Novel Reiterated Fnr-Type Proteins Control the Production of the Symbiotic Terminal Oxidase *cbb₃* in *Rhizobium etli* CFN42

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Symbiotic nitrogen-fixing bacteria express a terminal oxidase with a high oxygen affinity, the *cbb₃*-type oxidase encoded by the *fixNOQP* operon. Previously, we have shown that, in *Rhizobium etli* CFN42, the repeated *fixNOQP* operons (*fixNOQPd* and *fixNOQPF*) have a differential role in nitrogen fixation. Only the *fixNOQPd* operon is required for the establishment of an effective symbiosis; microaerobic induction of this operon is under the control of at least three transcriptional regulators, FixKf, FnrNd, and FnrNchr, belonging to the Crp/Fnr family. In this work, we describe two novel Crp/Fnr-type transcriptional regulators (StoRd and StoRf, symbiotic terminal oxidase regulators) that play differential roles in the control of key genes for nitrogen fixation. Mutations either in *stoRd* or *stoRf* enhance the microaerobic expression of both *fixNOQP* reiterations, increasing also the synthesis of the *cbb₃*-type oxidase in nodules. Despite their structural similarity, a differential role of these genes was also revealed, since a mutation in *stoRd* but not in *stoRf* enhanced both the expression of *fixKf* and the nitrogen-fixing capacity of *R. etli* CFN42.

The establishment of an effective symbiotic association between rhizobia and legumes requires the expression of several bacterial and plant genes. In nitrogen-fixing bacteria, oxygen concentration regulates expression of genes related to microaerobic and symbiotic life styles. Although symbiotic nitrogen-fixing bacteria are aerobic organisms, they are exposed to an oxygen-limited environment inside the nodules, which allows nitrogenase activity to be preserved.

The high-energy demand for nitrogen fixation is fulfilled through the expression of a terminal oxidase with a high oxygen affinity, the *cbb₃*-type oxidase, encoded by the *fixNOQP* operon (Mandon et al. 1994; Preisig et al. 1993). This oxidase lacks a copper-containing subunit II (Cu_A) and has a novel subunit composition and content of prosthetic groups. Subunit I, FixN, is a heme *b* and copper-containing subunit, FixO and FixP are *c*-type cytochromes containing single- and dihemes, respectively, and FixQ is a hydrophobic protein whose role is unknown (Preisig et al. 1993, 1996a; Zufferey et al. 1996, 1998).

In all symbiotic nitrogen-fixing bacteria analyzed, a *fixGHIS* operon is located immediately downstream of the *fixNOQP* operon. Based on sequence similarities and structural and ge-

netic analysis, it has been proposed that the proteins encoded by the *fixGHIS* operon participate in the uptake, metabolism, and assembly of the copper required for *cbb₃*-oxidase synthesis (Kahn et al. 1989; Preisig et al. 1996b).

The expression of both *fixN* and *fixG* operons is coregulated and induced microaerobically by a regulatory protein belonging to the Crp/Fnr family (Clark et al. 2001; Fischer 1994; Girard et al. 2000; Gutiérrez et al. 1997; López et al. 2001; Moris et al. 2004). Fnr is a protein that contains an O₂-sensitive [4Fe-4S]²⁺ cluster that directly senses oxygen. In *Escherichia coli*, it functions as a global regulator that controls the expression of up to 100 genes in response to oxygen limitation, and its active DNA-binding form is a homodimer containing two [4Fe-4S]²⁺ clusters (Kang et al. 2005; Kiley and Beinert 2003). The protein binds by a helix-turn-helix (HTH) motif to the promoter region of target genes, repressing the expression of genes for aerobic respiration and inducing genes involved in alternative forms of respiration.

The consensus DNA binding site for *E. coli* Fnr is a symmetrical motif (TTGAT-N4-ATCAA) called the FNR box or anaerobox (Kiley and Beinert 1999, 2003; Körner et al. 2003). Fnr homologs have been identified in a wide number of bacteria. Some of these proteins conserve the N-terminal cysteine-rich motif, which binds the Fe-S cluster, while others, like the members of the FixK group, lack it. Based on genome analysis, the number of members of the Crp-Fnr family now includes 369 members distributed in 14 phylogenetic branches (Körner et al. 2003). The FnrN group of regulators includes putative proteins with a slight modification of the conserved cysteine motif of Fnr, allowing the assembly of a [4Fe-4S] cluster and redox sensing. On the other hand, regulators belonging to the FixK branch are structurally homologous to FnrN but lack the N-terminal cysteine-rich motif. A characteristic frequently found in sequenced bacterial genomes is the existence of multiple members of the Crp-Fnr regulators in the same host (Körner et al. 2003).

Rhizobium etli CFN42 fixes nitrogen in association with bean plants, and its genome contains a chromosome and six large plasmids (pCFN42a to pCFN42f) whose sizes range from 184.4 to 642.5 kb (González et al. 2006). Plasmid d corresponds to the symbiotic one (pSym) and contains several genes involved in nodulation and nitrogen-fixation processes. However, some important regulatory elements present on the pSym in other *Rhizobium* species, such as *fixL* and *fixJ*, are not encoded on this replicon in *R. etli* CFN42. The *fixL* gene,

which controls the expression of *fixKf*, is located on pCFN42f, in a region containing copies of *fixK*, *fixNOQP*, and *fixGHIS* genes. Additionally, both *fixNOQP* iterations (*fixNOQPd* and *fixNOQPF*) are controlled by a novel *fixL*-*fixKf* cascade, for which the transcriptional activator with functional homology to FixJ remains to be described (Girard et al. 2000). A similar situation has been described in *Rhizobium leguminosarum* bv. *viciae* VF39 (Patschkowski et al. 1996).

Expression analysis of the reiterated *fixNOQP* operons in CFN42 showed that both *fixNOQPF* and *fixNOQPd* require a FixK transcriptional regulator (FixKf) but exhibit a differential dependence for FixL. Expression of *fixNOQPF* is fully dependent on FixL, while expression of the *fixNOQPd* operon (encoding the functional symbiotic terminal oxidase) can proceed at adequate levels in the absence of FixL, indicating additional regulatory controls for *fixNOQPd* (Girard et al. 2000). The functional characterization of the two *R. etli* *fmrN* homologs, *fmrNd* and *fmrNchr* (located on pSym and chromosome, respectively) indicated that both proteins participate, together with FixL and FixKf, in the transcriptional control of the *fixNOQPd* operon. The action of the *fmrNchr* regulatory protein is only detected in the absence of FixL, suggesting that FixL negatively regulates (either directly or indirectly) the expression of this protein (López et al. 2001).

