JOURNAL OF BACTERIOLOGY, Dec. 2001, p. 6999–7006 Vol. 183, No. 24
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.24.6999–7006.2001

Regulation of Gene Expression in Response to Oxygen in *Rhizobium etli*: Role of FnrN in *fixNOQP* Expression and in Symbiotic Nitrogen Fixation

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Received 31 May 2001/Accepted 7 September 2001

Previously, we reported finding duplicated *fixNOQP* operons in *Rhizobium etli* CFN42. One of these duplicated operons is located in the symbiotic plasmid (*fixNOQP*d), while the other is located in a cryptic plasmid (*fixNOQP*f). Although a novel FixL-FixKf regulatory cascade participates in microaerobic expression of both *fixNOQP* duplicated operons, we found that a mutation in *fixL* eliminates *fixNOQP*f expression but has only a moderate effect on expression of *fixNOQP*d. This suggests that there are differential regulatory controls. Interestingly, only the *fixNOQP*d operon was essential for symbiotic nitrogen fixation (L. Girard, S. Brom, A. Dávalos, O. Lopez, M. Soberón, and D. Romero, Mol. Plant-Microbe Interact. 13:1283–1292, 2000). Searching for potential candidates responsible for the differential expression, we characterized two *fnrN* homologs (encoding transcriptional activators of the cyclic AMP receptor protein [CRP]-Fnr family) in *R. etli* CFN42. One of these genes (*fnrN*d) is located on the symbiotic plasmid, while the other (*fnrN*chr) is located on the chromosome. Analysis of the expression of the *fnrN* genes using transcriptional fusions with *lacZ* showed that the two *fnrN* genes are differentially regulated, since only *fnrN*d is expressed in microaerobic cultures of the wild-type strain while *fnrN*chr is negatively controlled by FixL. Mutagenesis of the two *fnrN* genes showed that both genes participate, in conjunction with FixL-FixKf, in the microaerobic induction of the *fixNOQP*d operon. Participation of these genes is also seen during the symbiotic process, in which mutations in *fnrN*d and *fnrN*chr, either singly or in combination, lead to reductions in nitrogen fixation. Therefore, *R. etli* employs a regulatory circuit for induction of the *fixNOQP*d operon that involves at least three transcriptional regulators of the CRP-Fnr family. This regulatory circuit may be important for ensuring optimal production of the cbb_3, terminal oxidase during symbiosis.

Bacteria belonging to the family *Rhizobiaceae* may establish specific symbiotic relationships with their legume host plants. The bacteria elicit formation of new organs, the root nodules, in which differentiated bacterial cells (bacteroids) reduce atmospheric nitrogen to ammonia, thus supplying the host plants with combined nitrogen. Since nitrogen fixation is an energy-consuming process, effective symbioses depend on operation of a respiratory chain with a high affinity for O_2, closely coupled to ATP production. This requirement is fulfilled by a special three-subunit terminal oxidase (cytochrome terminal oxidase cbb_3), which was first identified in *Bradyrhizobium japonicum* as the product of the *fixNOQP* operon (19, 24, 25). Functional duplicated genes of the *fixNOQP* operon have been found in the *Rhizobiaceae*. For instance, both *Sinorhizobium meliloti* (28) and *Rhizobium leguminosarum* bv. viciae possess two copies of the *fixNOQP* operon, which are regulated in similar ways; both copies are required for optimal symbiotic nitrogen fixation (30).

In *S. meliloti*, expression of *fixNOQP* is regulated mainly through an O_2-sensing cascade comprised of the *fixL* and *fixJ* gene products; this cascade activates expression of the *fixK* gene, which leads to expression of the *fixNOQP* operon (2, 3, 9, 12, 18). *fixK* encodes a transcriptional activator belonging to the cyclic AMP receptor protein (CRP)-Fnr family (2). Interesting variations of this basic regulatory scheme have been found in other rhizobial strains. For instance, *R. leguminosarum* bv. viciae VF39 lacks conventional homologs of FixJ and FixL and instead has an unusual FixL homolog which combines structural features observed in both the sensor and responsive elements of a two-component regulator system. This FixL homolog is also involved in ex planta *fixNOQP* expression but seems to lack a significant role during symbiosis (30). *R. leguminosarum* bv. viciae VF39 contains FixK (30) and another transcriptional activator of the CRP-Fnr family, FnrN which activates the two *fixNOQP* copies (8, 15, 16, 30). In contrast to FixK, FnrN has a region with a high level of similarity to a domain in the *Escherichia coli* Fnr protein involved in O_2 sensing, suggesting that the FnrN transcriptional activity may be negatively modulated by O_2 (8). Both FixK and FnrN activate *fixNOQP* expression by binding to a DNA sequence located in the promoter region (TTGAT-N_4-ATCAA) called the anaerobox (2, 8, 11). Furthermore, *R. leguminosarum fnrN* is able to complement an *S. meliloti fixK* mutant for *fixNOQP* expression (8, 16), suggesting that the two proteins activate transcription in similar ways (8, 15). Additional variations are found in *R. leguminosarum* bv viciae UPM791, in which two *fnrN* genes and no *fixL*, *fixJ*, or *fixK* homologs are present (15,

