APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2004, p. 1287–1296 Vol. 70, No. 3
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.3.1287–1296.2004

Regulatory Role of *Rhizobium etli* CNPAF512 *fnrN* during Symbiosis

Martine Moris, Bruno Dombrecht, Chuanwu Xi, Jos Vanderleyden, and Jan Michiels*

Centre of Microbial and Plant Genetics, Katholieke Universiteit Leuven, B-3001 Heverlee, Belgium

Received 8 July 2003/Accepted 19 November 2003

The *Rhizobium etli* CNPAF512 *fnrN* gene was identified in the *fixABCX rpoN*$_2$ region. The corresponding protein contains the hallmark residues characteristic of proteins belonging to the class IB group of Fnr-related proteins. The expression of *R. etli fnrN* is highly induced under free-living microaerobic conditions and during symbiosis. This microaerobic and symbiotic induction of *fnrN* is not controlled by the sigma factor RpoN and the symbiotic regulator *nifA* or *fixLJ*, but it is due to positive autoregulation. Inoculation of *Phaseolus vulgaris* with an *R. etli fnrN* mutant strain resulted in a severe reduction in the bacteroid nitrogen fixation capacity compared to the wild-type capacity, confirming the importance of FnrN during symbiosis. The expression of the *R. etli fixN*, *fixG*, and *arcA* genes is strictly controlled by *fnrN* under free-living microaerobic conditions and in bacteroids during symbiosis with the host. However, there is an additional level of regulation of *fixN* and *fixG* under symbiotic conditions. A phylogenetic analysis of the available rhizobial FnrN and FixK proteins grouped the proteins in three different clusters.

Soil bacteria belonging to the genera *Rhizobium*, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Sinorhizobium* (collectively referred to as rhizobia) elicit the formation of nodules on the roots of their leguminous hosts. In these specialized organs, the bacteria are released into the plant cells and differentiate into bacteroids that fix atmospheric nitrogen into ammonia that can be assimilated by the host plant. The nodules provide the microoxic conditions required for functioning of the oxygen-sensitive nitrogenase enzyme complex.

In rhizobia, NifA activates transcription of several nitrogen fixation genes in conjunction with σ^{54} RNA polymerase (12). In *Rhizobium etli* CNPAF512, NifA is strictly required for nitrogen fixation activity in nodules of *Phaseolus vulgaris* and controls the expression of several genes involved in nitrogen fixation, including *nifH*, *iscN*, and *orf180-rpoN*$_2$ (13, 33, 34). Transcription of the *R. etli nifA* gene itself occurs independent of the oxygen status of the cell (33). Two *rpoN* genes encoding the alternative σ factor, σ^{54}, have been characterized in *R. etli* and have been shown to be differentially regulated (34, 35). During free-living growth, RpoN$_1$ is required for growth on several nitrogen and carbon sources (35). There is a severe decrease in nitrogen fixation after inactivation of *rpoN*$_2$ (34), indicating the essential role of this gene in bacteroids (12). The NifA enhancer-binding protein controls transcription activation of *rpoN*$_2$ under free-living microaerobic conditions and during symbiosis (34). Besides *nifA*, *fixLJ* regulatory genes were identified in *R. etli* CNPAF512. The *fixL* gene encodes a protein lacking heme-binding capacity (9, 10). FixLJ is involved in microaerobic *nifH* expression, but in contrast to the situation in *Sinorhizobium meliloti* and *Azorhizobium caulinodans* (25, 26), the expression of *nifA* is not dependent on FixLJ. Nitrogen fixation in the *R. etli fixLJ* mutant bacteroids is reduced (10).

In this paper, we describe identification of a third nitrogen fixation regulatory protein, FnrN, in *R. etli* CNPAF512. This protein is homologous to the oxygen-responsive transcriptional Fnr regulator of *Escherichia coli* involved in the regulation of genes with functions in anaerobic respiration (reviewed in references 30 and 53). *E. coli* Fnr and rhizobial FnrN and FixK proteins belong to the same family of homologous transcriptional regulators, the cyclic AMP receptor protein Crp-Fnr family, which is divided into three classes (15). The first class includes the Fnr protein of *E. coli* and homologous proteins involved in oxygen control of various cellular processes and is further divided into four subgroups (15, 59). It has been proposed that Fnr proteins that belong to class IB of this protein family sense the redox status with a strictly conserved cysteine-rich domain in the N terminus and an additional, conserved cysteine residue in the central part of the polypeptide. These cysteine residues may contribute to the formation of an iron-binding domain (15, 57). The cysteine motif of the class IB proteins differs from that of the proteins belonging to class IA, like *E. coli* Fnr (15, 57). In contrast to FnrN, rhizobial FixK regulators (class IC of the Crp-Fnr family) lack the N-terminal and central cysteine residues. It has been speculated that the activity of these proteins is not directly controlled by oxygen (3, 15, 27). The oxygen regulation of *S. meliloti fixK* occurs at the transcriptional level through the FixLJ system (8, 20, 48). Both FnrN and FixK regulators contain a helix-turn-helix motif in the C-terminal region that is involved in DNA binding (49). The promoter regions of the target genes that are bound by FixK or FnrN dimers contain a conserved motif (TTGA-C--GATCAA-G), called the anaerobox (15). Vollack et al. (59) proposed a change in the tripartite classification (15) and suggested an additional subgroup, class ID, comprising the Dnr proteins. Together with the FixK-like class IC proteins, these proteins lack the N-terminal cysteine motif.

Here, we describe identification, localization, and functional analysis of the *R. etli* CNPAF512 *fnrN* gene, whose product is a member of class IB of the Crp-Fnr family.

* Corresponding author. Mailing address: Centre of Microbial and Plant Genetics, K.U. Leuven, Kasteelpark Arenberg 20, B-3001 Heverlee, Belgium. Phone: (32-16) 321631. Fax: (32-16) 321963. E-mail: jan.michiels@agr.kuleuven.ac.be.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was routinely cultivated in

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Escherichia coli DH5α	F80*dlacZ*ΔM15 Δ(*lacZYA-argF*) *recA endA hsdr supE*	Gibco-BRL
Rhizobium etli strains		
CNPAF512	Nal^r, wild type	34
FAJ1154	Nm^r *rpoN$_1$*::Ω-Km	35
FAJ1169	Nm^r *rpoN$_2$*::Ω-Km	34
FAJ1170	Nm^r Sp^r *rpoN$_1$*::Ω-Sp *rpoN$_2$*::Ω-Km	34
FAJ1182	Nm^r *fnrN*::Ω-Km, same orientation	This study
FAJ1183	Nm^r *fnrN*::Ω-Km, opposite orientation	This study
Rp1000	Nm^r *nifA*::*aphII*	33
RpFAJ1002	Nm^r *fixL*::Ω-Km	10
RpFAJ1004	Nm^r *fixJ*::Ω-Km	10
CMPG8007	Nm^r *fixO*::mTn*5gusA*	This study
CMPG8169	Nal^r Nm^r *fixG*::mTn*5gusA*	60
CMPG8170	Nal^r Nm^r *fixI*::mTn*5gusA*	60
Sinorhizobium meliloti GMI347-CS112	Sm^r Nm^r Rf^r Gm^r GMI347 with pCS112 (pSUP202Gm containing the *fixN-lacZ* fusion of pGMI931) integrated into pSym	6
Plasmids		
pUC18	Ap^r, cloning vector	38
pUC18Not	Ap^r, cloning vector	24
pLAFR1	Tc^r, broad-host-range vector	17
pLAFR3	Tc^r, broad-host-range vector	50
pHP45Ω-Km	Ap^r Km^r	14
pJQ200-UC1	Gm^r *sacB*	43
pWM6	Ap^r Nm^r *uidA2*	32
pFAJ1172	Tc^r, *fnrN* gene in pLAFR1	34
pFAJ1174	Ap^r, a 4.2-kb *Not*I fragment containing *fnrN* cloned in pUC18Not	34
pFAJ1175	Tc^r, *fnrN* gene, *rpoN$_2$-gusA* fusion, and *orf180* gene in pLAFR1	34
pFAJ1176	Tc^r, *orf180-gusA* fusion in pLAFR3	34
pFAJ1178	Km^r, pLAFR3 containing *fnrN-gusA*	This study
pFAJ1192	Km^r, pLAFR3 containing *fixG-gusA* fusion	This study
pFAJ1193	Km^r, pLAFR3 containing *fixN-gusA* fusion	This study
pFAJ1319	Tc^r, *arcA-gusA* fusion in pLAFR3	11
pGMI931	Tc^r, *S. meliloti fixN-lacZ*	7

Luria-Bertani medium (36) at 37°C. *R. etli* CNPAF512 and mutant strains were grown on TY (0.5% tryptone, 0.3% yeast extract, 7 mM $CaCl_2$) or yeast extract-mannitol plates (58) at 30°C or in liquid defined acid minimal salts medium (40) supplemented with 10 mM mannitol and 10 mM NH_4Cl. *S. meliloti* was grown on Luria-Bertani medium supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$. Antibiotics were added to the media at the following concentrations: nalidixic acid, neomycin, kanamycin, and gentamicin, 30 μg/ml; ampicillin, 100 μg/ml; and tetracycline, 1 μg/ml (final concentration) for *R. etli* or 10 μg/ml (final concentration) for *E. coli*.

