Differential Regulation of *fixN*-Reiterated Genes in *Rhizobium etli* by a Novel *fixL*–*fixK* Cascade

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Among the complexities in the regulation of nitrogen fixation in the Rhizobiaceae are reiteration of regulatory components as well as variant roles for each component between species. For *Rhizobium etli* CFN42, we reported that the symbiotic plasmid (pCFN42d) contains a key regulatory gene (*fixKd*) and genes for a symbiotic cytochrome oxidase (*fixNOQPd*). Here we discuss the occurrence of reiteration of these genes (*fixKf* and *fixNOQpf*) and the finding of an unusual *fixL* homolog on a plasmid previously considered cryptic (pCFN42f). The structure of the deduced FixL polypeptide is suggestive of a fusion of the receiver and transmitter modules of a two-component regulatory system as described in *R. leguminosarum* bv. *viciae* VF39. Gene fusion analysis, coupled with mutation of each regulatory element, revealed that free-living expression of FixKf was dependent fully on FixL. In contrast, synthesis of FixKd was not detected under the conditions tested. The FixKf protein is needed for microaerobic expression of both *fixN* reiterations, whereas the FixKd protein appears to be dispensable. Interestingly, expression of the *fixN* reiterations exhibits a differential dependence for FixL, where transcription of *fixNf* was suppressed in the absence of FixL but expression of *fixNd* still showed significant levels. This suggests the existence of a FixL-independent mechanism for expression of the *fixNd* reiteration. Surprisingly, mutations in *fixL*, *fixKd*, or *fixKf* (either singly or in combination) did not alter symbiotic effectiveness. A mutation in *fixNd* (but not in *fixNf*) was, however, severely affected, indicating a differential role for these reiterations in nitrogen fixation.

Additional keywords: *Rhizobium*–legume symbiosis.

Studies of the regulation of nitrogen fixation in *Rhizobium* spp. have been greatly aided by the identification of several common regulatory steps. The genes involved in nitrogen fixation are commonly organized into species-specific clusters (*nif*). In addition to *nif* genes, the so-called *fix* genes in rhizo-

bia are needed for nitrogen fixation (*fix*). For most *Rhizobium* species, two regulatory cascades (FixL–FixJ–FixK and NifA–RpoN) have been described.

In several organisms, including *R. meliloti*, *Bradyrhizobium japonicum*, and *Azorhizobium caulinodans*, FixLJ and FixK proteins are the first effectors in the regulation of nitrogen fixation gene expression. FixL and FixJ are members of the two-component regulatory systems, where FixL is a hemo-protein with histidine kinase activity that senses oxygen concentrations (David et al. 1988). At low oxygen concentrations, FixL catalyzes its own phosphorylation with ATP. FixL–P then transfers the phosphate to the transcription factor FixJ in an oxygen-independent reaction (Gilles-Gonzalez and Gonzalez 1993). Phosphorylation of FixJ induces its dimerization (Da Re et al. 1999), which is required for binding to target promoters, thus triggering a cascade of gene expression required for nitrogen fixation. FixJ–P acts, in turn, as a positive regulator of the *fixK* gene. In general, the FixK proteins activate transcription of the *fixN* operon and have a negative effect on their own synthesis and, in *R. meliloti*, on NifA (Batut et al. 1989). The FixK protein is a transcriptional regulator belonging to the Crp–Fnr protein family. FixK and Fnr transcriptional regulators bind to a conserved nucleotide sequence (5′-TTGA-N6-TCAA-3′) in the promoter region of their target genes (Waelkens et al. 1992).

A common function of the FixLJ–FixK cascade is the regulation of the *fixNOQP* genes and, perhaps, the *fixGHIS* genes. The *fixNOQP* operon encodes a cytochrome *c* that contains a heme-copper oxidase with a high affinity for oxygen. This oxidase is induced specifically under oxygen limitation, and its kinetic characteristics make it suited for operation under the microaerobic conditions prevailing in the nodule (Preisig et al. 1993). The presence of a *fixGHIS*-like operon that is downstream of the *fixNOQP* genes is a general characteristic in *Rhizobium* spp. Oxygen limitation induces *fixGHIS* expression through the FixLJ–FixK regulatory cascade. In addition to the role of FixGHIS proteins in copper uptake and metabolism, it has been proposed that in *B. japonicum*, these proteins are required in the assembly and/or stability of the *cbb*₃-type heme-copper oxidase (Preisig et al. 1996).

