Characterization of a new internal promoter (P₃) for *Rhizobium leguminosarum* hydrogenase accessory genes *hupGHIJ*

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_Synthesis of the *Rhizobium leguminosarum* [NiFe] hydrogenase requires the participation of 16 accessory genes (hupCDEFGHIJKhypABFCDEX) besides the genes encoding the structural proteins (hupSL). Transcription of hupSL is controlled by a −24/−12-type promoter (P₁), located upstream of hupS and regulated by NifA. In this work, a second −24/−12-type promoter (P₃), located upstream of the hupG gene and transcribing hupGHIJ genes in *R. leguminosarum* pea (*Pisum sativum* L.) bacteroids, has been identified in the *hup* gene cluster. Promoter P₃ was also active in *R. leguminosarum* free-living cells, as evidenced by genetic complementation of hydrogenase mutants. Both NifA and NtrC activated P₃ expression in the heterologous host _Klebsiella pneumoniae_. Also, P₃ activity was highly stimulated by _K. pneumoniae_ NifA in _Escherichia coli_. This NifA activation of P₃ expression only required the ς₅₄-binding site, and it was independent of any cis-acting element upstream of the ς₅₄ box, which suggests a direct interaction of free NifA with the RNA polymerase holoenzyme. P₃-dependent *hupGHIJ* expression in pea nodules started in interzone II/III, spanned through nitrogen-fixing zone III, and was coincident with the NifA-dependent *nifH* expression pattern. However, P₃ was dispensable for *hupGHIJ* transcription and hydrogenase activity in pea bacteroids due to transcription initiated at P₁. This fact and the lack of an activator recruitment system suggest that P₃ plays a secondary role in symbiotic *hupGHIJ* expression._

INTRODUCTION

Aerobic bacteria, such as _Alcaligenes* (*Ralstonia*), _Pseudomonas*, _Bradyrhizobium*, _Rhizobium* or _Azotobacter_, oxidize hydrogen through a [NiFe] membrane-bound hydrogenase using oxygen as a final electron acceptor to obtain energy through oxidative phosphorylation. The synthesis of active [NiFe] hydrogenases requires, in addition to hydrogenase structural genes, the participation of an array of accessory genes involved in the synthesis and assembly of metal clusters (for reviews, see Friedrich & Schwartz, 1993; Casalot & Rousse, 2001; Vignais _et al_., 2001). In most cases, hydrogenase gene expression is regulated by a multicomponent regulatory system consisting of proteins involved in H₂ sensing, signal transduction and DNA transcription (Bernhard _et al_., 2001; Elsen _et al_., 1996).

In nitrogen-fixing, endosymbiotic bacteria such as _Bradyrhizobium japonicum_ and _Rhizobium leguminosarum_, H₂ is generated by nitrogenase itself, and a particular coupling occurs between hydrogen oxidation and nitrogen fixation inside the legume nodules. Although _B. japonicum_ contains a H₂-sensing and signal transduction system, mediated by the proteins HoxA, HupT and HupUV, which allows these bacteria to express hydrogenase in free-living conditions (Van Soom _et al_., 1993b, 1999; Black _et al_., 1994), the induction of the hydrogenase system in soybean nodules does not require H₂, and it has been proposed that the FixK₂ protein could be the activator that co-ordinates hydrogenase and nitrogenase expression (Durmowicz & Maier, 1998).

The expression of hydrogenase activity by _R. leguminosarum_ bv. _viciae_ has only been observed in symbiosis with legumes, and the regulation of this expression has been studied in detail (Ruiz-Argüeso _et al_., 2001). _R. leguminosarum_ bv. _viciae_ strain UPM791 contains a large gene cluster of 18 genes, hupSLCDEFGHIJKhypABFCDEX, which are needed for hydrogenase synthesis (Fig. 1a). Although several transcriptional units were initially defined by symbiotic complementation analysis (Leyva _et al_., 1990; Hidalgo _et al_., 1992), only two major promoters have been characterized within this gene cluster. First, a NifA-dependent −24/−12-type promoter (P₁), responsible for symbiotic activation of at least the hydrogenase structural genes hupSL, was identified upstream of hupS (Hidalgo _et al_., 1992; Brito _et al_., 1997). This NifA-dependent promoter constrains the expression of...
of hydrogenase activity to symbiotic cells, and results in a temporal and spatial co-expression of nitrogenase and hydrogenase structural genes in pea nodules (Brito et al., 1995). Second, an Fnr-type promoter (P5), located upstream of hypB within the hypA gene, activates the hypBFCDEX operon in pea bacteroids and also in vegetative cells in response to microaerobic conditions (Hernando et al., 1995). Two copies of an fnrN gene have been identified in R. leguminosarum bv. viciae, and their transcription is autoregulated (Colombo et al., 2000). FnrN controls both hydrogenase and nitrogenase activities by regulating the expression of the hypBFCDEX and fixNOQPfixGHIS operons, respectively (Gutiérrez et al., 1997).