DNA sequence analysis. DNA sequencing was performed with both strands of overlapping pUC18 subclones by using Cy5-labeled universal and synthetic oligonucleotide primers. Double-stranded DNA was sequenced by the dideoxynucleotide chain termination method with an automated sequencer (ALF; Pharmacia-LKB, Uppsala, Sweden). Computer-assisted sequence analyses were performed by using the ContigExpress software package (Informax Inc.).

Partial DNA sequence analysis of mutants CMPG8007, CMPG8169, and CMPG8170. The partial DNA sequences of the inactivated genes of transposon mutants CMPG8007, CMPG8169, and CMPG8170 were determined (60; this study). The corresponding insertions were cloned as *Xho*I fragments in the *Sal*I site of pUC18. The DNA sequences bordering the transposon insertion were determined by using a primer annealing at the 5′ end of the *gusA* gene in the mini-Tn*5* transposon (5′-CGGTACCTGACTAGCTAAGGAG-3′), reading outward from the *gusA* gene, or a synthetic primer based on the sequences obtained.

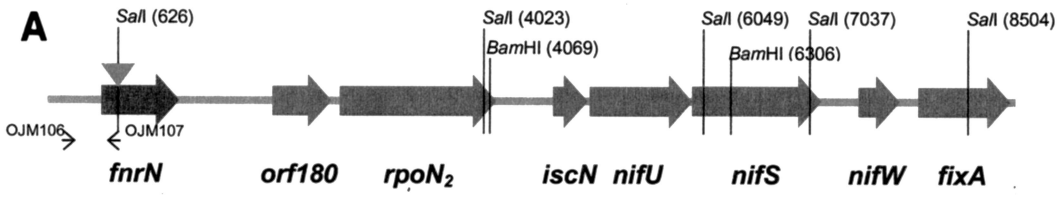
Construction of a rhizobial FnrN dendrogram. The amino acid sequences of 26 different FnrN and FixK proteins were aligned by using the ClustalW program (52). For representation purposes the alignment was imported into the GeneDoc program.

A tree was constructed with the Treecon for Windows (version 1.3b) software package (56). The distances between the sequences were calculated by using the Poisson correction method (insertions and deletions were taken into account) with a bootstrap analysis with 1,000 replications. The tree topology was inferred by the neighbor-joining method. The Phylip package (version 3.6; http://evolution.genetics.washington.edu/phylip.html) was used to construct the most parsimonious tree from 1,000 bootstrapped samples. Fnr of *E. coli* (accession number gi26108071) was used as an outgroup to root the tree.

β-Glucuronidase assays. Qualitative and quantitative analyses of β-glucuronidase activity were performed as described elsewhere (33). Cultures of *R. etli* were grown aerobically and microaerobically (0.3% O_2) at 30°C. Cultures were grown in liquid defined acid minimal salts medium (40) supplemented with 10 mM mannitol and 10 mM NH_4Cl. The data were analyzed for statistical differences by using Tukey's test ($P < 0.05$).

Construction of an *R. etli* CNPAF512 *fnrN* mutant. Plasmid pFAJ1174 was constructed previously and contains the *R. etli rpoN$_2$*, *orf180*, and *fnrN* genes (34). This plasmid was digested with *Sal*I (Fig. 1) and ligated with the 1.8-kb *Bam*HI fragment from pHP45Ω-Km after blunting of both fragments, which inactivated the *fnrN* gene. The resulting 6-kb *Not*I fragment was cloned into the *Not*I site of pJQ200-UC1 (43), and the resulting construct was subsequently used to mutagenize *R. etli* CNPAF512 as previously described (10). Insertion of the mutation was verified by Southern blot hybridization by using the appropriate probes. The resulting *R. etli fnrN* mutants were designated FAJ1182 and FAJ1183; in these mutants the *fnrN* gene and the resistance gene read in the same direction and in opposite directions, respectively.

Isolation and localization of *fixN* and *fixG*. To localize the *fixNOQP* genes, a search for a cosmid clone carrying this region was performed. Based on database sequences (accession number U76906), primers were designed to amplify a 200-bp *fixN* fragment (primer OJM135 [5′-CTGATTAATTAAAGTAAGCGG GCGGTGCCAAAG-3′] and primer OJM137 [5′-CTGAGAATTCTTCTGTCG TGTAATTCATGATG-3′]). By using colony PCR four overlapping cosmid clones (p2D2, p2G7, p8C7, and p19H4) were isolated from a genomic library (34) containing the *fixN* region. Partial sequence analysis of several *Eco*RI and



B

```
GAATTCCGGGAAAAGCTCGAAGCGTGCTACTCCTTTGTCCTGAATGTCAGTTCTACCAGC     60
CCGACGACACCACCCTAAGAGTCGGCGGCAATCTGCCGATTGGGTGTTGTCTGACAGCAA    120
TGGATATGGTGGCCTACCGTCCGCCGAGACGGAAGGCGATGGCCGCAGAAAGCCCGCTCA    180
AGCGGCGGGCCGAAAATGCCGTCCGAAACAAATCCACAAAACTGCCCTGCCTGGCACGGC    240
AAGAGCGTGGGTACGGAGATTTGATCTGGATCAAATCGCAATCCTGATGAAATTGATAGC    300
CATCAAAGACGCGCTGGAAGAAATAGGGATAGCCGAAGTCGCAACGGCCGCGTGGTCGTC    360
TCGGGCATGTCCGGCATAGGCGGTTTTCGAAGGGATCAGGACATGGACGTCGCACGCGGT    420
                                          M  D  V  A  R  G
GAGGTTCTCGTGATTAGCACTTCCGTCGCTTGCCGTTCCTGCCAAGCGCGGCACGGCGTC    480
 E  V  L  V  I  S  T  S  V  A  C  R  S  C  Q  A  R  H  G  V
GTTTGCGGCGCCTTGTCGGCTGGCCAGCTTAGCGAACTTGGCCGCCACTCGCTGCGCCGC    540
 V  C  G  A  L  S  A  G  Q  L  S  E  L  G  R  H  S  L  R  R
AAGGTCGACGC                                                      600
 K  V  D  A
```

FIG. 1. (A) Detailed physical map of the *R. etli* symbiotic region containing the *fnrN* gene. The insertion site for the Ω-Km interposon in the *fnrN* gene is indicated by a grey triangle. Oligonucleotide primers (small arrows) are described in Materials and Methods. (B) Promoter region of *R. etli fnrN*. Two putative anaeroboxes and a purine-rich DNA sequence resembling a ribosome-binding site are underlined once and twice, respectively. The *Sal*I restriction site, used to insert the Ω-Km interposon, is indicated by boldface type.

*Sal*I fragments confirmed the presence of the *fixN* and *fixG* genes. This region was shown to be located close to the previously described *nifHDK*$_3$ gene located on a 4.2-kb *Eco*RI fragment (33).

Construction of *gusA* fusions. (i) *fnrN-gusA*. A 0.6-kb *fnrN* promoter fragment was amplified by PCR with *Pwo* DNA polymerase by using oligonucleotides OJM106 (5′-CTGAGGATCCGCGGCCGCTTTGTCCTGAATGTCAGTTC-3′; *Bam*HI and *Not*I recognition sites are underlined) and OJM107 (5′-AAAG AGATCTCCGATCCTTGTGCAATGATCTC-3′; *Bgl*II recognition site is underlined). The resulting fragment was digested with *Bam*HI and *Bgl*II and cloned into pFAJ1171 (55), which fused the first 59 codons of *fnrN* to the *gusA* gene. To facilitate cloning into pLAFR3, the Ω-Km cassette from pHP45Ω-Km was amplified by PCR by using primers OJM059 (5′-ACTTGGATCCACGCCTTCCT CTCCGAATGC-3′; *Bam*HI site is underlined) and OJM060 (5′-ACTTGGAT CCGAATTCCGTGCGCGTCAGCCAGTTGG-3′; *Bam*HI and *Eco*RI sites are underlined), digested with *Bam*HI, and inserted into the *Eco*RI site located downstream of *gusA* after blunting of both fragments. The resulting plasmid contained an intact *Eco*RI site bordering the 3′ end of the Ω-Km cassette. Finally, a *Bam*HI-*Eco*RI fragment that was approximately 4 kb long and contained *fnrN-gusA*-Ω-Km^r was cloned into the broad-host-range vector pLAFR3, resulting in pFAJ1178.

