Host-Dependent Expression of *Rhizobium leguminosarum* bv. *viciae* Hydrogenase Is Controlled at Transcriptional and Post-Transcriptional Levels in Legume Nodules

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The legume host affects the expression of *Rhizobium leguminosarum* hydrogenase activity in root nodules. High levels of symbiotic hydrogenase activity were detected in *R. leguminosarum* bacteroids from different hosts, with the exception of lentil (*Lens culinaris*). Transcription analysis showed that the NifA-regulated *R. leguminosarum* hydrogenase structural gene promoter (*P*r) is poorly induced in lentil root nodules. Replacement of the *P*r promoter by the FnrN-dependent promoter of the fixN gene restored transcription of *hup* genes in lentil bacteroids, but not hydrogenase activity. In the *P*^fixN^-*hupSL* strain, additional copies of the *hup* gene cluster and nickel supplementation to lentil plants increased bacteroid hydrogenase activity. However, the level of activity in lentil still was significantly lower than in pea bacteroids, indicating that an additional factor is impairing hydrogenase expression inside lentil nodules. Immunological analysis revealed that lentil bacteroids contain reduced levels of both hydrogenase structural subunit HupL and nickel-binding protein HypB. Altogether, results indicate that hydrogenase expression is affected by the legume host at the level of both transcription of hydrogenase structural genes and biosynthesis or stability of nickel-related proteins HypB and HupL, and suggest the existence of a plant-dependent mechanism that affects hydrogenase activity during the symbiosis by limiting nickel availability to the bacteroid.

Additional keywords: *hup* and *hyp* genes, nitrogen fixation

A group of α-Proteobacteria collectively designated as rhizobia are able to establish symbiotic associations with legume plants. In these associations, the plant develops a new organ, the root nodule, in which bacteria differentiate into bacteroids and fix atmospheric dinitrogen, thus benefiting the plant. The symbiotic process involves a complex molecular dialogue between plant and bacterial partners; the main signals mediating this dialogue (flavonoids, lipochitoooligosaccharides, lipopolysaccharides, and exopolysaccharides) have been elucidated during the last two decades of active research (Frias et al. 2003; Long 2001). The analysis of plant mutants affected in nodule initiation and development has led to the identification of some of the plant proteins involved in signal transduction for nodule formation (Limpens and Bisseling 2003; Stacey et al. 2006). However, other plant factors involved in the process are still unknown. Grafting experiments between model legumes have proven the existence of uncharacterized shoot-derived factors required for symbiotic progression in indeterminate nodules (Lohar and VandenBosch 2005). There is increasing evidence indicating that specific plant factors affect the bacterium at the later stages in the symbiosis. As has been shown recently, the plant host controls bacteroid differentiation inside the nodule in galegoid plants and induces the endoreduplication of bacterial genome within nodules (Mergaert et al. 2006).

*Rhizobium leguminosarum* bv. *viciae* is able to induce efficient symbiosis with legume plants belonging to the tribe Fabae (genera *Pisum*, *Vicia*, *Lathyrus*, and *Lens*). The development of effective symbiotic associations of *R. leguminosarum* bv. *viciae* with this group of legume plants indicates that both partners contain compatible determinants for successful nodule formation and bacteroid differentiation. However, not all symbiotic combinations are equivalent, and differences in host plant preference for specific *R. leguminosarum* bv. *viciae* genotypes have been reported (Hynes and O’Connel 1990; Laguerrera et al. 1993). Within the same group of *Rhizobium*–legume associations, at least one symbiosis-related trait, namely oxidation of nitrogenase-evolved hydrogen, is strongly affected by the host plant, and permissive (*Pisum* and *Vicia* spp.) and nonpermis-sive (*Lens* spp.) hosts for hydrogenase expression have been known for a long time (López et al. 1983). Similar differences in permissivity also were observed among different pea cultivars (Bedmar et al. 1983), and further work using graftings between permissive and nonpermissive pea cultivars suggested the presence of plant shoot factors controlling hydrogenase expression in nodules (Bedmar and Phillips 1984). The existence of a plant host effect on hydrogen oxidation has been observed also in other rhizobial hydrogen oxidation systems, such as those from *Bradyrhizobium* sp. (*Lupinus*) and *R. tropici* (Murillo et al. 1989; Navarro et al. 1993).

Symbiotic hydrogen oxidation in *Rhizobium* spp. is mediated by an [NiFe] hydrogenase that allows recycling of hydrogen generated as a byproduct during nitrogen fixation (Palacios et al. 1993).
et al. 2005). This trait, designated Hup (from H₂ uptake), improves the energy efficiency of the nitrogen-fixation process and results in increases in plant productivity (Baginsky et al. 2005; Evans et al. 1988). Biosynthesis of [NiFe] hydrogenase is a complex process requiring approximately 18 genes (hypSLCDEFGHIJKhupABFCDEX) that encode, in addition to the structural subunits HupS and HupL, 16 accessory proteins. Both subunits undergo proteolytic processing during the synthesis of the mature enzyme. The roles for most of the remaining proteins are known, either as a result of direct analysis of the Hup system or, in many cases, by comparison with the hydrogenase accessory proteins from Escherichia coli or other bacteria: there are proteins participating in electron transfer (HupC) (Dross et al. 1992), HupL subunit proteolytic processing (HupD) (Rossmann et al. 1995), small subunit processing (HupGHJ) (Manyani et al. 2005), and the incorporation of nickel and iron into the active center of the enzyme (HypABFCD) (Böck et al. 2006). Two main promoters, designated P₁ and P₅, are involved in the regulation of hydrogenase gene expression: P₁ is a promoter dependent on NifA, the master regulator for nitrogen-fixation genes that also controls the expression of hypSL and downstream genes (Brito et al. 1994), whereas P₅ controls the expression of the hypBFCDEX operon and is activated by FnrN, a transcriptional activator responsive to low oxygen tensions (Hernando et al. 1995).