No other functional promoters have been characterized in the R. leguminosarum bv. viciae hydrogenase gene cluster. An R. leguminosarum mutant containing a Tn5 insertion within the hupH gene was used to define, by mutant complementation analysis, a transcriptional unit (hup III), which included the hupGHIJ genes (Leyva et al., 1990; Rey et al., 1992; Hidalgo et al., 1992). Genes hupGHIJ encode proteins of unknown molecular functions (Rey et al., 1992) but that are required for hydrogenase synthesis in R. leguminosarum (Rey et al., 1992; Brito et al., 1994) and Ralstonia eutropha (Bernhard et al., 1996). The hyaE and hyaF genes, homologous to hupG and hupH, are also needed for hydrogenase I synthesis in Escherichia coli (Menon et al., 1991). In this work, a −24/−12-type internal promoter (P3) that determines transcription of the hupGHIJ genes in Rhizobium leguminosarum symbiotic and free-living cells has been identified and characterized.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Bacterial strains and plasmids used in this work are listed in Table 1. R. leguminosarum strains (Leyva et al., 1987) were routinely grown in tryptone/yeast extract (Beringer, 1974) or yeast/mannitol (YMB; Vincent, 1970) medium at 28°C. Klebsiella pneumoniae strains were grown at 30°C in Luria–Bertani (LB) medium, or in K medium.
supplemented with 25 mM ammonium acetate (Imperial et al., 1984). E. coli strains were grown in LB medium. Antibiotics were added at the following concentrations (µg ml⁻¹): tetracycline, 12 (6 for Rhizobium); kanamycin, 50; ampicillin, 100; chloramphenicol, 20. Expression of K. pneumoniae NifA from pMJJ20 was induced aerobically in E. coli ET8000 at 28°C as described previously (Morett & Buck, 1988). NifA from Sinorhizobium meliloti was expressed anaerobically from pRM1W541.10 in E. coli ET8000. Plasmids were introduced into R. leguminosarum by conjugation using E. coli S17.1 as the donor strain (Parry et al., 1994), and transconjugants were selected in Rhizobium minimal medium (O’Gara & Shanmugam, 1976) supplemented with the corresponding antibiotic.

DNA manipulation and analysis. Plasmid DNA preparations, restriction enzyme digestions, agarose and polyacrylamide gel electrophoresis, DNA cloning, and transformation of DNA into E. coli cells were carried out using standard protocols (Sambrook et al., 1989). Genomic DNA from R. leguminosarum was extracted as described previously (Leyva et al., 1987). For Southern hybridizations, DNA probes were labelled with digoxigenin (Roche Molecular Biochemicals) and hybridization bands were visualized by luminography. DNA sequencing was carried out using the Rhodamine Terminator Cycle Sequencing Ready Reaction kit and an ABI377 automatic sequencer (PE Biosystems) or using a sequenase kit (Sequenase Version 2.0; United States Biochemical).

Plasmid construction. To generate transcriptional hupGH::lacZ fusions, a 2.3 kb XbaI–KpnI fragment from cosmid pAL618 was cloned in vector pMP220 in both orientations with respect to the lacZ gene, yielding plasmids pHIL30 and pHIL330. Plasmid pSPDI was generated by cloning a 1.6 kb HindIII fragment from pHIL30 in vector pSPV4. Plasmids pSPD2, pSPD3 and pSPD5 contain deletion fragments that were generated by PCR using SPD2 (5’-ATTTGGGCGGCGGATGAAAGAGA-3’), SPD3 (5’-AGATGT-TGGCGATATTTTCCT-3’) and SPD5 (5’-ATCTGGGGCCCTTGCGG-CGCCCCTG-3’) as upper primers, SPD3R (5’-GCAACCCAAAAACC-3’) as lower primer and pHIL30 DNA as template. The resulting PCR fragments of 654, 600 and 464 bp, respectively, were cloned in pCR2.1-TOPO generating TOPO-SPD plasmids. Then, the 414, 360 and 224 bp HindIII fragments from TOPO-SPD2, TOPO-SPD3 and TOPO-SPD5 were fused to the lacZ gene in vector pSPV4, resulting in plasmids pSPD2, pSPD3 and pSPD5, respectively.
To generate plasmid vector derivatives pEK26 and pSKA, a 745 bp HindIII–KpnI fragment from the hupGH genes and the entire PMJ220 plasmid as an EcoRI fragment, respectively, were cloned in pBluescript vector. pSKDG was the plasmid resulting from cloning a 1.6 kb HindIII fragment that spanned the hupD to hupG genes and contained the P3 promoter into the pBluescript SK+ vector.