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference
Rhizobium etli strains		
CFN42	Wild-type strain	27
CE3	Str^r derivative of CFN42	23
CFNX89	Derivative of CE3 cured of plasmid pCFN42d	4
CFNX636	CE3 derivative, *fixL*::*loxP* Sp	13
CFNX637	CE3 derivative, *fixKf*::ΩKm	13
CFNX642	CFNX636 derivative, *fixL*::*loxP*	This study
IBTOL12	CE3 derivative, *fnrN*d::ΩKm	This study
IBTOL14	CE3 derivative, *fnrN*chr::ΩSp	This study
IBTOL15	IBTOL12 derivative, *fnrN*d::ΩKm *fnrN*chr::ΩSp	This study
IBTOL16	CFNX642 derivative, *fixL*::*loxP fnrNchr*::ΩSp	This study
IBTOL17	CFNX642 derivative, *fixL*::*loxP fnrN*d::ΩKm	This study
IBTOL18	CFNX642 derivative, *fixL*::*loxP fnrN*chr::ΩSp *fnrN*d::ΩKm	This study
Rhizobium leguminosarum bv. viciae VF39	Sm^r	8
Escherichia coli strains		
XL1-Blue	*recA1 endA1 gyrA96 hsdR17 supE44 lac* [F′ *proAB lacI*^{q} *lacZ*Δ*M15* Tn*10* (Tc^r)] *thi*	6
S17-1	F$^-$ *pro-82 thi-1 endA1 hsdR17 supE44 recA13*, chromosomally integrated RP-4-2 (Tc::Mu) (Km::Tn*7*)	31
Plasmids		
pBluescript SK$^+$	Ap^r, sequencing vector	Stratagene
pRK2013	ColE1 *mob*$^+$ Tra$^+$ (RK2) Km^r	10
pMP220	Transcriptional *lacZ* fusion vector, Tc^r	34
pJQ200 SK$^+$	Gm^r *sacB*, suicide vector	26
pOL17	pJQ200 derivative, *fnrN*d::ΩKm	This study
pOL18	pJQ200 derivative, *fnrN*chr::ΩSp	This study
pJMS8	pRK7813::Cre, Tc^r	Martínez-Salazar, unpublished data
pOLfix10	pMP220 derived, *fixN*d-*lacZ* gene fusion	33
pOL15	pMP220 derived, *fnrN*d-*lacZ* gene fusion	This study
pOL16	pMP220 derived, *fnrN*chr-*lacZ* gene fusion	This study

16). Both *fnrN* genes are involved in activation of the *fixNOQP* operon (15, 16). In this strain, expression of both *fnrN* genes is autoregulated, thus ensuring equilibrated expression of *fnrN* in response to microaerobic conditions (7).

In *Rhizobium etli* CFN42, there are also two *fixNOQP* operons; one is located in the symbiotic plasmid (*fixNOQP*d) (33), and the other is located in a cryptic plasmid called pCFN42f (*fixNOQP*f) (13). Only the *fixNOQP*d operon is required for establishment of an effective symbiosis (13). Possible regulators of *fixNOQP* expression are located in plasmid pCFN42f (an *fixL* gene, encoding an unusual homolog of FixL, as well as the *fixK*f gene) and in the symbiotic plasmid (*fixK*d) (13, 33). Mutagenesis of these genes showed that both FixL and FixKf are needed for microaerobic induction of the *fixNOQP*f operon. Differential regulatory requirements were observed for microaerobic expression of the *fixNOQP*d operon; expression of this operon is completely dependent on FixKf but is only moderately affected by a mutation in *fixL*. A mutation in *fixK*d did not affect expression of the *fixNOQP* operons (13). None of the regulatory genes identified so far are indispensable for symbiotic nitrogen fixation (13).

To explain the differential control of *fixNOQP*d by FixL and FixKf, we postulated the existence of an additional transcriptional activator for *fixNOQP*d expression, whose expression should be negatively controlled by FixL (13). Here we describe finding two *R. etli fnrN* duplicated genes. We determined by mutagenesis and analysis of appropriate transcriptional fusions that these genes, together with FixL, are involved in induction of *fixNOQP*d expression and in symbiotic nitrogen fixation. Our work also revealed some of the features inherent in regulation of both *fnrN* genes; these features involve autoregulation and differential responses to the other regulatory genes identified previously.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used are listed in Table 1. *R. etli* and *R. leguminosarum* bv. viciae were cultured at 30°C in peptone yeast extract medium (PY) (23) or in yeast extract succinate medium (32). *E. coli* was grown at 37°C in Luria broth. Plasmids were transferred to *R. etli* strains by biparental mating using *E. coli* S17-1 as the donor strain. Antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1}; gentamicin, 10 μg ml^{-1}; kanamycin, 30 μg ml^{-1}; nalidixic acid, 20 μg ml^{-1}; rifampin, 50 μg ml^{-1}; spectinomycin, 100 μg ml^{-1}; and tetracycline, 5 μg ml^{-1}. When needed, sucrose was added at a concentration of 20% (wt/vol).

Growth conditions and β-galactosidase measurements. *R. etli* strains were cultured on PY plates for 4 days. Cells scraped from these plates were used to inoculate 250-ml Erlenmeyer flasks containing 50 ml of PY, and the cultures were incubated at 30°C for 24 h. Microaerobic cultures of *R. etli* were prepared by diluting the active inoculum to an initial optical density at 540 nm of 0.05 in 50 ml of yeast extract succinate medium in 150-ml serum stopper bottles; the cultures were flushed with a continuous stream (1,200 ml min^{-1}) of a sterile gas mixture (98% N_2, 2% O_2) for 5 min, sealed, and then incubated for 8 h at 30°C with shaking (200 rpm). β-Galactosidase activity was determined by measuring *o*-nitrophenol production as described previously (20); activities were expressed in micromoles of *o*-nitrophenol produced per minute per milligram of protein.

DNA manipulations. Cloning, restriction mapping, transformation, plasmid isolation, random priming, Southern blotting, and hybridization were performed by using standard protocols (20). Both DNA strands were sequenced either by employing appropriate subclones or by primer walking. The initial phases of sequencing were done at an automated DNA sequencing facility at the Institute of Biotechnology, Cuernavaca, Mexico; sequencing needed for gap filling was performed by the dideoxynucleotide chain termination method (29), using Sequenase 2.0 (Amersham Ltd.). Computer-assisted sequence analysis was performed with the Gene Works 2.5.1 program suite from Oxford Molecular Group Inc. Searches for homology with sequences in the GenBank database were done

with the BLAST programs (1, 14) running at the National Center for Biotechnology Information server.

PCR cloning of the *R. leguminosarum* bv. viciae *fnrN* gene. The *fnrN* gene of *R. leguminosarum* bv. viciae VF39 was obtained by PCR amplification. To do this, we designed a 27-mer forward primer complementary to a region located 418 bp upstream from the ATG of *fnrN* (7) (nucleotides 136 to 154; GenBank accession no. X55788), to facilitate additional cloning steps, a 9-bp extension, containing a built-in *Eco*RI restriction site (underlined) at the 5′ end was added, as follows: 5′-GGAATTCCATCGAATGTAGCGGTCACG-3′. The following reverse primer (27 bp) also contained an *Eco*RI site (underlined) and was complementary to a region 380 bp downstream of the stop codon of *fnrN* (nucleotides 1675 to 1692): 5′-GGAATTCCATCAGCATCGGCAAGCAGA-3′. The amplification reaction mixtures (total volume, 50 μl) typically contained each primer at a final concentration of 250 nM, 50 ng of total DNA of *R. leguminosarum* bv. viciae VF39, each deoxynucleoside triphosphate (dNTP) at a concentration of 200 μM, and 2 U of *Taq* polymerase. PCR amplifications were done with a Perkin-Elmer 480 DNA thermal cycler by using the following cycling regimen: a single denaturation step (2 min at 94°C), followed by 20 cycles consisting of 1 min at 60°C (annealing), 2 min at 73°C (extension), and 1 min at 95°C (denaturation), and then a final extension step (3 min at 73°C). The resulting 1,556-bp PCR product was digested with *Eco*RI and cloned into plasmid pBluescript SK$^+$ previously digested with *Eco*RI. This fragment was mapped and was shown to correspond to *R. leguminosarum* bv. viciae *fnrN* on the basis of its sequence (data not shown).