Nickel is an obvious requirement for the synthesis of Rhizobium hydrogenase. Insertion of nickel into the structural HupL apoprotein is absolutely required to obtain a mature and active HupL subunit (Böck et al. 2006). In this context, the availability of nickel ions is a major factor affecting symbiotic hydrogenase activity in *Rhizobium leguminosarum* bv. *viciae* in association with pea (*Pisum sativum*) plants (Brito et al. 1994; Ureta et al. 2005). This requirement also was observed in other symbiotic associations such as those involving combinations of Lotus spp. with *Mesorhizobium loti* strains into which the *R. leguminosarum* bv. *viciae* Hup system was transferred (Brito et al. 2000). In addition to nickel availability, a reduced level of transcription from the *R. leguminosarum* bv. *viciae* hypSL promoter (P₁) has been shown to limit hydrogenase activity in some heterologous systems, such as *Sinorhizobium meliloti–Medicago sativa* (Brito et al. 2000). However, in that report, it could not be ascertained whether the nickel-dependent limitation and the reduced transcription level were due to bacterial or plant factors. The development of our knowledge about the *R. leguminosarum* Hup system has provided tools to analyze the molecular basis of a host-dependent control over a bacterial function. In this work, we analyze the interaction of *R. leguminosarum* with two different legumes (pea and lentil) so that we can study the effect of the legume host on symbiotic hydrogenase expression within the same rhizobial strain. From the available data, we conclude that legume host affects *R. leguminosarum* hydrogenase expression at both transcriptional and post-transcriptional levels.

**RESULTS**

**Lentil (Lens culinaris) plants do not allow symbiotic expression of hydrogenase activity from *R. leguminosarum* bv. *viciae* hup⁺ strains.**

In order to investigate the effect of different legume hosts on *R. leguminosarum* bv. *viciae* hydrogenase expression, hydrogen metabolism and nitrogenase activity of strain UPM791 were tested in nodules and bacteroids from pea (*P. sativum*), lentil (*L. culinaris* cv. Magda), *Lathyrus sativus*, *L. odoratus*, *V. ervilia*, *V. monanthos*, *V. sativa*, and *V. villosa* plants (Table 1). Strain UPM791 induced high levels of hydrogen-uptake hydrogenase activity in bacteroids from all legume hosts tested, with the exception of lentil. Consequently, hydrogen evolved from *Pisum*, *Vicia*, and *Lathyrus* nodules was hardly detectable, whereas lentil nodules exhibited a high rate of hydrogen evolution. To rule out a specific cultivar effect, five lentil cultivars (Castilla, Americana, Salamanca, Pardina, and Magda) were inoculated with *R. leguminosarum* UPM791. In all these symbiotic associations, lentil nodules fixed nitrogen efficiently but evolved large amounts of hydrogen. Consistent with these data, hydrogenase activities were undetectable in bacteroids from all lentil cultivars tested (Table 2).

Because provision of nickel to the *R. leguminosarum*–pea symbiosis increases bacteroid hydrogenase activity (Brito et al. 1994), we investigated whether the observed lack of hydrogenase activity in lentil bacteroids was due to nickel limitation. To this end, pea (*P. sativum* cv. Frisson) and lentil (*L. culinaris* cv. Magda) plants inoculated with *R. leguminosarum* hup⁺ strains isolated from different geographic origins were grown in nutrient solutions without Ni added or supplemented with 85 μM nickel chloride. Strains UPM791 and UPM990 correspond to strains 128C53 and 128C30 of the Nitragin collection, whereas strains UPM1131 and UPM1135 were isolated from a natural Ni-rich ultramafic soil from Italy and from an industrial-waste-contaminated soil from Germany, respectively (Fernández et al. 2005). In the absence of added Ni, bacteroids from pea nodules produced by these *R. leguminosarum* strains exhibited high levels of hydrogenase activity, whereas very low activity levels were detected in bacteroids from lentil nodules (Table 3). In the presence of nickel, pea bacteroids exhibited significant increases of hydrogenase activity compared with values obtained from plants grown without nickel supplementation (Table 3). In contrast, only very small increments in hydrogen oxidation activity were observed in bacteroids from

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**Table 1. Hydrogenase and nitrogenase activities of nodules and bacteroids from different legume host plants in symbiosis with *Rhizobium leguminosarum* bv. *viciae* UPM791**