(ii) *fixN-gusA*. PCR amplification with *Pwo* DNA polymerase by using primers OJM151 (5′-CTGAGGATCCAGAAAGCAGCTGCGTCATAC-3′; *Bam*HI site is underlined) and OJM152 (5′-CTGATCTAGACGCTGCGACCGCGAT CACCATC-3′; *Xba*I site is underlined) yielded a *fixN* promoter fragment that was approximately 240 bp long. This fragment was cloned as a *Bam*HI-*Xba*I fragment in pUCNot. The resulting construct was digested with *Xba*I and blunt end ligated to the 3.8-kb *Bam*HI fragment from pWM6 containing a promoterless *gusA*-Km^r cassette. Finally, the 4-kb fragment was digested with *Not*I, blunted, and ligated in the blunted *Bam*HI site of the broad-host-range plasmid pLAFR3. The resulting plasmid was designated pFAJ1193 (*fixN-gusA*).

(iii) *fixG-gusA*. A 230-bp *fixG* promoter fragment was obtained by performing PCR with *Pwo* DNA polymerase and primers OJM153 (5′CTGAAAGCTTGA GCCGATAGTTTCAGCTCC-3′; *Hin*dIII site is underlined) and OJM154 (5′-CTGATCTAGAACGCGAACGTGGTCAATGTC-3′; *Xba*I site is underlined). The resulting PCR fragment was cloned as a *Hin*dIII-*Xba*I fragment in pUCNot. The resulting construct was digested with *Xba*I and blunt end ligated to the 3.8-kb *Bam*HI fragment from pWM6 containing a promoterless *gusA*-Km^r cassette. Finally, the 4-kb insert was removed as a *Not*I fragment and blunt end ligated in the *Bam*HI site of the broad-host-range plasmid pLAFR3. The resulting plasmid was designated pFAJ1192 (*fixG-gusA*).

Plant culture and acetylene reduction assay. Seeds of *P. vulgaris* cv. Limburgse vroege were sterilized by rinsing them with ethanol for 3 min and with 15% sodium hypochlorite for 13 min. Next, the seeds were washed 10 times in sterile water. Seeds were germinated for 3 days on water agar plates (15 g/liter) in the dark at 30°C. The seedlings were transferred to 250-ml conical bottles filled with 150-ml agar slants (1.2 g of agar/150 ml) of Snoeck medium, which is optimized for in vitro growth of common bean (46).

Plants were inoculated and grown essentially as described by Michiels et al. (34). For expression analysis during symbiosis, bacteroids from 3-week-old nodules were purified from plant material by differential centrifugation (34). The nitrogen fixation capacity was determined by the acetylene reduction assay as described by Michiels et al. (34). The acetylene reduction assay data and symbiotic expression data were analyzed for statistical differences by using Tukey's test ($P < 0.05$).

Nucleotide sequence accession number. The nucleotide sequence of the *R. etli fnrN* gene locus has been deposited in the DDBJ-EMBL-GenBank nucleotide sequence databases under accession no. AJ005696.

RESULTS

Cloning and DNA sequence of *R. etli fnrN*. Nucleotide sequence analysis of the upstream region of the previously identified *orf180-rpoN*$_2$ operon (34) revealed the presence of an open reading frame (ORF) (Fig. 1A). This ORF encodes a

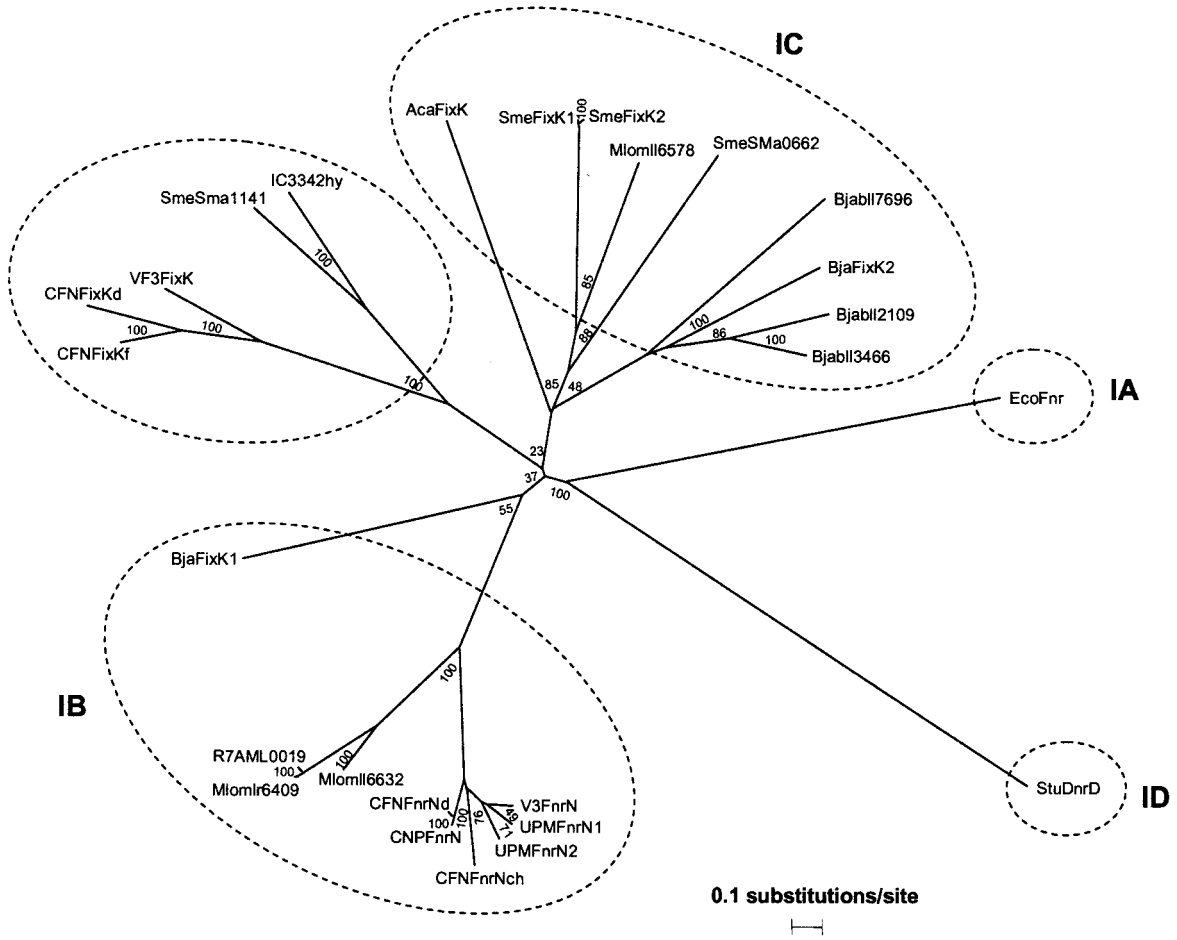


FIG. 2. Phylogenetic relationships of rhizobial FnrN and FixK proteins. The following proteins were included in the analysis: *R. etli* CNPAF512 FnrN (CNPFnrN); *R. etli* CFN42 FnrNd (CFNFnrNd) (accession number gi12083693) (31); *R. leguminosarum* bv. viciae strain VF39 FnrN (VF3FnrN) (accession number gi120623) (6); *R. leguminosarum* bv. viciae UPM791 FnrN1 (UPMFnrN1) (accession number gi619722) (23); *R. leguminosarum* bv. viciae UPM791 FnrN2 (UPMFnrN2) (accession number gi2114416 (22); *R. etli* CFN42 FnrNchr (CFNFnrNch) (accession number gi3462873) (31); *B. japonicum* FixK1 (BjaFixK1) (accession number gi95165) (1); *M. loti* strain MAFF303099 mll6632 (Mloml16632) (accession number gi13475537) (28); *A. caulinodans* FixK (AcaFixK) (accession number gi38702) (27); *B. japonicum* FixK2 (BjaFixK2) (accession number gi3021322) (37); *S. meliloti* FixK1 (SmeFixK1) (accession number gi14523782) (3); *S. meliloti* FixK2 (SmeFixK2) (accession number gi14523500) (2); *R. etli* CFN42 FixKf (CFNFixKf) (accession number gi9857982) (21); *R. etli* CFN42 FixKd (CFNFixKd) (accession number gi1679718) (47); *R. leguminosarum* bv. viciae VF39 FixK (VF3FixK) (accession number gi1240048) (39); *Rhizobium* strain IC3342 *orf4* (IC3342hy) (accession number gi152262) (54); mlr6409 (Mlomlr6409) (accession number gi13475362), mml6632 (Mloml16632) (accession number gi13475537), and mll6578 (Mloml16578) (accession number gi14026245) from *M. loti* strain MAFF303099 (28); *M. loti* strain R7A ML0019 (R7AML0019) (accession number gi20803846) (51); SMa0662 (SmeSMa0662) (accession number gi16262801) and Sma1141 (SmeSMa1141) (accession number gi16263070) from *S. meliloti* (2); and bll7696 (Bjabll7696) (accession number gi27382807), bll3466 (Bjabll3466) (accession number gi27378577), and bll2109 (Bjabll2109) (accession number gi27350363) from *B. japonicum* (29). Details of the construction are described in Materials and Methods. *E. coli* Fnr (EcoFnr) (accession number gi26108071) and *Pseudomonas stutzeri* DnrD (StuDnrD) (accession number gi4585795) were included to represent members of classes IA and IB, respectively, of the Crp-Fnr family. The numbers at the nodes indicate the percentages of trees in 1,000 bootstrap analyses that supported the topology.

