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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Previously, we reported finding duplicated *fixNOQP* operons in *Rhizobium etli* CFN42. One of these duplicated operons is located in the symbiotic plasmid (*fixNOQPd*), while the other is located in a cryptic plasmid (*fixNOQPh*). Although a novel FixL-FixKf regulatory cascade participates in microaerobic expression of both *fixNOQP* duplicated operons, we found that a mutation in *fixL* eliminates *fixNOQPh* expression but has only a moderate effect on expression of *fixNOQPd*. This suggests that there are differential regulatory controls. Interestingly, only the *fixNOQPh* 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*) is located in the symbiotic plasmid, while the other (*fnrNchr*) 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* is expressed in microaerobic cultures of the wild-type strain while *fnrNchr* 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 *fixNOQPd* operon. Participation of these genes is also seen during the symbiotic process, in which mutations in *fnrN* and *fnrNchr* either singly or in combination, lead to reductions in nitrogen fixation. Therefore, *R. etli* employs a regulatory circuit for induction of the *fixNOQPd* 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* 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 O₂, closely coupled to ATP production. This requirement is fulfilled by a special respiratory chain (13, 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 O₂-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 O₂ sensing, suggesting that the FnrN transcriptional activity may be negatively modulated by O₂ (8). Both FixK and FnrN activate *fixNOQP* expression by binding to a DNA sequence located in the promoter region (TTGAT-3'-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,
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 (fixNOQPd) (33), and the other is located in a cryptic plasmid called pCFN42f (fixNOQpf) (13). Only the fixNOQPd operon is required for establishment of an effective symbiosis (13). Possible regulators of fixNOQP expression are located in symbiotic plasmid pCFN42f (an fixL gene, encoding an unusual homolog of FixL, as well as the fixKf gene) and in the symbiotic plasmid (fixKd) (13, 33). Mutagenesis of these genes showed that both FixL and FixKf are needed for microaerobic induction of the fixNOQP operon. Differential regulatory requirements were observed for microaerobic expression of the fixNOQPd operon; expression of this operon is completely dependent on FixKf but is only moderately affected by a mutation in fixL. A mutation in fixKd 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 fixNOQPd by FixL and FixKf, we postulated the existence of an additional transcriptional activator for fixNOQPd 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 fixNOQPd 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% N₂, 2% O₂) 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 Inc.). 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

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### Table 1. Bacterial strains and plasmids

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<th>Strain or plasmid</th>
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<tr>
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References:

[13] This study
with the BLAST programs (1, 14) running at the National Center for Biotechnology Information. Sequence information.

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. X57588), to facilitate additional cloning steps, a 9-bp extension, containing a built-in EcoRI restriction site (underlined) at the 5’ end was added, as follows: 5’-GGA[TCT]CATGAA[GTAGT]GCG[CAGC[AGC[AGA]-3’. The following reverse primer (27 bp) also contained an EcoRI site (underlined) and was complementary to a region 380 bp downstream of the stop codon of fnrN (nucleotides 1675 to 1692): 5’-GGAATTC[CATCAGCTCGCA[ACGAGCA]-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 μl 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 1,536-bp PCR product was digested with EcoRI and cloned into pBluescript SK+ previously digested with EcoRI. 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::lacZ transcriptional fusion, plasmid pMP220 (containing a promoterless lacZ gene) was digested with EcoRI and PstI, and then the EcoRI end was filled in with Klenow polymerase and dNTPs as described previously (20). The resulting fragment was ligated to a 683-bp EcoRV-PstI fragment (containing the fnrN promoter), resulting in pOL16. A plasmid harboring an fnrN::lacZ fusion (pOL15) was constructed similarly by inserting a 548-bp EcoRI-SalI fragment (containing the fnrN promoter) into pMP220 previously digested with EcoRI and PstI. Before ligation, fragments were made compatible by filling in both the SalI end in fnrN and the PstI end in pMP220.

Construction of fnrN chr and fnrN mutants. To introduce a mutation into fnrNchr, a 1.3-kb BamHI-EcoRI fragment containing fnrNchr was cloned into plasmid pSK Bluescript. In the resulting plasmid, an fnrNchr::SpI deletion-substitution allele was generated by removing a 141-bp PstI fragment from fnrNchr (coding 90 to 132) and then filling in the PstI ends inserting a 2-kb HindIII-HindIII ISpI interposon (previously treated with Klenow polymerase and dNTPs to fill in the restriction sites). A suicide plasmid derivative useful for homogenization was constructed by excising a BamHI-EcoRI fragment containing the fnrNchr::SpI allele, treating it with Klenow polymerase to fill in the restriction sites, and then ligating it into Smal-digested pQ200SK+ (26), which resulted in plasmid POL18. Homogenization of the fnrNchr::SpI 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::SmI allele was generated by inserting an HindIII-HindIII ISpI cartridge into the SalI site of fnrN (coding 47); this was accomplished by filling in both fragments with Klenow polymerase, followed by ligation. The fragment containing the fnrN::SmI allele was then removed by EcoRI digestion, filled in by treatment with both fragments, and ligated with Smal-restricted pQ200SK+, 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::SpI Km-fnrNchr::SpI alleleic combination (strain IBTOL15) was constructed by performing a biparental cross, using E. coli S17-1/POL17 as the donor and R. etli IBTOL12 (fnrN::SmI) as the recipient; double recombinants were selected as NaI KM SP sucrose-resistant transconjugants.