In this work, we demonstrate that both *fixNOQP* reiterations as well as the *fixKf* gene are under additional regulatory controls by two novel Crp/Fnr-type transcriptional regulators, herein named StoRd and StoRf. Inactivation of either of these genes enhances the microaerobic expression of both *fixNOQP* iterations, increasing also the synthesis of the *cbb₃*-type oxidase in nodules. Despite their structural similarity, a differential role of these genes was also revealed, since a mutation in *stoRd* but not in *stoRf* enhanced both the expression of *fixKf* and the nitrogen-fixing capacity of *R. etli* CFN42.

RESULTS

Structural analysis of the *fixGHIS* reiterated regions in *R. etli*.

Analysis of the structural arrangement of the reiterated *fixGHIS* operons in *R. etli* revealed the presence of additional open reading frames (ORF) located downstream of each operon. *R. etli* ORF RHE_PD00288 (NP_659754) and RHE_PF00499 (YP_473115). These ORF were annotated as possible Fnr-like transcriptional regulators, based on their significant similarity to known CRP/Fnr members (González et al. 2003, 2006), as well as with novel Fnr-related proteins from *Rhizobium leguminosarum* bv. *viciae* 3841 (YP_765307, YP_770490, and YP_769304), *Agrobacterium tumefaciens* C58 (NP_355346 and NP_533066), *Sinorhizobium meliloti* 1021 (NP_435903),

and *Paracoccus denitrificans* (AAA69977). The polypeptides encoded by RHE_PD00288 and RHE_PF00499 display identifiable N-terminal domains, with a high similarity to domains that bind cyclic nucleotides, such as the catabolite gene activator protein as well as a C-terminal domain that contains an HTH DNA binding domain (Kiley and Beinert 1999).

Sequence analysis of the two reiterated *fixGHIS* regions shows that the intergenic region between the *fixSd* and *RHE_PD00288* and *fixSf* and *RHE_PF00499* coding regions comprises only 31 bp. This proximity suggests that these additional ORF may be part of the *fixGHIS* operons. This genome arrangement was not previously reported. A search carried out in sequenced genomes from the order Rhizobiales shows that this structural organization of *R. etli* *fixGHIS* operons is shared with *Sinorhizobium meliloti* 1021, in which ORF Sma1207 is located 56 bp downstream of *fixS1*. This ORF was annotated as a FixK-like regulatory protein, also a member of the CRP/Fnr family (Capela et al. 2001; Galibert et al. 2001). A similar arrangement was also detected in *R. leguminosarum* bv. *viciae* 3841, in which both copies of the *fixGHIS* operon have an additional ORF downstream, belonging to the CRP/Fnr family (Young et al. 2006).

To clarify if ORF *RHE_PD00288* and *RHE_PF00499* are part of the *fixGHIS* operons, two different experimental strategies were followed. Presence of functional promoters in this region was ascertained by generating β -glucuronidase (GUS) transcriptional fusions with several overlapping fragments from the putative *fixGHISd*-*RHE_PD00288* operon, as described below (Fig. 1A). The resulting plasmids (pMGB3, pMGB4, pMGB5, and pMGB6) were mobilized into *R. etli* CE3 wild-type strain by conjugation. GUS-specific activities were determined for each fusion in microaerobic cultures. As shown in Figure 1B, a high GUS-specific activity was obtained under microaerobic conditions only with the strain harboring plasmid pMGB3 (containing a sector preceding *fixGd*). In contrast, the other sectors display a very low expression under microaerobic conditions, indicating the absence of functional promoters. The presence of a single functional promoter for the whole region is consistent with the proposed operon organization for *R. etli* *fixGHISd*-*RHE_PD00288*.

Further proof of this operon arrangement was obtained by evaluating the expression of both *RHE_PD00288* and *RHE_PF00499* by reverse transcription-polymerase chain reaction (RT-PCR). Insertional mutations within each *fixG* coding region abolished the expression of *RHE_PD00288* or *RHE_PF00499* (data not shown). The absence of a cDNA product corresponding to *RHE_PD00288* in a *fixGd::loxSp* mutant and of *RHE_PF00499* in the *fixGf:: Ω Km* mutant indicates that a mutation in either *fixG* exerts a polar effect on the expression on its corresponding downstream ORF. These results demonstrate that, in *R. etli*, both *fixGHISd*-*RHE_PD00288* and *fixGHISf*-*RHE_PF00499* constitute operons.

Expression pattern of *fixGd* and *fixGf* reiterated regions.

To evaluate the participation of different regulatory proteins in the control of the *fixG* reiterated operons in *R. etli*, upstream regions of each copy were fused to the *uidA* reporter gene, generating pMGB3 (*fixGd::uidA*) and pMGB7 (*fixGf::uidA*). The microaerobic expression of these fusions was analyzed in the wild-type strain and in different mutant backgrounds, such as *fixL*, *fixKf*, and *stoRd* (strains CFNX636, CFNX637, and CFNX713, respectively).

Table 1 shows that microaerobic expression of *fixGf* (lines 1 and 4) and *fixGd* (lines 5 and 8) remains at the same level in both wild type and CFNX713. This expression was drastically diminished in the *fixKf:: Ω Km* mutant background (Table 1, lines 2 and 6), indicating that the FixKf protein is needed as a

Table 1. Microaerobic expression of *fixGf* and *fixGd* genes in different *Rhizobium etli* regulatory mutants

Strain	Strain genotype	Fusion genotype	Specific Activity ($\pm$ SD) ^a
CE3/pMGB7	Wild-type	<i>fixGf::uidA</i>	202 $\pm$ 20
CFNX637/pMGB7	<i>fixKf::ΩKm</i>	<i>fixGf::uidA</i>	16 $\pm$ 7
CFNX636/pMGB7	<i>fixL::loxSp</i>	<i>fixGf::uidA</i>	10 $\pm$ 11
CFNX713/pMGB7	<i>RHE_PD00288::loxSp</i>	<i>fixGf::uidA</i>	189 $\pm$ 3
CE3/pMGB3	Wild-type	<i>fixGd::uidA</i>	180 $\pm$ 26
CFNX637/pMGB3	<i>fixKf::ΩKm</i>	<i>fixGd::uidA</i>	15 $\pm$ 7
CFNX636/pMGB3	<i>fixL::loxSp</i>	<i>fixGd::uidA</i>	114 $\pm$ 8
CFNX713/pMGB3	<i>RHE_PD00288::loxSp</i>	<i>fixGd::uidA</i>	199 $\pm$ 12

^a Mean β -glucuronidase (GUS)-specific activity. Values are expressed as nanomoles per minute per milligram of protein. Data are the mean of two replicates from two independent experiments.

positive transcriptional regulator for expression of both *fixG* reiterations. Notably, expression of the *fixGf* fusion is fully dependent on the presence of an active *fixL* gene (Table 1, line 3) while the *fixGd* fusion is still expressed in a *fixL::* Ω Sp mutant strain (Table 1, line 7). Thus, expression of the *fixGd* and *fixGf* operons displays a differential dependence for FixL, similar to that previously found for the *R. etli fixN* reiterations (Girard et al. 2000).

