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Hydrogenase genes from *Rhizobium leguminosarum* bv. viciae are controlled by the nitrogen fixation regulatory protein NifA

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ABSTRACT ***Rhizobium leguminosarum*** **bv. viciae expresses an uptake hydrogenase in symbiosis with peas (*Pisum sativum*) but, unlike all other characterized hydrogen-oxidizing bacteria, cannot express it in free-living conditions. The hydrogenase-specific transcriptional activator gene *hoxA* described in other species was shown to have been inactivated in *R. leguminosarum* by accumulation of frameshift and deletion mutations. Symbiotic transcription of hydrogenase structural genes *hupSL* originates from a −24/−12 type promoter ($hupS_p$). A regulatory region located in the −173 to −88 region was essential for promoter activity in *R. leguminosarum*. Activation of $hupS_p$ was observed in *Klebsiella pneumoniae* and *Escherichia coli* cells expressing the *K. pneumoniae* nitrogen fixation regulator NifA, and in *E. coli* cells expressing *R. meliloti* NifA. This activation required direct interaction of NifA with the essential −173 to −88 regulatory region. However, no sequences resembling known NifA-binding sites were found in or around this region. NifA-dependent activation was also observed in *R. etli* bean bacteroids. NifA-dependent $hupS_p$ activity in heterologous hosts was also absolutely dependent on the RpoN σ-factor and on integration host factor. Proteins immunologically related to integration host factor were identified in *R. leguminosarum*. The data suggest that $hupS_p$ is structurally and functionally similar to nitrogen fixation promoters. The requirement to coordinate nitrogenase-dependent H_2 production and H_2 oxidation in nodules might be the reason for the loss of HoxA in *R. leguminosarum* and the concomitant NifA control of *hup* gene expression. This evolutionary acquired control would ensure regulated synthesis of uptake hydrogenase in the most common H_2-rich environment for rhizobia, the legume nodule.**

Hydrogen gas that is evolved during nitrogen fixation in legume root nodules can be recycled through the activity of an H_2-uptake (Hup) system synthesized by certain strains of rhizobia. This H_2 recycling system, whose first component is a membrane-bound, dimeric, and nickel- and iron-containing hydrogenase, has a potential to increase legume productivity (1, 2). The first report on the isolation of genes encoding the hydrogenase structural subunits in symbiotic bacteria was published in 1983 (3). Since then, a cluster of genes containing, in addition to the structural genes (*hupSL*), an array of at least 16 genes coding for accessory proteins required for the synthesis of an active hydrogenase has been identified and sequenced in *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bv. viciae (4, 5). Many of these genes are similar to those found in other H_2-oxidizing bacteria such as *Alcaligenes eutrophus*, *Rhodobacter capsulatus*, *Azotobacter vinelandii*, and *Escherichia coli* (6, 7). In *R. leguminosarum* bv. viciae UPM791, the hydrogenase gene cluster (*hupSLCDEFGHIJK hypABFCDEX*) is located in the large pSym plasmid, which also contains the *nif* and *fix* genes (8).

Considering the complexity of hydrogenase gene clusters and metabolic diversity of the H_2-oxidizing microorganisms, multiple factors and regulatory circuits are expected to control hydrogenase gene expression. In free-living *B. japonicum* cells, induction of hydrogenase activity requires microaerobic conditions, H_2, and nickel. These three environmental factors regulate hydrogenase activity at the transcriptional level by acting at the same *cis* site in the promoter of the hydrogenase structural genes (9). This *cis* element is located upstream of binding sites for σ^{54} and integration host factor (IHF) proteins, characteristic of positively regulated −24/−12-type promoters present in many nitrogen fixation genes (10). A strict requirement for RpoN (σ^{54}), but not for IHF, has been demonstrated in *B. japonicum* (11).

A transcriptional activator, HoxA, that may work in concert with σ^{54}-dependent RNA polymerases in the induction of hydrogenase structural genes has been found in *A. eutrophus* (12) and *B. japonicum* (13). Because HoxA is a member of the family of two-component regulatory systems (13), other gene products can be predicted to be involved in environmental signal sensing and transduction. Two genes, *hupU* and *hupV*, located upstream of hydrogenase structural genes in *B. japonicum* (14) and *R. capsulatus* (15) encode proteins with strong homology to the small and large subunits of the hydrogenase, respectively. A role for *hupUV* as a sensing complex for nickel levels and other environmental factors (O_2 or H_2) was proposed. In *R. capsulatus* the *hupUV* genes form an operon with *hupT*, which is involved in repression of hydrogenase synthesis (16).

