (*PhupSL*) expression showed that *hup* gene transcription is activated by NifA, the key regulator of the nitrogen fixation process (8). The fact that NifA-dependent promoters, such as *nif* promoters, are not induced in free-living conditions confines *R. leguminosarum hup* gene expression to the nodule environment.

The requirement for symbiotic hydrogenase expression has hampered the characterization of the hydrogenase system in *R. leguminosarum* bv. viciae. Experiments related to hydrogenase purification, the analysis of its regulation, and the elucidation of specific functions for the *hup* and *hyp* gene products have been delayed due to this fact. In an attempt to overcome this drawback, we have engineered *R. leguminosarum* strain UPM791 to obtain hydrogenase activity in microaerobic free-living cells. Interestingly, the modified strain also displays higher levels of hydrogenase activity in symbiotic conditions.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this work are listed in Table 1. *R. leguminosarum* strain UPM791 (24) was routinely grown in tryptone-yeast extract (2) or yeast-mannitol (YMB) (47) media at 28°C. *E. coli* strains were grown in Luria-Bertani medium. Antibiotics were added at the following concentrations: tetracycline, 5 µg·ml⁻¹; kanamycin, 50 µg·ml⁻¹; ampicillin, 100 µg·ml⁻¹; spectinomycin, 50 µg·ml⁻¹. Cosmid pAL618 is a pLAFR1 derivative carrying the hydrogenase gene cluster from UPM791 in a 20-kb DNA fragment (23). Strain SPF25 is a UPM791 derivative in which the NifA-dependent *PhupSL* promoter has been replaced by the microaerobically expressed *PfixN* promoter (see below). Cosmid pALPF1 is a pAL618 derivative carrying the *PfixN::hupSL* construct. Plasmids were introduced into *R. leguminosarum* by conjugation, and transconjugants were selected in *Rhizobium* minimal medium (Rm) (28) supplemented with the corresponding

antibiotic. For stoppered-tube hydrogenase induction assays with free-living microaerobic cells, cultures were aerobically grown in Rm or YMB medium to an optical density at 600 nm (OD₆₀₀) of 0.2 in a 200-ml flask, which was then tightly capped, evacuated, and flushed several times with 1% O₂ and incubated for 16 h at 28°C. For hydrogenase induction in the fermentor (BIOFLO C30; New Brunswick Scientific, Edison, N.J.), *R. leguminosarum* cultures were aerobically grown to an OD₆₀₀ of 0.35 in a flask and then incubated in the fermentor at 28°C, 400-rpm agitation, and continuous flow of 0.8% O₂.

DNA manipulation techniques. Plasmid DNA preparations, restriction enzyme digestions, agarose gel electrophoresis, DNA cloning, and transformation of DNA into *E. coli* cells were carried out by standard protocols (36). Genomic DNA of *R. leguminosarum* was extracted as previously described (24). For Southern hybridizations, DNA probes were labeled with digoxigenin and the hybridizing bands were visualized with a chemiluminescent DIG detection kit as described by manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). DNA sequencing was carried out using the rhodamine terminator cycle sequencing ready reaction kit and an ABI377 automatic sequencer (PE Biosystems, Foster City, Calif.).