However, the relative participation of *fixN* and *fixG* reiterated genes in nitrogen fixation is different. While *fixNd* encodes the terminal oxidase that is functional during symbiosis, the contribution of *fixNf* is almost null; for *fixG*, the presence of either reiteration is sufficient to fully support the capacity of *R. etli* to fix nitrogen in association with bean plants (data not shown).

Participation of *R. etli* ORF *RHE_PD00288* and *RHE_PF00499* in the expression of *fix* genes.

To evaluate the participation of these putative new Fnr-type regulators (dubbed *stoRd* and *stoRf*, respectively, after symbiotic terminal oxidase regulator) in *fix* gene regulation, plasmids pGL6 and pLG1 (Girard et al. 2000), harboring either *R. etli fixKf::* Ω *Tc-fixL::uidA* or *fixKf::uidA* transcriptional fusions, were introduced into strains CE3 (wild type), CFNX713 (*stoRd::loxSp*), and CFNX714 (*stoRf::* Ω Km). As shown in Table 2 (lines 1 to 3), microaerobic expression of *fixL* was not affected by mutations in either *stoRd* or *stoRf*. Microaerobic

expression of *fixKf* in strain CFNX713 (line 5) was nearly 50% higher than that observed in either the wild-type (line 4) or the CFNX714 strain (line 6). These results indicate that *StoRd* negatively regulates the expression of this gene, whereas *StoRf* does not participate in *fixKf* microaerobic expression.

To analyze whether expression of FixKf target genes was affected by the absence of *stoRd* or *stoRf* gene products, plasmids containing *fixNf::uidA* and *fixNd::uidA* transcriptional fusions (plasmids pLG2 and pLG4, respectively) were introduced into *R. etli* CE3 (wild type) and *StoR* mutants (CFNX713 and CFNX714). As shown in Table 2, a nearly 50% increase in the expression of *fixNf* was observed in both the CFNX713 and CFNX714 mutant backgrounds, compared with the wild type (lines 7, 8, and 9). Expression of *fixNd* was also augmented in both mutants, being 100% higher in a *stoRf* background (CFNX714, line 12) or 40% higher in a *stoRd* strain (CFNX713, line 11) than in the wild-type strain (line 10). Thus, we propose that the Fnr-type regulators encoded by *R. etli stoRd* and *stoRf* are negative transcriptional regulators for the symbiotic terminal oxidase in *R. etli* CFN42.

Production of covalently-bound heme proteins in bacteroids.

To evaluate if the enhancement in expression of the *fix-NOQPd* operon (encoding the symbiotic *cbh3* terminal oxidase) observed in the absence of the *stoRd* and *stoRf* gene products translates to an increment in the synthesis of the ter-

Plasmid	Fragment in plasmid (bp)	Mean β -glucuronidase-specific activity* ($\pm$ SD)
pMGB1	<i>XbaI-XbaI</i> (5933 bp)	ND**
pMGB3	<i>XbaI-EcoRI</i> (853 bp)	180 $\pm$ 25
pMGB4	<i>BamHI-BamHI</i> (2448 bp)	25 $\pm$ 14
pMGB5	<i>SmaI-EcoRV</i> (2467 bp)	25 $\pm$ 12
pMGB6	<i>EcoRI-XhoI</i> (767 bp)	10 $\pm$ 12

*In nm min⁻¹mg of protein⁻¹. Data are the mean of two replicates from two independent experiments.

**Not determined (not a Gus fusion)

Fig. 1. Expression analysis of the symbiotic plasmid *fixGHIS* region. For consistency with the remainder of the paper, *RHE_PD00288* was renamed as *stoRd*. **A**, Schematic representation of the putative operon. Open reading frames and transcriptional directions are indicated. Small black squares indicate putative promoter elements. Small arrows indicate primers used for polymerase chain reaction amplification (Up pd *XbaI* to Lw pd *XbaI* and Up *stoR* *gus* to Lw *stoR* *gus* for pMGB1 and pMGB6, respectively). Lines below indicate the restriction fragments cloned in plasmid pBBMCS53 to generate transcriptional fusions. Restriction sites: B, *BamHI*; E, *EcoRI*; RV, *EcoRV*; S, *SmaI*; and X, *XbaI*. **B**, Microaerobic expression of different gene fusions in the *Rhizobium etli* wild-type strain.

minal oxidase in nodules, we determined the production of *c*-type cytochromes in membrane fractions purified from bacteroids induced by the wild-type, *stoRd::loxSp*, *stoRf::ΩKm*, and *stoRd::loxSp-stoRf::ΩKm* strains.

As shown in Figure 2 (lane 1), bacteroids from the wild-type strain produce a 32- and a 27-Kda *c*-type cytochrome, corresponding to FixP and FixO (Soberón et al. 1999). The concentration of these cytochromes in bacteroids from *R. etli* mutant strain CFNX713 (*stoRd::loxSp*) was nearly twofold of that observed in wild-type bacteroids (Fig. 2, lanes 1 and 3). Interestingly, concentrations of *c*-type cytochromes in bacteroids from strains CFNX714 (*stoRf::ΩKm*) and CFNX715 (*stoRd::loxSp-stoRf::ΩKm*) were only modestly increased, being nearly 0.5-fold more than those produced by the wild-type strain (compare lane 1 with lanes 2 and 4). Our analysis shows that null mutations in *stoRd* and *stoRf* provoke significant increases in the production of the *cbb₃* terminal oxidase in *R. etli* bacteroids. These results are fully consistent with the proposed role of the StoR proteins as negative transcriptional regulators for the symbiotic terminal oxidase.