Transcription mapping. The location of the 5’ end of hupG mRNA was determined by primer extension analysis as described by Hidalgo et al. (1992). For this assay, two synthetic oligonucleotides, HUPPG2 (5′-TCATGCAGCATGGTAGGTG-3′) and HUPPG3 (5′-TGCCGGTTGAAAACAC-3′), were used. These primers were complementary to the mRNA sequence corresponding to amino acids 14–20 and 39–45, respectively, of HupG. Total RNA for this analysis was isolated from aerobic cells of E. coli ET8000(pHL320, pMJ220) and ET8000(pSPD5, pMJ220) as described previously (Summers, 1970). The synthetic primers were labelled with [γ-32P]ATP, and the corresponding DNA extension products obtained by reverse transcriptase reaction from RNA were visualized by autoradiography.

Site-directed mutagenesis of the P3 promoter. Site-directed mutagenesis of the RpoN-binding sequence was carried out on plasmid pSKDG using the Quick Change™ Site Directed Mutagenesis Kit (Stratagene) and following the manufacturer’s protocol. Two synthetic, complementary oligonucleotides carrying the corresponding mutations (underlined) were used to generate the promoter mutation. First, the oligonucleotide 5′-CTACTTTTCTCAGTCACCCA-CGCCGTTTGAG-3′ and its complementary primer were used to mutagenize the –24 position and, second, oligonucleotide 5′-CACCCACCGGTCTTGGAGTCATCATCTTCCG-3′ and its complementary primer were used to introduce the mutation in the –12 position. To confirm the presence of correct mutations in the resulting plasmid, pSKP3, the P3 promoter region was sequenced. The 1.6 kb HindIII fragment carrying the mutated promoter was cloned in pK18mobsacB (Schäfer et al., 1994) together with an additional 1.5 kb HindIII-EcoRI DNA fragment containing the rest of the hupG gene, and the hupHIJ genes. The resulting plasmid, pK18P3, was constructed into R. leguminosarum UPM791 by conjugation, and the P3 promoter was replaced by double crossover using the sacB system. Correct replacement of the promoter was confirmed by Southern blot experiments using a 654 bp EcoRI fragment as a DNA probe for the P3 promoter and taking advantage of the Xhol restriction site introduced with the mutation at the –12 position.

In situ hybridization of nif and hup mRNAs in pea nodules. Root pea (Pisum sativum L. cv. Rondo) nodules were harvested 16 days after inoculation with R. leguminosarum bv. viciae strain UPM791 or derivative mutant ALS1 (HupS). Nodule sections (7 μm thick) were prepared as described by Yang et al. (1991). The hupGH antisense and sense probes were prepared from pEK26. The nifH RNA antisense probe was obtained as described by Yang et al. (1991). The hupGH and nifH RNA probes were obtained and labelled in vitro with [35S]UTP using the appropriate (T3 or T7) RNA polymerase phage system (Van de Wiel et al., 1990). Nodule sections were hybridized with the RNA probes and developed after 3 or 4 weeks exposure as described by Van de Wiel et al. (1990). Micrographs were taken in bright field and bright field with epipolarization.

Plant tests and enzyme assays. Pea (P. sativum L. cv. Frisson) plants were used as the host for R. leguminosarum bv. viciae strains. Conditions for plant inoculation and growth have been described previously (Leyva et al., 1987), and plant nutrient solution was supplemented with 20 μM NiCl2. Nitrogenase derepression in K. pneumoniae was carried out as indicated by Imperial et al. (1984). β-Galactosidase activities in Rhizobium and pea bacteroids were determined as described by Miller (1972). Hydrogenase activity in bacteroid suspensions was measured by an amperometric method with oxygen as the terminal electron acceptor (Ruiz-Argüeso et al., 1978). The protein content of bacteroid suspensions was measured by the bicinchoninic acid method (Smith et al., 1985) after alkaline digestion in 1 M NaOH at 90°C for 10 min and with BSA as a standard.