Induction of hydrogenase structural genes in *R. leguminosarum* has consistently been detected only in symbiotic cells but not in free-living cells grown under conditions known to induce hydrogenase synthesis in other H_2-oxidizing bacteria (17). *In situ* hybridization assays demonstrated that *hup* genes are temporally and spatially coexpressed with *nif* genes in pea nodules (18). In this report, we show that *R. leguminosarum* bv. viciae contains a defective *hoxA* gene and that the *hupS* promoter is activated by NifA proteins from *Klebsiella pneumoniae*, *R. meliloti*, and *R. etli*. Activation occurs through NifA binding to a *cis* element located between positions −173 and −88. This activation, which requires both a σ^{54} factor and IHF proteins, indicates that the hydrogenase gene system has become part of the nitrogen fixation regulon in *R. leguminosarum*.

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Abbreviations: IHF, integration host factor; UAS, upstream activating sequences; KpNifA and RmNifA, NifA proteins from *Klebsiella pneumoniae* and *Rhizobium meliloti*, respectively.

Data deposition: The sequences reported in this paper have been deposited in the GenBank and EMBL databases [accession nos. X52974 (*hoxA*) and U36924 ($hupS_p$)].

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MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. *Rhizobium leguminosarum* bv. viciae UPM791 (Str^R Hup^+; ref. 8), *R. etli* CE3 (provided by L. Girard, Universidad Nacional Autónoma de México), *Bradyrhizobium japonicum* CB1809 (provided by J. Vanderleyden, John Innes Centre, Norwich, U.K.), and *K. pneumoniae* UNF122 (provided by M. J. Merrick, Catholic University of Leuven, Belgium) were used as wild-type strains. *R. etli* Fix^- strains CFNX247 (*nifA*Δ::Sp/Sm; ref. 19) and CFNX249 (*nifHa*::Sp/Sm *nifHb*Δ::Km) were a gift of L. Girard. *K. pneumoniae* strains UNF1789 (*ntrC209*), UNF2561 (*rpoN71*), and CK263 (*nifA2263*; ref. 20) were provided by M. J. Merrick. *K. pneumoniae* UN1655 (*nifE4701*; ref. 21) was a gift of G. P. Roberts (University of Wisconsin, Madison). *Escherichia coli* strains ET8000 and its *himD* (IHF^-) derivative SE1000 have been described (22). References to other plasmids are in figure legends or tables. Growth conditions for *Rhizobium* and *Bradyrhizobium* were as previously described (23).

General Procedures. Plasmid DNA preparation, restriction enzyme digestion, DNA cloning, DNA sequencing, agarose and polyacrylamide gel electrophoresis, and autoradiography were performed by standard methods (24). Serial deletions of plasmid pHL 315 were obtained by exonuclease III–nuclease S1 treatment. The coding probability of nucleotide sequences was analyzed by the method of Gribskov *et al.* (25) with a *R. leguminosarum* codon usage table. Immunoblot analysis was carried out as described by Rey *et al.* (26).

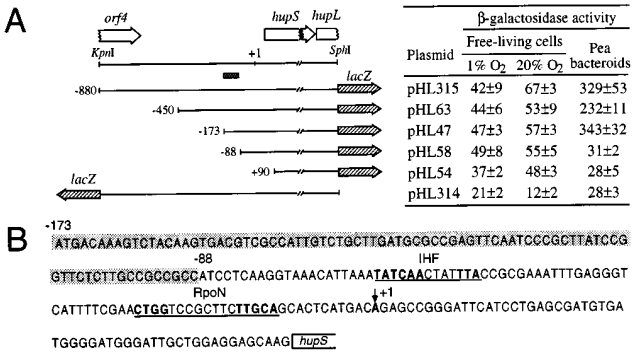
Bacteroid Preparation and Enzyme Assays. Pea (*Pisum sativum* L. cv. Frisson) and bean (*Phaseolus vulgaris* L. cv. Contender) plants were used as hosts for *R. leguminosarum* bv. viciae and *R. etli*, respectively. Conditions for plant inoculation and growth, and bacteroid preparation, were as previously described (8). Transcriptional activity was monitored by measuring β-galactosidase activity (27) in cells carrying *lacZ* gene fusions to the promoters of interest. For *E. coli* assays, the *K. pneumoniae nifA* gene encoded by plasmids pMJ220 or pMJ221 was induced with isopropyl-β-D-thiogalactopyranoside (2 mM) for 2 hr before activity determinations. Derepression and assay of nitrogenase activity in *K. pneumoniae* cultures were carried out as previously described (28).

Purification of His_6 Fusion Proteins. Fusion proteins containing the His_6 tag encoded by pRSETb (29)-derived plasmids pHis1 and pHis2 (Fig. 2*B*) were expressed in *E. coli* cells using the T7 polymerase/promoter system and [^{35}S]methionine/cysteine labeling (30), and purified by denaturing Ni(II)-nitrilotriacetic-agarose chromatography as described by Rey *et al.* (26).