Construction of the *PfixN::hupSL* fusion. A 320-bp DNA fragment containing the *PfixN* promoter of *R. leguminosarum* was amplified by PCR using primers PF1 (5'-GGATCCTGCTCACACATAAACCTG-3') and PF2 (5'-CATATGGT CGTCTCAATGCGCCG-3'). The 1,550-bp DNA region upstream of the *hupSL* promoter (*PhupSL*) and corresponding to *'orf3orf4* (8) was amplified with primers M7D (5'-AAGGTGGATAGCAAAT-3') and IM2 (5'-CGGGATCC AGCCCTCCGGTACCAAG-3'), whereas a 1,756-bp DNA fragment carrying the *hupSL'* genes was obtained with primers DH1 (5'-CATATGGCAACTGC CGAGAC-3') and DH2 (5'-TCTAGACTTGACGATCTCCTTCTG-3'). These DNA fragments were independently cloned in the PCR2.1-TOPO vector (Invitrogen BV, Groningen, The Netherlands), resulting in constructs pPF1, pIM1, and pDH1, respectively. Correct DNA amplification was confirmed by sequencing. The DNA region containing the *hupSL'* genes (from plasmid pDH1) was digested at the ATG start site of *hupS* and cloned in frame with the *fixN* promoter in pPF1, resulting in plasmid pPF2. For this cloning, we took advantage of the *NdeI* sites generated with primers PF2 and DH1. The *'orf3orf4* region from pIM1 was inserted into the *EcoRI/BamHI* sites upstream of the *PfixN* promoter in plasmid pPF2. The resulting 3,626-bp insert was cloned in pK18 *mobsac* (38), producing plasmid pKPF1, which was introduced in *R. leguminosarum* UPM791. Transconjugants where *PhupSL* was replaced by the *fixN* promoter by double crossover were identified by Southern blotting experiments using a 320-bp DNA probe from *PfixN* and genomic DNA from candidate strains. To generate cosmid pALPF1, plasmid pKPF1 was transformed into *E. coli* C2110(pAL618) and the double crossover was selected by using the *sacB* system. Cosmids carrying the *PfixN* promoter were identified by Southern hybridizations. An *EcoRI/XbaI* DNA fragment carrying the *PfixN::hupSL* fusion was also cloned in plasmid pSPV4 (29) in front of the promoterless *lacZ* gene, resulting in plasmid pSPF1.

Plant tests and enzyme assays. Inoculation of pea (*Pisum sativum* L. cv. Frisson) seedlings with *R. leguminosarum* strains and plant growth under bacteriologically controlled conditions were carried out as previously described (24, 33). The nitrogen-free plant nutrient solution was supplemented with 20 µM NiCl₂ from day 10 after seedling inoculation. Pea bacteroids from 21-day-old nodulated plants were prepared as described by Leyva et al. (24). Hydrogenase activity in bacteroid suspensions and free-living cells was measured by an amperometric method with oxygen as the terminal electron acceptor (33). Hydrogen evolution in intact nodules was determined by gas chromatography (5). The protein contents of bacteroids and free-living cells were measured by the bicinchoninic acid method (42) with the modifications described by Brito et al. (6).

Cell fractionation of *R. leguminosarum* cultures. A 400-ml overnight microaerobic culture was collected by centrifugation, washed, and resuspended in 4 ml of 20 mM Tris-HCl, pH 8.0, buffer containing a protease inhibitor mixture (complete-Mini; Roche Molecular Biochemicals). Cells were broken by three passages through a French pressure cell at 12,000 lb/in², and the cell lysate was centrifuged for 20 min at 13,000 × g. The resultant supernatant (S10) was removed and centrifuged in a TL-100 ultracentrifuge (Beckman Inc., Palo Alto, Calif.) at 135,000 × g for 1 h at 4°C. The supernatant (S100) was transferred to another tube, and the membrane fraction (MB) was resuspended in the same volume of Tris buffer. Both fractions were cleared by an additional centrifugation at 135,000 × g for 1 h at 4°C. All fractions were aliquoted and stored at -40°C.

Immunological detection of Hup and Hyp proteins. Immunological detection of HupL and HypB in free-living cells, bacteroids, and cell fractions was carried out by immunoblotting as described previously (6) using antibodies raised against *B. japonicum* HupL and *R. leguminosarum* HypB. Both antisera were used at a 1:2,000 dilution.