Construction of *lacZ* gene fusions. To generate an *fnrN*chr-*lacZ* transcriptional fusion, plasmid pMP220 (containing a promoterless *lacZ* gene) was digested with *Eco*RI and *Pst*I, and then the *Eco*RI end was filled in with Klenow polymerase and dNTPs as described previously (20). The resulting fragment was ligated to a 683-bp *Eco*RV-*Pst*I fragment (containing the *fnrN*chr promoter), resulting in pOL16. A plasmid harboring an *fnrN*d-*lacZ* fusion (pOL15) was constructed similarly by inserting a 548-bp *Eco*RI-*Sal*I fragment (containing the *fnrN*d promoter) into pMP220 previously digested with *Eco*RI and *Pst*I. Before ligation, fragments were made compatible by filling in both the *Sal*I end in *fnrN*d and the *Pst*I end in pMP220.

Construction of *fnrN*chr and *fnrN*d mutants. To introduce a mutation into *fnrN*chr, a 1.3-kb *Bam*HI-*Eco*RI fragment containing *fnrN*chr was cloned into plasmid pSK Bluescript. In the resulting plasmid, an *fnrN*chr::ΩSp deletion-substitution allele was generated by removing a 141-bp *Pst*I fragment from *fnrN*chr (codons 90 to 132) and then filling in the *Pst*I ends and inserting a 2-kb *Hin*dIII-*Hin*dIII ΩSp^r interposon (previously treated with Klenow polymerase and dNTPs to fill in the restriction sites). A suicide plasmid derivative useful for homogenotization was constructed by excising a *Bam*HI-*Eco*RI fragment containing the *fnrN*chr::ΩSp allele, treating it with Klenow polymerase to fill in the restriction sites, and then ligating it into *Sma*I-digested pJQ200SK$^+$ (26), which resulted in plasmid pOL18. Homogenotization of the *fnrN*chr::ΩSp allele was carried out by mobilizing this construct into *R. etli* CE3; double recombinants were selected on PY containing sucrose and spectinomycin, which generated strain IBTOL14.

An *fnrN*d::ΩKm allele was generated by inserting an *Hin*dIII-*Hin*dIII ΩKm cartridge into the *Sal*I site of *fnrN*d (codon 47); this was accomplished by filling in both fragments with Klenow polymerase, followed by ligation. The fragment containing the *fnrN*d::ΩKm allele was then removed by *Eco*RI digestion, filled in by treatment with Klenow polymerase, and ligated with *Sma*I-restricted pJQ200SK$^+$, which resulted in plasmid pOL17. This construct was mobilized into *R. etli* CE3, and double recombinants were selected in the presence of sucrose and kanamycin, which generated strain IBTOL12.

A derivative carrying the *fnrN*d::ΩKm-*fnrN*chr::ΩSp allelic combination (strain IBTOL15) was constructed by performing a biparental cross, using *E. coli* S17-1/pOL17 as the donor and *R. etli* IBTOL12 (*fnrN*d::ΩKm) as the recipient; double recombinants were selected as Nal^r Km^r Sp^r sucrose-resistant transconjugants.

To construct a derivative carrying an unmarked *fixL* mutant allele, we took advantage of the special characteristics of the previously described *fixL*::*loxP* Sp mutant (13). In this mutant, the spectinomycin resistance determinant is flanked by two synthetic *loxP* sites. In vivo site-specific recombination between the *loxP* sites, catalyzed by the Cre recombinase, leads to high-frequency excision of the spectinomycin resistance determinant (J. Martínez-Salazar, unpublished data), leaving an unmarked 189-bp insertion in *fixL* (*fixL*::*loxP*). To generate such an allele, a broad-host-range plasmid encoding the Cre recombinase (pJMS8) was introduced by conjugation into *R. etli* CFNX636 (*fixL*::*lox* Sp). Transconjugants resulting from the cross displayed high-frequency loss of the spectinomycin resistance determinant. Removal of pJMS8 from these Sp^s derivatives was accomplished by screening for Tc^s segregants, which resulted in strain CFNX642 (*fixL*::*loxP*).

Homogenotization of appropriate *fnrN* mutant alleles was accomplished by transferring the corresponding plasmids into *R. etli* CFNX642 (*fixL*::*loxP*), which resulted in double mutants IBTOL16 (*fixL*::*loxP fnrN*d::ΩKm) and IBTOL17 (*fixL*::*loxP fnrN*chr::ΩSp) and triple mutant IBTOL18 (*fixL*::*loxP fnrN*d::ΩKm *fnrN*chr::ΩSp).

To verify that the desired gene replacements had occurred, DNA blots of all of the derivatives were analyzed by Southern hybridization with the appropriate *fnrN* and cassette probes.

Nitrogen fixation determination. To measure acetylene reduction, *Phaseolus vulgaris* cv. Negro Jamapa seeds were surface sterilized with diluted sodium hypochlorite and germinated on moist sterile filter paper. Three-day-old seedlings were transferred to plastic pots filled with sterile vermiculite and inoculated with 1 ml of the appropriate bacterial strain (grown in PY); plants were grown in a greenhouse under irrigation with a nitrogen-free nutrient solution (33). For nitrogenase determinations, excised root systems were incubated for 40 min at room temperature in sealed glass vials containing acetylene at a final concentration of 10% in the gas phase. Ethylene production was measured with a Varian 3300 gas chromatograph fitted with a Varian 4290 integrator (33).