Although the general characteristics of this regulatory system constitute a useful conceptual framework to understand the regulation of nitrogen fixation in *Rhizobium* spp., there are

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important differences among the species. In particular, it has been recognized that the connectivity among the different cascades, the specific regulatory role of each element, and its target genes may vary within the different nitrogen fixers (Fischer 1994; Kaminski et al. 1998). Additional differences are imposed by the presence of reiterated DNA sequences (Romero et al. 1997). Reiteration of *fix* genes has been reported for the *fixK* gene in *R. meliloti* (Renalier et al. 1987) and *B. japonicum* (Anthamatten et al. 1992; Nellen-Anthamatten et al. 1998) and for *fixNOQP* genes in *R. meliloti* (Renalier et al. 1987) and *R. leguminosarum* bv. *viciae* (Schlüter et al. 1997). Studies of *B. japonicum* determined that the *fixLJ*-linked *fixK* gene (called *fixK₂*) is important for nitrogen fixation and acts as a general regulator for genes that are induced by low oxygen levels (Nellen-Anthamatten et al. 1998). In *R. leguminosarum* bv. *viciae*, both copies of *fixNOQP* genes are required for optimal nitrogen fixation (Schlüter et al. 1997).

More radical departures from the classical regulatory scheme have been reported for *R. leguminosarum* bv. *viciae* VF39 and *R. etli* CNPAF512 (formerly *R. leguminosarum* bv. *phaseoli*). In each case, the deduced amino acid sequences for the corresponding FixL polypeptides reveal novel features. In *R. leguminosarum* bv. *viciae* VF39, the *fixL* homolog is located in a plasmid other than the symbiotic one. In addition to the sequence motifs found in other FixL proteins, this protein possesses a fourth additional domain that is homologous to the receiver domain present in response regulators. Furthermore, a structural homolog for the *fixJ* gene is absent in this organism.

Mutations in the *fixL* gene displayed a Fix⁺ phenotype (Patschkowski et al. 1996). The central oxygen-sensing domain in *R. etli* CNPAF512 FixL protein does not conserve the heme-binding motif present in other FixL proteins. Mutants in CNPAF512 *fixL* gene are able to fix nitrogen (D'hooghe et al. 1995; D'hooghe et al. 1998).

During our studies of genome structure and recombinational dynamics in the type strain *R. etli* (CFN42), we identified most of the *nif* and *fix* genes already described on the symbi-

otic plasmid (pSym or pCFN42d) (Girard et al. 1991; Soberón et al. 1999b). Important regulatory elements described in other *Rhizobium* species such as *fixL* and *fixJ*, however, are not encoded on the pSym. Here we show that the only copy of *fixL* present in this strain is located on a plasmid previously considered cryptic (pCFN42f). In this plasmid there is a *fix*-reiterated region containing copies of *fixK*, *fixNOQP*, and *fixG* genes. Detailed expression analysis and mutation of the regulatory elements present in each reiteration were carried out in order to establish a possible regulatory model for *fix* genes in *R. etli* CFN42.

RESULTS

Cloning and sequence of a *fix*-reiterated region located on pCFN42f.