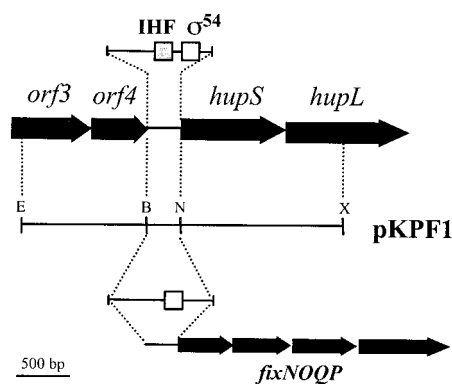


FIG. 1. Construction of a microaerobically expressed hydrogenase system in *R. leguminosarum* bv. *viciae* UPM791. The σ^{54} -dependent promoter of *hupSL* expressed in symbiosis (top) was replaced by the FnrN-dependent *PfixN* promoter from *R. leguminosarum* UPM791. This construct was introduced into the pK18 *mobsac* plasmid, producing pKPF1. This plasmid was used to exchange the *PhupSL* promoter in strain UPM791 and in cosmid pAL618, obtaining strain SPF25 and cosmid pALPF1, respectively. Arrows, genes flanking the *PhupSL* promoter and the *fixNOQP* genes; white and grey boxes (top), σ^{54} and integration host factor binding sequences in *PhupSL*; bottom box, FnrN consensus binding site (anaerobox). B, *Bam*HI; E, *Eco*RI; N, *Nde*I; X, *Xba*I.

RESULTS

Construction of a *hup* gene system expressing hydrogenase activity in microaerobic free-living cells of *R. leguminosarum* bv. *viciae* UPM791. To obtain hydrogenase activity in free-living cells of *R. leguminosarum* UPM791, we replaced the NifA-dependent *hupSL* promoter (*PhupSL*) by a promoter known to be induced in free-living cells of *R. leguminosarum* UPM791. Since it was known that the *hypBFCDEX* operon of the UPM791 hydrogenase cluster is induced in microaerobiosis by FnrN (13, 14), we chose an FnrN-regulated promoter. The *fixNOQP* operon from *R. leguminosarum* UPM791 had previously been cloned in our laboratory and had been shown to be controlled by FnrN (13). A 320-bp DNA region containing the *fixN* promoter (*PfixN*) was amplified by PCR and cloned in front of the start site of the *hupS* gene (Fig. 1; see Materials and Methods). A DNA region upstream of *PhupSL* covering *'orf3orf4'* was also amplified by PCR and cloned upstream of the *PfixN* promoter in plasmid pKPF1, a derivative of pK18 *mobsac*. This construction was transferred by conjugation into *R. leguminosarum* strain UPM791, and replacement of *PhupSL* by *PfixN* was selected by using sucrose sensitivity, mediated by the *sacB* system. Correct promoter insertion in the resulting strain, SPF25, was confirmed by Southern blotting experiments and by sequencing the promoter fusion. In parallel, and by a similar approach, pKPF1 was used to replace *PhupSL* with *PfixN* in cosmid pAL618, which carries the entire *hup* gene cluster, resulting in cosmid pALPF1.

Induction of hydrogenase activity in aerobic and microaerobic cultures of UPM791, SPF25, and merodiploid strains UPM791(pAL618) and SPF25(pALPF1) was measured (Table 2). Strain SPF25 expressed hydrogenase activity in microaerobic free-living cells at a level similar to that observed in pea bacteroids from wild-type strain UPM791 (23). A sixfold increase in hydrogenase activity in microaerobic cells of SPF25(pALPF1), compared with that in SPF25 cells, was ob-