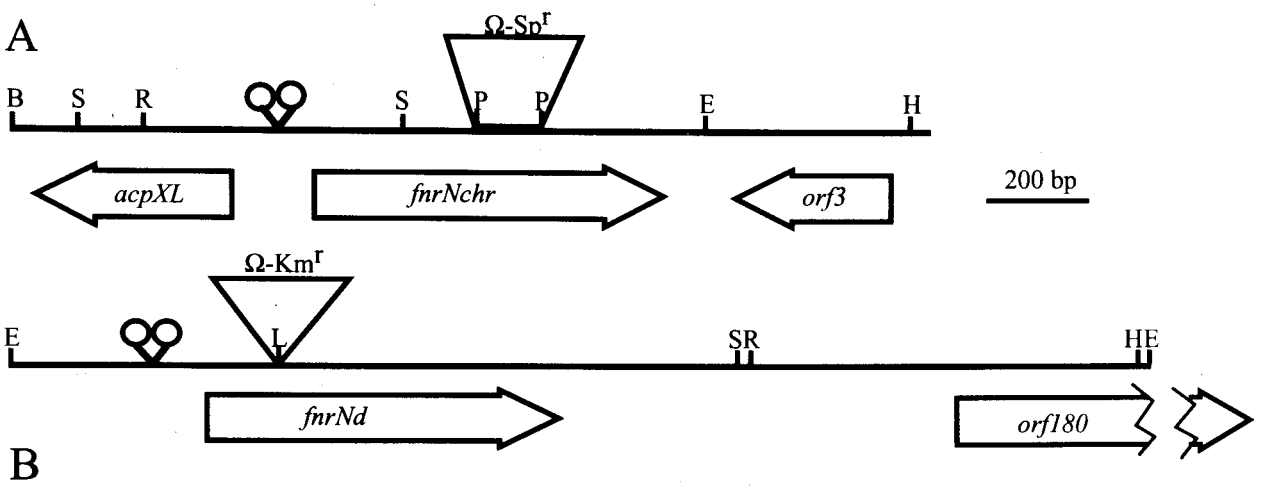
Nucleotide sequence accession numbers. The nucleotide sequences determined in this study for the *fnrN*chr and *fnrN*d loci have been deposited in the GenBank database under accession numbers AF083916 and AF083917, respectively.

RESULTS

Duplication of the *fnrN* genes in *R. etli* CFN42. To ascertain it an Fnr-like protein participates in differential regulation of the *fixN* duplicated genes, we decided to search for *fnrN* homologs in *R. etli*. To do this, blotted plasmid profiles from a CFN42 streptomycin-resistant derivative (CE3) and a strain lacking the symbiotic plasmid (CFNX89) (4) were subjected to high-stringency hybridization by using an *R. leguminosarum fnrN* gene as the probe (see Materials and Methods). Two hybridization sequences, one corresponding to the chromosome and the other corresponding to the symbiotic plasmid, were observed in the wild-type strain, while the strain cured of plasmid pCFN42d hybridized only to chromosomal DNA (data not shown). These data suggested that there are two *fnrN* homologs in *R. etli*, one located in the symbiotic plasmid (pCFN42d) and the other located on the chromosome.

To isolate both *fnrN* homologs, a CFN42 cosmid library (17) was screened by hybridization with an *R. leguminosarum fnrN* probe. Two nonoverlapping cosmids were identified by this procedure. One cosmid carried the chromosomal homolog (*fnrN*chr), and the other harbored the pCFN42d homolog (*fnrN*d), as determined by hybridization. A 1.9-kb *Bam*HI-*Hin*dIII fragment carrying the chromosomal *fnrN* homolog (*fnrN*chr) was cloned, as was a 2.3-kb *Eco*RI fragment carrying *fnrN* from pCFN42d (*fnrN*d). These fragments were completely sequenced. Figure 1A shows physical maps of the two regions sequenced.

In the chromosomal region, three open reading frames (ORFs) were identified. The middle ORF (*fnrN*chr) encoded a polypeptide that was 239 amino acids long and exhibited extensive similarity with FnrN proteins from *R. leguminosarum*. FnrNchr exhibited 83 and 79% identity with *R. leguminosarum* bv. viciae VF39 FnrN and FnrN2, respectively, and 25% identity with Fnr from *E. coli*. Upstream of *fnrN*chr there was another ORF, which encoded a polypeptide that was 92 amino acids long and which was is transcribed in a divergent fashion. This polypeptide was very similar (98% identity) to AcpXL (5) from *R. leguminosarum* (previously designated ORF∗) (8), which is an acyl carrier protein involved in the synthesis of lipid A in *R. leguminosarum*. Downstream of *fnrN*chr and also in a

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FnrNd    ---MDVARSEVLVIGTSVACRSCQARHGVVCGALSAGQLSELGRHSLRRK.VDAGCEIIAQGSENSFYSNITRGVVKLCKVMSDGRQQIVGLQFAPDFVGRPFVRESTLS..AEAATDAE   114
FnrNchr  ---MDVASNEFFEQGTPVPCRSCQARHGVVCGALSNAQLKELNRHSMRRK.IGAGSEIIAQGSESSFYSNIMRGVVKLCKMMPDGRQQIVGLQFAPDFVGRPFVRETTLS..AEAATDAE   114
FnrN     ---MDVAHSGVLEVGIPVACRSCQARHGVVCGVLSSGQLKDLGRHSLRRK.VDAGCEIIAQGSESSFYSNIMSGVVKLCKVMPDGRHEIVGLQFAPDFVGRPFVRESTLS..AEAATDAE   114
FnrN2    ---MDVARSKVSDTGTPVACASCQARHGVVCGALWSGQLRELGRQSLRRT.IDAGSEIIAQGSESSFYSNIMRGVVKLCKVMPDGRHEIVGLQFAPDFVGRPFVPESMLS..AQAATDAE   114
FNR      MIPEKRIIRRIQSGGCAIHCQDCSISQLCIPFTLNEHELDQLDNIIERKKPIQKGQTLFKAGDELKSLYAIRSGTIKSYTITEQGDEQITGFHLAGDLVGFDAIGSGHHPSFAQALETSM   120