RESULTS

Deletion Analysis of the $hupS_p$ Regulatory Region. Symbiotic transcription at the *hupS* promoter ($hupS_p$) initiates 56 bp upstream of the *hupS* gene (31). Upstream of the transcription start site, a σ^{54}-binding sequence characteristic of −24/−12-type promoters was identified, suggesting a positive activation of $hupS_p$. To localize potential *cis*-acting sequences required for symbiotic expression of $hupS_p$, a series of *hupSL–lacZ* fusions containing sequential 5′ deletions of a DNA region spanning 880 bp upstream of the transcription start site were generated as shown in Fig. 1. Fusion-containing plasmids were transferred into *R. leguminosarum* UPM791 strain and analyzed for β-galactosidase activity both in aerobically and microaerobically grown vegetative cells and in pea bacteroids (Fig. 1).

β-galactosidase activities associated to plasmid pHL315, which contains the largest promoter fragment, were very low in free-living cells, regardless of the oxygen level. They were comparable to those associated to plasmid pHL54, which has been deleted of all promoter sequences, and were considered as background levels. In contrast, pea bacteroids from strain UPM791 (pHL315) showed a 12-fold increase in their β-galactosidase activity, as compared with that associated to plasmid pHL54. These results indicate a symbiotic requirement for $hupS_p$ expression and are consistent with the previous observation of a lack of transcription of *hupS* in vegetative cells (17). Deletion of 430 (pHL63) and 707 bp (pHL47) from the 5′ end of the insert DNA in plasmid pHL315 had no effect on β-galactosidase activities. However, deletion of a further 85-bp fragment (pHL58) resulted in complete abolishment of symbiotic $hupS_p$ expression. This indicates that sequences located downstream of position −173, relative to the transcriptional start site, contain *cis*-acting elements involved in transcription of hydrogenase genes.



Plasmid	β-galactosidase activity: Free-living cells 1% O_2	Free-living cells 20% O_2	Pea bacteroids
pHL315	42±9	67±3	329±53
pHL63	44±6	53±9	232±11
pHL47	47±3	57±3	343±32
pHL58	49±8	55±5	31±2
pHL54	37±2	48±3	28±5
pHL314	21±2	12±2	28±3

FIG. 1. Analysis of the *hupS* promoter. (*A*) Deletion analysis. The restriction and genetic maps of the promoter region are shown at the top left of the figure. The *hupS* transcriptional start site is indicated as position +1. 5′ deletion fragments were cloned in front of the promoterless *lacZ* gene in plasmid pMP220 (32) to generate plasmids of the pHL series. The position of the 5′ end with respect to the transcription start site is indicated. The hatched bar below the restriction map indicates the 85-bp DNA region required for *hupSL* transcription. β-galactosidase activities of aerobically and microaerobically grown cells and of pea bacteroids from *R. leguminosarum* bv. viciae UPM791 derivative strains carrying the pHL plasmids are shown on the right. Values (Miller units) are the average of three independent determinations ± SE. No differences in symbiotic stabilities of pHL plasmids were observed. (*B*) DNA sequence (GenBank/EMBL accession nos. U36924 and X52974). Regions similar to the RpoN (σ^{54}) promoter consensus sequence YTGGCAC-N_5-TTGCA (33) and to IHF-binding site WATCAA-N_6-TTR (34) are underlined, and identical nucleotides are shown in boldface. The 85-bp region essential for $hupS_p$ activation is shadowed.

***R. leguminosarum* bv. viciae UPM791 Contains an Inactive *hoxA* Locus.** Both in *A. eutrophus* (6) and *B. japonicum* (13) hydrogenase gene expression is under the control of a *hup*-specific transcriptional activator encoded by the *hoxA* gene. In these organisms, *hoxA* is located immediately downstream of genes equivalent to *R. leguminosarum hypX*. Sequence analysis of this region in *R. leguminosarum* bv. viciae UPM791 revealed the absence of any ORF long enough to encode a HoxA-like protein (Fig. 2*A*). However, three smaller ORFs encoding amino acid sequences with similarity to different regions of HoxA were identified. The first of them (ORF1, 411 bp), overlaps with *hypX* by the sequence ATGA, is preceded by a plausible Shine–Dalgarno motif, and exhibited a high coding probability along its whole length. The derived amino acid sequence of ORF1 (136 aa) showed high similarity (60.7% and 57.4% identity) with the N-terminal region of HoxA from *B. japonicum* and *A. eutrophus*, respectively (data not shown). Two other ORFs (ORF2, spanning from position 396 to 1253, and ORF3, from position 556 to 1119) were identified downstream of ORF1 (Fig. 2*A*). These ORFs contained alternating regions of high and low coding probability and are not preceded by plausible Shine–Dalgarno sequences. The derived