RESULTS

Promoter activity of the intergenic region upstream of hupG (P3)

Inspection of the R. leguminosarum hup gene cluster showed that the hup and hyp genes are tightly arranged (Fig. 1a; Ruiz-Argüeso et al., 2001), except for a 168 bp intergenic region between the hupF and hupG genes. Analysis of this region revealed the existence of a highly conserved σ54-binding sequence (5′-TGCCGAC N9 TTGCA-3′), characteristic of −24/−12-type promoters, 46 bp upstream of the hupG start codon, and of a sequence with a similar similarity to the consensus motif for binding of the integration host factor (IHF) (Fig. 1b; Hidalgo et al., 1992). The presence of these sequence motifs in the hupF–hupG region suggested the existence of a promoter (P3) responsible for transcription of hupG and downstream genes, although no recognizable upstream activating sequences (UASs) could be identified in the region. Promoter activity of this region was assayed in R. leguminosarum by means of lacZ gene fusions (Table 2). Low but reproducible activity above background was observed in a hupGH::lacZ fusion (pHL320), both in free-living cells (aerobic and microaerobic conditions) and in pea bacteroids. A different construct (pSPD1) encoding a hupGH::lacZ fusion was used as substrate for site-directed mutagenesis of the −24/−12 box in the P3 region (see Methods). The resulting plasmid (pSPD1P3), incorporating a mutated ACCCAGCGCTCTCGA −24/−12 box instead of the wild-type sequence TGCCGACGCCGTTCG, did not show detectable promoter activity under any of the conditions tested (Table 2), thus proving that the −24/−12 box is part of the P3 promoter.

P3 promoter functionality in free-living cells and bacteroids

A genetic complementation test was performed to determine whether transcription from P3 can result in synthesis of adequate levels of the downstream gene products. Plasmid pALPF1 (Brito et al., 2002) encodes a functional hup cluster that can be induced in free-living cells under the control of the microaerobic regulator FnrN to generate high levels of hydrogenase activity (Brito et al., 2002; Table 3). The same high levels of hydrogenase activity were induced when pALPF1 was introduced into strain UPM791P3 (Table 3). This strain incorporates the mutation in the P3 σ54-binding sequence present in pSPD1P3 (see Methods). An in-frame deletion in the pALPF1 hupH gene, one of the genes downstream of P3, resulted in a derivative plasmid which determined high levels of
Table 2. Expression analysis of the P3 promoter in R. leguminosarum bv. viciae UPM791

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Fusion</th>
<th>Promoter feature</th>
<th>β-Galactosidase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pea nodule bacteroids</td>
</tr>
<tr>
<td>pHL320</td>
<td>hupGH::lacZ</td>
<td>Wild-type</td>
<td>68 ± 20</td>
</tr>
<tr>
<td>pHL330</td>
<td>hupGH::lacZ</td>
<td>Reverse</td>
<td>22 ± 11</td>
</tr>
<tr>
<td>pSPD1</td>
<td>hupG::lacZ</td>
<td>Wild-type</td>
<td>67 ± 17</td>
</tr>
<tr>
<td>pSPD1P3</td>
<td>hupG::lacZ</td>
<td>Mutated σ54 box</td>
<td>20 ± 7</td>
</tr>
<tr>
<td>pSPD2</td>
<td>hupG::lacZ</td>
<td>Wild-type</td>
<td>84 ± 16</td>
</tr>
<tr>
<td>pSPD3</td>
<td>hupG::lacZ</td>
<td>Wild-type</td>
<td>79 ± 13</td>
</tr>
<tr>
<td>pSPD5</td>
<td>hupG::lacZ</td>
<td>Deleted σ54 box</td>
<td>18 ± 9</td>
</tr>
</tbody>
</table>

*Values (Miller units) are expressed as the means of three assays ± SD.

free-living hydrogenase activity in strain UPM791 (Table 3). This hydrogenase activity was due to complementation in trans of the ΔhupH mutation by the chromosomal hupH copy in strain UPM791 transcribed from P3, since introduction of plasmid pALPFΔhupH in strain UPM791P3 resulted in a severe reduction in hydrogenase activity. Similar results were obtained with an in-frame deletion in the downstream gene hupJ (Table 3), indicating that P3 is responsible for transcription of the hupGHJ genes.