<table>
<thead>
<tr>
<th>Legume host</th>
<th>Bacteroid hydrogenase activityb</th>
<th>Nodule H₂ evolutionc</th>
<th>Nodule C₂H₂ reductiond</th>
<th>RE⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pisum sativum</em></td>
<td>2,780 ± 70</td>
<td>0.43 ± 0.01</td>
<td>26.0 ± 0.3</td>
<td>0.98</td>
</tr>
<tr>
<td><em>Lens culinaris</em></td>
<td>≤20</td>
<td>4.52 ± 0.42</td>
<td>9.7 ± 0.1</td>
<td>0.53</td>
</tr>
<tr>
<td><em>Lathyrus sativus</em></td>
<td>1,590 ± 70</td>
<td>0.09 ± 0.04</td>
<td>15.3 ± 0.2</td>
<td>0.99</td>
</tr>
<tr>
<td><em>L. odoratus</em></td>
<td>890 ± 20</td>
<td>2.78 ± 0.53</td>
<td>24.0 ± 0.4</td>
<td>0.88</td>
</tr>
<tr>
<td><em>Vicia sativa</em></td>
<td>1,740 ± 160</td>
<td>0.43 ± 0.06</td>
<td>25.1 ± 0.7</td>
<td>0.98</td>
</tr>
<tr>
<td><em>V. villosa</em></td>
<td>7,640 ± 1,120</td>
<td>0.20 ± 0.06</td>
<td>8.1 ± 0.9</td>
<td>0.98</td>
</tr>
<tr>
<td><em>V. ervilia</em></td>
<td>1,850 ± 60</td>
<td>&lt;0.01</td>
<td>13.7 ± 0.1</td>
<td>1.00</td>
</tr>
<tr>
<td><em>V. monanthos</em></td>
<td>5,460 ± 600</td>
<td>0.11 ± 0.1</td>
<td>5.60 ± 0.3</td>
<td>0.98</td>
</tr>
</tbody>
</table>

a Values are the average of at least two independent replicates ± standard error.

b Expressed as nmol H₂ h⁻¹ per milligram of protein.

c Expressed as μmol H₂ h⁻¹ (fresh weight of nodules).

d Expressed as μmol C₂H₂ h⁻¹ g⁻¹ (fresh weight of nodules).

RE = relative efficiency (1 – H₂ evolution/C₂H₂ reduction).
lentil plants grown in the nickel-supplemented nutrient solution. Following these results, we decided to test the possibility that nickel was not transported to the nodules in lentil plants.

We measured the level of nickel in the nodule cytosol fraction obtained after crushing whole nodules and spinning down cell debris and bacteroids (discussed below). In this analysis, we determined the level of nickel along with those of three additional metal elements (Mn, Cu, and Mo) as controls (Table 4).

The data obtained indicate that both pea and lentil nodules contained similar levels of nickel when grown on no-nickel-added solutions. Nodule cytosol fractions obtained from plants grown in nutrient solutions containing 85 µM NiCl₂ showed a marked increase (approximately 10x) compared with the no-nickel-added situation, and this increase was similar in pea and lentil plants. In all cases, the concentrations of Mn, Cu, and Mo were comparable in both hosts, an indication that the soluble fractions prepared also were comparable. We conclude from these data that the plant cytoplasm surrounding the symbiosomes contain similar levels of nickel in both hosts.

We investigated the status of the hydrogenase structural protein HupL in bacteroids by immunological assays. Crude extracts from UPM791 and UPM1131 bacteroids obtained from pea plants grown without nickel supplementation showed a prominent band corresponding to the 66-kDa unprocessed form of HupL (Fig. 1, lane 1). Nickel addition to pea plants induced an increase on the amount of the lower, processed band that results from nickel-dependent maturation of the unprocessed polypeptide (Fig. 1, lane 3). In contrast, no proteins corresponding to the HupL processed or unprocessed polypeptides were detected in bacteroids from lentil plants in any nickel condition tested (Fig. 1, lanes 2 and 4). Altogether, these results indicate that lentil plants severely restrict symbiotic hydrogenase activity of _R. leguminosarum_ Hup⁺ strains, and that this limitation is highly specific to this host. Immunological analysis indicates that hydrogenase structural proteins are either not synthesized or unstable in lentil bacteroids.

### The NifA-dependent hupSL promoter is not induced in lentil nodules.

The _R. leguminosarum_ _hup_ and _hyp_ genes are regulated by two different activators, NifA and FnrN, that control promoters P₁ and P₅ located upstream of _hupS_ and _hypB_ genes, respectively (Brito et al. 1997; Hernando et al. 1995). To investigate whether the lack of HupL protein in lentil bacteroids was due to a low level of _hupL_ gene expression, the activity of _R. leguminosarum_ P₁ promoter was analyzed in pea and lentil bacteroids. To this aim, we used plasmid pHL55, a pAL618 derivative carrying a _hupL::lacZ_ transcriptional fusion (Palacios et al. 1990). High levels of β-galactosidase activity were observed in UPM791(pHL55) pea bacteroids, whereas bacteroids induced by the same strain in lentil plants exhibited only basal levels of activity (Fig. 2A). These low values were similar to those shown by aerobic vegetative cells, where the P₁ promoter is not induced. Because _hupSL_ genes are transcribed only in bac-

### Table 2. Hydrogenase and nitrogenase activities of _Rhizobium leguminosarum_ bv. _viciae_ UPM791 bacteroids and nodules from different lentil cultivars

