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FnrN Controls Symbiotic Nitrogen Fixation and Hydrogenase Activities in *Rhizobium leguminosarum* Biovar viciae UPM791

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Rhizobium leguminosarum **bv. viciae UPM791 contains a second copy of the *fnrN* gene, which encodes a redox-sensitive transcriptional activator functionally homologous to *Escherichia coli* Fnr. This second copy (*fnrN2*) is located in the symbiotic plasmid, while *fnrN1* is in the chromosome. Isolation and sequencing of the *fnrN2* gene revealed that the deduced amino acid sequence of FnrN2 is 87.5% identical to the sequence of FnrN1, including a conserved cysteine-rich motif characteristic of Fnr-like proteins. Individual *R. leguminosarum fnrN1* and *fnrN2* mutants exhibited a Fix^+ phenotype and near wild-type levels of nitrogenase and hydrogenase activities in pea (*Pisum sativum* L.) nodules. In contrast, an *fnrN1 fnrN2* double mutant formed ineffective nodules lacking both nitrogenase and hydrogenase activities. Unlike the wild-type strain and single *fnrN1* or *fnrN2* mutants, the *fnrN1 fnrN2* double mutant was unable to induce micro-oxic or bacteroid activation of the *hypBFCDEX* operon, which encodes proteins essential for hydrogenase synthesis. In the search for symbiotic genes that could be controlled by FnrN, a *fixNOQP* operon, putatively encoding a micro-oxically induced, bacteroid-specific cbb_3-type terminal cytochrome oxidase, was isolated from strain UPM791 and partially sequenced. The *fixNOQP* operon was present in a single copy located in the symbiotic plasmid, and an anaerobox was identified in the *fixN* promoter region. Consistent with this, a *fixNOQP′-lacZ* fusion was shown to be highly induced in micro-oxic cells of the wild-type strain. A high level of micro-oxic induction was also observed in single *fnrN1* and *fnrN2* mutants, but no detectable induction was observed in the *fnrN1 fnrN2* double mutant. The lack of expression of *fixNOQP* in the *fnrN1 fnrN2* double mutant is likely to cause the observed Fix^- phenotype. These data demonstrate that, contrary to the situation in other rhizobia, FnrN controls both hydrogenase and nitrogenase activities of *R. leguminosarum* bv. viciae UPM791 in the nodule and suggest that this strain lacks a functional *fixK* gene.**

Legume root nodules are the site of the N_2-fixing symbiosis established between a group of aerobic soil bacteria, collectively designated rhizobia, and legume plants. Nodules provide the appropriate micro-oxic environment required to protect the bacterial oxygen-sensitive nitrogenase complex against O_2 damage. The low but constant O_2 concentration inside the nodule is maintained by the high O_2 affinity-low O_2 release rate of the legume leghemoglobin system. Multiple genes of rhizobial metabolism, including *nif* and *fix* genes, are induced in the nodule in response to the micro-oxic environment (2, 12, 50).

In a large number of diazotrophic organisms, NifA is the central nitrogen fixation transcriptional activator protein. NifA promotes transcription of *nif* and *fix* genes from −24/−12-type promoters by binding to upstream activating sequences (UAS) and by operating in concert with σ^{54} RNA polymerase (33). In *Sinorhizobium meliloti* (formerly *Rhizobium meliloti* [9]), *nifA* gene expression is controlled by the two-component *fixLJ* regulatory system (8, 55). FixL is an O_2-regulated hemoprotein with kinase and phosphatase activities that phosphorylates FixJ, the transcriptional activator component (14). Genes homologous to *fixLJ* have also been identified in *Bradyrhizobium japonicum* and in *Azorhizobium caulinodans*, but in these organisms, unlike the situation in *S. meliloti*, NifA expression is not controlled by FixLJ (22, 34, 53, 54). In all these three species, *fixLJ* genes are required for expression of *fixK* (*fixK2* in *B. japonicum*) genes under micro-oxic conditions (3, 12, 23).

FixK proteins are members of the Crp/Fnr family of homologous regulatory proteins. This family has been divided into three classes based on overall amino acid sequence similarities and functional differences (12). Class I includes the Fnr protein from *Escherichia coli* and homologous proteins involved in oxygen control of various cellular processes. They activate O_2-regulated genes by binding to a DNA sequence motif (anaerobox) present in all target genes (12). Most of the Fnr-like proteins included in class I contain a conserved Cys-X_{2-3}-Cys-X_{5-7}-Cys arrangement at their N termini and a fourth conserved Cys residue in the central domain of the protein that may play a role in redox sensing similar to that of the corresponding motif in the *E. coli* Fnr protein (16). FixK proteins (class IC) are an exception and do not contain the Cys motif. Consequently, it is likely that the regulatory activity of FixK proteins is not redox controlled and that their synthesis needs to be regulated at the transcriptional level by the FixLJ system. A common function of FixK proteins in the three species is their control of the *fixNOQP* operon (20, 30, 37). The *fixNOQP* operon encodes a cbb_3-type cytochrome oxidase that is induced under micro-oxic conditions and is essential for respiration during symbiotic nitrogen fixation (37).