Further evidence for the functionality of the P3 promoter was obtained by studying the distribution of hupGH mRNA in pea nodules by in situ hybridization analysis. In pea nodules, the hupSL genes are co-induced with nif genes by means of NifA (Brito et al., 1995; Brito et al., 1997). Sections from pea root nodules induced by R. leguminosarum strain UPM791 were hybridized with 35S-labelled antisense nifH and hupGH probes (details are described in Methods). Accumulation of transcripts specific for hupGH genes was clearly observed in the cell layer corresponding to the interzone II/III and in the nitrogen-fixing zone III (as defined by Vasse et al., 1990; see Fig. 2a). This hybridization pattern was similar to that observed with the nifH antisense probe in an adjacent section of the same nodule (Fig. 2b). We also analysed hupGH expression in sections from nodules infected with the R. leguminosarum mutant AL51. This mutant harbours a Tn5 insertion in the hupS gene. The insertion blocks transcription from P1, the hupS promoter, and does not result in de novo transcription from internal Tn5 promoters, as judged from in situ hybridization analysis with a hupL probe located downstream the insertion point (Brito et al., 1995). Transcripts corresponding to the hupGH genes were also detected in these nodules sections with a pattern of hybridization similar to that detected in the wild-type strain UPM791 (Fig. 2c). From these results we concluded that hupGH expression occurs at the interzone II/III and at nitrogen-fixing zone III where the nif genes are also expressed. Since hupGH expression was also observed in the absence of transcription from P1, these results show that a promoter region between hupL and hupGH (P3 promoter) is functional in symbiotic conditions.

Table 3. Effect of P3 on free-living hydrogenase activity of R. leguminosarum bv. viciae UPM791

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Genotype (genome/plasmid)</th>
<th>Hydrogenase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPM791</td>
<td>pALPF1</td>
<td>Wild-type</td>
<td>&lt;10</td>
</tr>
<tr>
<td>UPM791</td>
<td>pALPF1ΔhupH</td>
<td>Wild-type/hup+</td>
<td>4940 ± 530</td>
</tr>
<tr>
<td>UPM791P3</td>
<td>pALPF1</td>
<td>P3 mutation/hup+</td>
<td>4680 ± 780</td>
</tr>
<tr>
<td>UPM791P3</td>
<td>pALPF1ΔhupH</td>
<td>Wild-type/ΔhupH</td>
<td>6530 ± 2550</td>
</tr>
<tr>
<td>UPM791</td>
<td>pALPF1ΔhupF</td>
<td>Wild-type/ΔhupF</td>
<td>350 ± 110</td>
</tr>
<tr>
<td>UPM791</td>
<td>pALPF1ΔhupF</td>
<td>Wild-type/ΔhupH</td>
<td>5990 ± 1850</td>
</tr>
<tr>
<td>UPM791</td>
<td>pALPF1ΔhupF</td>
<td>P3 mutation/ΔhupF</td>
<td>910 ± 450</td>
</tr>
</tbody>
</table>

*Values [nmol H2 oxidized h⁻¹ (mg protein)⁻¹] are the mean of three assays ± SD.
†Plasmid pALPF1 is a pAL618 derivative in which the NifA-dependent P1 promoter has been replaced by an FnrN-dependent promoter (fixN promoter) resulting in microaerobic free-living hydrogenase induction.
Heterologous activation of P₃ by NifA and other regulators

Since the symbiotic pattern of expression of the P₃ promoter region resembled that of NifA-dependent promoters (P₁ hup promoter and nifH promoter; Fig. 2), we investigated the possibility that P₃ was also activated by NifA. The fact that nodules generated by NifA⁻ mutants are aberrant (Brito et al., 1997), together with the low β-galactosidase activity associated with P₃ promoter fusions (Table 2), determined that no conclusive results could be obtained with rhizobial NifA⁻ mutants (data not shown). Instead, β-galactosidase activity associated with a hupGH::lacZ fusion was studied in K. pneumoniae in the presence or absence of NifA (Table 4). Under both aerobic and anaerobic conditions, significant β-galactosidase activities were observed in the wild-type strain. Interestingly, hupGH expression was reduced by threefold in the σ₅₄ and ntrC mutants as compared with the wild-type strain. In the absence of NifA, P₃ could be activated by NtrC (or other regulators present in the cell) as shown by the β-galactosidase activities associated with the nifA mutant strain. Supplementation with NifA from K. pneumoniae in plasmid pMJ220 stimulated by more than eightfold the β-galactosidase activity in the wild-type strain and also in the nifA and ntrC mutants, but not in the rpoN mutant, demonstrating that the RNA polymerase σ₅₄ factor was strictly required for activation of P₃ in K. pneumoniae.