FnrNd    ICVFPRNLLERMILETRELQRSLHAQALNELDAAREWMLTLGRRTAEEKVASLLHLITAHAE..TATSTAFDLPL SRAEIADFLGLTIETVSRQLTRL RKEGVIRIENIRHITVPDMDAL 232
FnrNchr  ICVFPRNLLDRMIGETPELQRRLDDQALNELARPRPTDAELGRRTAEEKVASLLHLIATHAEPQTAISTAFDLPL SRAEIADFLGLTIETVSRQMTRL RKWGVILIENSRHIVVPDLDEL 234
FnrN     ICVFPRSLLDRMISETPELQRSLHDQALKELDAAREWIVTLGRRTAEEKVASLLHLIATHAEPQTATSTAFDLPL SRAEIADFLGLTIETVSRQMTRL RKSGVIRIENFRHIIVPDMDEL 234
FnrN2    ICVFPRDLLDRMISETPKLQRTLYDQALKELDAAREWMLTLGRRTAEEKVASLLHLIATHAEPQTATTTSFDLPL SRAEIADFLGLTIETVSRKLTKL RKSGVIRIENFRHIIVPDMDEL 234
FNR      VCEIPFETLDDLSGKMPNLRQQMMRLMSGEIKGDQDMILLLSKKNAEERLAAFIYNLSRRFAQRGFSPREFRLTM TRGDIGNYLGLTVETISRLLGRF QKSGMLAVKG.KYITIENNDAL 239

FnrNd    AKKISG----- 238
FnrNchr  ERISA------ 239
FnrN     ERMISA----- 240
FnrN2    ERMISA----- 240
FNR      AQLAGHTRNVA 250
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FIG. 1. *fnrN* genes and proteins of *R. etli*. (A) Physical and genetic map of the *R. etli fnrN* genes. The positions of the potential Fnr target site sequences are indicated by open circles. The open arrows represent genes discussed in the text. The restriction sites used in subcloning for sequencing are shown, as follows: E, *Eco*RI; S, *Sac*I; L, *Sal*I; H, *Hin*dIII; R, *Eco*RV. (B) Alignment of the predicted amino acid sequences encoded by the *fnrN*chr and *fnrN*d genes from *R. etli* with the amino acid sequences of FnrN and FnrN2 from *R. leguminosarum* bv. viciae (GenBank accession no. AA86478 and AAB58263, respectively) and Fnr from *E. coli* (GenBank accession no. P03019). The four cysteine residues probably involved in oxygen sensing are indicated by boldface type and underlining. The helix-turn-helix motif is enclosed in a box.

divergent orientation, there was a third ORF (*orf3*), which was 106 residues long and exhibited significant similarity (92% identity) with ORF114 of *R. leguminosarum* (8). The overall organization of the *fnrN*chr region is similar to the organization found in *R. leguminosarum* (8).

Nucleotide sequence analysis of the region corresponding to pCFN42d revealed the presence of an ORF (*fnrN*d) that encoded a polypeptide which was 240 amino acids long and was very similar to several members of the CRP-Fnr family. FnrNd exhibited 85 and 81% identity with *R. leguminosarum* bv. viciae VF39 FnrN and FnrN2, respectively, and 27% identity with Fnr from *E. coli*. Downstream of *fnrN*d there was a partial ORF that was 108 residues long and encoded a polypeptide which was similar (96% identity) to the amino-terminal end of ORF180 from *R. etli* CNPAF512 (22). *orf180* is also located on the symbiotic plasmid of *R. etli* CNPAF512 and is cotranscribed with *rpoN*2 (21). Both *fnrN* genes are preceded by two conserved anaerobox sequences (Fig. 1A), suggesting that transcription of these genes is activated in response to low oxygen concentrations.

Figure 1B shows an amino acid alignment for *R. etli* FnrNchr and FnrNd, *R. leguminosarum* FnrN1 and FnrN2, and *E. coli* Fnr. As expected for bona fide members of the Fnr family, the FnrNchr and FnrNd polypeptides contained the redox-sensitive module involved in oxygen sensing, formed by four conserved cysteine residues at positions 17, 20, 28, and 116, as well as the carboxy-terminal helix-turn-helix motif involved in DNA binding (Fig. 1B).

Regulatory genes controlling *fnrN*d and *fnrN*chr expression. Previously, it was shown that the *fixL* mutant was able to moderately induce *fixNOQP*d expression in microaerobic cultures, in contrast to a mutant with a mutation in the *fixK* gene located in plasmid pf (*fixK*f), which exhibited no *fixNOQP*d expression (13). This result was previously explained by arguing that FixL, through a regulatory branch independent of FixKf, repressed another transcriptional activator for *fixNOQP*d (13). To evaluate the participation of different regulatory genes (including *fixL* and *fixK*f) in expression of the *fnrN*chr and *fnrN*d genes, the promoter regions of these genes were fused with a promoterless *lacZ* gene, as described in Materials and Methods. Plasmids pOL15 (*fnrN*d *lacZ*) and pOL16 (*fnrN*chr *lacZ*) were then introduced separately into *R. etli* wild-type and appropriate mutant strains, and β-galactosidase activities were determined in microaerobic cultures as described in Materials and Methods.

Only background levels of microaerobic expression of a *fnrN*chr-*lacZ* fusion were observed in the wild-type strain or in a strain harboring the *fnrN*chr::ΩSp mutation (Fig. 2A). In contrast, high levels of expression of the *fnrN*chr-*lacZ* fusion were observed in an *fnrN*d::ΩKm mutant background or in a