R. etli *stoR* reiterations as regulators for nitrogen fixation.

To analyze if null mutations in *stoR* reiterated genes modify the capacity of *R. etli* CFN42 to fix nitrogen in symbiosis, bean plants were inoculated with *R. etli* strains CE3 (wild-type), CFNX713 (*stoRd::loxSp*), CFNX714 (*stoRf::ΩKm*), and CFNX715 (*stoRd::loxSp-stoRf::ΩKm*) and were evaluated for nitrogenase activity, plant dry weight, and total nitrogen content (Table 3). In all cases, the highest nitrogenase activity was observed in plants at 18 days after inoculation. Specific nitrogenase activity was nearly doubled in plants inoculated with a *stoRd::loxSp* mutant, as compared with the wild-type strain (Table 3). This increase in specific nitrogenase activity is also reflected in a 30% higher shoot dry weight and in a 45% higher plant nitrogen content observed (at 45 days after inoculation) in the *stoRd::loxSp* mutant (Table 3). The enhanced nitrogen-fixation capacity detected here indicates that *stoRd* plays a crucial repressive role during the symbiosis of *R. etli* with bean plants.

Considering the high structural similarity of *stoRf* with *stoRd* and similar effects in terms of overproduction of the symbiotic terminal oxidase, the effects of a *stoRf::ΩKm* strain on nitrogen fixation in beans were somewhat surprising. Compared with the wild-type strain, plants inoculated with strain CFNX714 (*stoRf::ΩKm*) displayed significant decreases in all parameters, including specific nitrogenase activity, shoot dry weight, and total nitrogen content (Table 3). Thus, *stoRf*

appears to be needed for full expression of nitrogen-fixing ability. Interestingly, the deleterious effects of loss of *stoRf* on nitrogen fixation were compensated upon introduction of a mutation in *stoRd*. As shown in Table 3, strain CFNX715 (*stoRd::loxSp-stoRf::ΩKm*) showed levels of specific nitrogenase activity and total nitrogen content similar to the ones observed with strain CFNX713 (*stoRd::loxSp*). These results indicate that both *stoRd* and *stoRf* are needed for the fine-tuning (in a negative and a positive way, respectively) of genes crucial for nitrogen fixation of *R. etli* on bean plants.

DISCUSSION

In this work, we demonstrate that the products of two novel reiterated genes, *stoRd* and *stoRf*, have a differential role in controlling the expression of crucial genes for an efficient nitrogen fixation in *R. etli*. Common to both genes is that their inactivation lead to an enhanced expression of both the *fixNOQPd* and the *fixNOQPF* operons, translating into a high expression of the symbiotic terminal oxidase in nodules. However, a null mutation in *stoRd* (but not in *stoRf*) provoked an increased expression of *fixKf*. A further instance of differential gene control by these genes is inferred from their opposing effects on symbiotic nitrogen fixation, in which a null mutation in *stoRd* instigated a significant enhancement in nitrogen fixation on bean plants while inactivation of *stoRf* diminished the levels of nitrogen fixation.

Since the *stoRd* and the *stoRf* gene products are Fnr-like proteins with typical DNA-binding domains, it is tempting to speculate that their common actions on the expression of *fixNd*

Fig. 2. Heme *c*-staining membrane proteins of bacteroids from nodules of *Phaseolus vulgaris* inoculated with *Rhizobium etli*. Each lane contains 20 µg of membrane proteins purified from wild type (CE3) (lane 1); *stoRf::ΩKm* (CFNX714) (lane 2); *stoRd::loxSp* (CFNX713) (lane 3); and *stoRd::loxSp-stoRf::ΩKm* (CFNX715) (lane 4) bacteroids.

Table 2. Role of StoRd and StoRf in regulation of *fix* genes in *Rhizobium etli*

Strain	Strain genotype	Fusion genotype	Specific Activity (±SD) ^a
CE3/pLG6	Wild-type	<i>fixKf::ΩTc-fixL::uidA</i>	78 ± 4
CFNX713/pLG6	<i>stoRd::loxSp</i>	<i>fixKf::ΩTc-fixL::uidA</i>	80 ± 7
CFNX714/pLG6	<i>stoRf::ΩKm</i>	<i>fixKf::ΩTc-fixL::uidA</i>	79 ± 7
CE3/pLG1	Wild-type	<i>fixKf::uidA</i>	123 ± 11
CFNX713/pLG1	<i>stoRd::loxSp</i>	<i>fixKf::uidA</i>	190 ± 26
CFNX714/pLG1	<i>stoRf::ΩKm</i>	<i>fixKf::uidA</i>	128 ± 14
CE3/pLG2	Wild-type	<i>fixNf::uidA</i>	230 ± 34
CFNX713/pLG2	<i>stoRd::loxSp</i>	<i>fixNf::uidA</i>	344 ± 25
CFNX714/pLG2	<i>stoRf::ΩKm</i>	<i>fixNf::uidA</i>	306 ± 17
CE3/pLG4	Wild-type	<i>fixNd::uidA</i>	319 ± 30
CFNX713/pLG4	<i>stoRd::loxSp</i>	<i>fixNd::uidA</i>	524 ± 33
CFNX714/pLG4	<i>stoRf::ΩKm</i>	<i>fixNd::uidA</i>	741 ± 62

^a Mean β-glucuronidase-specific activity. Values are expressed as nanomoles per minute per milligram of protein. Data are the mean of two replicates from at least four independent experiments.

Table 3. Participation of StoR regulators in nitrogen fixation

Strain	Relevant genotype	Specific nitrogenase activity ^a	Shoot dry weight ^b	Total nitrogen content ^c
CE3	Wild type	51 ± 21	2.3 ± 0.7	70 ± 6
CFNX713	<i>stoRd::loxSp</i>	96 ± 25*	3.1 ± 0.6*	102 ± 2*
CFNX714	<i>stoRf::ΩKm</i>	38 ± 19*	1.6 ± 0.6*	45 ± 13*
CFNX715	<i>stoRd::loxSp-stoRf::ΩKm</i>	80 ± 22*	2.5 ± 0.9	96 ± 32*

^a Micromoles ethylene per gram of nodule per hour. Data are the average of two different experiments (consisting of 10 plants for each treatment), analyzed at 18 days postinoculation (dpi). Asterisks (*) indicate that the means of the samples are different from the wild type at the 0.05 level ($P = 0.05$, Student's *t*-test).

^b Grams per plant. Data are the average of two different experiments (consisting of 10 plants for each treatment), analyzed at 45 dpi.