To construct a derivative carrying an unmarked fnrN mutant allele, we took advantage of the special characteristics of the previously described fnrN::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 fnrN (fnrN::loxP). To generate such an allele, a broad-host-range plasmid encoding the Cre recombinase (pJMS8) was introduced by conjugation into R. etli CFNX636 (fnrN::loxP Sp). Transconjugants resulting from the cross displayed high-frequency loss of the spectinomycin resistance determinant. Removal of pJMS8 from these Sp derivatives was accomplished by screening for TeC segregants, which resulted in strain CFNX642 (fnrN::loxP). Homogenization of appropriate fnrN mutant alleles was accomplished by transferring the R. etli containing plasmids into R. etli CFNX642 (fnrN::loxP), which resulted in double mutants IBTOL16 (fxl::loxP fnrN::SmI Km) and IBTOL17 (fxl::loxP fnrNchr::SpI) and triple mutant IBTOL18 (fxl::loxP fnrN::SpI Km fnrNchr::SpI).

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 cassettes 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 fnrNchr and fnrN 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 fnxN 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 (CFNX39) (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 (fnrNchr), and the other harbored the PCFN42d homolog (fnrNd), as determined by hybridization. A 1.9-kb BamHI-HindIII fragment carrying the chromosomal fnrN homolog (fnrNchr) was cloned, as was a 2.3-kb EcoRI fragment carrying fnrN from pCFN42d (fnrNd). 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 (fnrNchr) 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 fnrNchr there was another ORF, which encoded a polypeptide that was 92 amino acids long and which was 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 fnrNchr and also in a
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 (fnrNd) 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 chrd 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 rpoN2 (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 fnrNd and fnrNchr expression. Previously, it was shown that the fiXL mutant was able to moderately induce fnxNOQPd expression in microaerobic cultures, in contrast to a mutant with a mutation in the fiXK gene located in plasmid pf (fiXKf), which exhibited no fnxNOQPd expression (13). This result was previously explained by arguing that FixL, through a regulatory branch independent of FixKf, repressed another transcriptional activator for fnxNOQPd (13). To evaluate the participation of different regulatory genes (including fiXL and fiXKf) in expression of the fnrNchr and fnrNd genes, the promoter regions of these genes were fused with a promoterless lacZ gene, as described in Materials and Methods. Plasmids pOL15 (fnrNd lacZ) and pOL16 (fnrNchr 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 fnrNchr-lacZ fusion were observed in the wild-type strain or in a strain harboring the fnrNchr::ISp mutation (Fig. 2A). In contrast, high levels of expression of the fnrNd-lacZ fusion were observed in an fnrNd::I Km mutant background or in a
strain carrying the \( fixL::loxP \) mutant allele, although twofold-higher levels of induction were observed in the \( fixL \) mutant than in the \( fnrN \) mutant (Fig. 2A). These results show that FnrNd and, more importantly, FixL negatively control expression of \( fnrNchr \), confirming our previous prediction about the repressive role of FixL in another transcriptional activator of the \( fnrNchr \) operon (13).

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

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

**Expression of \( fnrNchr \) 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 \( fixKf \) (13) Thus, it is conceivable that the loss of a repressive effect on \( fnrNchr \) expression observed in a \( fixL \) mutant might be attributable to a loss of \( fixKf \), which should act as a negative regulator of \( fnrNchr \) expression. According to this hypothesis, a mutation in \( fixKf \) should result in induction of \( fnrNchr \) at levels as high as those seen in the \( fixL \) mutant. To ascertain if this was the case, the levels of expression of the \( fnrNchr-lacZ \) transcriptional fusion were determined for an \( fixKf \) mutant (CFNX637) that was characterized previously (13). Figure 2A shows that contrary to the hypothesis, expression of the \( fnrNchr-lacZ \) fusion in the \( fixKf \) 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 \( fnrNchr \) expression is not exerted through FixKf but operates through a separate regulatory branch.

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

**FnrNchr participates in differential regulation of \( fnrNchr \) expression.** The expression characteristics exhibited by the \( fnrNchr \) 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 fixNOQPd operon (13). To evaluate the role of the fnrn genes in expression of the fixNOQPd operon, plasmid pOL10, containing an fixNd-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 fixNd-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 or fnrnchr; however, the fnrnNd fnrnchr 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 fixNd-lacZ fusion twofold (13). The levels of expression of this fusion in a fixL fnrnNd 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 fixNOQPd operon.

In contrast, the fixL fnrnNchr double mutant, as well as the fixL fnrnNchr fnrnNd triple mutant, exhibited levels of induction of the fixNd-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 fixNOQPd under microaerobic free-living culture conditions. However, FnrNd also participates in fixNOQPd expression to some extent, as suggested by the reduction in the levels of fixNOQPd expression in the double fnrn mutant.

Nitrogenase activities of R. etli strains with mutations in fnrnchr and/or fnrnNd. 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 fnrnchr, in fnrnNd, in both fnrnchr and fnrnNd, or in fnrnchr, fnrnNd, afxL, and nitrogenase activities were determined at different times after inoculation. Figure 3 shows that all of the mutant strains except the fnrnchr fnrnNd 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 plants. Also, these data show that both FnrN proteins, in conjunction with FixL, participate in maintaining nitrogenase activity during symbiosis.

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 cbb3 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 environmental signal deter-
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 fnrNchr 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 fixNOQPd 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. vicie 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 fixNOQPd 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 fnrNchr 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, fnrNchr fnrNd 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 fixKf and to repress expression of fnrNchr (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 (fnrNchr, fnrNd, fixNOQPf, fixNOQPd) 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.

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