26-kDa protein that is very similar (80% or more identity) to the known FnrN proteins. The ORF was therefore designated *fnrN*. A putative ribosome-binding site is located 7 bp upstream from the proposed start codon (Fig. 1B). Two DNA sequence motifs, 5′-TTGATCTGGATCAAA-3′ and 5′-TTGATAGCCATCAAAG-3′, located 96 and 128 bp upstream of the ORF (Fig. 1B) strongly resemble (identical nucleotides are underlined) the consensus rhizobial FnrN- and FixK-binding site or anaerobox (TTGA-C--GATCAA-G) (15), suggesting that there is (auto)regulation by an Fnr-like protein. When this *fnrN* gene was used as a probe, no other signal was detected by Southern blot hybridization with genomic DNA.

An alignment of rhizobial FnrN and FixK proteins was constructed (proteins were selected by using a threshold value of 30% amino acid identity with FnrN of *R. etli* CNPAF512). Based on this alignment, a phylogenetic tree was constructed (Fig. 2). The phylogenetic relationship illustrates that there are different groups of rhizobial FnrN and FixK proteins. The known FnrN proteins, together with FixK1 of *Bradyrhizobium japonicum* and several database entries for *Mesorhizobium loti*

TABLE 2. Expression of *S. meliloti fixN-lacZ* in *S. meliloti* and *R. etli*

Strain[a]	Plasmid	β-Glucuronidase activity (Miller units)[b]	
		Aerobic conditions	Microaerobic conditions
GMI347-CS112		30 (3)	11 (9)
GMI347-CS112	pFAJ1172	25 (3)	969 (151)
GMI347-CS112	pFAJ1175	21 (9)	407 (76)
CNPAF512	pGMI931	27 (4)	1,033 (36)
FAJ1182	pGMI931	29 (6)	23 (3)
FAJ1183	pGMI931	22 (2)	8 (2)

[a] The *S. meliloti* strain used was GMI347-CS112 (*fixJ* mutant, chromosomal *fixN-lacZ*), and the *R. etli* strains used were CNPAF512 (wild type), FAJ1182, and FAJ1183 (*fnrN* mutant).
[b] The values in parentheses are standard deviations. The data are the means of at least four replicates, and the results were confirmed independently in separate experiments.

TABLE 4. Expression of *R. etli fnrN-gusA* (pFAJ1178) in *R. etli* wild-type and mutant backgrounds

Strain	Relevant genotype	β-Glucuronidase activity (Miller units)[a]		
		Aerobic conditions	Microaerobic conditions	Symbiotic conditions
CNPAF512	Wild type	79 (33)	474 (175)	1,025 (169)
Rp1000	*nifA*	51 (29)	380 (71)	1,033 (99)
FAJ1182	*fnrN*	40 (6)	59 (17)	82 (12)
FAJ1183	*fnrN*	23 (5)	66 (12)	50 (22)
FAJ1154	$rpoN_1$	81 (20)	267 (67)	1,039 (145)
FAJ1169	$rpoN_2$	56 (38)	451 (104)	968 (131)
RpFAJ1002	*fixL*	51 (29)	470 (108)	1,008 (194)
RpFAJ1004	*fixJ*	60 (24)	453 (131)	ND[b]

[a] The symbiotic expression data are the means of at least 10 replicates, and the results were confirmed independently in separate experiments. The free-living expression data are the means of at least four replicates. The values in parentheses are standard deviations.
[b] ND, not determined.

strain MAFF303099 and strain R7A, contain the cysteine signature, Cys-X_2-Cys-X_2-R-X_4-Cys-X_{87}-Cys-X-F, defined for class IB Fnr-related proteins (15) (data not shown) and form a cluster that is distinct from the more divergent group of FixK proteins. The FixK proteins are divided into two separated clusters, which are distinct from *E. coli* Fnr (a member of class IA of the Crp-Fnr family) and *Pseudomonas stutzeri* DnrD (a member of class ID) (Fig. 2). This tripartite division of the rhizobial FnrN and FixK proteins was confirmed by constructing a parsimonious tree (data not shown).

Expression of *R. etli fnrN* in *S. meliloti*. To investigate whether the cloned *fnrN* gene codes for an active protein, this gene was transferred into *S. meliloti* strain GMI347-CS112 (6). This *S. meliloti fixJ* mutant is unable to induce a chromosomally integrated, *fixK*-dependent *fixN-lacZ* fusion. Table 2 shows that the *fixN-lacZ* fusion in GMI347-CS112 was highly activated under microaerobic conditions (0.3% oxygen) in the presence of either pFAJ1172 or pFAJ1175. No expression was observed under aerobic conditions. These results indicate that the *R. etli fnrN* gene is actively expressed in *S. meliloti* and can functionally substitute for the *fixK* gene in expression of *fixN*. Since GMI347-CS112 is *fixJ*, expression of *R. etli fnrN* in *S. meliloti* occurs independent of the FixLJ system.

Phenotypes of *R. etli fnrN* mutants. To investigate *fnrN* function, *R. etli fnrN* mutant strains (FAJ1182 and FAJ1183) were constructed by site-specific mutagenesis (see Materials and Methods). The symbiotic phenotypes of these mutants were determined (Table 3). No effect of the mutation on nodule number was observed when the data were compared with data for the wild-type strain. Nitrogen fixation was expressed in terms of acetylene reduction activity. The acetylene reduction activities of both *fnrN* mutants were approximately 80% lower than the activity of the wild type.

TABLE 3. Nitrogenase activities and nodulation of the *R. etli* wild type and *fnrN* mutants

Strain	Relevant genotype	Acetylene reduction activity (μmol of ethylene/plant/h)[a]	No. of nodules/plant[a]
CNPAF512	Wild type	9.5 (2.8)	199 (31)
FAJ1182	*fnrN*	2 (1.0)	204 (50)
FAJ1183	*fnrN*	2.3 (1.0)	ND[b]

[a] The data are the means of at least 10 replicates, and the results were confirmed independently in separate experiments. The values in parentheses are standard deviations.
[b] ND, not determined.

Expression analysis of *R. etli fnrN*. To study the regulation of *R. etli fnrN*, a translational *fnrN-gusA* fusion (pFAJ1178) was constructed (see Materials and Methods). This fusion was introduced into the *R. etli* wild type and into the following regulatory mutants: Rp1000 (*nifA*), FAJ1154 and FAJ1169 (*rpoN*), FAJ1182 and FAJ1183 (*fnrN*), RpFAJ1002 (*fixL*), and RpFAJ1004 (*fixJ*). Expression of the fusion was assayed under aerobic, microaerobic, and symbiotic conditions (Table 4). Expression of *R. etli fnrN* was induced during microaerobic growth and in bacteroids. The level of expression was reduced to aerobic background levels in both *fnrN* mutants, while it reached wild-type levels in *R. etli nifA*, *rpoN*, *fixL*, and *fixJ* mutants. The observed autoregulation is in agreement with the presence of two putative FnrN-binding sites in the *fnrN* promoter region. The *R. etli* FnrN protein is probably oxygen sensitive (see above), which could explain why positive autoregulation occurred only in microoxic environments.

Isolation and phenotypes of *R. etli fixG*, *fixO*, and *fixI* mutants. In a search for genes activated by low oxygen tension, three symbiotic mutants (CMPG8007, CMPG8169, and CMPG8170) were identified in an independent analysis based on screening of an *R. etli* CNPAF512::mTn*5gusA* library (60, 61; Moris, unpublished results). The partial DNA sequences of the inactivated genes of these mutants were determined as described in Materials and Methods. From the analysis of these partial sequences, the identities of the genes could be determined. The transposon insertion of mutant strain CMPG8169 was localized after codon 53 of the *fixG* gene. The sequence of the promoter and part of the *fixG* gene is shown in Fig. 3A. A sequence resembling an anaerobox was found 50 bp upstream from the presumptive ATG start codon. The presence of this sequence suggests that there was regulation by an Fnr-like protein. In mutant CMPG8170, the transposon was inserted after codon 377 of the *fixI* gene (the codon numbers are the *Rhizobium leguminosarum* bv. viciae *fixI* codon numbers). Analysis of the partial DNA sequence flanking the transposon in mutant CMPG8007 revealed that it was inserted after