^c Milligrams of N per plant. Total nitrogen content of dry samples of plants was determined for triplicate in each of three replicate plants for each treatment at 45 dpi.

and *fixNf* are instrumental in the early steps of the cascade for activation of nitrogen fixation. Upon closer scrutiny however, this alternative does not hold, due to the fact that the products of these genes do not control *fixL* while expression of *fixKf* is controlled only by *stoRd* but not by *stoRf*. Thus, the most likely explanation for their similar effects on the symbiotic terminal oxidase is that both bind to the regulatory regions of *fixNd* and *fixNf*, directly repressing their expression, perhaps by hindering the access of FixKf. Further proof of this proposal awaits the purification of StoRd and StoRf and demonstration of specific binding to the promoter region of *fixNf* and *fixNd*. Experiments are underway toward this objective.

Any plausible explanation of the differential control of *fixKf* by StoRd has to consider the fact that *fixKf* and *fixNf* are located closely in the genome (as diverging transcriptional units), sharing a common regulatory region. We have postulated that both StoRd and StoRf are able to bind to the regulatory region of *fixNf*; thus, if both proteins were recognizing the DNA in the same way, the expression of *fixKf* should be affected similarly upon inactivation of *stoRd* or *stoRf*. Our results clearly show that StoRd (but not StoRf) has a repressive effect on *fixKf* expression. Therefore, it is likely that these proteins bind to similar but not identical sequences. The binding of StoRd would have a negative effect on the expression of *fixKf*, probably by modulating FixL-dependent activation. We have previously shown that expression of *fixKf* is also subjected to negative autoregulation (Girard et al. 2000). Interestingly, the expression levels of *fixKf* observed in a *stoRd::loxSp* mutant, although high, are still fourfold lower than the level observed in a *fixKf::ΩKm* mutant. This suggests that *fixKf* expression is subject to a two-tiered negative control mediated by StoRd and FixKf.

An intriguing aspect of our data relates to the opposing roles of loss of *stoRd* and *stoRf* on symbiotic nitrogen fixation. These contrasting roles could be explained by theorizing that *stoRd* represses a set of nitrogen fixation genes including but not limited to both copies of *fixNOQP*. In contrast, it is likely that *stoRf* controls, either directly or indirectly, a different set of genes than *stoRd* does. Identification of the target genes controlled by both regulators will be pursued in the near future through microarray analysis of these mutants.

Figure 3 shows our current model for the participation of FixK, FnrN, and Fnr-like proteins in the regulation of nitrogen fixation genes in *R. etli*, incorporating the proposed negative roles of *stoRd* and *stoRf* on *fixNOQP* expression found here. Control of nitrogen fixation genes by repressors, albeit unusual, is not without precedent. For instance, in most rhizobial species, FixK displays negative autoregulation (Batut et al. 1989; Fischer 1994). Besides the cases of negative autoregulation, the only cases of bona fide repressor control of nitrogen-fixation genes are StoRd and StoRf in *R. etli* and FixT in *S. meliloti*; in the last case, FixT represses transcription of *nifA* and *fixK*, preventing the formation or accumulation of the phosphorylated form of FixL (Foussard et al. 1997; Garnerone et al. 1999). In this regard, it is germane to recall that both *R. leguminosarum* bv. *viciae* 3841 and *S. meliloti* 1021 harbor convincing homologs to the *stoR* genes. The analysis of a mutation in the *S. meliloti* homolog (Sma1207) was published recently (Bobik et al. 2006). This mutant displays a wild-type phenotype in regard to nitrogen fixation, indicating that control mediated by *stoR* genes operates in a species-specific way. We are presently evaluating whether mutations in the *R. leguminosarum* bv. *viciae* homologs have similar effects on nitrogen fixation, given their higher phylogenetic relatedness to *R. etli*.

Regulation of nitrogen fixation genes in Rhizobiales shows a series of intricacies of daunting complexity. Among these are: i) positive control in response to environmental conditions used under relatively common circumstances for the organism,

as exemplified by *fixL-fixJ*, *fixK*, and *nifA*, to mention but a few; ii) reiteration of regulatory components, which allows diversification of regulatory pathways or survival even in the presence of mutation, as *fixK* and *rpoN* in both *Bradyrhizobium japonicum* and *R. etli*; iii) sure-fire or cross-talk circuits in which the regulation of important genes is maintained, albeit in a modified state, as *fixL* vs. *fnrNchr* in *R. etli*, and iv) negative control, commonly used under relatively unusual circumstances for the organism, as exemplified by StoRd and StoRf in *R. etli* and FixT in *S. meliloti*. It is clear that such a complex combination of regulatory mechanisms is the product of the evolutionary use of an energy-demanding process under a variety of environmental conditions, contributing to shape these circuits as we see them today.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used are listed in Table 4. *Rhizobium* strains were grown at 30°C in PY rich medium (Noel et al. 1984) or in Y minimal medium (MMY) with succinate (10 mM) and ammonium chloride (10 mM) as carbon and nitrogen sources, respectively (Bravo and Mora 1988). *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium. Antibiotics were added at the following concentrations

Fig. 3. Model for the participation of FixK, FnrN, and Fnr-like proteins in the regulation of nitrogen fixation genes in *Rhizobium etli*. The dotted arrow indicates conditional control in a *fixL* background. Positive and negative regulation is indicated by plus and minus signs, respectively.

($\mu\text{g ml}^{-1}$): carbenicillin (Cb), 100 (*E. coli*); chloramphenicol (Cm), 15 (*E. coli*); gentamicin (Gm), 15 (*E. coli* and *R. etli*); kanamycin (Km), 30 (for both *E. coli* and *R. etli*); nalidixic acid (Nal), 20 (*R. etli*); spectinomycin (Sp), 100 (*E. coli* or *R. etli*). When required, sucrose was added at 12.5% (wt/vol). For transcriptional fusion analysis, cultures were grown to mid-exponential phase in PY medium. Cells were collected by centrifugation (at $6,000 \times g$ in a Sorval SS34 rotor), were washed with sterile MMY, and were concentrated 100-fold. Twenty milliliters of MMY containing 10 mM of succinate and 10 mM of ammonium chloride as carbon and nitrogen sources were inoculated with these suspensions at an initial optical density at 540 nm of 0.05. For growth under microaerobic conditions, cultures were incubated in 150-ml bottles closed with an airtight stopper and flushed with several volumes of an oxygen/argon (1:99, vol/vol) mixture. Cultures were grown with shaking (200 rpm) for 10 h at 30°C (Girard et al. 1996, 2000).

Microbiological and recombinant DNA methods.