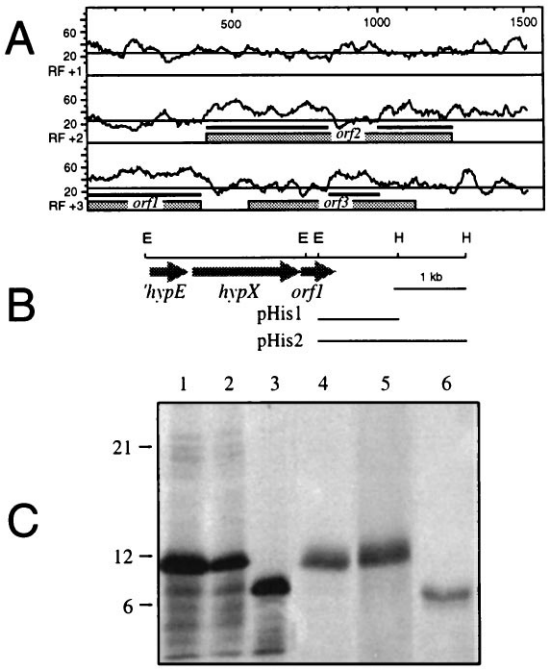


FIG. 2. Analysis of the *hoxA* locus. (*A*) Coding potential of the *ca.* 1.5 kb of DNA downstream of *hypX* along the three reading frames (RF). Open reading frames 1, 2, and 3 are indicated by hatched boxes. Thick bars indicate ORF regions of high (30–65%) amino acid similarity to the *B. japonicum* HoxA protein. (*B*) Fusion constructs. Fragments cloned in the His_6 vector pRSETb (29) to yield pHis1 and pHis2 are shown below the physical and genetic map of the *hypX-hoxA*-like region. Restriction enzymes: E, *Eco*RI; H, *Hin*dIII. (*C*) Expression analysis of plasmids pHis1 and pHis2 in *E. coli*. [^{35}S]methionine/cysteine-labeled extracts (lanes 1–3) and purified hexahistidine-containing proteins (lanes 4–6) were identified by $NaDodSO_4$/PAGE and autoradiography. Lanes: 1 and 4, pHis1; 2 and 5, pHis2; 3 and 6, pRSETb control. The position of molecular mass markers, in kDa, is indicated on the left.

amino acid sequences from the regions with high coding probability exhibited high similarity (over 40% identity) with the corresponding regions of *B. japonicum* and *A. eutrophus* HoxA (data not shown), including the conserved ATP-binding motif GXXGXGKE in ORF2. No other ORFs encoding amino acid sequences with similarity to the C-terminal end of HoxA, including the DNA-binding helix-turn-helix motif (12), were identified downstream of ORF2. These results suggest that frameshift and deletion mutations have accumulated in the *hoxA* gene, inactivating it. In addition, Southern blot experiments did not reveal the presence of *hoxA* duplicates in the *R. leguminosarum* UPM791 genome.

To eliminate the possibility that the frameshifts originate from persistent sequencing artifacts, the actual molecular size of the ORF1 translation product was determined. To this effect, two different DNA fragments spanning the sequenced region (Fig. 2*B*) were cloned in the His_6 vector pRSETb to generate fusion constructs encoding a predicted 114-aa product consisting of a 42 aa His_6 N terminus and a 72-aa ORF1 C terminus. A major product of the expected size (*ca.* 12 kDa) was identified after T7 RNA polymerase-dependent expression and [^{35}S]methionine/cysteine-selective labeling (Fig. 2*C*, lanes 1 and 2) of both constructs. This product could be purified by Ni(II)-chelate affinity chromatography (Fig. 2*C*, lanes 4 and 5), thus showing that it contained the His_6 tag. Since no translation products larger in size could be identified or isolated, these results show that the pseudo-HoxA protein encoded by *R. leguminosarum* UPM791 is a truncated, defective product, presumably as a result of the frameshift mutations identified by DNA sequencing.

NifA from *Klebsiella pneumoniae* and *Rhizobium meliloti* Activate the *hupS* Promoter. Hydrogenase and nitrogenase genes are temporally and spatially coexpressed in pea nodules (18); in addition, $hupS_p$ contains RpoN- and IHF-binding motifs characteristic of *nif* promoters at appropriate distances from the transcription initiation site (Fig. 1*B*; ref. 31). These results prompted us to investigate whether NifA, the central regulatory component for nitrogen fixation, is involved in symbiotic transcriptional activation of hydrogenase structural genes, despite the fact that no sequences resembling NifA upstream activating sequences (UAS) ($TGT\text{-}N_{10}\text{-}ACA$; ref. 35) were found in, or around, the $hupS_p$ region absolutely required for symbiotic transcription (Fig. 1*B*). Experiments with *R. leguminosarum nifA* mutants were inconclusive because Nif^- pea nodules are structurally and developmentally aberrant (18).