Previous data from our laboratory (Girard et al. 1991) have shown that the symbiotic plasmid of *R. etli* CFN42 (pCFN42d or pSym) shares a homologous region with plasmid pCFN42f. Hybridization and sequencing analysis revealed that this region in pCFN42d contains five open reading frames (ORF) identified as the *R. etli* *fixKd* and *fixNOQPd* operon (Soberón et al. 1999b). In order to ascertain whether the corresponding region in pCFN42f carries structural homologs of these genes, *Eco*RI-restricted genomic DNAs from strains CE3 (an Sm^r derivative of CFN42) (Table 1) and its derivatives CFNX89 and CFNX186 (lacking plasmids pCFN42d or pCFN42f, respectively) were subjected to high-stringency Southern hybridization with the *fixKd* copy as a probe. Two *fixK*-hybridizing regions (4.2 and 9.5 kb) were seen in the genome of strain CE3 (Fig. 1A, lane 1). These signals correspond to the *fixKd* region (9.5 kb) and a new *fixKf* homolog (4.2 kb) on the basis of their presence or absence in strains lacking the pSym (Fig. 1A, lane 2) or pCFN42f (Fig. 1A, lane 3). Assignment of these homologs to specific plasmids was corroborated by hybridization of a set of *Agrobacterium tumefaciens* transconjugants that harbor each of the different plasmids of strain CE3 (Flores et al. 1987) with the same *fixKd* probe (data not shown). Similar hybridization experiments clearly showed the existence of homologs to *fixP* (Fig. 1B) and *fixN* (data not shown) on pCFN42f.

To isolate the *fix* homologous region of pCFN42f, a CFN42 cosmid library was screened by hybridization with a *fixKd* probe. Of the three cosmid clones isolated by this procedure, one carried the *fixKd* (Fig. 1A, lane 5) and *fixPd* genes (Fig. 1B, lane 5), as evaluated by hybridization. This clone was not analyzed further. The remaining overlapping clones (cos772 and cos594) carried the *fixKf* homolog (Fig. 1A, lanes 4 and 6). Hybridization with the *fixPd* region as a probe revealed that only cos772 harbors the corresponding homolog on pCFN42f (Fig. 1B, lanes 4 and 6).

Subcloning and complete sequence analysis of a 4,869-bp region of pCFN42f in these cosmids revealed three complete ORF with homology to *fix* gene products. A 1,620-bp-long ORF (540 amino acid) was designated *fixNf* on the basis of its high similarity to *fixN* sequences of several species. The predicted *fixNf* polypeptide shows a high similarity to FixN polypeptides in different *Rhizobium* species, including *R. etli* (84%, *fixNd* reiteration, accession number U76906), *R. leguminosarum* bv. *viciae* (*fixNd* and *fixNc*, 84% and 82%, respectively; accession numbers Z80339 and Z80340), *R.*

Fig. 1. High-stringency hybridization analysis of total DNA of *Rhizobium etli* CE3 (wild type, lanes 1); CFNX89 (lacking pSym, lanes 2); CFNX186 (lacking pCFN42f, lanes 3), and cosmid clones cos594 (lanes 4), cos668 (lanes 5), and cos772 (lanes 6) with **A**, pSym copies of *fixK* and **B**, *fixP* genes as probes. Size of hybridizing *Eco*RI fragments (in kb) is indicated.

meliloti (81%, accession number Z21854), *A. caulinodans* (79%, accession number X74410), and *B. japonicum* (74%, accession number AJ005001). A second ORF of 780-bp (starting with a valine instead of a methionine codon transcribed in opposite direction to *fixNf*) encodes a polypeptide, 260 amino acid long, that possesses a high similarity to FixK polypeptides of *R. etli* (*fixKd*, 79%, accession number U76906) and *R. leguminosarum* bv. *viciae* (76%, accession number Z70305). This similarity is reduced somewhat compared with other FixK polypeptides such as *R. meliloti* (54%, accession number Z21854), *B. japonicum* FixK₁ and FixK₂ (51% and 50%, respectively; accession numbers M86805 and AJ005001), and *A. caulinodans* (45%, accession number X59071).