Total and plasmid DNA isolation, digestion with restriction enzymes, cloning, agarose gel electrophoresis, and *E. coli* transformation were performed using standard protocols (Sambrook et al. 1989). Enzymes used for DNA restriction and modification were purchased from either Invitrogen (Carlsbad, CA, U.S.A.)

or New England Biolabs (Beverly, MA, U.S.A.) and were used according to the instructions of the manufacturer.

Conjugative transfer of plasmids from *E. coli* to *Rhizobium* spp. was done routinely by triparental matings, using either pRK2013 or pRK2073 (Table 4) as helpers. For determination of plasmid profiles, a modified Eckhardt procedure was used (Hynes and McGregor 1990).

For Southern hybridizations, DNA was digested with appropriate restriction enzymes, was electrophoresed in 1% (wt/vol) agarose gels, and was blotted onto nylon (Hybond N+). Hybridization was carried out under high stringency conditions, using Amersham's Rapid-hyb buffer (Amersham, U.K.). Specific probes were normally obtained by PCR and were labeled with $\alpha^{32}\text{P}$ -CTP by random priming, using Amersham's Rediprime system.

PCR amplification.

Specific PCR primers were designed using the Oligo 6.0 software and were purchased from Unidad de Síntesis Química [Instituto Biotecnología, Universidad Nacional Autónoma de México (UNAM)]. PCR amplifications were done in a Mastercycler 5330 (Eppendorf, Hamburg, Germany). Pfu DNA polymerase was used in PCR reactions, with a cycling regime that includes a denaturing step at 94°C for 1 min, followed by 30

Table 4. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference
<i>Rhizobium etli</i>		
CFN42	Wild type (type strain)	Quinto et al. 1982
CE3	Sm ^r derivative of CFN42	Noel et al. 1984
CFNX89	CE3 derivative, lacking the pSym	Brom et al. 1992
CFNX186	CE3 derivative, lacking pCFN42f	Brom et al. 1992
CFNX636	CE3 derivative, <i>fixL::loxSp</i>	Girard et al. 2000
CFNX637	CE3 derivative, <i>fixKf::ΩKm</i>	Girard et al. 2000
CFNX641	CE3 derivative, <i>fixNf::loxSp-fixNd::ΩKm</i>	Girard et al. 2000
CFNX713	CE3 derivative, <i>RHE_PD00288::loxSp</i>	This study
CFNX714	CE3 derivative, <i>RHE_PF00499::ΩKm</i>	This study
CFNX715	CE3 derivative, <i>RHE_PD00288::loxSp- RHE_PF00499::ΩKm</i>	This study
CFNX716	CE3 derivative, <i>fixGd::loxSp</i>	This study
CFNX717	CE3 derivative, <i>fixGf::ΩKm</i>	This study
CFNX718	CE3 derivative, <i>fixGd::loxSp-fixGf::ΩKm</i>	This study
<i>Escherichia coli</i>		
DH5α	<i>supE44ΔlacU169(φ80lacΔM15)hsdR17recA1endA1gyrA96 thi-1relA1</i>	Sambrook et al. 1989
BW21038	<i>DE3 lacX74 uidA (ΔMlu1)::pir⁺ miniTn5 Lac4 (Cm^r lac^f lacZ ΔM15) recA1 ΔphnC⁺DEFGHIJKLMN)33-30 ΔphoA532</i>	Metcalfe et al. 1996
HB101	<i>supE44 hsdS20 (r_Bm_B) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i>	Sambrook et al. 1989
Plasmids		
pSK ⁺	Cloning vector Ap ^r	Stratagene, La Jolla, CA, U.S.A.
pMOSBlueT	Cloning vector for PCR products, Ap ^r	Amersham
pSUP202	Suicide cloning vector Ap ^r Cm ^r Tc ^r	Simon et al. 1983
pJQ200mp18	Suicide cloning vector Gm	Quandt and Hynes 1993
pBBMCS53	<i>ΔplacZ</i> pBBR1MCS-5 derivative, carrying the promoterless <i>uidA</i> gene from pWM5, Gm ^r	Girard et al. 2000
pRK2073	Conjugation helper plasmid Sm/Sp ^r	Better and Helinski 1983
pRK2013	Conjugation helper plasmid Km ^r	Figurski and Helinski 1979
pLG1	pBBMCS53 derivative <i>fixKf::uidA</i>	Girard et al. 2000
pLG2	pBBMCS53 derivative <i>fixNf::uidA</i>	Girard et al. 2000
pLG4	pBBMCS53 derivative <i>fixNd::uidA</i>	Girard et al. 2000
pLG502	pSUP202 derivative carrying 2.0-kb <i>HindIII</i> ΩKm cassette	L. Girard, unpublished data
pMGB1	pCR2.1 TOPO:: <i>fixGHISd-stoR</i>	This study
pMGB2	pCR2.1 TOPO:: <i>fixGHISf- RHE_PF00499</i>	This study
pMGB3	pBBMCS53 derivative <i>fixGd::uidA</i>	This study
pMGB4	pBBMCS53 derivative <i>fixGd-fixId:: uidA</i>	This study
pMGB5	pBBMCS53 derivative <i>fixId- RHE_PD00288::uidA</i>	This study
pMGB6	pBBMCS53 derivative <i>RHE_PD00288::uidA</i>	This study
pMGB7	pBBMCS53 derivative <i>fixGf::uidA</i>	This study
pMGB8	pSUP202 derivative, <i>fixGd::loxSp</i>	This study
pMGB9	pJQ200mp18 derivative, <i>fixGf::ΩKm</i>	This study
pMGB10	pLG502 derivative, <i>RHE_PD00288::loxSp</i>	This study
pMGB11	pJQ200mp18 derivative, <i>RHE_PF00499::ΩKm</i>	This study

^a Antibiotic abbreviations: Km = kanamycin, Sp = spectinomycin, Ap = ampicillin, Cm = chloramphenicol, Tc = tetracycline, Gm = gentamycin, and Sm = streptomycin.

cycles of 94°C for 30 s, 45 to 60°C (according to the primer's melting temperature) for 30 s, and 72°C for 90 s. A final elongation step was made at 72°C for 5 to 10 min, according to the length of the DNA fragment to be amplified. When necessary, PCR products were generated with a GeneAmp XL PCR kit (Perkin-Elmer, Branchburg, NJ, U.S.A.), with an initial denaturation step at 94°C for 2 min; the cycling regime afterwards consisted of 16 cycles of 94°C for 30 s and 68°C for 10 min, followed by 12 cycles of 94°C for 30 s and 68°C for 11 min.

RT-PCR analysis.