The putative NifA-dependence of $hupS_p$ expression was tested in the heterologous genetic background of *Klebsiella pneumoniae* cells under diazotrophic conditions. To this end, plasmid pHL315 was transferred into *K. pneumoniae* wild-type and *ntrC*, *rpoN*, *nifA,* and *nifE* mutants, and the β-galactosidase activity of the resulting strains was determined in cells derepressed anaerobically for nitrogenase (Table 1). High levels of β-galactosidase activity were detected in nitrogenase-derepressed *K. pneumoniae* wild-type and *nifE* strains, both of which express the *nifA* gene. In contrast, only background levels of β-galactosidase activity were observed in *nifA*, *ntrC*, and *rpoN* strains, none of which expresses NifA. These results strongly suggest that KpNifA is able to activate the $hupS_p$ promoter. The dependence of $hupS_p$ promoter activity on the RpoN gene product was tested in *K. pneumoniae* UNF2561 *rpoN* (Table 1). Since *nifA* transcription is also dependent on RpoN, KpNifA was expressed under the control of the *lac* promoter using plasmid pMJ220 (35). This plasmid was able to restore $hupS_p$ activity in the *nifA* and *ntrC* mutants, but not in the *rpoN* mutant (Table 1). This result demonstrates that σ^{54} is required for NifA-dependent $hupS_p$ activity and that NtrC is not implicated in $hupS_p$ activation.

It is unlikely that $hupS_p$ activation in wild-type *K. pneumoniae* requires signals resulting from nitrogenase activity (e.g., H_2 evolution), since it was also observed in strain UNF1665 (*nifE4701*), which induces no nitrogenase activity, and in aerobically grown cells containing plasmid pMJ220. However, to exclude indirect effects derived from the nitrogen-fixing system, we tested the proposed NifA-dependent

Table 1. Expression of $hupS_p$ in *K. pneumoniae*

		β-Galactosidase, Miller units			
Strain*	Genotype	Anaerobic	Aerobic	Anaerobic (+pMJ220†)	Aerobic (+pMJ220)
UNF122	wild type	964	105	1,695	3,188
CK263	*nifA*	69	70	1,706	2,487
UNF2561	*rpoN*	24	16	13	17
UNF1789	*ntrC*	34		2,216	3,210
UNF1665	*nifE*	1,125			

*All strains contain plasmid pHL315 (*hupSL–lacZ*).
†pMJ220 expresses the KpNifA protein (35).

hupS$_p$ activation in *E. coli*, a nondiazotrophic bacterium. Activation of *hupS*$_p$ in *E. coli* was detected only when KpNifA, encoded by plasmid pMJ220, was present (Table 2). A similar behavior was observed for the NifA-dependent *K. pneumoniae* and *R. meliloti nifH* promoters (plasmids pIZ263 and pMB210, respectively), which were included as controls. These experiments were repeated in *E. coli* cells expressing RmNifA (Table 2). Clear differences in specificity between the two NifA proteins were observed, with RmNifA promoting very poor activation of the *K. pneumoniae nifH* promoter. However, good *hupS*$_p$ activation was observed in the presence of any of the two NifA proteins. These results again indicate an activation of *hupS*$_p$ by NifA.

NifA-Dependent Transcription from *hupSp* Requires Binding to Upstream *cis*-Acting DNA Sequences. The requirement for binding of KpNifA and RmNifA to upstream activating sequences in *hupS*$_p$ for transcriptional activation was investigated by three different approaches. First, activation of *hupS*$_p$ was examined in *E. coli* cells containing plasmid pMJ221. This plasmid expresses an altered form of the KpNifA protein (KpNifA*) incapable of binding to UAS due to an amino acid substitution (Y512F) in the helix-turn-helix DNA-binding motif at the C terminus of the protein (37). KpNifA* can promote transcription by interacting directly with the RNA polymerase–σ^{54} complex in those promoters, such as that for *nifH* in *R. meliloti*, whose activation is not absolutely dependent on the presence of UAS (38). As shown in Table 2, KpNifA* did not promote transcription from *hupS*$_p$. In a control experiment, KpNifA* was capable of activating the *R. meliloti nifH* promoter, but not the *K. pneumoniae nifH* promoter.