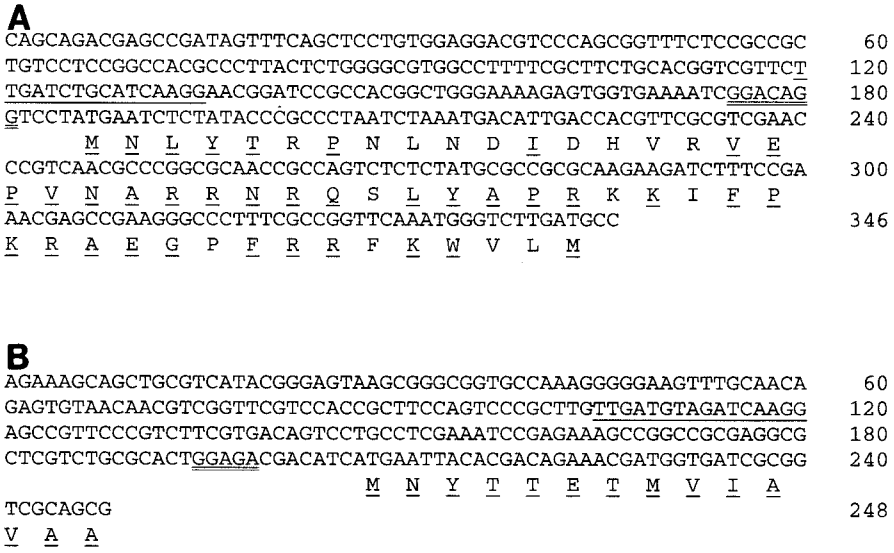


FIG. 3. DNA sequences of the promoter regions and 5′ ends of the *fixG* (A) and *fixN* (B) genes of *R. etli* CNPAF512. Putative anaeroboxes and ribosome-binding sites are underlined once and twice, respectively. The deduced amino acid sequence is indicated below the DNA sequence. Amino acids conserved in the *R. leguminosarum* bv. viciae FixG and FixN sequences are underlined.

codon 23 of *fixO*. Expression of the *R. etli fixG*::*gusA*, *fixI*::*gusA*, and *fixO*::*gusA* fusions was clearly induced during symbiosis (Table 5). All three mutants displayed reduced acetylene reduction activity (27 to 95% reductions compared to the wild-type acetylene reduction activity) (60; Moris unpublished results). In order to test directly the *fnrN*-dependent expression of these genes, plasmid-borne *fixN-gusA* and *fixG-gusA* fusions were constructed.

Regulation of *R. etli fixN*, *fixG*, and *arcA* expression. As *fixG* is generally linked with *fixNOQP* genes in rhizobia, a search for a cosmid clone carrying this region was performed (as described in Materials and Methods). The presence of the *fixN* and *fixG* genes was confirmed by partial sequence analysis of fragments of the identified cosmids. This region was shown to be located close to the previously described $nifHDK_3$ gene located on a 4.2-kb *Eco*RI fragment (33). Analysis of the DNA sequence of the *fixN* promoter revealed the presence of an anaerobox 86 bp upstream from the ATG start codon (Fig. 3B). To study the regulation of expression of *fixN* and *fixG*, transcriptional *gusA* fusions with the promoters of both genes were constructed. The pFAJ1192 (*fixG-gusA*) and pFAJ1193 (*fixN-gusA*) fusions were introduced into the *R. etli* wild-type strain and into regulatory mutants, and expression of the fusions was analyzed under free-living aerobic and microaerobic conditions and during symbiosis (Table 6). Both *fixN* and *fixG* were induced under microaerobic and symbiotic conditions compared to the expression under aerobic conditions. A similar expression pattern was observed for *R. etli* mutant strains CMPG8169, CMPG8170, and CMPG8007 carrying *fixG-gusA*, *fixI-gusA*, and *fixO-gusA* insertions (Table 5). Clear differences in the extent of induction and the effect of mutations in regulatory genes on *fixN* and *fixG* expression were noticed (Table 6). During symbiosis, expression of pFAJ1192 was upregulated in *rpoN*, *nifA*, and *fixL* mutants compared to the wild-type expression, indicating that there was symbiosis-specific regulation. On the other hand, the β-glucuronidase activity of pFAJ1193 was lower in these mutants than in the wild-type strain during symbiosis. The β-glucuronidase activity of both fusions was reduced to aerobic background levels in the *R. etli fnrN* strain under all conditions tested (free-living microaerobic and symbiotic conditions). Based on these data, distinct and complex regulation of *fixG* and *fixN* expression during symbiosis is hypothesized. Similarly, expression of the *S. meliloti fixN-lacZ* fusion plasmid pGMI931 in *R. etli* was induced only under low oxygen tension (Table 2), and expression of this plasmid did not occur in the *R. etli fnrN* mutant.

TABLE 5. Expression of *fixG*::*gusA*, *fixI*::*gusA*, and *fixO*::*gusA* in *R. etli*[a]

Strain	Relevant genotype	β-Glucuronidase activity (Miller units)[b]		
		Aerobic conditions	Microaerobic conditions	Symbiotic conditions
CMPG8169	*fixG*	35 (1)	106 (7)	182 (57)
CMPG8170	*fixI*	0 (0)	56 (22)	4,729 (1,058)
CMPG8007	*fixO*	27 (0.4)	1,285 (186)	1,931 (269)

[a] Cultures were grown in acid minimal salts medium supplemented with 10 mM succinate and 10 mM NH_4Cl.
[b] See Table 4, footnote *a*.

The arginine deiminase pathway in *R. etli* which is active in nitrogen-fixing bacteroids (11) is encoded by the *arcABC* genes, which are located 1 kb upstream from the *R. etli fixLJ* genes. Approximately 700 bp upstream from the *R. etli arcA* gene, we identified a sequence motif (TTGATCCGGCT CAATG) with strong similarity to an anaerobox (nucleotides conserved in the consensus are underlined) (15), suggesting that there was regulation by an Fnr-like protein, as it is the case in *Pseudomonas aeruginosa* (18). We tested whether inactivation of the *R. etli fnrN* gene affects expression of the *R. etli arcA-gusA* fusion plasmid pFAJ1319 (Table 6). The *arcA-gusA* fusion was

TABLE 6. Expression of *R. etli fixG-gusA* (pFAJ1192), *fixN-gusA* (pFAJ1193), *arcA-gusA* (pFAJ1319), *rpoN$_2$-gusA* (pFAJ1175), and *orf180-gusA* (pFAJ1176) in *R. etli* wild-type and mutant backgrounds

Strain[a]	Plasmid	β-Glucuronidase activity (Miller units)[b]		
		Aerobic conditions	Microaerobic conditions	Symbiotic conditions
CNPAF512 (wild type)	pFAJ1192 (*fixG-gusA*)	31 (1)	324 (42)	134 (25)
FAJ1170 (*rpoN$_1$ rpoN$_2$*)	pFAJ1192	39 (1)	345 (56)	1,318 (190)
RP1000 (*nifA*)	pFAJ1192	15 (6)	358 (38)	979 (103)
FAJ1183 (*fnrN*)	pFAJ1192	43 (12)	38 (15)	27 (3)
RpFAJ1002 (*fixL*)	pFAJ1192	61 (12)	263 (52)	453 (152)
CNPAF512 (wild type)	pFAJ1193 (*fixN-gusA*)	6 (1)	16 (4)	1,219 (153)
FAJ1170 (*rpoN$_1$ rpoN$_2$*)	pFAJ1193	6 (1)	16 (2)	604 (148)
RP1000 (*nifA*)	pFAJ1193	7 (2)	22 (5)	644 (70)
FAJ1183 (*fnrN*)	pFAJ1193	7 (1)	8 (2)	20 (14)
RpFAJ1002 (*fixL*)	pFAJ1193	8 (2)	29 (4)	403 (75)
CNPAF512 (wild type)	pFAJ1319 (*arcA-gusA*)	0 (0)	39 (6)	ND[c]
FAJ1182 (*fnrN*)	pFAJ1319	0 (0)	0 (1)	ND
FAJ1183 (*fnrN*)	pFAJ1319	0 (0)	0 (1)	ND
RpFAJ1002 (*fixL*)	pFAJ1319	0 (0)	27 (6)	ND
CNPAF512 (wild type)	pFAJ1175 (*rpoN$_2$-gusA*)	ND	390 (49)	ND
FAJ1183 (*fnrN*)	pFAJ1175	ND	467 (56)	ND
FAJ1170 (*rpoN$_1$ rpoN$_2$*)	pFAJ1175	ND	7 (3)	ND
CNPAF512 (wild type)	pFAJ1176 (*orf180-gusA*)	ND	49 (7)	ND
FAJ1183 (*fnrN*)	pFAJ1176	ND	52 (11)	ND
FAJ1170 (*rpoN$_1$ rpoN$_2$*)	pFAJ1176	ND	6 (3)	ND

[a] The relevant genotype is indicated in parentheses.
[b] See Table 4, footnote *a*.
[c] ND, not determined.

induced under microaerobic conditions, and this induction was strictly dependent on the presence of a functional FnrN protein.