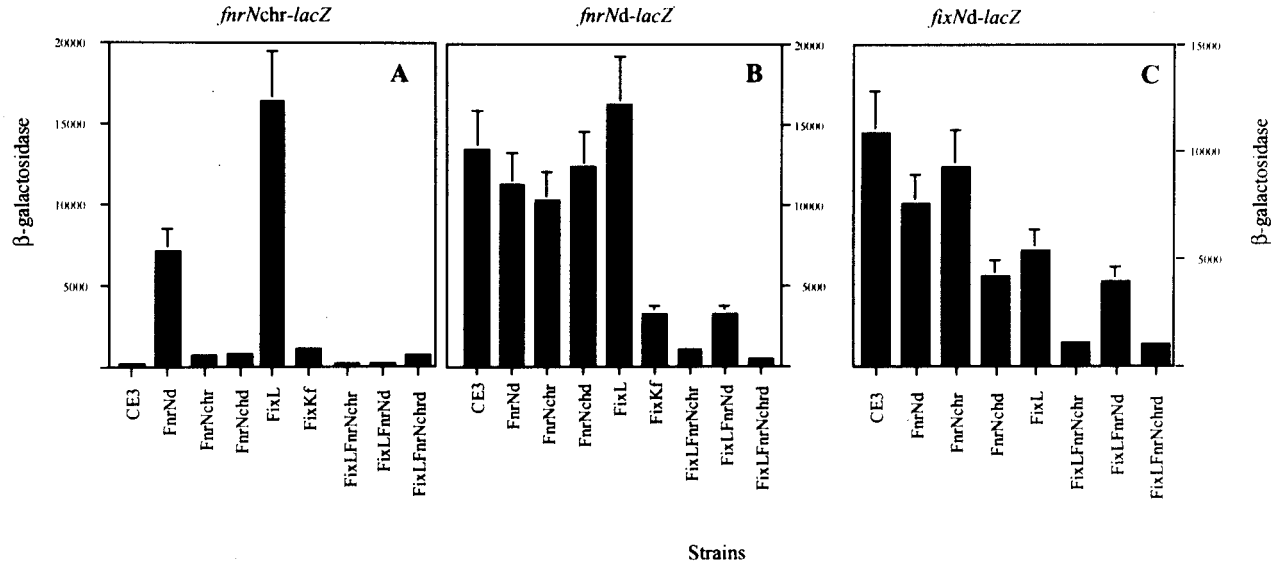

FIG. 2. Expression of *fnrN*chr, *fnrN*d, and *fixNOQP*d genes in different *R. etli* strains. (A) Expression of *fnrN*chr-*lacZ* fusion (pOL16) in different *R. etli* strains cultured microaerobically. (B) Expression of the *fnrN*d-*lacZ* fusion (pOL15) in different *R. etli* strains grown microaerobically. Data are means obtained with three independent cultures; the error bars indicate standard deviations. (C) Expression of the *fixN*d-*lacZ* fusion (pOL10) in different *R. etli* strains cultured microaerobically. β-Galactosidase activities are expressed in micromoles of *o*-nitrophenol produced per minute per milligram of protein. FnrNchrd, double *fnrN* mutant.

strain carrying the *fixL*::*loxP* mutant allele, although twofold-higher levels of induction were observed in the *fixL* mutant than in the *fnrN*d mutant (Fig. 2A). These results show that FnrNd and, more importantly, FixL negatively control expression of *fnrN*chr, confirming our previous prediction about the repressive role of FixL in another transcriptional activator of the *fixNOQPd* operon (13).

Further regulatory roles for both *fnrN*d and *fnrN*chr can be inferred from the behavior of multiple-mutant derivatives. For instance, in the *fixL fnrN*d double mutant (IBTOL17), an *fnrN*chr-*lacZ* fusion was not expressed; this unexpected result suggests that in the absence of FixL, FnrNd could also be involved in positive control of the *fnrN*chr gene (Fig. 2A). Similarly an *fnrN*d *fnrN*chr double mutant (IBTOL15) or an *fixL fnrN*chr double mutant (IBTOL16) exhibited background levels of expression, as observed for the *fnrN*chr-*lacZ* fusion (Fig. 2A). These results suggest the possibility that in the absence of either *fnrN*d or *fixL*, FnrNchr is involved in its own induction. It has been shown previously that in *R. leguminosarum* bv. viciae *fnrN* gene expression is subject to both positive and negative autoregulation (7).

Unlike *fnrN*chr, an *fnrN*d-*lacZ* fusion exhibited high levels of expression in a wild-type background; these high levels were not affected by the presence of single mutations in *fnrN*chr, *fnrN*d, or *fixL* or even by the presence of the *fnrN*d *fnrN*chr double mutation (Fig 2B). The lack of an effect of any of the single mutations on *fnrN*d expression may have been due to redundant positive functions for each gene, because in the *fixL fnrN*d double mutant the levels of expression of *fnrN*d *lacZ* were reduced fourfold, while in the *fixL fnrN*chr double mutant and in the *fixL fnrN*d *fnrN*chr triple mutant the levels of expression of *fnrN*d *lacZ* were reduced 14- and 36-fold, respectively (Fig. 2B). These results show that FixL, FnrNchr, and FnrNd are all involved in positive regulation of *fnrN*d expression under microaerobic conditions.

Expression of *fnrN*chr is controlled by FixL through a regulatory branch independent of FixKf. Previously, we reported that a mutation in *fixL* results in a significant reduction in the level of transcription of *fixK*f (13) Thus, it is conceivable that the loss of a repressive effect on *fnrN*chr expression observed in a *fixL* mutant might be attributable to a loss of *fixK*f, which should act as a negative regulator of *fnrN*chr expression. According to this hypothesis, a mutation in *fixK*f should result in induction of *fnrN*chr at levels as high as those seen in the *fixL* mutant. To ascertain if this was the case, the levels of expression of the *fnrN*chr-*lacZ* transcriptional fusion were determined for an *fixK*f mutant (CFNX637) that was characterized previously (13). Figure 2A shows that contrary to the hypothesis, expression of the *fnrN*chr-*lacZ* fusion in the *fixK*f mutant was not significantly induced compared with the levels observed for the wild-type strain. This result suggests that the negative effect of FixL on *fnrN*chr expression is not exerted through FixKf but operates through a separate regulatory branch.

To find out if the positive regulation of FixL on *fnrN*d was exerted through FixKf, we also determined the levels of expression of the *fnrN*d-*lacZ* transcriptional fusion in an *fixK*f mutant background. The levels of induction of this fusion were fivefold lower in the *fixK*f mutant than in the wild-type strain (Fig. 2B), indicating that the positive regulation of FixL on *fnrN*d is exerted through FixKf. A mutation in *fixK*d had no effect on expression of the two *fnrN* genes (data not shown).

FnrNchr participates in differential regulation of *fixNOQP*d expression. The expression characteristics exhibited by the *fnrN*chr gene, namely, (i) lack of expression in a wild-type background, (ii) negative control of expression by FixL, and

(iii) control through a regulatory branch independent of FixKf, make this gene a good candidate for the hypothetical regulatory gene responsible for differential regulation of the *fixNOQP*d operon (13). To evaluate the role of the *fnrN* genes in expression of the *fixNOQP*d operon, plasmid pOL10, containing an *fixN*d-*lacZ* transcriptional gene fusion (32), was introduced separately into different mutant backgrounds. β-Galactosidase activity was determined in cultures grown under microaerobic conditions as described in Materials and Methods.