Second, the NifA-dependent activation of *hupS*$_p$ was tested in *E. coli* cells harboring *hupS*$_p$ deletion derivative plasmids pHL47 and pHL58 (Table 2). As it was the case in *R. leguminosarum* bacteroids (Fig. 1*A*), high levels of β-galactosidase activity were associated to plasmid pHL47 but not to plasmid pHL58, thus showing that the same 85-bp upstream DNA fragment, located between positions -173 and -88, is also required for KpNifA- and RmNifA-dependent transcription from *hupS*$_p$.

Third, the effect of multiple copies of *hupS*$_p$ on *K. pneumoniae* nitrogenase activity was determined. If NifA activates transcription from *hupS*$_p$ through direct binding, titration of NifA will result in decreased *nif* gene expression and nitrogenase activity (39). To test this hypothesis, restriction fragments containing the intact or deleted forms of *hupS*$_p$ cloned into the multicopy pBC KS vector were introduced into *K. pneumoniae* and tested for inhibition of nitrogenase (Table 3). Plasmids pBC315 and pBC47, which contain 880 and 173 bp of DNA upstream of the transcription initiation site, respectively, caused a 90% inhibition of the wild-type level of nitrogenase activity. Meanwhile, plasmid pBC58, which is deleted upstream of position -87, caused no nitrogenase inhibition. This suggests that nitrogen fixation inhibition, and hence NifA binding, is specifically associated with sequences located within positions -173 to -88 of the *hupS* promoter region.

Table 3. Inhibition of *K. pneumoniae* nitrogenase activity by *R. leguminosarum hupS*$_p$ sequences

Strain/plasmid*	*hupS*$_p$ sequences (bp upstream of +1)	Nitrogenase† activity	% inhibition
UNF122	None	0.53 ± 0.15	0
UNF122 (pBC KS)	None	0.56 ± 0.18	0
UNF122 (pBC315)	880	0.06 ± 0.05	91
UNF122 (pBC47)	173	0.06 ± 0.04	90
UNF122 (pBC58)	88	0.54 ± 0.10	0

*Inserts from plasmids pHL315, pHL47, and pHL58 were cloned in pBC KS to yield pBC315, pBC47, and pBC58, respectively.
†Values [μmol of C_2H_2 reduced $\cdot h^{-1} \cdot$ (ml of culture)$^{-1}$] are the average of four independent determinations ± SE.

Symbiotic NifA-Dependent Expression of *hupS*$_p$. The NifA requirement for symbiotic expression of *hupS*$_p$ was tested in the *R. etli* bean system. Contrary to the *R. leguminosarum* pea system, bean nodules from plants inoculated by Nif$^-$ strains were, at least up to the third week postinoculation, structurally similar to Fix$^+$ nodules (data not shown). The *hupS*$_p$ reporter plasmid pHL315 was introduced in the *R. etli* CE3 wild-type strain, and mutants CFNX247 (*nifA*Δ::Sp/Sm) and CFNX249 (*nifHa*::Sp/Sm *nifHb*Δ::Km), and bacteroid β-galactosidase activities were determined 3 weeks after inoculation. The *hupS* promoter was expressed in wild-type bacteroids (140 Miller units), but a 6-fold decrease in *hupS*$_p$ expression (24 Miller units) was observed in CFNX247 *nifA* bacteroids. The normal levels of *hupS*$_p$ activation (147 Miller units) observed with strain CFNX249 (*nifHa nifHb*) show that the results obtained with the *nifA* mutant are not due to the Fix$^-$ phenotype but specifically to the lack of active NifA.

Integration Host Factor Mediates NifA-Dependent Transcription. The IHF has been shown to stimulate NifA-mediated activation of *nifH* transcription by binding to a region located between the NifA UAS and the σ^{54}-binding site (10). Based on sequence similarities, an IHF-binding site was proposed in *hupS*$_p$ (Fig. 1*B*; ref. 31). The involvement of IHF in NifA-dependent *hupS*$_p$ activation was tested in the *E. coli* IHF$^-$ mutant SE1000 (*himD*). Only background transcription from *hupS*$_p$ was detected in this strain in the presence of plasmid pMJ220, which constitutively expresses KpNifA (data not shown). A similar behavior was observed with a *K. pneumoniae nifH–lacZ* fusion construct, whose activation is known to depend on NifA and IHF (10). These results suggest that a *R. leguminosarum* equivalent to IHF might play a role in hydrogenase gene transcription. However, no such protein has been reported in *R. leguminosarum*. By using antibodies raised against *E. coli* IHF, we were able to show the presence of two immunoreactive protein bands of M_r 11 and 13 kDa in cell-free extracts from *R. leguminosarum* bv. viciae UPM791 pea bacteroids (Fig. 3, lane 3). These proteins were similar in size to the *E. coli* IHF proteins (Fig. 3, lane 1) and to IHF-like proteins from *Bradyrhizobium japonicum* (ref. 11; Fig. 3, lane 2), and may correspond to the α and β subunits of the *R. leguminosarum* IHF complex.