<table>
<thead>
<tr>
<th>Lentil cultivar</th>
<th>Bacteroid hydrogenase activity</th>
<th>Nodule H₂ evolution</th>
<th>Nodule C₂H₂ reduction</th>
<th>RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castellana</td>
<td>&lt;50</td>
<td>5.24 ± 0.31</td>
<td>13.37 ± 0.25</td>
<td>0.61</td>
</tr>
<tr>
<td>Americana</td>
<td>&lt;50</td>
<td>2.07 ± 0.22</td>
<td>7.3 ± 0.1</td>
<td>0.72</td>
</tr>
<tr>
<td>Salamanca</td>
<td>&lt;50</td>
<td>2.50 ± 0.27</td>
<td>9.20 ± 0.18</td>
<td>0.73</td>
</tr>
<tr>
<td>Pardina</td>
<td>&lt;50</td>
<td>1.68 ± 0.19</td>
<td>5.70 ± 0.24</td>
<td>0.71</td>
</tr>
<tr>
<td>Magda</td>
<td>&lt;50</td>
<td>1.55 ± 0.06</td>
<td>7.08 ± 0.43</td>
<td>0.78</td>
</tr>
</tbody>
</table>

a Values are the average of at least two independent replicates ± standard error.

b Expressed as nmol H₂ h⁻¹ per milligram of protein.

c Expressed as µmol H₂ h⁻¹ g⁻¹ (fresh weight of nodules).

d Expressed as µmol C₂H₂ h⁻¹ g⁻¹ (fresh weight of nodules).

e RE = relative efficiency (1 – H₂ evolution/C₂H₂ reduction).

### Table 3. Bacteroid hydrogenase activity of _Rhizobium leguminosarum_ Hup⁺ strains as a function of nickel addition

<table>
<thead>
<tr>
<th>Strain</th>
<th>No Ni²⁺ supplementation</th>
<th>Ni²⁺ supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pea</td>
<td>Lentil</td>
</tr>
<tr>
<td>UPM791</td>
<td>1.237 ± 20</td>
<td>60 ± 2</td>
</tr>
<tr>
<td>UPM990</td>
<td>635 ± 160</td>
<td>&lt;50</td>
</tr>
<tr>
<td>UPM1131</td>
<td>316 ± 10</td>
<td>50</td>
</tr>
<tr>
<td>UPM1135</td>
<td>952 ± 10</td>
<td>190 ± 10</td>
</tr>
</tbody>
</table>

a Expressed as H₂ h⁻¹ per milligram of protein. Hydrogenase activities were obtained using oxygen as electron acceptor. Values are the average of at least two independent replicates ± standard error.

b NiCl₂ was added to the plant nutrient solution to 85 µM final concentration.

### Table 4. Concentration of nickel and other elements in nodule cytosol fractions from pea and lentil as a function of Ni added to plants

<table>
<thead>
<tr>
<th>Host</th>
<th>Ni added (µM)</th>
<th>Concentration of elements in nodule cytosol fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ni</td>
</tr>
<tr>
<td>Pea</td>
<td>0</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>0.71 ± 0.18</td>
<td>2.69 ± 0.42</td>
</tr>
<tr>
<td>Pea</td>
<td>85</td>
<td>4.85 ± 1.20</td>
</tr>
<tr>
<td>Lentil</td>
<td>85</td>
<td>5.30 ± 0.23</td>
</tr>
</tbody>
</table>

a Values (µM) are the average of two independent replicates.
teroid cells from the active nitrogen fixation zone inside pea nodules (Brito et al. 1995), we investigated the level of hup gene expression in this specific zone in lentil nodules. For this purpose, the β-galactosidase activity associated to P1 promoter expression was monitored by histochemical staining of pea and lentil nodule sections. In agreement with β-galactosidase activities, pea nodules induced by strain UPM791 (pHL55) were strongly stained in the nitrogen fixation zone (Fig. 2B). In contrast, lentil nodules induced by the same strain developed a pale blue color (Fig. 2C), similar to that observed in control lentil nodules produced by strain UPM791 (Fig. 2D). To rule out the possibility that the deficient P1 promoter expression could be due to plasmid instability inside the nodule, activity of P1 promoter also was analyzed using a genomic transcriptional fusion. To this aim, plasmid pVSL1, carrying a 1,756-bp DNA fragment internal to hupSL genes fused to the lacZ gene, was introduced by single recombination into UPM791. β-Galactosidase activity assays indicated that the genomic hupL::lacZ fusion was highly induced in pea but not in lentil bacteroids (Fig. 2E).

We also analyzed whether hyp gene expression was affected in lentil bacteroids. To this aim, transcription from the FnRN-dependent P5 promoter was monitored by histochemical staining in plasmid pH14 carrying a hypB::lacZ transcriptional fusion. In contrast to P1 promoter expression, the hypB::lacZ fusion reached similar values of β-galactosidase activity in bacteroids from both legume hosts (Fig. 2A).

Altogether, analysis of gene expression showed a differential induction of P1 and P5 promoters in lentil bacteroids. Although the FnRN-dependent P5 promoter is efficiently expressed in lentil nodules, transcription of the P1 promoter is impaired, and this deficiency might explain the lack of hydrogenase structural protein HupL and the reduced level of hydrogenase activity in this host plant.