served (Table 2). No hydrogen uptake in microaerobic UPM791 or UPM791(pAL618) cells or in aerobic cultures of any strain was detected. The presence of HupL and HypB proteins in these cultures was analyzed by immunoblotting experiments (data not shown). In microaerobic SPF25 cells, the HupL antibody cross-reacted with a 65-kDa band corresponding to the form of processed and active HupL, and this band was more intense in microaerobic cells of merodiploid strain SPF25(pALPF1). In addition, a prominent band corresponding to the unprocessed form was detected in this culture. No HupL immunoreactive bands were observed in microaerobic or aerobic cells of UPM791 and UPM791(pAL618). As expected, the HypB antiserum specifically recognized a 39-kDa protein in microaerobic cells of all strains (data not shown). The FnrN dependence of microaerobic hydrogenase induction was tested in strain DG2 (mutant *frrN1* and *frrN2*); no hydrogenase activity was observed in this strain (data not shown). The effect of nickel addition to the culture medium on microaerobic hydrogenase activity was also measured. Supplementation of the culture medium with 1 μ M NiCl₂ did not significantly increase the levels of hydrogenase activity in our experimental conditions. In fact, addition of 10 μ M NiCl₂ had an adverse effect on hydrogenase activity, whereas a final concentration of 20 μ M NiCl₂ in the culture medium completely abolished hydrogen uptake (data not shown). Addition of hydrogen (5%) to the induction atmosphere was also tested and did not affect hydrogenase activity levels (data not shown).

To better characterize hydrogenase induction in microaerobic free-living cells, SPF25(pALPF1) hydrogenase activity was monitored for 24 h in cultures continuously sparged with 0.8% O₂ (Fig. 2A). Hydrogen uptake was clearly detected after 4 h and reached a maximum at 10 h. No increase was observed after 24 h of microaerobiosis. This is twice the activity observed in stoppered cultures, probably because in the latter condition the oxygen supply limits rhizobial metabolism. Transcription of hydrogenase genes during the time course of induction was analyzed by using a *PfixN::hupSL-lacZ* transcriptional fusion. Expression of the *PfixN::hupSL* promoter was detected after 2 h of microaerobic incubation (data not shown). Also, the status of the HupL subunit during the experiment was studied by immunoblot assays (Fig. 2B). The immunoblot revealed that the inactive, unprocessed HupL form also appeared after 2 h of microaerobiosis. One hour later, both the unprocessed and processed HupL forms were observed; their appearance correlates with the increase in the level of hydrogenase activity.

TABLE 2. Hydrogenase activity in *R. leguminosarum* free-living cells cultured under different oxygen tensions

Strain	Hydrogenase activity ^a at O ₂ tension (%) of:	
	20	1
UPM791	<10	<10
SPF25	<10	930 ± 190
UPM791 (pAL618)	<10	<10
SPF25(pALPF1)	<10	6,300 ± 1,290

^a Values of hydrogenase activity are expressed as nanomoles of hydrogen per hour per milligram of protein and are the averages of three assays ± standard deviations.

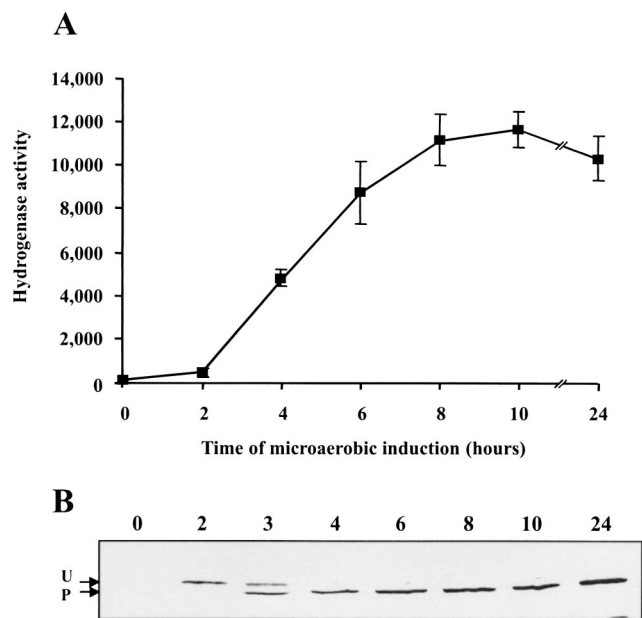


FIG. 2. Time course of microaerobic hydrogenase induction in *R. leguminosarum*. (A) SPF25(pALPF1) cells were induced in a fermentor and amperometrically analyzed for hydrogenase activity with oxygen as the electron acceptor. Values are given as nanomoles of hydrogen per hour per milligram of protein. (B) Immunodetection of HupL in SPF25(pALPF1) cell extracts at 0, 2, 3, 4, 6, 8, 10, and 24 h of microaerobic incubation. Arrows, unprocessed (U) and processed (P) forms of HupL. Bars, standard deviations.