As shown in Fig. 2C, a high level of expression of the *fixN*d-*lacZ* fusion was observed in a wild-type background. The levels of expression of this fusion were not significantly reduced by the introduction of single mutations in either *fnrN*d or *fnrN*chr; however, the *fnrN*d *fnrN*chr double mutant exhibited induction levels that were twofold lower than those of the wild-type strain. As reported previously, a mutation in *fixL* reduced the levels of expression of the *fixN*d-*lacZ* fusion twofold (13). The levels of expression of this fusion in a *fixL fnrN*d double mutant background were the same as the levels in the *fixL* background, thus eliminating the possibility that FnrNd is the regulator responsible for differential regulation of the *fixNOQP*d operon. In contrast, the *fixL fnrN*chr double mutant, as well as the *fixL fnrN*chr *fnrN*d triple mutant, exhibited levels of induction of the *fixN*d-*lacZ* fusion that were 10-fold lower than the levels of expression found in the wild-type strain (Fig. 2C). These results suggest that FixL-FixKf and FnrNchr are responsible for induction of *fixNOQP*d under microaerobic free-living culture conditions. However, FnrNd also participates in *fixNOQP*d expression to some extent, as suggested by the reduction in the levels of *fixNOQP*d expression in the double *fnrN* mutant.

Nitrogenase activities of *R. etli* strains with mutations in *fnrN*chr and/or *fnrN*d. To determine the roles of both FnrN proteins in nitrogen fixation, *P. vulgaris* plants were inoculated separately with the wild-type strain or strains harboring mutations in *fnrN*chr, in *fnrN*d, in both *fnrN*chr and *fnrN*d, or in *fnrN*chr, *fnrN*d, anf *fixL*, and nitrogenase activities were determined at different times after inoculation. Figure 3 shows that all of the mutant strains except the *fnrNchr fnrNd fixL* triple mutant were still able to fix nitrogen during symbiosis. However, all mutations had some effect on the temporal activity of nitrogenase. Interestingly, 32 days after inoculation nitrogenase activity was greatly affected in plants inoculated with the *fnrN* mutants (Fig. 3). These data show that a loss of FnrN proteins has a long-term effect on nitrogenase expression in planta. Also, these data show that both FnrN proteins, in conjunction with FixL, participate in maintaining nitrogenase activity during symbiosis.

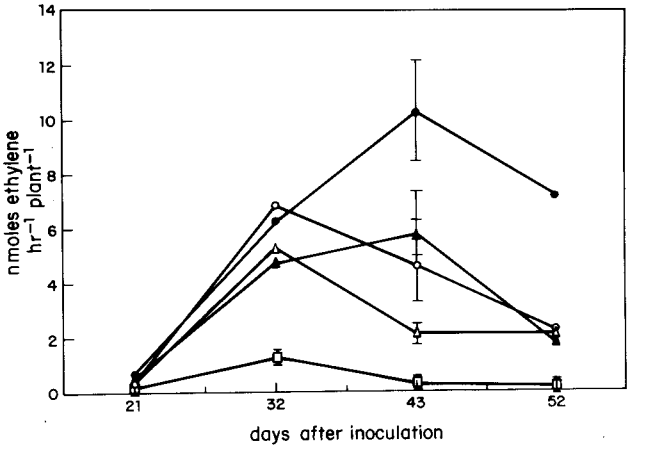

FIG. 3. Time course of acetylene reduction activity in plants inoculated with different *R. etli* strains. Plants were inoculated with CE3 (●), IBTOL14 (*fnrN*chr) (○), IBTOL12 (*fnrN*d) (▲), IBTOL15 (*fnrN*d *fnrN*chr) (△), and IBTOL18 (*fixL fnrN*d *fnrN*chr) (□). The acetylene reduction activities of four plants in each of two pots were determined on different days. The data are means based on the results for two pots (eight plants); the variations were 30% or less. The error bars indicate standard deviations.

DISCUSSION

Production of the symbiotic terminal oxidase by *Rhizobium* strains is a key process for achieving optimal symbiotic nitrogen fixation, since this terminal oxidase has a high affinity for oxygen and is efficiently coupled to the synthesis of ATP. We have shown previously that overexpression of the *fixNOQP* genes, which code for the cbb_3 type of symbiotic terminal oxidase, can enhance symbiotic nitrogen fixation in certain genetic backgrounds (33). Since nitrogen fixation is a microaerobic process, oxygen is a key enviromental signal determining expression of *fixNOQP*. Our previous studies of microaerobic control of the two *fixNOQP* operons (*fixNOQP*d and *fixNOQP*f) in *R. etli* CFN42 showed that both set of genes are controlled by an *fixL-fixK*f cascade without participation of an *fixJ* gene (13). Interestingly, expression of the operon most important for symbiotic nitrogen fixation, *fixNOQP*d, is completely dependent on FixKf but can proceed at adequate levels in the absence of FixL. This unexpected result was explained previously by proposing the existence of an additional transcriptional activator for *fixNOQP*d expression, whose expression should be under negative control by FixL in a regulatory branch independent of FixKf (13).

In this paper, we describe an analysis of expression of two *fnrN* genes (*fnrN*d and *fnrN*chr) in *R. etli* CFN42. In this study we also explored the role of these *fnrN* genes in controlling expression of the *fixNOQP*d operon. Figure 4 shows our current view of the circuit used for regulation of the *fixNOQP* genes under microaerobic conditions. In this model X represents the functional homolog of FixJ that has not been identified yet. Our results show that FnrNchr is a possible additional transcriptional activator for *fixNOQP*d because (i) it is not expressed in a wild-type background, (ii) it is negatively controlled by FixL through a regulatory branch independent of FixKf, and (iii) it is required for microaerobic expression of *fixNOQP*d in an *fixL* mutant background. Moreover, *fnrN*d also plays a role, albeit a minor one, in microaerobic expression of the *fixNOQP*d operon, as suggested by the behavior of an *fnrN*d *fnrN*chr double mutant (Fig. 2C). The effect of the mutations on *fnrN* genes, *fixK*f, and *fixL* could not be attributed to polar effects on downstream genes since in the case of the *fnrN* genes and *fixK*f (13) there are no downstream genes that could be cotranscribed, while in the case of *fixL* we have previously shown that *fixK*f is transcribed independently (13) Thus, microaerobic expression of the *fixNOQP*d operon in *R. etli* is subject to the direct regulatory input of three different activa-