An FnRN-dependent promoter induces transcription of hupSL genes, but does not increase hydrogenase activity in lentil bacteroids.

Because the FnRN-dependent P5 promoter is efficiently transcribed in lentil bacteroids, we investigated whether the replacement of the NiFeA-dependent hydrogenase structural gene promoter by an FnRN-dependent promoter induced hupSL gene transcription and, hence, hydrogenase activity in lentil bacteroids. To this aim, we took advantage of strain SPF25, an UPM791 derivative strain whose P1 promoter has been replaced by the FnRN-dependent promoter of the fixN gene. This substitution enables hydrogenase expression in microaerobic free-living cells (Brito et al. 2002). In order to test transcription of the FnRN-dependent fixN promoter controlling hupSL expression in pea and lentil bacteroids, a genomic transcriptional hupL::lacZ fusion was constructed in strain SPF25 using plasmid pVSL1. The resulting hupL::lacZ genomic fusion exhibited similar levels of β-galactosidase activity in pea and lentil bacteroids as well as in microaerobic free-living cells (Fig. 2E). These data indicate that legume host restriction on hupSL gene expression can be overcome when FnRN controls hupSL transcription.

The effect of hupSL promoter substitution on symbiotic hydrogenase activity was determined in SPF25 bacteroids from pea and lentil plants (Table 5). Replacement of P1 by the FnRN-dependent promoter resulted in higher levels of hydrogenase activity in bacteroids from both legume hosts, although values from lentil bacteroids were still significantly lower. In order to determine whether a higher gene copy number could increase the level of hydrogenase activity in lentil bacteroids, we measured hydrogen oxidation activity of strain SPF25 carrying plasmid pALPF1; this plasmid provides additional copies of the hydrogenase gene cluster under the control of the FnRN-dependent PfinN promoter (Brito et al. 2002). Hydrogenase activities in SPF25(pALPF1) bacteroids were higher than those observed in SPF25 bacteroids from pea and lentil plants (Table 5); these results suggest that extra copies of hyp and hyp genes increase the availability of functional proteins for hydrogenase biosynthesis in lentil nodules. However, values of hydrogenase activity of SPF25(pALPF1) in lentil still remained threefold lower than in pea bacteroids. For this reason, we investigated

Table 5. Bacteroid hydrogenase activity of Rhizobium leguminosarum SPF25 as a function of metal supplementation

<table>
<thead>
<tr>
<th>Strain, metal added</th>
<th>Bacteroid hydrogenase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pea</td>
</tr>
<tr>
<td>SPF25</td>
<td></td>
</tr>
<tr>
<td>No metal</td>
<td>1,600 ± 220</td>
</tr>
<tr>
<td>Ni</td>
<td>4,670 ± 440</td>
</tr>
<tr>
<td>Fe</td>
<td>2,550 ± 350</td>
</tr>
<tr>
<td>NiFe</td>
<td>5,010 ± 50</td>
</tr>
<tr>
<td>SPF25(pALPF1)</td>
<td></td>
</tr>
<tr>
<td>No metal</td>
<td>2,250 ± 160</td>
</tr>
<tr>
<td>Ni</td>
<td>10,090 ± 1,380</td>
</tr>
</tbody>
</table>

* Expressed as nmol H2 h⁻¹ per milligram of protein. Values are the average of at least two independent replicates ± standard error.

* When required, metals were added to the plant nutrient solution as NiCl2 or ferric citrate to 85 μM final concentration.
whether the reduced level of hydrogenase activity observed in lentil nodules was due to a potential metal deficiency that impaired maturation of hydrogenase structural subunits. To this end, pea and lentil plants inoculated with strain SPF25 or SPF25(pALPF1) were grown in nutrient solutions containing nickel, iron, or a mixture of both metals. Nickel addition strongly increased (threefold) hydrogenase activity of pea bacteroids (Table 5). More remarkably, nickel supplementation of lentil plants induced an eightfold increase of hydrogen oxidation activity in SPF25 bacteroids. Supplementing the nutrient solution with iron alone had a minor effect on hydrogenase activities, whereas the combination of nickel and iron ions resulted in levels of hydrogenase activity similar to those observed in a nutrient solution supplemented only with nickel (Table 5). The effect of nickel addition on bacteroid hydrogenase activity also was tested in plants inoculated with strain SPF25(pALPF1). In this case, the level of hydrogenase activity increased fourfold in response to nickel addition. Despite these increases, activity levels of both SPF25 and SPF25 (pALPF1) lentil bacteroids were still significantly lower than those in pea bacteroids (Table 5). These data support the idea that nickel availability is an important limiting factor for hydrogenase expression in the \textit{R. leguminosarum}–lentil symbiosis and, at the same time, that an additional factor, not replaceable by nickel addition, is impairing hydrogenase activity in lentil.