Only the processed HupL form was observed after 4 h of incubation.

Subcellular localization of hydrogenase in free-living microaerobic cells of *R. leguminosarum*. As a further characterization of the microaerobically induced hydrogenase activity we attempted the identification and localization of the active and inactive-hydrogenase forms in the cell. Cellular fractionation of *R. leguminosarum* pea bacteroids has been attempted in the past, but methodological problems, due to the fragility of bacteroids and the shortage of biological material, made this assay a tedious and complicated task that led only to contradictory results. Microaerobic free-living cells of strain SPF25(pALPF1) were fractionated as described in Materials and Methods. Fractions corresponding to the crude extract (S10), the soluble fraction (S100), and membrane fraction (MB) were analyzed for the presence of the processed and unprocessed HupL forms in immunoblot assays (Fig. 3). As expected, two HupL immunoreactive bands were observed in whole cells, as well as in the S10 crude extracts. The slow-migrating band, corresponding to the unprocessed HupL form, was mostly observed in the soluble S100 fraction, whereas the fast-moving, processed subunit was only found in the membrane fraction. This result indicates that the active hydrogenase of *R. leguminosarum* is inserted in the membrane, while the inactive form remains soluble in the cytoplasm. As a control for contamination of the cellular fractions, we confirmed by immunoblot assays with the HypB antiserum that HypB is found only in the soluble fraction, as it is expected for a cytoplasmic protein (Fig. 3).

Increased symbiotic hydrogenase activity of the FnrN-de-

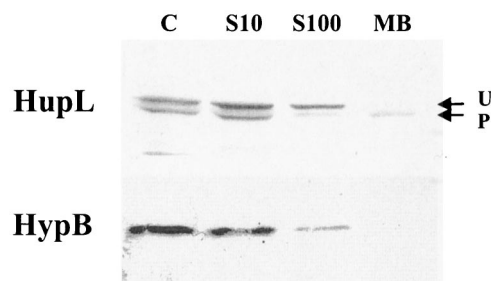


FIG. 3. Subcellular localization of HupL and HypB in microaerobic, free-living *R. leguminosarum* cells. SPF25(pALPF1) whole cells (C), crude extracts (S10), the soluble fraction (S100), and membranes (MB) were analyzed for the presence of HupL and HypB proteins by immunoblotting. Arrows, HupL unprocessed (U) and processed (P) forms.

pendent *hup* system. To ascertain that the *PhupSL* replacement had not impaired the hydrogen uptake ability in symbiosis, we measured the hydrogenase activity of SPF25 pea bacteroids. To do this, pea plants were inoculated with cultures of strains UPM791 and SPF25 and grown in a standard plant nutrient solution without nickel supplementation or supplemented with 20 μ M NiCl₂. Hydrogenase activity in 21-day-old bacteroids, with oxygen as the terminal electron acceptor, was measured (Table 3). SPF25 bacteroids displayed high levels of hydrogenase activity, indicating that the promoter replacement had not affected hydrogen uptake in symbiosis. In fact, SPF25 bacteroids showed a twofold increase in hydrogenase activity compared to the wild-type strain, UPM791, regardless the nickel supplementation. This increment in hydrogenase activity was correlated with an increase of the processed-HupL band as revealed by immunoassays carried out with HupL antiserum (data not shown). Hydrogen evolution assays with intact nodules showed that SPF25 bacteroids, even those from plants not exposed to nickel addition, were able to recycle virtually all hydrogen produced by the nitrogenase, whereas nodules from UPM791 grown in the same condition still evolved significant amounts of hydrogen (Table 3). This result suggests that our construction may improve symbiotic hydrogenase activity under the nickel-limiting conditions normally found in agricultural soils.