In E. coli, not only NifA from K. pneumoniae, but also NifA from S. meliloti under anaerobic conditions, were able to activate the P₃ promoter (Fig. 3). The requirement of the σ₅₄-binding signature for NifA-dependent P₃ activity under these conditions was also confirmed by using the mutant promoter present in pSPD1P₃ (Fig. 3).

We took advantage of the high level of NifA-dependent expression of P₃ in E. coli to determine the transcription start site. Total mRNA from aerobic cells of E. coli ET8000(pHL320, pMJ220) and two primers (HUPFG2 and HUPFG3) complementary to DNA regions internal to hupG were used in a primer extension experiment (Fig. 1b). As negative control, strain ET8000(pSPD5, pMJ220) which overexpressed NifA but lacked the DNA upstream of the hupG gene was assayed. A G base located 34 bases upstream of the hupG ATG was identified as the transcriptional initiation site using both oligonucleotides (Fig. 1c). This transcriptional initiation site placed the conserved GC sequence from the putative σ₅₄-binding box (5'-TGGCAC N₅ TTGCA-3') at position -13, which is consistent with the σ₅₄-dependent transcription of P₃.

No canonical NifA UASs were present in the P₃ promoter.

Fig. 2. Localization of nifH and hupGH transcripts in sections of pea nodules by in situ hybridization. Longitudinal sections from 16-day-old pea nodules inoculated with R. leguminosarum wild-type strain UPM791 (a, b) and hupS mutant ALS51 (c, d) hybridized with hupGH (a, c) and nifH (b) mRNA antisense probes, and a hupGH (d) sense probe as a control. Arrows indicate the same cell of the interzone II/III in adjacent sections that hybridizes with the hupGH (a) and the nifH (b) mRNA probes. (a) And (b), bright field plus epipolarization micrographs. (c) And (d), bright field micrographs. The section hybridized with the nifH probe was exposed for 3 weeks; those sections hybridized with the hupGH antisense and sense probes were exposed for 4 weeks.
However, since NifA-dependent expression of the hup structural promoter (P3) has been shown to depend on binding of NifA to non-canonical sequences (Brito et al., 1997), the presence of such putative activating sequences was investigated by deletion analysis of the DNA region upstream of hupG. This analysis was carried out in E. coli cells in the presence of K. pneumoniae NifA. Fig. 3 shows that NifA-dependent P3 activity did not require any DNA sequence located upstream of the putative IHF motif, thus suggesting that NifA is activating P3 from solution. Similar results were obtained for the native levels of P3 activity in R. leguminosarum pea bacteroids. When the same deletion plasmids were introduced in R. leguminosarum, no effect of these deletions on P3 activity was observed (Table 2), except for the deletion in plasmid pSPD5, which includes the σ^{54}-binding sequence.

Finally, the possible involvement of the putative IHF-binding sequence in P3 activation by NifA was ruled out after observing that the NifA-dependent β-galactosidase activity of the hupGH::lacZ fusion in pHL320 was not affected in E. coli SE1000, an IHF mutant (Table 5). As control of IHF-dependent activation, the reporter activity associated with pHL315 (hupSL::lacZ) was measured (Table 5; Brito et al., 1997). This activity was reduced sixfold in the IHF mutant strain.

Taken together, these results indicate that the presence of the putative σ^{54}-binding sequence was the only requirement

### Table 4. Expression analysis of the P3 promoter in K. pneumoniae cells grown under aerobic or anaerobic conditions

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Genotype</th>
<th>β-Galactosidase activity†</th>
<th>Aerobic</th>
<th>Anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>−NifA</td>
<td>+KpNifA†</td>
</tr>
<tr>
<td>UNF122</td>
<td>Wild-type</td>
<td>100 ± 10</td>
<td>715 ± 30</td>
<td>100 ± 50</td>
</tr>
<tr>
<td>CK263</td>
<td>nifA-negative</td>
<td>90 ± 10</td>
<td>720 ± 10</td>
<td>80 ± 40</td>
</tr>
<tr>
<td>UNF2651</td>
<td>rpoN-negative</td>
<td>30 ± 10</td>
<td>40 ± 10</td>
<td>30 ± 10</td>
</tr>
<tr>
<td>UNF1789</td>
<td>ntrC-negative</td>
<td>40 ± 10</td>
<td>940 ± 420</td>
<td>30 ± 10</td>
</tr>
</tbody>
</table>

*All strains contained a hupGH::lacZ fusion in pHL320.
†Values (Miller units) are the mean of three determinations ± SD.
‡NifA from K. pneumoniae (KpNifA) was supplied by pMJ220.