**Levels of HupL and HypB proteins are reduced in lentil bacteroids.**

In order to further understand the host-dependent differences in Hup activity, we investigated the status of hydrogenase proteins in SPF25 bacteroids by immunological assays. Immunoblots developed with an anti-HupL antiserum showed the presence of a band corresponding to the HupL unprocessed form in lentil bacteroids from plants grown without nickel supplementation (Fig. 3A, lane 2). However, this band was substantially fainter than the corresponding one in bacteroids from pea plants grown under the same conditions (Fig. 3A, lane 1). We also observed that nickel provision to the plants resulted in lower levels of the HupL unprocessed form and the appearance of a weak, faster-migrating band in SPF25 lentil bacteroids (Fig. 3A, lane 4) corresponding to the processed form. The amount of this protein correlated well with the low hydrogenase activities associated with these bacteroids. In the case of the merodiploid strain SPF25(pALPF1) cells, prominent bands corresponding to the slow- and fast-migrating HupL polypeptides were observed in lentil bacteroids (Fig. 3A, lane 6). Again, higher amounts of the HupL processed form were detected in SPF25(pALPF1) bacteroids obtained from lentil plants grown on a nickel-supplemented solution (Fig. 3A, lane 8). It has to be noted, however, that the total amount of HupL protein was higher in pea bacteroids, which is consistent with the activity data (Fig. 3A, lanes 5 and 7).

We previously demonstrated that hydrogenase structural genes are efficiently transcribed from the FnrN-dependent promoter; therefore, we investigated whether the reduced amount of HupL was due to a deficient synthesis of Hyp proteins required for NiFe cofactor biosynthesis and its incorporation into the HupL apoprotein. For this purpose, status of HypB and HypD proteins was analyzed in bacteroid cell extracts by immunological assays. As expected, prominent HypB-immunoreactive bands were detected in pea bacteroids (Fig. 3B, lanes 1, 3, 5, and 7). Surprisingly, no signal corresponding to this protein was detected in SPF25 lentil bacteroids regardless of the metal condition tested (Fig. 3B, lanes 2 and 4), whereas only a very weak anti-HypB-reacting signal was detected in SPF25(pALPF1) lentil bacteroids (Fig. 3B, lanes 6 and 8). In order to determine whether such an effect was specific for this protein, the level of HypD protein, encoded downstream of hypB and in the same transcriptional unit, also was analyzed. In contrast to HypB, the amount of HypD was unaffected by the plant host (Fig. 3C). These results indicate that a legume host factor or condition post-transcriptionally affects HupL and HypB proteins, decreasing the amount of these proteins in lentil bacteroids.

**DISCUSSION**

The data presented in this article indicate the existence of a host-dependent control of bacterial hydrogenase activity in lentil nodules acting at both transcriptional and post-transcriptional levels. Evidence for eukaryotic control on bacterial gene expression has been reported recently in the \textit{Rhizobium}–legume interaction; as has been shown by Mergaert and associates (2006), the legume host controls bacterial cell cycle and bacteroid differentiation in indeterminate nodules produced by galegoid legumes. Similarly, Miller and associates (2007) have shown the existence of a host-specific regulation of NifA-dependent symbiotic genes from \textit{R. leguminosarum} bv. \textit{trifolii} in association with different \textit{Trifolium} sp. In the case of bacterial hydrogenase expression, a marked plant-dependent control has been reported in the \textit{Rhizobium}–legume symbiosis (Bedmar et al. 1983; López et al. 1983; Murillo et al. 1989; Navarro et al. 1993) and also in epiphytic methylotrophic bacteria resident on the leaf surface from soybean plants (Holland and Polacco 1992). In neither of these cases has the control mechanism been elucidated.

Our analysis of hydrogenase gene transcription revealed that expression from the NifA-regulated \textit{P1} promoter is blocked in lentil bacteroids. The \textit{P1} promoter shows the structure of a $\sigma^{54}$ factor-type promoter and is regulated by NifA (Brito et al. 1997). NifA is known as the master activator of the nitrogen fixation process; together with the $\sigma^{54}$ and integration host factors, it regulates genes involved in nitrogenase synthesis (\textit{nif} and \textit{fix} genes) (Fischer 1994) along with hydrogenase genes (Brito et al. 1997). The lack of symbiotic transcription from the \textit{P1} promoter could be a consequence of deficient NifA transcription or activity in lentil nodules. However, inefficient production or activity of NifA or other regulatory proteins should be reflected in nitrogenase functioning. This was not the case, because the same \textit{R. leguminosarum} strains induce normal
Fix⁺ nodules in lentil plants, suggesting that NifA levels inside the lentil bacteroid are not limiting. The observed effect on transcription could be due to a more direct effect of some unidentified factor acting on the hupSL promoter. Data obtained in the analysis of host-specific expression of symbiotic genes in R. leguminosarum bv. trifolii suggest that an unidentified protein, acting in response to a host-specific factor, might bind to NifA-dependent promoters and interfere with their transcription (Miller et al. 2007). In our case, it is tempting to speculate that a protein, acting in response to a host-specific factor, might affect P₁ expression in a similar way.