Total RNA was purified from wild-type strain and *fixG* mutants grown under microaerobic conditions using High Pure RNA isolation kit (Roche, Mannheim, Germany). Single-stranded cDNA synthesis was performed using the ThermoScript RT-PCR system (Invitrogen). Specific 500-bp RT-PCR products for each reiteration (from nucleotide 37 to 535) were obtained using primers UpfmrLdRT (5'-CTGTTTCACGGCC TGGACA-3') and LwfmrLdRT (5'-GCCTGCCCTTACCGGA AAAGAATTC-3') for *RHE_PD00288* product and primers UpfmrLfRT (5'-CTGTTTCGACGGGCTGGATG-3') and LwfmrLfRT 5'-GCCTGCCGTACCGGAAGAATTC-3') for *RHE_PF00499*. After 35 cycles of amplification (94°C for 40 s; 60°C for 40 s; 72°C for 40 s) and a final elongation step at 72°C for 5 min, PCR products were analyzed by electrophoresis in agarose gels.

Plasmids and mutant strains construction.

For cloning of each reiterated *fixGHIS* region, two plasmids were generated. Amplification of the *fixGHIS* region from the symbiotic plasmid was done using total DNA from strain CFNX186 (lacking plasmid pCFN42f) as template with the specific primers Up pd-XbaI (5'-GATCTAGAGCCAAAGG AAAGGTG-3', *XbaI* site is underlined) and Lw pd-XbaI (5'-GCTCTAGACTGCACTTCCCTATTAAG-3', *XbaI* site is underlined). The resulting 5,965-bp product, containing the 3' end of *fixPd* (from nucleotide 670), the complete coding regions for *fixGd*, *fixHd*, *fixId*, *fixSd*, and *RHE_PD00288*, and 215 nucleotides downstream from the last gene, was cloned by T-A annealing into pCR 2.1-TOPO (Invitrogen), giving rise to pMGB1. Amplification of the *fixGHISf* region was achieved using total DNA from strain CFNX89 (lacking the symbiotic plasmid) as template and primers Up pf-XbaI (5'-GATCTAG AATACGACAGTCAAGGAGC-3', *XbaI* site is underlined) and Lw pf-XbaI (5'-GCTCTAGAGATAAGCATGACGTCC AG-3', *XbaI* site is underlined) as forward and reverse primers, respectively. The desired amplified fragment (5,714 bp) contains the 3' end of *fixPf*, the complete coding regions from *fixGHISf* to 97 nucleotides downstream of *RHE_PF00499*. This fragment was cloned into pCR 2.1-TOPO, resulting in plasmid pMGB2.

To construct *uidA* transcriptional fusions of the different overlapping fragments spanning the *fixGHISd* region, appropriate restriction fragments from pMGB1 (Fig. 1) were gel-purified and cloned separately in the broad-host plasmid pBBRMCS53 (Girard et al. 2000) carrying a promoterless *uidA* gene, resulting in plasmids pMGB3 to pMGB5 (Fig. 1). Generation of a *RHE_PD00288 (stoRd)::uidA* transcriptional fusion was done by amplifying the corresponding gene using primers Up stoR *gus* (5'-TCGAATTCGCTGCGATTAAATGC CTT-3', *EcoRI* site is underlined) and Lw stoR *gus* (5'-TC CTCGAGTTTTCCGGTAAGGCAGC-3', *XhoI* site is underlined); the 767-bp product was restricted with *EcoRI* and *XhoI* and was cloned into pBBRMCS53, giving rise to pMGB6 (Fig. 1). To construct a *fixGf::uidA* transcriptional fusion, a 739-bp PCR product was obtained using total DNA from strain CFNX89 as template, and primers Up pf-XbaI and Lw *fixGf*-

gus (5'-GATCTAGAAAATTCCTGCGGCCAGAT-3', *XbaI* site underlined) were cloned into pBBMCS53 to obtain plasmid pMGB7.

To introduce a mutation into *fixGd*, a 2,505-bp product was obtained by PCR utilizing primers Up *fixGd*-PstI (5'-CTGCA GCCAAAGGAAAGGTGGAAA-3', underlined, built-in *PstI* site) and Lw *fixGd*-PstI (5'-CTGCAGCCCTTCGCCCGTCTT-3', *PstI* site is underlined). The *PstI* sites in the primers were used to clone the fragment into the suicide vector pSUP202 (Simon et al. 1983). In the resulting plasmid, a *fixGd::loxSp* deletion-substitution allele was generated by deleting a 177-bp *BglII* fragment of *fixGd* (codons 35 to 94) and inserting in its place a *loxSp* interposon (Martínez-Salazar and Romero 2000), generating plasmid pMGB8.

Taking advantage of the two *EcoRI* sites present in plasmid pMGB2 (one in the vector pCR 2.1-TOPO and the other in the *fixGf* coding region), a 1,186-bp *EcoRI* fragment was purified and cloned into the pBluescript SK⁺ vector (Stratagene, La Jolla, CA, U.S.A.). Mutagenesis of the *fixGf* gene was achieved by introducing into the *BglII* site (nucleotide 281) a *BamHI* ΩKm cassette (Prentki and Krisch 1984). This plasmid was used as a template in PCR amplifications, employing primers Up *fixGf*-XhoI (5'-CTCGAGCGGCGGCACGTAAG GAGA-3', built-in *XhoI* site is underlined) and Lw *fixGf*-XhoI (5'-CTCGAGCGGCGCCATGGACACATA-3', built-in *XhoI* site is underlined). To construct a suicide plasmid useful for homogenization, the resulting 3,359-bp PCR product was digested with *XhoI* and was cloned into the single *SalI* site of pJQ200mp18 (Quandt and Hynes 1993), resulting in plasmid pMGB9.

Specific mutagenesis of *RHE_PD00288* was done, amplifying by PCR the corresponding genomic region from strain CFNX186, using the specific primers Up stoR-XhoI (5'-TACT CGAGGCTGCGATTAAATGCCTT-3', built-in *XhoI* site is underlined) and Lw stoR-XhoI (5'-TACTCGAGGCATGTTG GTGCTCGTTG-3', built-in *XhoI* site is underlined). The 1,185-bp PCR product (containing the 3' end of *fixSd*, the complete coding regions for *RHE_00288*, and the 5' end of the *RHE_PD00287* gene) was cloned into pMOSBlue (using the blunt-ended PCR cloning kit from Amersham). The resulting plasmid was modified by inserting a *loxSp* cassette digested with *BamHI* into the *BglII* site in *RHE_PD00288* (nucleotide 95). Using the *XhoI* sites in the PCR primers Up stoR-XhoI and Lw stoR-XhoI, the fragment containing the *RHE_PD00288::loxSp* allele was cloned into the *SalI* site in pLG502, giving rise to pMGB10.