In *R. leguminosarum* bv. viciae, no evidence for the presence of *fixLJ*-homologous genes has been found. However, Patschkowski et al. (36) have recently identified a *fixK* gene and an unusual *fixL*-homologous gene in an accessory plasmid of strain VF39. In addition to *fixK*, this strain also contains an

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fnr-like gene (*fnrN* [7]). A *fixK fnrN* double mutant exhibited a Fix^- phenotype in symbiosis with peas (36).

R. leguminosarum bv. viciae UPM791 is a hydrogenase-positive strain capable of recycling H_2 generated by the nitrogenase complex in pea nodules (45). A cluster of 18 genes (*hupSLCDEFGHIJK hypABFCDEX*) located in the symbiotic plasmid is required for synthesis of an active hydrogenase in this bacterium (references 27 and 43 and references therein). The *hypBFCDEX* operon is expressed in pea bacteroids and in microaerobically grown vegetative cells from an Fnr-type promoter (P_{5b}) located within the *hypA* gene (19). Expression of P_{5b} in the heterologous *S. meliloti* bacterial host was dependent on the presence of an active *fixK* gene. By complementing an *S. meliloti* $FixK^-$ mutant with an *R. leguminosarum* bv. viciae UPM791 genomic library, an *fnrN* gene that could replace *fixK* for activation of both *fixNOQP* and *hypBFCDEX* operons in *S. meliloti* was identified. However, mutagenesis of the *fnrN* gene had no effect on hydrogenase expression in *R. leguminosarum* UPM791 (19).

In this work, we report the identification of a second *fnrN* gene and of the *fixNOQP* operon, both located in the symbiotic plasmid of *R. leguminosarum* UPM791. Mutation of both the plasmid- and the chromosome-borne copies of the *fnrN* gene causes a Hup^- Fix^- phenotype. In the absence of a functional FnrN protein, expression of *hypBFCDEX* and *fixNOQP* operons is abolished. These data demonstrate that FnrN controls both hydrogenase and nitrogenase activities in the nodule and suggest the absence in this strain of a functional *fixK* gene.

MATERIALS AND METHODS

Chemicals. All restriction endonucleases, DNA-modifying enzymes, and nucleoside triphosphates were purchased from Boehringer Mannheim Gmbh (Mannheim, Germany) and were used according to the manufacturer's indications. Medium constituents were from Oxoid Ltd. (Basingstoke, United Kingdom). Antibiotics used in growth media were from Boehringer Mannheim Gmbh and Serva Feinbiochemica (Heidelberg, Germany). All other chemicals were of reagent or electrophoresis grade.

Bacterial strains, plasmids, and growth conditions. *R. leguminosarum* bv. viciae UPM791 (26), pSym-cured 128C53.5 (28), and YH17 (*fnrN1*::CK1 [19]) strains have been described previously. Cosmids pAL188 and pAL143, containing *fnrN2* and *fixNOQP*, respectively, were isolated from a previously constructed *R. leguminosarum* UPM791 genomic library (26). Plasmids pFK22, containing the *fnrN1* gene, and pHL202, containing a *hypB'-lacZ* transcriptional fusion, have also been described (19). A similar *fixNOQP'-lacZ* transcriptional fusion was constructed by cloning the 4.4-kb *Eco*RI fragment from pAL143 into promoter probe vector pMP220 (51) to yield pHL413. Plasmids were introduced into *Rhizobium* strains by triparental mating as previously described (27). *R. leguminosarum* strains were grown in media and under conditions previously described (27, 35). Incubation of *Rhizobium* cultures under micro-oxic conditions was carried out as described by Palacios et al. (35).

DNA manipulation and analysis. Preparation of plasmid DNA, restriction enzyme digestions, DNA cloning, PCR DNA amplification, as well as agarose and polyacrylamide DNA electrophoresis, and Southern hybridization of genomic DNA were performed by standard procedures (46). Eckhardt gels (11) were used for rhizobial-plasmid resolution. Nonradioactive bands labelled with digoxigenin (Boehringer Mannheim) were visualized either by luminography or by the use of a GS-250 Molecular Imager System (Bio-Rad, Hercules, Calif.). Nucleotide sequences were determined by the dideoxynucleotide chain termination method.

Construction of an *fnrN2* mutant. The Ω spectinomycin-streptomycin resistance cassette from plasmid pHP45Ω (40) was inserted in the 1.9-kb *Eco*RI fragment containing the *fnrN2* gene. The interrupted *fnrN2* gene was mobilized and exchanged into *R. leguminosarum* UPM791 and YH17 by means of the pK18*mobsacB* vector (47) to yield mutant strains DG1 (*fnrN2*::Ω) and DG2 (*fnrN1*::CK1 *fnrN2*::Ω).