Table 2. NifA-dependent expression of *R. leguminosarum hupS*$_p$ in *E. coli*

		β-Galactosidase, Miller units			
Plasmid	(fusion)	+RmNifA (pRmW541.10)†	+KpNifA (pMJ220)	+KpNifA* (pMJ221)	−NifA
pHL315	(*hupSL–lacZ*)	2,460	1,654	47	28
pHL47	(*hupSL–lacZ*)	1,549	1,518	71	40
pHL58	(*hupSL–lacZ*)	195	116	87	45
pIZ263	(*nifH–lacZ*)‡	126	5,401	3	5
pMB210	(*nifH–lacZ*)§	33,635	13,423	1,030	104

†pRmW541.10 expresses the RmNifA protein (36).
‡*K. pneumoniae nifH–lacZ* fusion.
§*R. meliloti nifH–lacZ* fusion.

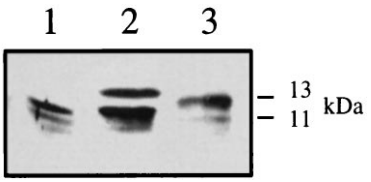


FIG. 3. Identification of proteins immunologically related to *E. coli* IHF in pea bacteroids from *R. leguminosarum*. Lanes: 1, purified *E. coli* IHF; 2, *B. japonicum* CB1809 extract; 3, *R. leguminosarum* bv. viciae UPM791 extract. Estimated molecular masses of the *R. leguminosarum* bands are indicated.

DISCUSSION

The *R. leguminosarum* bv. viciae uptake hydrogenase system is unique because it is only expressed in symbiosis with the host legume (ref. 17; Fig. 1*A*). This is in contrast to the situation with other root-nodule bacteria, such as *B. japonicum* (5) and *Azorhizobium caulinodans* (40), where the hydrogenase system can be induced under appropriate culture conditions (low pO_2, presence of H_2 and Ni^{2+}). Expression of *R. leguminosarum* hydrogenase structural genes (*hupSL*) in pea bacteroids is controlled from a promoter located upstream of *hupS* ($hupS_p$), which has the structure of a $-24/-12$-type, RpoN-dependent promoter (31). Promoters of this type require an enhancer-binding protein for activation (41). Two other lines of evidence support the idea that $hupS_p$ is positively regulated. (*i*) $hupS_p$ upstream sequences are required to drive expression of *hupSL--lacZ* fusions. Promoter deletion experiments showed that these sequences are located within a 85-bp DNA fragment spanning from position -173 to -88 (Fig. 1). This enhancer-like element is expected to be the binding site of the putative activating protein. (*ii*) Consensus IHF-binding sequences are present between the *cis*-acting element and the $-24/-12$ region. IHF proteins are involved in bending the DNA to facilitate the interaction of the activator protein with the RNA polymerase–σ^{54} complex (10).

Induction of the hydrogenase system under free-living conditions in other bacteria is known to require a hydrogenase-specific response regulator: HoxA in *B. japonicum* (13) and *A. eutrophus* (12), and HupR1 in *R. capsulatus* (42). *R. leguminosarum* bv. viciae UPM791 was shown to contain a *hoxA*-like locus, but the *hoxA* gene has been inactivated by the accumulation of frameshift mutations at the 5′ end and by an insertion or deletion at the 3′ end. As a result, the *hoxA*-like locus encodes a truncated protein product of only 136 aa residues instead of the 482–484 aa residues found in active HoxA proteins. This truncated protein lacks the key ATP-binding and C-terminal helix-turn-helix DNA-binding motifs. In addition, it is not required for hydrogenase gene expression, because plasmid pAL618, which does not contain the *hoxA*-like locus, is able to confer regulated hydrogenase activity to naturally Hup^- *Rhizobium* strains (23).

The symbiotic requirement for hydrogenase expression in *R. leguminosarum* bv. viciae and the structural similarity between $hupS_p$ and *nif* promoters, together with the fact that molecular hydrogen, the substrate of uptake hydrogenase, is an obligate by-product of the nitrogenase reaction (43), suggest that *hup* genes might be controlled by a symbiotic nitrogen fixation regulatory protein. Results from *in situ* hybridization experiments with pea nodules (18) support this possibility. In these experiments, a coinduction of *nifH*/*nifA* and *hupSL* genes was observed in a defined cell layer of the nodule interzone II-III. In this interzone, O_2 concentration abruptly drops to values below 100 nM (44), and this apparently triggers activation of *nif* genes. Because NifA is a key regulatory protein in the O_2-controlled activation of *nif* and *fix* genes (45), it was an obvious candidate for activation of $hupS_p$.