The observed lack of transcription of the P₁ promoter provides only a partial explanation for the reduced level of hydrogenase activity detected in lentil bacteroids, because replacement of the original NifA-dependent P₁ promoter by an FnrN-dependent promoter that clearly restored transcription of hydrogenase structural genes did not increase hydrogenase activity. Under these conditions, not limited by hupSL transcription, immunological analysis revealed reduced levels of HupL in lentil nodules. Assuming that similar transcription levels in pea and lentil bacteroids should lead to comparable levels of protein synthesis in both hosts, a likely possibility is that this protein was less stable in lentil nodules. A lower stability of HupL under low nickel conditions has been observed previously (Brito et al. 1994; Ureta et al. 2005). Addition of nickel did increase both the presence and activity of HupL, also indicating that HupL instability was related to nickel deficiency. More intriguing is the case of HypB protein. No HypB immunoreactive signal was detected in SPF25 lentil bacteroids, whereas a very low amount of this protein was detected in the corresponding merodiploid strain. This effect is apparently specific for this protein, because normal levels of HypD, encoded within the same operon (Rey et al. 1993), were found in both hosts under all conditions tested. HypD is a metal-binding protein involved in Fe coordination with the CN ligand, whereas HypB is a nickel-binding protein that participates in the nickel insertion into the active center (Böck et al. 2006). Again, protein function suggests a nickel-mediated effect on protein stability. Although HypB stability in the absence of nickel has not been measured specifically, the abnormal aggregation behavior of the purified recombinant protein (Rey et al. 1994) could be associated with a lack of stability "in vivo" under low intracellular nickel levels.

The analysis of pea and lentil nodule cytosol fractions indicates that the host-specific differences in hydrogenase activity were not due to differences in the concentration of nickel in the plant cytosol surrounding the bacteroids. Our working hypothesis to explain these differences points toward a limitation on the entry of this element into the bacteroid. Such limitation might be a consequence of host-specific differences on either the nickel chemical form or the presence or absence of adequate transport systems for this element, or a combination of both factors, that would result in low levels of nickel inside the lentil bacteroid. This low level of intracellular nickel might affect protein synthesis or stability and, hence, hydrogenase activity. In fact, it is possible that both the transcriptional and post-transcriptional hydrogenase control may be based on a single factor, nickel availability to the bacteroids. Nickel is a known regulatory factor of hydrogenase expression in the endosymbiotic bacterium B. japonicum (Kim and Maier 1990) and in other hydrogen-oxidizing microorganisms (Iwig et al. 2006). In R. leguminosarum UPM791 pea bacteroids, no nickel-dependent control of the P₁ promoter transcription was observed (Brito et al. 1994); however, the situation could be different within lentil nodules. Nickel transport and homeostasis are poorly understood in both plant and bacterial partners. On the bacterial side, several types of nickel transporters and nickel-responsive regulators have been identified (Mulrooney and Hausinger 2003). In the case of E. coli, it has recently been described that the nickel-responsive repressor RenR controls the expression of proteins involved in efflux and import of nickel (Iwig et al. 2006). This repressor also controls hydrogenase-3 activity in E. coli. It is possible that transcription of the P₁ promoter and expression of hydrogenase was controlled by a similar mechanism in Rhizobium spp. On the plant side, nickel is not present as a free ion in plants but in chelated forms with organic acids such as citrate or malate, or amino acids like histidine (Cataldo et al. 1978; Krämer et al. 1996). The chemical nature of these chelators varies between species (White 1981) and it is possible that, in the case of lentil, nickel may be bound to compounds not accessible to transporters located on either peribacteroid or bacteroid membranes. Regarding this, it is interesting to consider the data published by Bedmar and associates (1984) showing the existence of a shoot-derived factor affecting hydrogenase activity in root nodules. Such factor could be a specific nickel chelator.

To date, several cases of plant host control of bacterial gene expression have been reported. However, factors and mechanisms involved have not been elucidated yet. Whatever the nature of the legume host factors involved in hydrogenase expression control, they apparently are specific for the R. leguminosarum–lentil symbiosis. It would be interesting to determine whether the control exerted by the lentil host affects R. leguminosarum bacterial systems other than hydrogenase. In this context, global analyses of lentil bacteroid gene expression will help to identify other bacterial genes controlled by the legume host and provide insights on the eukaryotic mechanism or factor involved.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.**

Bacterial strains and plasmids used in this work are listed in Table 6. R. leguminosarum bv. viciae strains were grown routinely at 28°C in tryptone-yeast extract (TY) (Beringer 1974), Rhizobium minimal media (O’Gara and Shanmugan 1976), or yeast-mannitol media (Vincent 1970). Cosmid pAL618 is a pLAFR1 derivative carrying the hydrogenase gene cluster from R. leguminosarum UPM791 (Leyva et al. 1987). Plasmids pHLS5 and pHL14 are derivatives of plasmid pAL618 containing Tn3HoHo1 insertions that generate hupL::lacZ and hypB::lacZ transcriptional fusions, respectively (Palacios et al. 1990). Strain SPF25 derives from UPM791 by replacement of the native NifA-dependent hupSL promoter by the FnrN-dependent fixN gene promoter (Brito et al. 2002). Plasmid pALPF1 is a pAL618 derivative carrying the same promoter modification as SPF25. E. coli strains DH5α (Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.) and S17.1 (Simon et al. 1983) were used for cloning and mating purposes, respectively. Antibiotics were added at the following concentrations: tetracycline at 5 μg·ml⁻¹, kanamycin at 50 μg·ml⁻¹, and ampicillin at 100 μg·ml⁻¹.