Generation of an *R. leguminosarum* UPM791 *fixN* DNA probe. Two oligonucleotides (primer 1, 5′-AACGCGGTGGGCTTCTTCCT-3′; primer 2, 5′-ACGT GGCCGATGGTCCAGTC-3′) derived from the *S. meliloti fixN* sequence (GenBank/EMBL accession no. Z21854) and corresponding to conserved regions in an alignment of previously sequenced *fixN* genes from rhizobia were synthesized and used as primers for PCR amplification of total DNA from strain UPM791. The amplified 416-bp DNA fragment was sequenced to confirm that it corresponded to part of a *fixN* gene.

Plant tests and enzymatic assays. Pea (*Pisum sativum* L. cv. Frisson) plants were used as hosts for *R. leguminosarum* bv. viciae strains. Conditions for plant inoculation and growth have been described previously (26). Acetylene reduction and hydrogenase activities were determined as described by Ruiz-Argüeso et al. (45). β-Galactosidase activities in *Rhizobium* cell cultures and pea bacteroids were determined as described by Miller (32). Protein contents of cell or bacteroid suspensions were determined by the bicinchoninic acid method (49) after alkaline digestion in 1 N NaOH at 90°C for 10 min and with bovine serum albumin as a standard.

Immunoblot analysis. A HypB-specific antiserum (44) was used for immunoblot analysis of cell extracts by standard techniques (18). Briefly, cell extracts (total protein, 30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% polyacrylamide gels and resolved proteins were transferred to Immobilon-P membranes (Millipore, Bedford, Mass.) by wet electrotransfer. Blots were probed with a 1:1,500 dilution of the HypB-specific antiserum and a secondary goat anti-rabbit immunoglobulin G–alkaline phosphatase conjugate (Bio-Rad). A chromogenic substrate (bromochloroindolyl phosphate and nitroblue tetrazolium) was used as the developing reagent.

RESULTS

Identification of a second copy of the *fnrN* gene located in the symbiotic plasmid. An *fnrN* gene (hereafter referred to as *fnrN1*) was previously identified within a 2.6-kb *Eco*RI fragment in *R. leguminosarum* bv. viciae UPM791 (19). Southern hybridization experiments using total DNA from UPM791 and an *fnrN1* internal DNA fragment as the probe had suggested the existence of another *fnr*/*fixK*-like gene located within a 1.9-kb *Eco*RI fragment (19). By screening a genomic library from strain UPM791 with the same probe, a cosmid (pAL188) containing such a 1.9-kb *Eco*RI fragment was identified. The DNA from this fragment was isolated and sequenced. An open reading frame encoding a protein of 240 amino acid residues (aa) was identified. This protein was highly homologous (87.5% identity) to FnrN1 and to FnrN from *R. leguminosarum* bv. viciae VF39 (7) (87.9% identity) and homologous to a certain extent to FixK (28.3% identity) from strain VF39 (36) (Fig. 1). The corresponding gene was designated *fnrN2*, and its nucleotide sequence, together with the amino acid sequence of the encoded protein (FnrN2), was deposited in GenBank (accession no. U90520). An N-terminal cluster of three cysteine residues (positions 17, 21, and 28) and an internal cysteine residue (position 115) are conserved in FnrN1 and FnrN2 and in FnrN from strain VF39. These cysteines, which are present in Fnr-like proteins known to behave as oxygen sensors (16, 31), are absent in the FixK protein from strain VF39 (36). *R. leguminosarum* FnrN proteins showed only limited similarity with *E. coli* Fnr (23% sequence identity for both FnrN1 and FnrN2; data not shown).

Southern blot analysis of total DNA from the UPM791 wild-type strain and a pSym-cured derivative (128C53.5 [28]) revealed that the 1.9-kb *Eco*RI fragment hybridizing with the *fnrN2* probe is absent in this strain, indicating that the *fnrN2* gene is located in plasmid p128C53c, which also contains the symbiotic genes (28) (Fig. 2A, lanes 1 and 2). The plasmid location of *fnrN2* was confirmed by resolving the plasmids of strains UPM791 and 128C53.5 in Eckhardt gels and hybridizing them with the *fnrN2* internal DNA probe (Fig. 2B, lanes 3 and 4). This analysis also revealed that the *fnrN1* gene is not located in any of the four plasmids visualized in strain UPM791 and likely has a chromosomal location.

FnrN is essential for nodule hydrogenase and nitrogenase activities. In order to investigate the role of *fnrN1* and *fnrN2* genes in symbiotic H_2 uptake and N_2 fixation, an *fnrN2* mutant (DG1) and an *fnrN1 fnrN2* double mutant (DG2) were generated by insertion of spectinomycin resistance cassettes into the *fnrN2* genes of strains UPM791 and YH17, respectively, as described in Materials and Methods. The correct insertion of the spectinomycin resistance cassette in the genomes of the