Finally, we tested whether a mutation in the *fnrN* gene affects expression of the *rpoN2-gusA* fusion pFAJ1175 and the *orf180-gusA* fusion pFAJ1176 (34). Microaerobic activation of these fusions depends on the presence of functional NifA and RpoN proteins. Expression of these genes was not reduced in the *R. etli fnrN* mutant (Table 6).

DISCUSSION

The primary structure of *R. etli* CNPAF512 FnrN reveals that there is a conserved cysteine cluster at the N terminus (15, 57) which is also present in other proteins encoded by *fnrN*-like genes belonging to class IB (data not shown). These proteins form a separate cluster, as revealed by the phylogenetic analysis of various rhizobial FnrN and FixK sequences (Fig. 2). The phylogenetic analysis also revealed a division of the FixK proteins into two distinct groups (Fig. 2). The FixK proteins of *S. meliloti*, *A. caulinodans*, and *B. japonicum* (FixK2) belong to the same cluster. This group of proteins was previously called class IC of the Crp-Fnr family (15). Null mutations in the *fixK* genes of these rhizobia eliminate nitrogen fixation (3, 15, 27). This cluster also includes homologous FixK proteins of *M. loti*, *B. japonicum*, and *S. meliloti*. In addition, there is a distinct group containing FixK proteins of *R. leguminosarum* bv. viciae VF39, *R. etli* CFN42, *Rhizobium* sp. strain IC3342, and *S. meliloti* (Fig. 2). In contrast to the symbiotic phenotype of *S. meliloti*, *A. caulinodans*, and *B. japonicum fixK* mutants, *fixKd* and *fixKf* mutants of *R. etli* CFN42 and *fixK* mutants of *R. leguminosarum* bv. viciae do not exhibit a severe reduction in nitrogenase activity (21, 39).

The phylogenetic tree shows that the sequence of *R. etli* CNPAF512 FnrN is most closely related to the sequence of FnrNd of *R. etli* CFN42. However, there are significant functional differences between these proteins. Besides the occurrence of a second copy of the *fnrN* gene (*fnrNchr*) in *R. etli* CFN42 (together with duplication of *fixK* genes), the methods of regulation differ (see below) (31). Knocking out the *R. etli* CNPAF512 *fnrN* gene causes a severe symbiotic defect beginning at the start of nitrogen fixation, whereas the CFN42 *fnrNd* gene has a role in the late stages of the symbiosis. Loss of the *R. etli* CNPAF512 FnrN protein in the bacteroids results in an almost 80% decrease in the nitrogen fixation activity compared to the wild-type nitrogen fixation activity. In addition, the *fnrN* gene is highly induced in bacteroids. These results indicate that *R. etli* CNPAF512 FnrN plays a key role during symbiosis with common bean plants.

The methods of regulation of the *fnrN* genes belonging to class IB are different in the different rhizobia. The expression of *B. japonicum fixK1* is indirectly dependent on FixLJ through FixK2 (37). In *R. etli* CFN42, two copies of the *fnrN* genes (*fnrNchr* and *fnrNd*) were identified. Expression of these genes is differentially controlled by an unusual *fixL* gene under free-living microaerobic conditions without participation of a *fixJ* gene. *fnrNd* is positively regulated by FixL through FixKf. On the other hand, FixL negatively regulates *fnrNchr* independent of FixKf (31). In contrast to these observations, the free-living microaerobic and symbiotic induction of *R. etli* CNPAF512 *fnrN* was shown to be independent of *fixLJ*. Also, in *R. legu-*

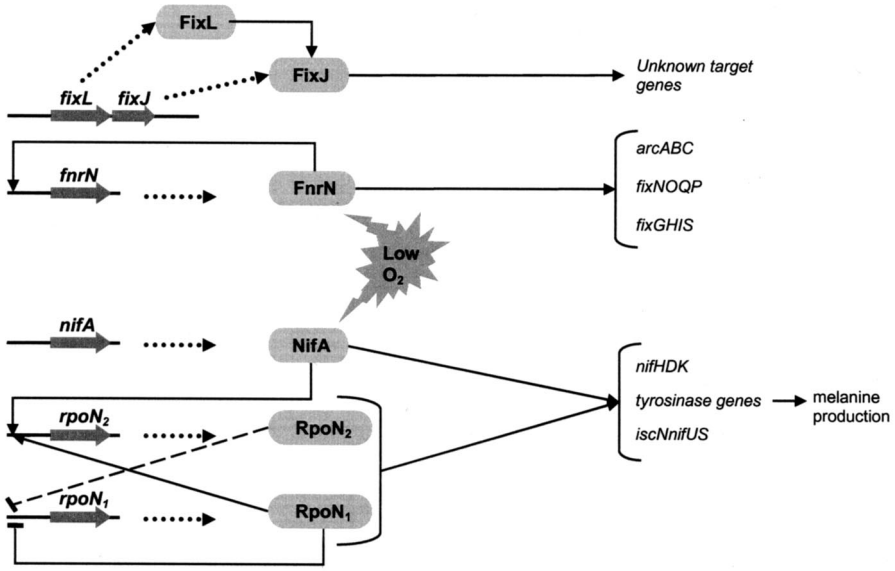


FIG. 4. Schematic overview of the free-living microaerobic regulation of *nif*, *fix*, and other symbiotically important genes of *R. etli* CNPAF512. Only strictly dependent relationships are indicated. $rpoN_1$ is essential during free-living growth, while $rpoN_2$ is required for symbiosis. Transcription of $rpoN_2$ is highly induced under free-living microaerobic conditions by $RpoN_1$ and NifA. Expression of $rpoN_1$ is negatively autoregulated (indicated by a blocked line); in addition, negative regulation of $RpoN_2$ for $rpoN_1$ expression is hypothesized (indicated by a blocked dashed line) (34). NifA and RpoN are strictly required for expression of *iscNnifUS* (13), for expression of *nifHDK*, and for the production of melanine (34). The target genes of FixLJ are unknown. FixLJ contributes to the regulation of *nifH*, but because of the strict dependence of *nifH* transcription on NifA and RpoN, it has been suggested that the regulation of FixLJ is indirect (10). The target genes of FnrN represented in this scheme were identified in this study.

minosarum bv. viciae VF39, *fnrN* is not controlled by FixK, but it has been proposed that FixK and FnrN act in parallel (39). In *R. leguminosarum* bv. viciae UPM791 no evidence for the presence of *fixLJ* orthologs has been found (22).

In *S. meliloti* and *A. caulinodans* no *fnrN* genes belonging to class IB were identified. The *A. caulinodans* and *S. meliloti fixK* genes are both regulated by FixLJ (3, 27). In addition, *S. meliloti* FixK induces the expression of *fixT*, whose product negatively regulates *fixK* expression by counteracting FixLJ activities (16, 19).

In microoxic conditions, *R. etli* CNPAF512 *fnrN* is autoactivated, resulting in a self-amplifying cascade. This autoregulation is consistent with the presence of anaeroboxes in the promoter region of *fnrN*. Positive autoregulation of *fnrN* also occurs in *R. leguminosarum* bv. viciae VF39 *fnrN* (4, 45). Likewise, in bacteroids of *R. leguminosarum* bv. viciae UPM791, autoregulation of *fnrN1* and *fnrN2* expression was observed. Analysis of the promoter region of the two *fnrN* genes revealed the presence of two anaeroboxes. Differential binding of FnrN1 on the anaeroboxes in the *fnrN1* promoter has been reported, resulting in both positive and negative autoregulation (5). Furthermore, in the promoters of the *R. etli* CFN42 *fnrN* genes two putative Fnr-binding sites are present, and complex regulatory interactions between the two *fnrN* genes have been observed (31). In contrast, no autoregulation of *B. japonicum* FixK1 was observed (1).

Three target genes of FnrN have been identified in *R. etli* CNPAF512: *fixN*, which is part of the *fixNOQP* operon coding for a cbb_3-type cytochrome oxidase (41); *fixG*, which is part of the *fixGHIS* operon and in *B. japonicum* is involved in the assembly and stability of the FixNOQP complex (42); and *arcA*, which encodes a protein with a function in the arginine deiminase pathway in *R. etli* (11). *R. etli* CNPAF512 FnrN is an essential positive regulator of *fixG* and *fixN* under free-living microaerobic conditions and in bacteroids and induces *arcA* under free-living microaerobic conditions. In contrast to the microaerobic regulation of *fixG* and *fixN* expression, the symbiotic expression of these genes is subject to an additional level of regulation. Besides the strict dependence on FnrN, symbiotic expression of these genes also involves fine-tuning by RpoN, NifA, and FixL (Table 6). Additional symbiotic regulatory mechanisms were also observed previously for the regulation of $rpoN_2$ expression, and it was proposed that these mechanisms include an unknown symbiosis-specific mechanism (34). Positive regulation of *fixN* by FnrN is also observed in other rhizobia. FnrN (together with FixL) from *R. leguminosarum* bv. viciae VF39 induces *fixNc* and *fixNd* under free-living microaerobic conditions (44). The microaerobic expression of *R. etli* CFN42 *fixNd* is mainly activated by FnrNchr and FixL-FixKf (21, 31). In *R. leguminosarum* bv. viciae UPM791 no genes homologous to *fixLJ* or *fixK* were found, but *fixN* is controlled by the two *fnrN* genes under microoxic conditions (22). In contrast, in *B. japonicum* the *fixNOQP* operon is regulated by FixK2 (and consequently also by FixJ) but not by FixK1 (37, 41).