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**Fig. 3.** Deletion analysis of the P3 promoter in E. coli. The physical and genetic map of a XhoI–KpnI (2337 bp) fragment of the R. leguminosarum UPM791 hup region is shown at the top. The position of the σ^{54}-binding site is indicated by vertical arrows and the putative IHF-binding sequence by a square. Horizontal lines indicate the DNA regions cloned in front of the reporter lacZ gene in pMP220 (pHL plasmids) or pSPV4 (pSPD plasmids). pSPD1P3 is a pSPD1-derivative construct containing a mutation in the RpoN-binding site. The number of nucleotides with respect to the transcription initiation site is indicated on the left of the cloned fragments. NifA from K. pneumoniae (KpNifA) and S. meliloti (SmNifA) was supplied from plasmids pMJ220 and pRmW541.10, respectively. The β-galactosidase activity associated with each fusion was determined in E. coli ET8000. Data (Miller units) are the mean of three determinations ± SD.
both for NifA-dependent promoter expression in E. coli and for native expression in pea bacteroids, and that P3 is not a typical \( \sigma^{34} \) promoter activated ‘at a distance’ by activators binding to UASs (Morett & Segovia, 1993).

**Promoter P3 is not required for hydrogenase activity in R. leguminosarum pea bacteroids**

To investigate the physiological relevance of P3 on hydrogenase expression in *R. leguminosarum*, strain UPM791P3, incorporating the mutation in the P3 \( \sigma^{34} \)-binding sequence, was used. Both wild-type and mutant strains were used as inocula for pea plants and hydrogenase activity of bacteroids was determined. No significant differences in the level of \( \mathrm{O}_2 \)-dependent hydrogenase activity from wild-type strain UPM791 and the mutant strain UPM791P3 were found \([4290 \pm 360 \text{ vs } 4230 \pm 620 \text{ nmol } \mathrm{H}_2 \text{ oxidized } h^{-1} (\text{mg protein})^{-1}] \). A dot-blot analysis confirmed the same level of hupG mRNA signal in wild-type and UPM791P3 mutant strains (data not shown). These results indicate that P3 is not essential for *R. leguminosarum* hydrogenase activity in pea nodules under the experimental conditions tested. Since the gene products of *hupGHIJ* are essential for hydrogenase activity (Rey *et al*., 1992; Brito *et al*., 1994), this result also implies the existence of transcription originated upstream of P3, probably from the P1 promoter.

**DISCUSSION**

In this work, a \(-24/-12\)-type promoter (P3) responsible for transcription of the *R. leguminosarum* hupGH and downstream genes has been identified upstream of hupG. This promoter is located within the only substantial (168 bp) intergenic space within the otherwise tightly grouped *hup–hyp* cluster. Transcriptional activity from this promoter was low as compared to that from the P1 hydrogenase structural promoter (~20%; Table 2, Brito *et al*., 1997). The fact that the same low but reproducible \( \beta \)-galactosidase activities associated with P3 were measured when fusion constructs were assayed in aerobic and microaerobic free-living cells or in pea bacteroids suggests that little specificity is associated with this promoter. However, promoter activity was absolutely dependent on integrity of the \(-24/-12\) region, and *in situ* localization of hupGH transcripts within pea nodules showed that expression of the hupGH genes was initiated in the interzone II/III, and simultaneously with expression of the nifH gene, as it was previously observed with hydrogenase structural genes hupSL (Brito *et al*., 1995). This expression pattern was also observed in nodules produced by mutant AL51 where transcription from P3, the NifA-dependent promoter of the hydrogenase structural genes, was interrupted, indicating that P3 was functional in pea nodule bacteroids and that its pattern of expression followed closely that of NifA-dependent genes. Since it is well established that expression of *nifA*, the transcriptional activator of nitrogenase genes, is induced sharply in this nodule zone (Soupéne *et al*., 1995), these results suggest that P3 expression in pea bacteroids is NifA-dependent. The use of an *R. leguminosarum* NifA mutant to confirm this possibility is complicated by the nitrogen-fixing deficiency of nodules from this type of mutants (Brito *et al*., 1997).