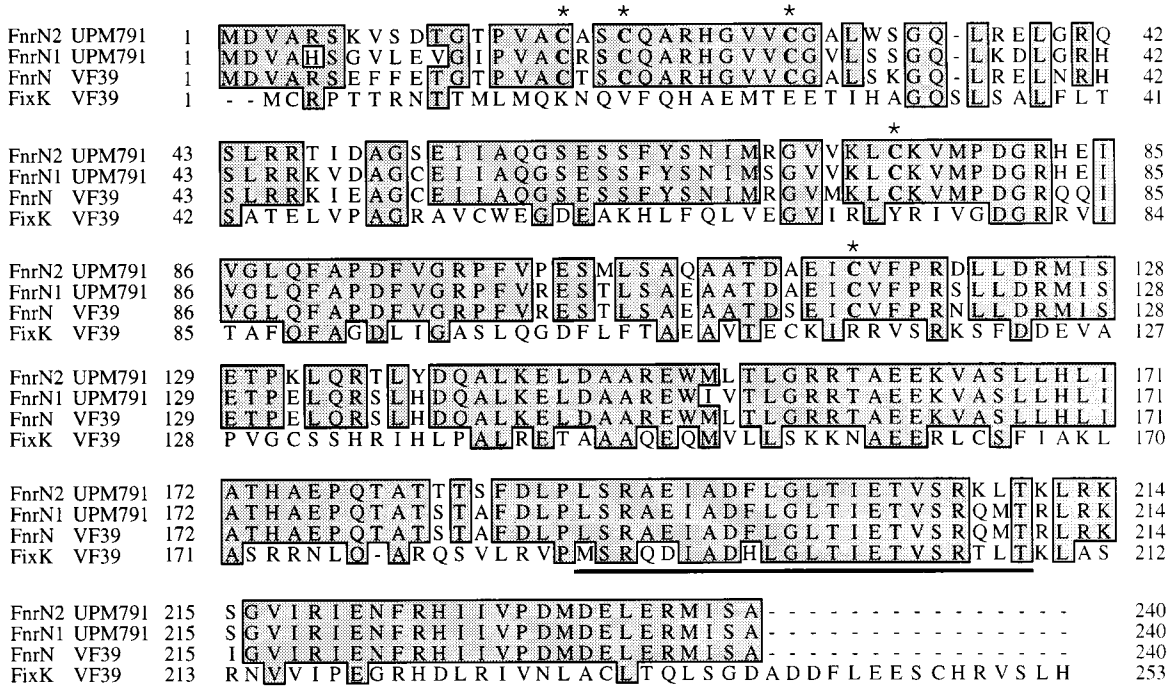


FIG. 1. Comparison of amino acid sequences of Fnr-like regulatory proteins from *R. leguminosarum* bv. viciae: FnrN2 from UPM791 (this work), FnrN1 from UPM791 (19), FnrN from VF39 (7), and FixK from VF39 (36). Residues conserved in at least three proteins are enclosed by shaded boxes. Cysteine residues conserved in FnrN proteins are marked by asterisks. The helix-turn-helix DNA-binding motif was defined according to the method of Dodd and Egan (10) and is underlined.

resulting mutant strains was confirmed by Southern blot hybridization, upon which the expected increase in size of the appropriate band was observed (Fig. 2A, lanes 3 to 5).

The *R. leguminosarum* bv. viciae wild-type and *fnrN* mutant strains were used as inocula for peas, and the symbiotic nitrogenase and hydrogenase activities were tested in nodules and bacteroids (Table 1). Both single mutants, YH17 (*fnrN1*) and DG1 (*fnrN2*), induced the formation of normal red root nodules exhibiting rates of nitrogenase activity (acetylene reduction) not much lower than those from the wild-type strain. Bacteroids from both mutants also showed high levels of oxygen- and methylene blue-dependent hydrogenase activities. In contrast, nodules produced by the double mutant DG2 were small and white and negligible nitrogenase activity was detected by the acetylene reduction test. Bacteroids from these nodules exhibited no detectable hydrogenase activities with either oxygen or methylene blue as the final electron acceptor. The Fix^- Hup^- phenotype of the DG2 mutant was complemented by introduction of a wild-type copy of the *fnrN1* gene (data not shown) cloned in plasmid pFK22 (19).

***fnrN* genes control *hyp* gene expression.** Previous work had identified an Fnr-type promoter upstream of *hypB* within the *hypA* gene and showed that the *hypBFCDE* operon is micro-oxically induced in an *S. meliloti* background by *S. meliloti* FixK or by *R. leguminosarum* FnrN1 (19, 35). To investigate the effect of *fnrN1* and *fnrN2* genes on *hyp* gene expression in free-living *R. leguminosarum*, the *hypB-lacZ* fusion plasmid pHL202 (19) was introduced into wild-type strain UPM791, single *fnrN* mutants YH17 and DG1, and double mutant DG2 and the β-galactosidase activities of the resulting transconjugants were determined under air or micro-oxic conditions (Table 2). The *hypB* promoter was highly expressed in the wild-type strain in response to micro-oxic conditions. In micro-oxic cells of both single *fnrN1* or *fnrN2* mutants, β-galactosidase activity associated with plasmid pHL202 was reduced to approximately 65% of that in the wild-type strain. In contrast, only background levels of β-galactosidase activity were observed in micro-oxic cells from the double *fnrN1 fnrN2* mutant. This result clearly demonstrates that FnrN1 and FnrN2 activate *hyp* gene expression in response to micro-oxic conditions.