To mutagenize *RHE_PF00499*, a 3,083-bp PCR product was synthesized employing the primers Up *fmrI*-XhoI (5'-CGCTCGAGGCGCTGCTGATGCTGAAG-3', built-in *XhoI* site is underlined) and Lw *fmrI* (5'-TATCCGACACCG AAC-3'). The *XhoI* site in the upper primer and the *BamHI* site present in the product were used to clone a 2,993-bp *XhoI*-*BamHI* fragment into pBluescript SK⁺ vector digested with *XhoI*-*BamHI*. In this plasmid, a *RHE_PF00499::ΩKm* deletion-substitution allele was generated by deleting a 586-bp sector (from nucleotide 70 to 656) by digestion with *HindIII* and inserting in its place a *HindIII*-*HindIII* ΩKm cartridge. The fragment containing the *RHE_PF00499::ΩKm* deletion-substitution allele was then removed by *XhoI*-*BamHI* digestion and was subcloned into *SalI*-*BamHI*-restricted pJQ200mp18 (Quandt and Hynes 1993) to give plasmid pMGB11.

Construction of mutants.

Introduction of all mutant alleles into *R. etli* was carried out by homogenization. To introduce the *fixGd::loxSp* deletion-substitution allele, *E. coli* DH5α/pMGB8 was mated in a triparental cross using *R. etli* CE3 as a recipient and HB101/pRK2013

as a helper. Double recombinants were selected as $\text{Nal}^r \text{Sp}^r \text{Tc}^s$ transconjugants. This procedure yielded strain CFNX716. Introduction of the *RHE_PD00288::loxSp* allele was done in the same way, except pMGB10 was used as donor plasmid. Double recombinants were selected as $\text{Nal}^r \text{Sp}^r \text{Km}^s$ transconjugants (strain CFNX713).

Homogenization of *fixGf::ΩKm* and *RHE_PF00499::ΩKm* mutant alleles was done using the *sacB* marker present in plasmid pJQ200mp18; this marker allows an easy selection for homogenization events by scoring for resistance to sucrose. *R. etli* strains containing either *fixGf::ΩKm* (strain CFNX717) or *RHE_PF00499::ΩKm* (strain CFNX714) alleles were constructed in this way, using plasmids pMGB9 or pMGB11 as donors, respectively.

For construction of a derivative harboring the *fixGf::ΩKm-fixGd::loxSp* (strain CFNX718) allelic combination, *E. coli* BW21038/pMGB9 was mated in a triparental cross using *R. etli* CFNX716 (*fixGd::loxSp*) as a recipient and *E. coli* HB101/pRK2013 as a conjugation helper. To generate a derivative carrying the *RHE_PD00288::loxSp-RHE_PF00499::ΩKm* allelic combination (strain CFNX715), *R. etli* CFNX713 (*RHE_PD00288::loxSp*) was mated with *E. coli* BW21038/pMGB11 and HB101/pRK2013 in a triparental cross. Double recombinants were selected as $\text{Nal}^r \text{Km}^r \text{Sp}^r$ and sucrose-resistant transconjugants. To verify that the desired gene replacements had occurred, both plasmid profiles as well as total DNA blots of all the derivatives were analyzed by Southern hybridization against the appropriate gene and cassette probes.

Measurement of GUS activity.

Microaerobic cultures of *R. etli* strains harboring transcriptional fusions were grown as described previously (Girard et al. 1996). Quantitative GUS activity was performed on 1.0-ml culture samples using 4-nitrophenyl β-D-glucuronide as substrate as previously described (Girard et al. 2000). Data were normalized to total cell protein concentration by the Lowry method over a second set of 1.0-ml samples. Specific activities are reported in nanomoles of product per minute per milligram of protein.

Bacteroid fractionation, protein gel electrophoresis, and haem c staining.

Nodules were harvested from 35-day-old plants, were freed of vermiculite, and were rinsed several times with sterile water. Bacteroids were prepared by grinding 3 g of fresh nodules with 4 ml of 50 mM potassium phosphate buffer (pH 7) containing 250 mM mannitol. The homogenate was filtered through four layers of cheesecloth and was centrifuged at $250 \times g$ at 4°C for 5 min to remove nodule debris. The resulting supernatant was centrifuged two times at $12,000 \times g$ at 4°C for 10 min and was washed twice in 50 mM potassium phosphate buffer (pH 7). Bacteroids were resuspended in 3 ml of 50 mM potassium phosphate buffer (pH 7) containing 100 μM 4-amidinophenylmethanesulfonyl fluoride, RNase (20 μg ml⁻¹), and DNase I (20 μg ml⁻¹). Cells were disrupted using a French pressure cell (SLM Aminco, Jessup, MD, U.S.A.). The cell extract was centrifuged at $20,000 \times g$ for 20 min to remove unbroken cells, and the supernatant was then centrifuged at $140,000 \times g$ for 1 h. The membrane pellet was resuspended in 100 μl of the same buffer.

Membrane protein aliquots (30 μg) were diluted in sample buffer (124 mM Tris-HCl [pH 7.0], 20% glycerol, 4.6% sodium dodecyl sulfate (SDS), and 50 mM 2-mercaptoethanol), incubated at room temperature for 10 min, separated at 4°C in SDS-12% polyacrylamide gel electrophoresis, transferred to a nitrocellulose filter, and stained for haem-dependent peroxidase activity by chemiluminescence, as described previously

(Vargas et al. 1993). Protein concentration was estimated by using the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA, U.S.A.) with bovine serum albumin as the standard.

Evaluation of nitrogen-fixation capacity.

Three-day-old *Phaseolus vulgaris* cv. Negro Jamapa seedlings were inoculated with the desired strain and were grown in a greenhouse as previously described (Peralta et al. 2004). At 18 days postinoculation (dpi), 10 plants were analyzed for nitrogenase activity (by acetylene reduction), nodule number, as well as nodule and plant dry weight. Nitrogen content determination in dried plant material was performed at 45 dpi by grinding the samples in a Knife Mill Grindomix GM 200 (Retsch, GmbH & Co, Haan, Germany) and determining total nitrogen content in a chemiluminescence-based nitrogen analyzer (model ANTEK 9000, Antek Instruments, Inc., Houston, TX, U.S.A.), as reported previously (Peralta et al. 2004). Bacteria were isolated from nodules and their identity was verified by their antibiotic resistance patterns.

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