Using heterologous *nifA* genes from *K. pneumoniae* (in *K. pneumoniae* and *E. coli*) and *R. meliloti* (in *E. coli*), NifA-dependent transcriptional activation of $hupS_p$ was observed (Tables 1 and 2). This activation required the $hupS_p$ upstream DNA sequences necessary for transcription in *R. leguminosarum*, and did not occur when an altered form of KpNifA, incapable of DNA binding, was used (Table 2). These results rule out an activation mechanism based on direct interaction of NifA with the RNA polymerase–σ^{54} complex (46) and suggest that NifA activates transcription by binding to enhancing elements in the $-173/-88$ $hupS_p$ region. However, this region did not contain any NifA UAS (5′-TGT-N_{10}-ACA-3′; ref. 35), nor was any present in the ≈4 kb of upstream DNA present in the Hup^+ plasmid pAL618 (47). It is well established that most NifA-regulated promoters contain this consensus UAS (45), although NifA activation through binding to promoter sequences differing from the consensus has been recently documented (19). An alternative explanation for the observed NifA-dependent transcriptional activation of $hupS_p$ is that, even in the heterologous *E. coli* host, NifA induces another transcriptional activator, which in turn activates $hupS_p$. This possibility was ruled out by performing multicopy *nif* inhibition experiments (Table 3). Introduction of multiple copies of $hupS_p$ resulted in specific inhibition of nitrogenase. When promoters containing NifA-binding sites are introduced in *K. pneumoniae*, *nif* gene expression and nitrogenase activity are inhibited due to titration of NifA. This observation has been used in the past to define sequences important for NifA binding (39, 48) and, in our case, it shows that NifA-mediated activation of $hupS_p$ occurs through direct binding to upstream DNA sequences.

Symbiotic expression analyses of $hupS_p$ in *nifA R. leguminosarum* mutants, both by *in situ* hybridization and by *lacZ* fusion assays in bacteroids, were inconclusive because Fix^- pea nodules are disorganized both in their structure and in their development (18). However, the *R. etli* bean system was found amenable to this type of analysis. Transcription activation of the *R. leguminosarum hupS* promoter was observed in *R. etli* wild-type and *nifH* bean bacteroids, but not in *nifA* bean bacteroids.

Two additional observations suggest that $hupS_p$ activation by NifA occurs by a mechanism similar to that of well characterized *nif* promoters (10): (*i*) Transcription from $hupS_p$ was abolished in a *rpoN* mutant (Table 1), and (*ii*) IHF was required for NifA-dependent activation in the heterologous host *E. coli*, and proteins immunologically related to IHF were detected in *R. leguminosarum* (Fig. 3), suggesting that IHF-mediated DNA bending is important for $hupS_p$ activation.

All the above results suggest that NifA binds to sequences in $hupS_p$ that differ from the consensus UAS. Identification of these sequences will require direct physical evidence of binding. Recent developments in the establishment of *in vitro* assays with active purified NifA protein (49) should facilitate this task.

The requirement to coordinate nitrogenase-dependent H_2 evolution and H_2 oxidation in nodules might be the reason for the NifA control of *hupSL* expression in *R. leguminosarum* and the concomitant evolutionary loss of HoxA. *B. japonicum*, the only other Hup^+ root-nodule bacterium studied in depth, is able to grow chemolithotrophically with H_2 and CO_2 by coordinately expressing ribulose-biphosphate carboxylase and hydrogenase in free-living conditions (50), and requires HoxA for free-living hydrogenase expression (13). In contrast, *R. leguminosarum* is not able to carry out a chemolithotrophic metabolism and induces hydrogenase activity only in symbiosis. It is possible that the H_2-uptake system of *R. leguminosarum* was originally under the control of a *hoxA*-like gene and hydrogenase activity was expressed in free-living conditions in a similar way as *B. japonicum.* However, adaptation to an ecological situation where the capacity for free-living H_2

oxidation was no longer advantageous may have led to the loss of a functional *hoxA* gene and probably also other genes, such as *hupT* and *hupUV*, which are potentially involved in signal transduction or in free-living sensing of H_2 or Ni. Alternatively, the structural and accessory genes required for synthesis of an uptake hydrogenase system, but not the missing regulatory genes, may have been recently acquired by *R. leguminosarum* as a cluster through lateral transfer, and maintained in the symbiotic plasmid. In any case, symbiotic control of *hup* expression through NifA would ensure regulated synthesis of uptake hydrogenase in the most common H_2-rich environment for rhizobia, the legume nodule.

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