The effect of FnrN on expression of the *hypB* promoter was also examined by determining the levels of HypB protein in bacteroids and micro-oxically grown cells (Fig. 3). Again, no expression of HypB protein was detected in the double *fnrN1 fnrN2* mutant strain DG2, either in micro-oxically grown cells or in bacteroids.

Together, these results show that *hyp* gene expression is controlled by the FnrN proteins in response to low oxygen concentrations. The inability to express Hyp proteins is likely to be the cause of the lack of hydrogenase activity observed in pea bacteroids from the double *fnrN1 fnrN2* mutant strain DG2.

Isolation of the *fixNOQP* operon from *R. leguminosarum* UPM791. Since the *fixNOQP* genes from different rhizobia are essential for bacteroid metabolism in the micro-oxic nodule environment (37, 39) and since their expression is O_2 regulated through *fixK* in those rhizobia (12), the *fixNOQP* operon was an obvious candidate as a target for regulation by FnrN1/FnrN2 in *R. leguminosarum* UPM791. In order to identify and isolate the *fixNOQP* genes from *R. leguminosarum* UPM791, we took advantage of the existence of highly conserved regions in FixN, a protein homologous to subunit I of the heme-copper oxidase superfamily (5, 13). Two oligonucleotides (see Materials and Methods) were designed and used as primers for PCR amplification of a DNA fragment internal to *fixN* from total DNA of strain UPM791. This fragment was used as a probe in Southern hybridization analysis of total-DNA restriction enzyme digests. A single band, corresponding to a 4.4-kb *Eco*RI

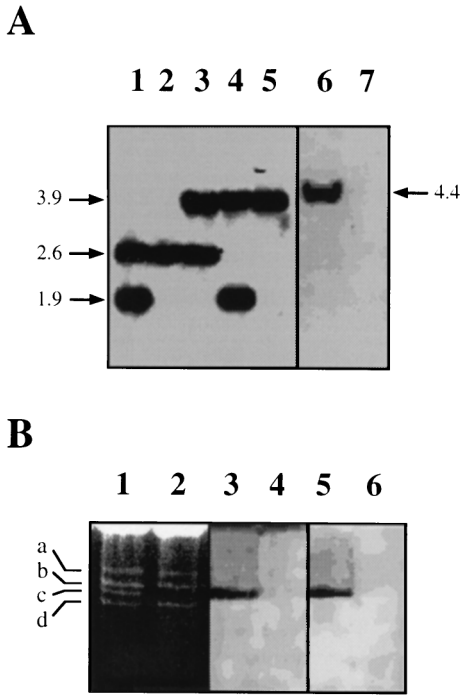


FIG. 2. Localization of *fnrN1*, *fnrN2*, and *fixN* in the genomes of *R. leguminosarum* UPM791 and derivative strains. (A) Southern hybridization of *Eco*RI-digested total DNA with an *fnrN2* (lanes 1 to 5) or a *fixN* (lanes 6 and 7) DNA probe. The estimated molecular sizes (in kilobases) of hybridizing fragments are shown on the left and right. Strains: UPM791 (lanes 1 and 6), 128C53.5 (lanes 2 and 7), DG1 (lane 3), YH17 (lane 4), and DG2 (lane 5). Insertions of the CK1 cassette in *fnrN1* (YH17 and DG2) and of the Ω cassette in *fnrN2* (DG1 and DG2) result in *fnrN*-containing fragments of identical expected size (3.9 kb). (B) Plasmid resolution in Eckhardt gels. Shown are ethidium bromide-stained gels (lanes 1 and 2) and corresponding hybridizations with *fnrN2* (lanes 3 and 4) and *fixN* (lanes 5 and 6) DNA probes. Strains: UPM791 (lanes 1, 3, and 5) and 128C53.5 (lanes 2, 4, and 6). Lowercase letters on the left denote plasmids identified in strain UPM791. The symbiotic plasmid (p128C53c [28]) has been cured in strain 128C53.5.

DNA fragment, was identified (Fig. 2A, lane 6). This band was absent from the pSym-cured strain 128C53.5 (Fig. 2A, lane 7). The plasmid location of *fixN* was confirmed by hybridizing Eckhardt-type gels with the same *fixN* probe (Fig. 2B, lanes 5 and 6). A cosmid clone, pAL143, containing the 4.4-kb *Eco*RI DNA fragment, was identified and isolated from an *R. leguminosarum* UPM791 genomic library (26).

A total of 3,176 bp from the 3′ end of the 4.4-kb *Eco*RI fragment was sequenced (GenBank accession no. U90521). Four contiguous and equally oriented genes, *fixN*, *fixO*, *fixQ*, and *fixP′* (5′-end fragment), were identified (Fig. 4A). The *fixN* gene encodes a 540-aa protein product which showed 80 to 98% sequence identity with FixN products from other rhizobia in the data banks (data not shown). The FixO (244 aa), FixQ (50 aa), and FixP′ (107 aa) gene products also showed sequence similarity to corresponding rhizobial FixO, FixQ, and FixP proteins. Analysis of the DNA sequence upstream of *fixN* identified a palindromic TTGAT-N_4-ATCAA sequence characteristic of Fnr/FixK binding sites (an anaerobox [12, 52]) located 80 bp upstream of the *fixN* gene start codon (Fig. 4B). This sequence was highly conserved in the promoter regions of *fixN* genes from other rhizobia and *Agrobacterium tumefaciens*, and an anaerobox consensus specific for FixN could be derived (Fig. 4B). No open reading frames with similarity to gene sequences in databases were identified upstream of *fixN* within the 4.4-kb *Eco*RI fragment.