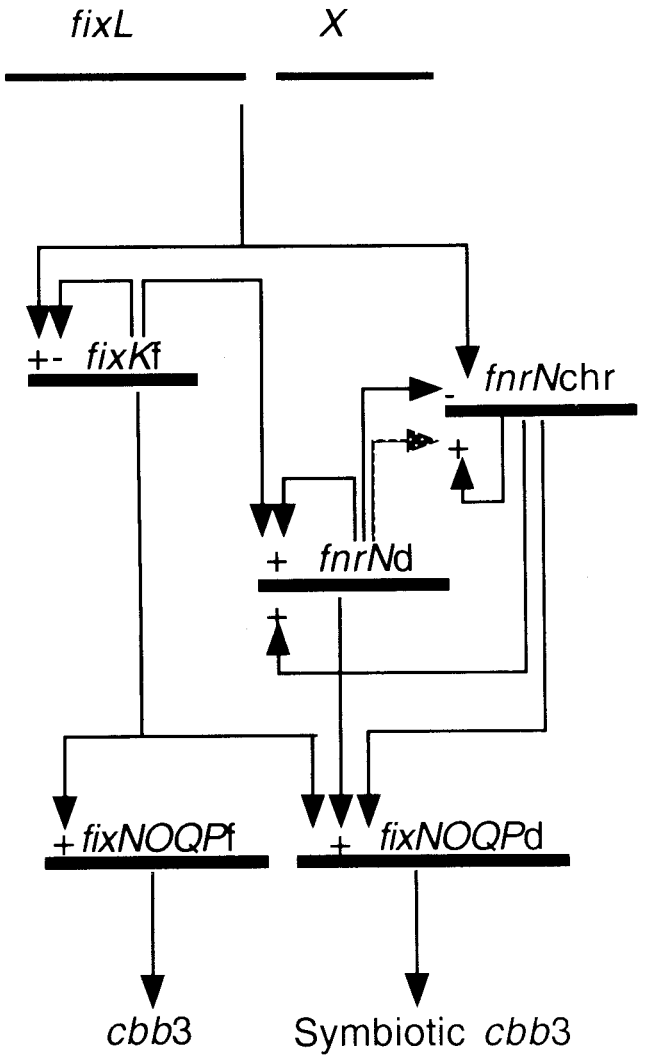

FIG. 4. Regulatory circuit for *fixNOQP* gene expression in *R. etli* CE3 under microaerobic conditions. The dotted arrow indicates conditional control in an *fixL* background. Positive regulation and negative regulation are indicated by plus and minus signs, respectively.

tors: FixKf, FnrNchr, and FnrNd (Fig. 4). However, although the levels of expression of the *fixNd-lacZ* fusion in the triple mutant were 11-fold lower than the levels of expression in the wild-type strain, supporting hypothesis that these regulators have an important role, they were 5-fold higher than the background levels. Therefore, we cannot exclude the possibility that additional signal transduction pathways participate in induction of microaerobic *fixNOQP* expression. In this regard, an anaerobic two-component signal transduction pathway (RegAB) regulates the expression of genes encoding a cbb_3 terminal oxidase in *Rhodobacter capsulatus* (36). However, our data indicates that the transcriptional regulators FixL, FnrNchr, and FnrNd are essential for efficient nitrogen fixation (Fig. 3). In particular, FnrN proteins have a major role in the late stage of symbiosis since single mutations in the *fnrN* genes have a severe effect on nitrogenase activity 32 days after plant inoculation (Fig. 3).

Our data shows that both FnrN genes are involved in positive and negative control of gene expression. Although dual control by a regulatory protein that acts on the same target gene (as exhibited by FnrNd acting on the *fnrN*chr gene) is rare, it is not without precedent. Transcriptional factors of the MerR family can act both positively and negatively when they are bound at a single site (35). A more similar example occurs in *R. leguminosarum* UPM791, in which both positive autoregulation and negative autoregulation have been observed for the *fnrN* genes (7).

The model for expression of *fixNOQP*d in *R. etli* (Fig. 4) combines elements from several systems. For instance, in *R. leguminosarum* UPM791, microaerobic expression of *fixNOQP* is achieved with participation of duplicated *fnrN* genes and without participation of FixL, FixJ, or FixK (7, 15). In *R. leguminosarum* bv. viciae VF39, control is achieved through an unusual FixL protein and FnrN without participation of a conventional FixJ or FixK protein (32). Finally, *B. japonicum* (22, 24, 25) and *S. meliloti* (2, 9, 18) are similar in the sense that they control *fixNOQP* expression mainly through a system in which FixL, FixJ, and FixK are used. Regulation of the *fixNOQP*d operon in *R. etli* is striking because of the number of putative interactions among regulatory proteins, including control by an unusual FixL protein, FixKf, FnrNchr, and FnrNd without participation of a conventional FixJ protein. Furthermore, this model also includes regulatory interactions between the two *fnrN* genes, as well as control of *fnrN*chr by FixL. We believe that this regulatory system should allow exquisite tuning of *fixNOQP* expression to cope with the demands imposed by the nitrogen fixation process. Indications that this is the case came from the nitrogenase activities of plants inoculated with the *fnrN* mutants. Although a complete loss of nitrogenase activity was observed only with the *fixL fnrN*chr *fnrN*d triple mutant, analysis of the temporal activity of nitrogenase revealed that the two *fnrN* genes are more important for supporting nitrogen fixation at late stages of the symbiosis (Fig. 3).

Current efforts in our group are devoted to determining which protein acts, together with FixL, to activate expression of *fixK*f and to repress expresssion of *fnrN*chr (X in Fig. 4). Also, it is important to understand the mechanisms by which the two *fnrN* genes and the two *fixNOQP* operons are differentially regulated. As pointed out previously, two anaerobox sequences were found in front of both *fnrN* genes. However, in the promoter regions of *R. etli* genes (*fnrN*chr, *fnrN*d, *fixNOQP*f, *fixNOQP*d) that are under the control of either FixKf or FnrN or both, the anaerobox sequences are identical to the consensus anaerobox sequence. Therefore, we still have to determine the affinities of binding of the different FixK and FnrN proteins to the different anaerobox sequences. Such efforts should provide a better understanding of the molecular events involved in the differential expression of these genes.

ACKNOWLEDGMENTS

This work was supported in part by the European Communities through International Scientific Co-operation Program contract CI1*-CT94-0042, by DGAPA contracts IN204697 and IN202599, and by CONACyT contracts 31561-N and 0028.

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