TABLE 3. Hydrogen metabolism in bacteroids and nodules of *R. leguminosarum* bv. viciae strains

Strain	Bacteroid hydrogenase activity ^a		Nodule H ₂ evolution ^b	
	-Ni	+Ni	-Ni	+Ni
UPM791	1,080 \pm 40	2,930 \pm 320	2.13 \pm 0.31	<0.25
SPF25	2,210 \pm 190	5,060 \pm 350	0.34 \pm 0.09	<0.25

^a Values are the averages of three assays \pm standard deviations and are expressed as nanomoles of hydrogen per hour per milligram of protein. +Ni, addition of 20 μ M NiCl₂ to the standard plant nutrient solution; -Ni, no nickel addition.

^b Data are the means of four independent determinations \pm standard deviations and are expressed as micromoles of hydrogen evolved per hour per gram of nodule fresh weight.

DISCUSSION

Hydrogenase systems from *B. japonicum* and *R. leguminosarum* bv. *viciae* have been extensively characterized in the last 10 years. Although the systems are similar in gene composition and organization, they differ markedly in their regulation (35). The *B. japonicum* hydrogenase is induced in soybean bacteroids and in microaerobic free-living cultures in the presence of hydrogen and nickel, whereas *R. leguminosarum* expresses hydrogenase activity only in symbiotic conditions. This requirement for symbiosis, likely due to the fact that the *PhupSL* promoter is controlled by NifA (8), has obviously hampered the study of the *R. leguminosarum* hydrogenase. In this paper, we develop a strategy to induce hydrogenase activity in free-living cells of *R. leguminosarum*. Replacement of the NifA-regulated *hupSL* promoter by the FnrN-dependent promoter of the *fixNOQP* operon led to hydrogenase activity in microaerobic free-living cells. Synthesis of hydrogenase polypeptides started as early as 2 h after microaerobic incubation, as revealed by the accumulation of the unprocessed HupL form. One hour later, low levels of hydrogenase activity, which were also correlated with the processing of the large subunit, were detected, and maximal hydrogenase activity was obtained after 10 h of microaerobiosis.

The results obtained in these experiments highlight two main points. First, microaerobic hydrogenase activity is not limited by nickel availability in our experimental conditions, since nickel traces in the culture medium are enough for maximum levels of hydrogenase activity in microaerobiosis. In contrast, maximum symbiotic hydrogenase activity in pea bacteroids is obtained only when the plant nutrient solution is supplemented with NiCl₂ (6). This effect is correlated with a nickel-dependent processing of the hydrogenase subunits and not with a nickel-dependent regulation of *PhupSL* (6). The different behaviors of microaerobic and symbiotic hydrogenase activity indicate that bacterial nickel transport does not limit hydrogenase activity in free-living cultures of *R. leguminosarum* and suggest that nickel limitation in symbiosis is probably exerted by the host plant.

Second, our results indicate that the *PhupSL* substitution places the expression of the entire *hup* gene cluster under the control of FnrN, given that microaerobiosis is the only requirement for expression of hydrogenase activity in free-living cells and that this activity is abolished in a *R. leguminosarum* *fnrN* double mutant. This is interesting, since two promoters putatively expressed in symbiosis, in addition to *PhupSL*, have been identified in the *hup* gene cluster of *R. leguminosarum*. The first one was found in front of the *hupGHJK* operon (22), and the second was found upstream of *hypA* and within *hupK* (15). No regulators for these two putative promoters have been identified, but the involvement of FnrN can be ruled out because they are expressed only in symbiosis. In addition, the *hupGHJK* and *hypA* gene products are essential for hydrogenase activity in symbiosis (15, 30), as well as in microaerobic free-living cells (B. Brito, unpublished data). This implies that the *PfixN::hupS* promoter should transcribe the *hupSLCDEF*, *hupGHJK*, and *hypA* operons in microaerobiosis without any transcriptional stop. This is not surprising since previous evidence indicates that an unusually large transcript of 17 kb from the promoter of the *hoxKG* genes of *Ralstonia eutropha* (ho-