TABLE 1. Hydrogenase and nitrogenase activities of wild-type *R. leguminosarum* UPM791 and *fnrN* mutants in symbiosis with peas

Strain	Relevant genotype	Bacteroid hydrogenase activity (mean ± SD)[a] with electron acceptor:		Nodule nitrogenase activity (mean ± SD)[b]
		O_2	Methylene blue	
UPM791	Wild type	5,310 ± 98	16,880 ± 221	16.1 ± 3.5
YH17	*fnrN1*	3,520 ± 128	10,370 ± 74	9.8 ± 1.3
DG1	*fnrN2*	4,660 ± 481	14,340 ± 250	12.3 ± 3.7
DG2	*fnrN1 fnrN2*	<10	<10	<0.1

[a] Values (nanomoles of H_2 oxidized · $hour^{-1}$ · milligram of $protein^{-1}$) are the averages of at least three determinations.

[b] Values (micromoles of C_2H_2 reduced · $hour^{-1}$ · gram [fresh weight] of $nodules^{-1}$) are the averages of at least three determinations.

TABLE 2. Effect of *fnrN* genes on the activation of *hypB* and *fixN* promoters in free-living cells of *R. leguminosarum* UPM791

Strain	Genotype	β-Galactosidase activity (Miller units) (mean ± SD)			
		hypB′-lacZ (pHL202)		*fixNOQP′-lacZ* (pHL413)	
		21% O_2	1% O_2	21% O_2	1% O_2
UPM791	Wild type	68 ± 8	1,960 ± 30	30 ± 5	1,450 ± 17
YH17	*fnrN1*	67 ± 9	1,310 ± 24	22 ± 3	1,090 ± 22
DG1	*fnrN2*	68 ± 8	1,270 ± 108	68 ± 7	1,380 ± 15
DG2	*fnrN1 fnrN2*	72 ± 3	36 ± 7	13 ± 3	16 ± 2

***fnrN* genes control *fixNOQP* expression.** In order to monitor expression of the *fixNOQP* operon, the 4.4-kb *Eco*RI fragment containing the *fixNOQP′* genes was cloned in the correct orientation upstream of the *lacZ* reporter gene in plasmid pMP220 to yield transcriptional fusion plasmid pHL413 (Fig. 4A). Expression in strains UPM791 (wild type), YH17 (*fnrN1*), DG1 (*fnrN2*), and DG2 (*fnrN1 fnrN2*) was analyzed (Table 2). Very low expression was observed under air, but micro-oxic conditions (0.8% O_2) induced high β-galactosidase activities in the wild-type strain, UPM791. Similar, although lower, activity levels were observed in single *fnrN* mutants. However, no significant expression was observed in the double *fnrN1 fnrN2* mutant strain DG2. This result indicates that micro-oxic expression of *fixNOQP* genes is under control of the *fnrN* genes in *R. leguminosarum* bv. viciae UPM791 and that the two *fnrN* genes are functionally equivalent and sufficient for *fixNOQP* activation.

DISCUSSION

Our interest in the micro-oxic induction of the hydrogenase accessory *hypBFCDEX* operon in *R. leguminosarum* bv. viciae UPM791 led to the identification of *fnrN* (*fnrN1*), an *fnr*-like regulatory gene (19). Although this gene was able to activate

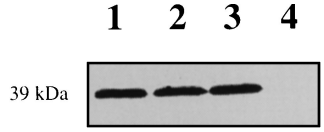
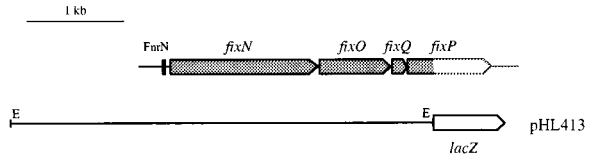


FIG. 3. Immunoblot analysis of HypB in pea bacteroids from *R. leguminosarum* UPM791 (lane 1), YH17 (lane 2), DG1 (lane 3), and DG2 (lane 4).