Taken together, the results presented here show that besides the previously identified regulatory cascades of nitrogen fixation genes controlled by NifA and FixL (10, 33), a third independent symbiotic regulator, FnrN, is operational in *R. etli* CNPAF512. This protein is involved in sensing a low-oxygen signal and in transducing the signal into a regulation cascade of a specific subset of nitrogen fixation genes (Fig. 4).

ACKNOWLEDGMENTS

We thank J. Batut and U. Priefer for providing plasmid pGMI931 and *S. meliloti* strain GMI347-CS112.

This work was supported by grants from the Research Council of Katholieke Universiteit Leuven (grant GOA/2003/09) and from the Fund for Scientific Research—Flanders (grant G.0108.01).

REFERENCES

1. **Anthamatten, D., B. Scherb, and H. Hennecke.** 1992. Characterization of a *fixLJ*-regulated *Bradyrhizobium japonicum* gene sharing similarity with the *Escherichia coli fnr* and *Rhizobium meliloti fixK* genes. J. Bacteriol. **174:**2111–2120.
2. **Barnett, M. J., R. F. Fisher, T. Jones, C. Komp, A. P. Abola, F. Barloy-Hubler, L. Bowser, D. Capela, F. Galibert, J. Gouzy, M. Gurjal, A. Hong, L. Huizar, R. W. Hyman, D. Kahn, M. L. Kahn, S. Kalman, D. H. Keating, C. Palm, M. C. Peck, R. Surzycki, D. H. Wells, K. C. Yeh, R. W. Davis, N. A. Federspiel, and S. R. Long.** 2001. Nucleotide sequence and predicted functions of the entire *Sinorhizobium meliloti* pSymA megaplasmid. Proc. Natl. Acad. Sci. **98:**9883–9888.
3. **Batut, J., M. L. Daveran-Mingot, M. D. J. Jacobs, A. M. Garnerone, and D. Kahn.** 1989. FixK, a gene homologous with *fnr* and *crp* from *Escherichial coli*, regulates nitrogen-fixation genes both positively and negatively in *Rhizobium meliloti.* EMBO J. **8:**1279–1286.
4. **Clark, S. R. D., I. J. Oresnik, and M. F. Hynes.** 2001. RpoN of *Rhizobium leguminosarum* bv. *viciae* strain VF39SM plays a central role in FnrN-dependent microaerobic regulation of genes involved in nitrogen fixation. Mol. Gen. Genet. **264:**623–633.
5. **Colombo, M. V., D. Gutierrez, J. M. Palacios, J. Imperial, and T. Ruiz-Argueso.** 2000. A novel autoregulation mechanism of *fnrN* expression in *Rhizobium leguminosarum* bv *viciae.* Mol. Microbiol. **36:**477–486.
6. **Colonna-Romano, S., W. Arnold, A. Schluter, P. Boistard, A. Puhler, and U. B. Priefer.** 1990. An Fnr-like protein encoded in *Rhizobium leguminosarum* biovar *viciae* shows structural and functional homology to *Rhizobium meliloti* FixK. Mol. Gen. Genet. **223:**138–147.
7. **David, M., M. L. Daveran, J. Batut, A. Dedieu, O. Domergue, J. Ghai, C. Hertig, P. Boistard, and D. Kahn.** 1988. Cascade regulation of *nif* gene expression in *Rhizobium meliloti.* Cell **54:**671–683.
8. **de Philip, P., J. Batut, and P. Boistard.** 1990. *Rhizobium meliloti* Fix L is an oxygen sensor and regulates *R. meliloti nifA* and *fixK* genes differently in *Escherichia coli.* J. Bacteriol. **172:**4255–4262.
9. **D'hooghe, I., J. Michiels, and J. Vanderleyden.** 1998. The *Rhizobium etli* FixL protein differs in structure from other known FixL proteins. Mol. Gen. Genet. **257:**576–580.
10. **D'hooghe, I., J. Michiels, K. Vlassak, C. Verreth, F. Waelkens, and J. Vanderleyden.** 1995. Structural and functional analysis of the *fixLJ* genes of *Rhizobium leguminosarum* biovar *phaseoli* CNPAF512. Mol. Gen. Genet. **249:**117–126.
11. **D'hooghe, I., C. Vander Wauven, J. Michiels, C. Tricot, P. de Wilde, J. Vanderleyden, and V. Stalon.** 1997. The arginine deiminase pathway in *Rhizobium etli*: DNA sequence analysis and functional study of the *arcABC* genes. J. Bacteriol. **179:**7403–7409.
12. **Dombrecht, B., K. Marchal, J. Vanderleyden, and J. Michiels.** 26 November 2002, posting date. Prediction and overview of the RpoN-regulon in closely related species of the *Rhizobiales*. Genome Biol. **3:**research0076.1-0076.11. [Online.] http://genomebiology.com.
13. **Dombrecht, B., M. Z. Tesfay, C. Verreth, C. Heusdens, M. C. Napoles, J. Vanderleyden, and J. Michiels.** 2002. The *Rhizobium etli* gene *iscN* is highly expressed in bacteroids and required for nitrogen fixation. Mol. Genet. Genomics **267:**820–828.
14. **Fellay, R., J. Frey, and H. Krisch.** 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for in vitro insertional mutagenesis of gram-negative bacteria. Gene **52:**147–154.
15. **Fischer, H. M.** 1994. Genetic regulation of nitrogen fixation in rhizobia. Microbiol. Rev. **58:**352–386.
16. **Foussard, M., A. M. Garnerone, F. Ni, E. Soupene, P. Boistard, and J. Batut.** 1997. Negative autoregulation of the *Rhizobium meliloti fixK* gene is indirect and requires a newly identified regulator, FixT. Mol. Microbiol. **25:**27–37.
17. **Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene **18:**289–296.
18. **Galimand, M., M. Gamper, A. Zimmermann, and D. Haas.** 1991. Positive Fnr-like control of anaerobic arginine degradation and nitrate respiration in *Pseudomonas aeruginosa.* J. Bacteriol. **173:**1598–1606.
19. **Garnerone, A. M., D. Cabanes, M. Foussard, P. Boistard, and J. Batut.** 1999. Inhibition of the FixL sensor kinase by the FixT protein in *Sinorhizobium meliloti.* J. Biol. Chem. **274:**32500–32506.
20. **Gilles-Gonzalez, M. A., G. S. Ditta, and D. R. Helinski.** 1991. A haemoprotein with kinase activity encoded by the oxygen sensor of *Rhizobium meliloti.* Nature **350:**170–172.
21. **Girard, L., S. Brom, A. Dávalos, O. Lopez, M. Soberón, and D. Romero.** 2000. Differential regulation of *fixN*-reiterated genes in *Rhizobium etli* by a novel *fixL-fixK* cascade. Mol. Plant-Microbe Interact. **13:**1283–1292.
22. **Gutierrez, D., Y. Hernando, J. M. Palacios, J. Imperial, and T. Ruiz-Argueso.** 1997. FnrN controls symbiotic nitrogen fixation and hydrogenase activities in *Rhizobium leguminosarum* biovar *viciae* UPM791. J. Bacteriol. **179:**5264–5270.
23. **Hernando, Y., J. M. Palacios, J. Imperial, and T. Ruiz-Argueso.** 1995. The *hypBFCDE* operon from *Rhizobium leguminosarum* biovar *viciae* is expressed from an Fnr type promoter that escapes mutagenesis of the *fnrN* gene. J. Bacteriol. **177:**5661–5669.
24. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172:**6557–6567.
25. **Hertig, C., R. Y. Li, A. M. Louarn, A. M. Garnerone, M. David, J. Batut, D. Kahn, and P. Boistard.** 1989. *Rhizobium meliloti* regulatory gene *fixJ* activates transcription of *R. meliloti nifA* and *fixK* genes in *Escherichia coli*. J. Bacteriol. **171:**1736–1738.
26. **Kaminski, P. A., and C. Elmerich.** 1998. The control of *Azorhizobium caulinodans nifA* expression by oxygen, ammonia and by the HF-I-like protein, NrfA. Mol. Microbiol. **28:**603–613.
27. **Kaminski, P. A., K. Mandon, F. Arigoni, N. Desnoues, and C. Elmerich.** 1991. Regulation of nitrogen fixation in *Azorhizobium caulinodans*: identification of a *fixK* like gene, a positive regulator of *nifA.* Mol. Microbiol. **5:**1983–1991.
28. **Kaneko, T., Y. Nakamura, S. Sato, E. Asamizu, T. Kato, S. Sasamoto, A. Watanabe, K. Idesawa, A. Ishikawa, K. Kawashima, T. Kimura, Y. Kishida, C. Kiyokawa, M. Kohara, M. Matsumoto, A. Matsuno, Y. Mochizuki, S. Nakayama, N. Nakazaki, S. Shimpo, M. Sugimoto, C. Takeuchi, M. Yamada, and S. Tabata.** 2000. Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti.* DNA Res. **7:**331–338.
29. **Kaneko, T., Y. Nakamura, S. Sato, K. Minamisawa, T. Uchiumi, S. Sasamoto, A. Watanabe, K. Idesawa, M. Iriguchi, K. Kawashima, M. Kohara, M. Matsumoto, S. Shimpo, H. Tsuruoka, T. Wada, M. Yamada, and S. Tabata.** 2002. Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. DNA Res. **9:**189–197.
30. **Kiley, P. J., and H. Beinert.** 1998. Oxygen sensing by the global regulator, FNR: the role of the iron-sulfur cluster. FEMS Microbiol. Rev. **22:**341–352.
31. **Lopez, O., C. Morera, J. Miranda-Rios, L. Girard, D. Romero, and M. Soberón.** 2001. Regulation of gene expression in response to oxygen in *Rhizobium etli*: role of FnrN in *fixNOQP* expression and in symbiotic nitrogen fixation. J. Bacteriol. **183:**6999–7006.
32. **Metcalf, W. W., and B. L. Wanner.** 1993. Evidence for a fourteen-gene, *phnC* to *phnP* locus for phosphonate metabolism in *Escherichia coli.* Gene **129:**27–32.
33. **Michiels, J., I. D'hooghe, C. Verreth, H. Pelemans, and J. Vanderleyden.** 1994. Characterization of the *Rhizobium leguminosarum* biovar *phaseoli nifA* gene, a positive regulator of *nif* gene expression. Arch. Microbiol. **161:**404–408.
34. **Michiels, J., M. Moris, B. Dombrecht, C. Verreth, and J. Vanderleyden.** 1998. Differential regulation of *Rhizobium etli rpoN2* gene expression during symbiosis and free-living growth. J. Bacteriol. **180:**3620–3628.
35. **Michiels, J., T. Van Soom, I. D'hooghe, B. Dombrecht, T. Benhassine, P. de Wilde, and J. Vanderleyden.** 1998. The *Rhizobium etli rpoN* locus: DNA sequence analysis and phenotypical characterization of *rpoN*, *ptsN*, and *ptsA* mutants. J. Bacteriol. **180:**1729–1740.
36. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Nellen-Anthamatten, D., P. Rossi, O. Preisig, I. Kullik, M. Babst, H. M. Fischer, and H. Hennecke.** 1998. *Bradyrhizobium japonicum* FixK2, a crucial distributor in the FixLJ-dependent regulatory cascade for control of genes inducible by low oxygen levels. J. Bacteriol. **180:**5251–5255.
38. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26:**101–106.
39. **Patschkowski, T., A. Schluter, and U. B. Priefer.** 1996. *Rhizobium leguminosarum* bv *viciae* contains a second *fnr*/*fixK*-like gene and an unusual *fixL* homologue. Mol. Microbiol. **21:**267–280.
40. **Poole, P. S., N. A. Schofield, C. J. Reid, E. M. Drew, and D. L. Walshaw.** 1994. Identification of chromosomal genes located downstream of *dctD* that affect the requirement for calcium and the lipopolysaccharide layer of *Rhizobium leguminosarum.* Microbiology **140:**2797–2809.
41. **Preisig, O., D. Anthamatten, and H. Hennecke.** 1993. Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen-fixing endosymbiosis. Proc. Natl. Acad. Sci. **90:**3309–3313.
42. **Preisig, O., R. Zufferey, and H. Hennecke.** 1996. The *Bradyrhizobium japonicum fixGHIS* genes are required for the formation of the high-affinity cbb(3)-type cytochrome oxidase. Arch. Microbiol. **165:**297–305.
43. **Quandt, J., and M. F. Hynes.** 1993. Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. Gene **127:**15–21.
44. **Schluter, A., T. Patschkowski, J. Quandt, L. B. Selinger, S. Weidner, M.**