**DNA manipulation techniques and plasmid constructions.**

Plasmid DNA preparations, restriction enzyme digestions, and DNA transformations into E. coli were carried out by standard protocols (Sambrook et al. 1989). Plasmids were introduced into R. leguminosarum strains by conjugation and transconjugants were selected in Rhizobium minimal medium supplemented with the corresponding antibiotics. The pSVL1 construction is a pVIK112-based plasmid that carries a fusion of the R. leguminosarum hupSL genes with a promoterless lacZ gene. For this construction, an internal 1,756-bp DNA fragment of hupSL genes was cloned in the Smal/XbaI restric-
tion sites of pVIK112. Plasmid pVSL1 was introduced by conjugation in strains UPM791 and SPF25 and transcriptional fusions were obtained through single recombination with the genomic hupSL genes. The single cross-over was verified by Southern hybridization analysis.

**Plant tests and enzyme assays.**

Pea (*P. sativum* L., cv. Frisson), lentil (*Lens culinaris* cvs. Castilla, Americana, Salamanca, Pardina, and Magda), *Lathyrus sativus*, *L. odoratus*, *Vicia ervilia*, *V. monanthos*, *V. sativa*, and *V. villosa* seeds were surface-sterilized and planted in Leonard jar-type cultures under bacteriologically controlled conditions (Ruiz-Argüeso et al. 1978). Plants were grown in a nitrogen-free plant nutrient solution that contained (per liter) KCl, 74 mg; KH₂PO₄, 175 mg; MgSO₄ · 7H₂O, 246 mg; CaSO₄, 345 mg; Fe(III) citrate, 1.8 mg; MnSO₄ · 7H₂O, 1 mg; NiCl₂, 1 mg; H₂BO₃, 700 μg; ZnSO₄ · 5H₂O, 110 μg; CuSO₄ · 5H₂O, 39 μg; and (NH₄)₆Mo₇O₂₄ · 4H₂O, 5 μg. To study the effect of metals (Ni and Fe) on hydrogenase activity, we used nutrient solutions without Ni added or supplemented with 85 μM nickel chloride. In these studies, Fe was added as ferric citrate. Enzymatic assays were carried out with nodules or bacteroids from 21-day-old plants. Hydrogen evolution and acetylene reduction in intact nodules were determined by gas chromatography (Ruiz-Argüeso et al. 1978). Hydrogenase activity in bacteroid suspensions was measured using an amperometric method with oxygen as electron acceptor (Ruiz-Argüeso et al. 1978). Protein content of bacteroids was determined by the bicinchoninic acid method (Smith et al. 1985). β-Galactosidase staining of nodule cytosol was stored at –70°C in plastic tubes until analysis. Assays of β-galactosidase activity were carried out with nodules or bacteroids from 21-day-old plants. Hydrogen evolution and acetylene reduction in intact nodules were determined by gas chromatography (Ruiz-Argüeso et al. 1978). Hydrogenase activity in bacteroid suspensions was measured using an amperometric method with oxygen as electron acceptor (Ruiz-Argüeso et al. 1978). Protein content of bacteroids was determined by the bicinchoninic acid method (Smith et al. 1985) with the modifications described by Brito and associates (1994). β-Galactosidase activity was determined in *R. leguminosarum* free-living cultures and bacteroids from 21-day-old plants as described by Miller (1972). β-Galactosidase staining of nodule sections was carried out in nodules from 21-day-old pea and lentil plants as previously described by Boivin and associates (1990).  

**Metal determination in root nodule cytosol.**

Metal determinations were made using ultrapure reagents and HCl-treated material essentially as described previously (Becana and Klucas 1992). After excision, 0.5 g of root nodules were washed with MilliQ-grade water, placed in 12-ml plastic tubes, and crushed in 100 mM potassium phosphate buffer (pH 7). Extracts were centrifuged sequentially at 120,000 × g to remove plant and bacteroid cells, respectively. Aliquots of the 8-ml supernatant (containing the nodule cytosol) were stored at –70°C in plastic tubes until analysis. Analysis of metals (Ni, Mn, Cu, and Mo) was performed in an inductively coupled plasma mass spectrometry system (ICP-MS Elan 6000; Perkin Elmer, Waltham, MA, U.S.A.) at the Servicio Interdepartamental de Investigación center of the Universidad Autónoma of Madrid. Analysis of metals in the buffer revealed no detectable amounts of Ni and Cu, and low amounts of Mn (0.33 μM) and Mo (0.02 μM).

**Immunological detection of Hup and Hyp proteins.**

Immunological detection of Hup and Hyp proteins in bacteroid crude extracts was carried out by Western blot assays using antisera raised against HupL from *Bradyrhizobium japonicum*, HypB from *R. leguminosarum*, and HypD from *E. coli*. Immunological assays were performed as previously described (Brito et al. 2000). Briefly, crude extracts were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis in 10% acrylamide gels and transferred onto Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.). Filters were incubated with antisera (at dilutions 1:1000 for HupL and HypB and 1:100 for HypD) and with a secondary goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate antibody (Bio-Rad Laboratories, Inc. Hercules, CA, U.S.A.). A chromogenic substrate (bromochloroindolyl phosphate-nitro blue tetrazolium) was used as developing reagent.

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**LITERATURE CITED**