B

R. leguminosarum UPM791	*fixN*	tcg**TTGAT**G**TGGATCAAGGAGC**aat-N71 -ATG
R. leguminosarum VF39	*fixNc*	acg**TTGAT**G**TAGATCAAGGAGC**aat-N69 -ATG
R. etli	*fixN*	ttg**TTGAT**G**TAGATCAAGGAGC**cgt-N80 -ATG
R. leguminosarum VF39	*fixNd*	tcg**TTGAC**GCA**GATCAAGGCGC**aat-N69 -ATG
S. meliloti	*fixN*	cac**TTGAT**C**TGGATCAAGGTGC**cca-N84 -ATG
A. tumefaciens	*fixN*	ttc**TTGAT**C**TGGATCAAGGTG**Gcgc-N105-ATG
A. caulinodans	*fixN*	cct**TTGAC**T**TGTATCAAGGTG**Aaac-N68 -ATG
B. japonicum	*fixN*	atc**TTGATT**TCA**ATCAA**TTCC**C**cgc-N48 -ATG
	fixN consensus	**TTGAT-T-GATCAAGG-GC**

FIG. 4. Organization of the *R. leguminosarum* UPM791 *fixNOQP* operon. (A) Shaded boxes and solid lines in the genetic map denote sequenced regions. The putative FnrN-binding site (anaerobox) is marked by a black bar. The structure of the *fixNOQP′-lacZ* plasmid pHL413 is also indicated. (B) Alignment of anaeroboxes present in the promoter regions of *fixN* promoters from rhizobia and *A. tumefaciens*, derived from the corresponding GenBank/EMBL sequences: *R. leguminosarum* UPM791 *fixN* (this work), *R. leguminosarum* VF39 *fixNc* (Z80340), *Rhizobium etli fixN* (U76906), *R. leguminosarum* VF39 *fixNd* (Z80339), *S. meliloti fixN* (Z21854), *A. tumefaciens fixN* (Z46239), *A. caulinodans fixN* (X74410), and *B. japonicum fixN* (L07487). The distances from the anaeroboxes to the translational start site of *fixN* are indicated. A consensus for *fixN* anaeroboxes is also indicated.

the *fnr*-type promoter of the *hypBFCDEX* operon in the heterologous *S. meliloti* host, mutational inactivation of *fnrN1* had no effect on symbiotic *hyp* expression or hydrogenase activity and caused only a 35% reduction in *hyp* expression in free-living organisms under micro-oxic conditions. In this work we show that this phenotype resulted from the existence of a second, highly homologous *fnr*-like gene (*fnrN2*), which we have now isolated and sequenced. The two deduced protein products, FnrN1 and FnrN2, contain identical Cys-X_2-Cys-X_7-Cys motifs at their N termini. This cysteine cluster is characteristic of Fnr-like proteins belonging to class IB, and it is not present in FixK proteins (class IC [12]). Gene *fnrN2* was located in the symbiotic plasmid (pSym), whereas *fnrN1* could not be located in any of the known plasmids in strain UPM791, which suggests that it has a chromosomal location. The close sequence similarity of FnrN1 and FnrN2 (87.5% identity), together with the apparent functional interchangeability of the two genes (see below), makes it likely that a second copy of the *fnrN* gene (either the chromosomally encoded or the pSym-encoded one) arose from a duplication of the original gene. Nevertheless, the location of these genes in different replicons may make their contributions to the general pool of FnrN protein not exactly equivalent, and we have obtained preliminary evidence for a differential expression of these genes (unpublished data).

As was the case with *fnrN1*, a mutation in the *fnrN2* gene had little effect on symbiotic hydrogenase activity. In contrast, the double *fnrN1 fnrN2* mutant produced white, small, non-nitrogen-fixing nodules (Fix^-) which lacked any detectable nitrogenase or hydrogenase activity. Control of the hydrogenase system by FnrN was probably exerted at the level of *hypBFCDEX* expression, since no HypB protein was detected in nodules from the *fnrN1 fnrN2* mutant. Lack of HypB protein was not a result of the Fix^- phenotype, since pea bacteroids from a $NifA^-$ *R. leguminosarum* mutant contained immunodetectable levels of HypB (4). In addition, unlike the situation for the wild type and single *fnrN1* or *fnrN2* mutants, no micro-oxic induction of the *hyp* operon was detected in vegetative cells of the double *fnrN1 fnrN2* mutant. The proposed FnrN-dependent control of the *hyp* operon is consistent with the presence in the *hypB* promoter (P_{5b}) of a consensus Fnr-binding motif (anaerobox) centered at position −41 relative to the transcription start site (19). Although the *hyp* operon is conserved in other H_2-oxidizing bacteria, a similar control of *hyp* genes by Fnr-like proteins has so far been described only for the *E. coli hyp* operon, where *hypBCDE* transcription can take place, under some conditions, from an Fnr-dependent promoter (29).