Kramer, L. Zhou, M. F. Hynes, and U. B. Priefer. 1997. Functional and regulatory analysis of the two copies of the *fixNOQP* operon of *Rhizobium leguminosarum* strain VF39. Mol. Plant-Microbe Interact. **10:**605–616.

45. **Schluter, A., T. Patschkowski, G. Unden, and U. B. Priefer.** 1992. The *Rhizobium leguminosarum* FnrN protein is functionally similar to *Eschericha coli* FNR and promotes heterologous oxygen dependent activation of transcription. Mol. Microbiol. **6:**3395–3404.
46. **Snoeck, C., C. Verreth, I. Hernandez-Lucas, E. Martinez-Romero, and J. Vanderleyden.** 2003. Identification of a third sulfate activation system in *Sinorhizobium* sp. strain BR816: the CysDN sulfate activation complex. Appl. Environ. Microbiol. **69:**2006–2014.
47. **Soberón, M., O. Lopez, C. Morera, M. L. Girard, M. L. Tabche, and J. Miranda.** 1999. Enhanced nitrogen fixation in a *Rhizobium etli ntrC* mutant that overproduces the *Bradyrhizobium japonicum* symbiotic terminal oxidase cbb_3. Appl. Environ. Microbiol. **65:**2015–2019.
48. **Soupène, E., M. Foussard, P. Boistard, G. Truchet, and J. Batut.** 1995. Oxygen as a key developmental regulator of *Rhizobium meliloti* N_2 fixation gene expression within the alfalfa root nodule. Proc. Natl. Acad. Sci. **92:** 3759–3763.
49. **Spiro, S.** 1994. The FNR family of transcriptional regulators. Antonie Leeuwenhoek **66:**23–36.
50. **Staskawicz, B., D. Dahlbeck, N. Keen, and C. Napoli.** 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea.* J. Bacteriol. **169:**5789–5794.
51. **Sullivan, J. T., J. R. Trzebiatowski, R. W. Cruickshank, J. Gouzy, S. D. Brown, R. M. Elliot, D. J. Fleetwood, N. G. McCallum, U. Rossbach, G. S. Stuart, J. E. Weaver, R. J. Webby, F. J. De Bruijn, and C. W. Ronson.** 2002. Comparative sequence analysis of the symbiosis island of *Mesorhizobium loti* strain R7A. J. Bacteriol. **184:**3086–3095.
52. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
53. **Unden, G., and J. Schirawski.** 1997. The oxygen-responsive transcriptional regulator FNR of *Escherichia coli*: the search for signals and reactions. Mol. Microbiol. **25:**205–210.
54. **Upadhyaya, N. M., K. F. Scott, W. T. Tucker, J. M. Watson, and P. J. Dart.** 1992. Isolation and characterization of *Rhizobium* (IC3342) genes that determine leaf curl induction in pigeon pea. Mol. Plant-Microbe Interact. **5:**129–143.
55. **Vande Broek, A., M. Lambrecht, K. Eggermont, and J. Vanderleyden.** 1999. Auxins upregulate expression of the indole-3-pyruvate decarboxylase gene in *Azospirillum brasilense.* J. Bacteriol. **181:**1338–1342.
56. **Van de Peer, Y., and R. De Wachter.** 1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput. Applic. Biosci. **10:**569–570.
57. **Van Spanning, R. J., A. P. De Boer, W. N. Reijnders, H. V. Westerhoff, A. H. Stouthamer, and Van Der Oost J.** 1997. FnrP and NNR of *Paracoccus denitrificans* are both members of the FNR family of transcriptional activators but have distinct roles in respiratory adaptation in response to oxygen limitation. Mol. Microbiol. **23:**893–907.
58. **Vincent, J. M.** 1970. A manual for the practical study of root-nodule bacteria. Blackwell Scientific Publications, Ltd., Oxford, United Kingdom.
59. **Vollack, K. U., E. Hartig, H. Korner, and W. G. Zumft.** 1999. Multiple transcription factors of the FNR family in denitrifying *Pseudomonas stutzeri*: characterization of four *fnr*-like genes, regulatory responses and cognate metabolic processes. Mol. Microbiol. **31:**1681–1694.
60. **Xi, C., G. Dirix, J. Hofkens, F. C. Schryver, J. Vanderleyden, and J. Michiels.** 2001. Use of dual marker transposons to identify new symbiosis genes in *Rhizobium.* Microb. Ecol. **41:**325–332.
61. **Xi, C., M. Lambrecht, J. Vanderleyden, and J. Michiels.** 1999. Bi-functional *gfp*- and *gusA*-containing mini-Tn*5* transposon derivatives for combined gene expression and bacterial localization studies. J. Microbiol. Methods **35:**85–92.