mologous to *hupSL* of *R. leguminosarum*) spans the whole hydrogenase cluster (40). Our results suggest that the *hupGHJK* and *hypA* promoters are somehow dispensable and only required to reinforce transcription from the main promoter, *PhupSL*. The relevance of the *hyp* promoter in *R. leguminosarum* is still an open question that is currently being elucidated by generating point mutations that specifically inactivate the two overlapping promoters expressing the *hyp* operon (15).

The availability of strain SPF25 provides a fast and easy system to generate hydrogenase-induced *R. leguminosarum* cells and opens new perspectives for the characterization of this enzyme and the molecular functions of Hup and Hyp products. As a first step in the *R. leguminosarum* hydrogenase characterization, we carried out the localization of the hydrogenase in the cell. Although this type of information has been obtained for hydrogenases from other microorganisms, it remained elusive in *R. leguminosarum* due to the constrictions imposed by the symbiosis. We now have been able to localize the processed and unprocessed hydrogenase forms in the cell compartments. Cellular fractionation of microaerobic free-living cells of SPF25(pALPF1) showed that the unprocessed HupL protein is found in the soluble fraction corresponding to the cytoplasm, whereas the processed HupL subunit is mostly associated with the membrane. This result is in agreement with observations reported for *Ralstonia eutropha* (19), *Azotobacter vinelandii* (26), and *E. coli* hydrogenase-1 (27) and fits with the current model of hydrogenase maturation (9). In this model, incorporation of metal groups leads to the processing of the HupL precursor, which is targeted to the membrane as a complex with the HupS subunit by the Tat (twin-arginine translocation) system (31).

Several strategies to improve and extend hydrogenase activity in different rhizobial strains have been followed in our and other laboratories (1, 3, 17, 20, 21, 25). Recently a novel *hup* minitransposon has been reported to generate new hydrogen-recycling strains of *Rhizobiaceae* (1). This minitransposon has been successfully applied to introduce the Hup trait in *R. leguminosarum*, *Rhizobium etli*, *Sinorhizobium meliloti*, and *Mesorhizobium loti* Hup⁻ strains. Different levels of hydrogenase activity were obtained depending on the rhizobial background and the legume host partner. Previous studies established that *hupSL* activation by NifA and nickel availability to the bacteroid could be major factors limiting hydrogenase activity in certain rhizobial strains (5). As soils with high nickel content are rare in nature and since addition of nickel to the soil is not an acceptable agricultural practice, the selection of *Rhizobium*-legume partners with optimal hydrogenase activity in poor-nickel conditions has been proposed as a way to improve nitrogen fixation through the activity of an efficient hydrogenase enzyme (5). Here, we propose an alternative strategy based on the replacement of the NifA-dependent promoter of *hupSL*. We demonstrate that SPF25 expressing the *hup* genes from an FnrN-dependent promoter shows an increased hydrogenase activity in symbiosis. This results in the recycling of all hydrogen produced by nitrogenase even in plants grown without nickel addition. This increment in hydrogen uptake might be due to an enhanced transcription of the *hup* and, probably, the *hyp* operons by *PfixN*. In this situation, a higher production of Hup and Hyp proteins would lead to improved nickel incor-

poration in the hydrogenase. This effect has already been shown in bacteroids that carry extra copies of the *hup* and *hyp* genes and that are exposed to low nickel concentrations (6). It will be interesting to know whether the promoter substitution causes an increase in hydrogenase activity in other rhizobial strains that have been shown to be limited in their hydrogen uptake ability.

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