The well-established control of *fixNOQP* operon expression by FixK in *S. meliloti*, *A. caulinodans*, and *B. japonicum* (20, 30, 37) prompted us to search for a similar gene cluster in *R. leguminosarum* bv. viciae UPM791 as a candidate target for FnrN regulation of symbiotic nitrogen fixation. The *fixNOQP* operon encodes a cbb_3-type cytochrome oxidase that is induced under micro-oxic conditions and is essential for respiration during symbiotic nitrogen fixation (37, 39). A single copy of a *fixNOQP* gene cluster highly homologous to those characterized for other rhizobia was identified in the symbiotic plasmid of strain UPM791. Expression analysis of a *fixNOQP′-lacZ* fusion in the wild type, the single *fnrN1* and *fnrN2* mutants, and the double *fnrN1 fnrN2* mutant demonstrated that the *R. leguminosarum fixNOQP* operon is expressed under micro-oxic conditions under the control of FnrN. As was the case with the *hyp* operon, single *fnrN1* or *fnrN2* mutations caused only a slight (10 to 25%) decrease in the levels of *fixNOQP* induction under micro-oxic conditions, demonstrating that the two copies of the *fnrN* gene are equally functional. Again, and consistent with FnrN regulation, an Fnr-binding motif (anaerobox) was present upstream of the *fixN* coding region. Although no direct demonstration of the essentiality of *fixNOQP* for symbiotic nitrogen fixation is available for *R. leguminosarum* bv. viciae, it is likely that lack of *fixNOQP* expression might be sufficient to explain the observed Fix^- phenotype in the double *fnrN1 fnrN2* mutant. However, it is clear that other targets for regulation by FnrN may exist in *R. leguminosarum*. In all rhizobia studied so far, the *fixNOQP* operon is followed by a *fixGHIS* gene cluster (21, 30, 37). These genes are required for synthesis of an active *fixNOQP*-encoded cbb_3 cytochrome oxidase (38). An anaerobox is present in the *fixG* promoter region, and, at least for *B. japonicum*, expression has been shown to be strictly dependent on micro-oxic or anaerobic conditions (38). Partial sequencing of the pSym region located downstream of *fixNOQP* suggests that a *fixGHIS* cluster also exists in *R. leguminosarum* UPM791 (17). In addition, the existence of a chromosomal copy of *fnrN* suggests that there may be non-symbiotic genes controlled by FnrN.

The Fix^- phenotype and the absence of *fixNOQP* expression exhibited by the *fnrN1 fnrN2* mutant strongly suggest that *R. leguminosarum* bv. viciae UPM791 lacks a functional *fixK* gene. Unlike those of *B. japonicum*, *S. meliloti*, and *A. caulinodans*, the *R. leguminosarum* UPM791 *fixNOQP* operon is activated by FnrN rather than by FixK. This result is also in contrast with the recent identification of a functional *fixK* gene and an atypical *fixL* gene in *R. leguminosarum* VF39 (36). In this strain, where a single copy of the *fnrN* gene has been detected (7), mutation of both *fixK* and *fnrN* genes is required to confer a Fix^- phenotype. Strain VF39 also differs from UPM791 in that it carries two copies of the *fixNOQP* operon, one in plasmid pRleVF39c, immediately upstream of *fixK* (36, 42), and a second copy in the symbiotic plasmid (pRleVF39d [41]). The absence of a plasmid similar to pRleVF39c in strain UPM791 could account for both differences between strains.

The existence of two *fnr*/*fixK* genes appears to be rather frequent in rhizobia: *S. meliloti* carries a functional duplication

of the *fixK* gene in its symbiotic plasmid (3); *B. japonicum* possesses an *fnr*-type gene (*fixK1*) in addition to the symbiotically important *fixK*-like gene (*fixK2* [1, 12]); *R. leguminosarum* VF39 also carries an *fnr*-like (*fnrN*) and a *fixK* gene (7, 36); and finally, *R. leguminosarum* UPM791, as shown in this work, possesses two *fnr*-like (*fnrN1* and *fnrN2*) genes. The available evidence suggests that rhizobial Fnr-like and FixK proteins recognize identical sequences in their cognate promoters and that they are interchangeable to a large extent (1, 6, 7, 12, 19, 36). The main difference between Fnr-like and FixK proteins might be at the level of redox control. If, as has been amply shown with the Fnr protein from *E. coli* (15, 16, 24, 25), Fnr-like rhizobial proteins (FnrN, FnrN1, FnrN2, and FixK1) contain a redox-sensitive Fe-S cluster bound by their conserved Cys motif, their transcriptional activity should be dependent on the redox potential of the cell. This has been shown to be the case for *R. leguminosarum* FnrN, FnrN1, and FnrN2 (17, 48). On the other hand, the activity of FixK proteins, which lack Cys ligands and, presumably, an, Fe-S cluster, should not be redox sensitive (12). This would explain the existence of an oxygen-sensing regulatory system (FixLJ) governing *fixK* transcription.

Since oxygen appears to be the key regulatory signal in the development of rhizobial nitrogen fixation symbioses (12, 50), it has been hypothesized that rhizobia have evolved the specialized *fixLJK* cascade system for fine-tuning control of nitrogen fixation-related functions, in opposition to the "classical" Fnr-like systems present in a large number of bacteria, where they govern a diverse range of nondiazotrophic functions (12). Our results with *R. leguminosarum* UPM791 demonstrate, however, that this is not always the case and that at least one member of the *Rhizobium* type species has evolved an efficient diazotrophic system where redox control is based on Fnr. Our results and those of Patschkowski et al. (36) also suggest that there is a much larger variability in the organization of redox controls of rhizobial symbiotic functions than previously suspected (12) and that this plasticity might be in large part the result of plasmid-facilitated genetic exchange.

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