The P3 promoter was activated by NifA from *K. pneumoniae* both in *K. pneumoniae* and in *E. coli*. In *K. pneumoniae* it was also activated by NtrC or other uncharacterized regulators, since a nifA mutation affected P3 expression less drastically than a ntrC mutation. The high levels of P3 activity obtained in *E. coli* or *K. pneumoniae* in the presence of extra copies of *nifA* allowed further characterization of the promoter. No binding sequences located upstream of the \( \sigma^{34} \)-binding box, either UASs or IHF-binding sites, were required for P3 activation, suggesting that P3 is activated by NifA from solution by direct interaction with the P3-bound \( \sigma^{34} \)-RNA polymerase. Instances of promoter activation by NifA from solution have been reported. The nifLA operon from *K. pneumoniae* is activated by high concentrations of NifA even though the nifL promoter lacks the conserved UAS postulated as the target for NifA (Drummond *et al*., 1983). The removal of UASs from the *S. meliloti* nifH promoter results in normal expression in alfalfa nodule bacteroids (Better *et al*., 1985). Similarly, the *R. leguminosarum* glnB gene promoter is activated by NtrC in the absence of UASs (Chiurazzi & Iaccarino, 1990).

The above data are also consistent with the observed lack

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**Table 5. Effect of IHF on P3 promoter expression in E. coli**

Assays were carried out with stationary-phase cultures in order to maximize IHF levels.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Fusion</th>
<th>( \beta )-Galactosidase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ET8000 (wild-type)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-\text{NifA}) + (+\text{KpNifA})†</td>
</tr>
<tr>
<td>pHL315</td>
<td>hupSL::lacZ</td>
<td>45 ± 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6760 ± 1500</td>
</tr>
<tr>
<td>pHL320</td>
<td>hupGH::lacZ</td>
<td>30 ± 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1020 ± 260</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SE1000 (IHF⁺)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-\text{NifA}) + (+\text{KpNifA})†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 ± 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1290 ± 90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 ± 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>900 ± 140</td>
</tr>
</tbody>
</table>

*Values (Miller units) are the mean of three determinations ± SD.
†NifA from *K. pneumoniae* (KpNifA) was supplied by pSKA.
of activator specificity (Pérez-Martín & de Lorenzo, 1995) and imply that P3 can be activated from solution by any σ54-RNA polymerase activator present.

The same results were obtained for symbiotic expression of P3 in R. leguminosarum. However, in view of the observed pattern of P3 expression in the nodule and of the fact that the ntrC gene is not expressed in pea bacteria (Szeto et al., 1987), it is likely that the observed symbiotic activation of P3 is mediated by NifA.

The specific function of hupGHIJ gene products is unknown, but the need of these proteins for hydrogenase activity in R. leguminosarum has been established (Rey et al., 1992; Brito et al., 1994). Genes homologous to hupGHIJ are also present in the hydrogenase gene cluster from other nitrogen-fixing bacteria such as Azotobacter vinelandii and B. japonicum, and in Ralstonia eutropha and Rhodobacter capsulatus (Casalot & Rousset, 2001). A hupF–hupG intergenic space long enough to accommodate a promoter similar to P3, and containing a conserved σ54-binding sequence at the expected distance from hupG, has only been found in the endosymbiotic bacterium B. japonicum (Van Soom et al., 1993a; Fu & Maier, 1994). Symbiotic expression of the hupSL gene products is critical for expression of accessory genes (hoxOQRT), and implies that P3 can be activated from solution by any NifA-dependent-type promoters. In Ralstonia eutropha, no promoter region has been identified upstream of the hupGHIJ-homologous genes (hoxOQRT), and transcription originating from the membrane-bound hydrogenase structural gene promoter has been demonstrated (Schwartz et al., 1999).

Although the hupGHIJ genes are preceded by the symbiotically functional P3, this promoter does not seem to be essential for their expression in the mature bacteroid within the nodule, as shown by the results obtained with the σ54-box mutant UPM791P3. Transcription from P3, the hydrogenase structural gene promoter, is probably responsible for the expression of these genes in the UPM791P3 strain. These results suggest that the P3 promoter would only be relevant for expression of accessory genes hupGHIJ under conditions where transcription originating from the upstream promoter P1 becomes limiting for hydrogenase synthesis. Since transcription of the hupGHIJ genes from the P1 promoter produces transcripts of over 7 kb in length, any condition affecting the levels of these transcripts (diminished processivity of RNA polymerase, increased mRNA degradation) or their translation (ribosome processivity) would probably have a more severe effect on distal genes. Irrespective of what these conditions may be, it is noteworthy that the unique molecular adaptation of the hydrogenase gene cluster of Rhizobium leguminosarum UPM791 to symbiotic expression within the bacteroid not only includes the NifA-dependent expression of the P1 hydrogenase structural gene promoter (Brito et al., 1997) but also that of the internal, secondary P3 promoter.

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