A third ORF that starts 24 bp downstream of the *fixKf* termination codon was identified. This ORF (1,923 bp, 641 amino acid) was designated *fixL* on the basis of its high similarity (88%) to the unusual *fixL* gene from *R. leguminosarum* bv. *viciae* (accession number Z70305). The deduced amino acid sequence of the *R. etli* FixL protein also displays moderate similarity to the FixL proteins of other species such as *R.*

meliloti (49%, accession number P10955), *A. caulinodans* (49%, accession number P26489), *B. japonicum* (45%, accession number S13330), and *R. leguminosarum* bv. *phaseoli* (34%, accession number U27314). The *fixL* gene of *R. etli* CFN42 is similar to the *fixL* gene of *R. leguminosarum* bv. *viciae* (Patschkowski et al. 1996) and shows several sequence characteristics that are a mixture of those found in the transmitter and response elements of two-component regulatory systems (Fig. 2). Among these, the *fixL* gene of *R. etli* lacks the N-terminal hydrophobic segments, showing a putative motif involved in heme binding. The transmitter motif of a two-component system (including a conserved histidine that is probably involved in autophosphorylation), a sequence motif (N-X₁₅₋₄₅-DXGXG-X₂₀₋₅₀-GXG) characteristic of the histidine kinase protein family (Stock et al. 1989), and a segment with homology to the receiver domain typical of two-component response regulators are also present in FixL of *R. etli*.

In addition to these similarities, partial sequence analysis of the region downstream of *fixNf* revealed possible homologs of the *fixO*, *fixQ*, *fixP*, and *fixG* genes (data not shown). Downstream of the *fixL* gene there is a probable homolog of the *azu*

Table 1. 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 the pCFN42f	Brom et al. 1992
CFNX636	CE3 derivative, <i>fixL::loxSp</i>	This work
CFNX637	CE3 derivative, <i>fixKf::ΩKm</i>	This work
CFNX638	CE3 derivative, <i>fixKd::ΩSp</i>	This work
CFNX639	CE3 derivative, <i>fixKf::ΩKm-fixKd::ΩSp</i>	This work
CFNX640	CE3 derivative, <i>fixNf::loxSp</i>	This work
IBTmfixNd ^r	CE3 derivative, <i>fixNd::ΩKm</i>	This work
CFNX641	CE3 derivative, <i>fixNf::loxSp-fixNd::ΩKm</i>	This work
<i>Escherichia coli</i>		
DH5α	<i>supE44 ΔlacU169 (φ80lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Sambrook et al. 1989
BW21038	DE3 <i>lacX74 uidA (ΔMlu1)::pir⁺ miniTn5 Lac4 (Cm^r lac^r lacZ ΔM15) recA1 Δ(phnC?DEFGHIJKLMNOP)33-30 ΔphoA532</i>	Metcalf et al. 1996
BW20767	RP4-2 <i>tet::Mu-1 kan::Tn7 integrant leu-63::IS10 recA1 creC510 hsdR17 endA1 zbf-5 uidA(ΔMlu1)::pir⁺ thi</i>	Metcalf et al. 1996
HB101	<i>supE44 hsdS20 (r_B-m_B) recA13 ara-14 proA2 lacY1 galk2 rpsL20 xyl-5 mtl-1</i>	Sambrook et al. 1989
Plasmids		
pSK ⁺	Sequencing vector Ap ^r	Stratagene, La Jolla, CA, U.S.A.
pWM91	<i>sacRB</i> suicide vector Ap ^r	Metcalf et al. 1996
pMOSBlueT	Cloning vector for PCR products, Ap ^r	Amersham
pSUP202	Suicide cloning vector Ap ^r Cm ^r Tc ^r	Simon et al. 1983
pJQ200SK ⁺	Suicide cloning vector Gm ^r	Quandt and Hynes 1993
pWM5	Plasmid with <i>uidA</i> cassette	Metcalf and Wanner 1993
pBBR1MCS-5	Broad-host-range cloning vector, Gm ^r	Kovach et al. 1995
pBBMCS53	Δ <i>placZ</i> pBBR1MCS-5 derivative, carrying the promoterless <i>uidA</i> gene from pWM5, Gm ^r	This work
pLG1	pBBR1MCS5 derivative <i>fixKf::uidA</i>	This work
pLG2	pBBR1MCS5 derivative <i>fixNf::uidA</i>	This work
pLG3	pBBR1MCS5 derivative <i>fixKd::uidA</i>	This work
pLG4	pBBR1MCS5 derivative <i>fixNd::uidA</i>	This work
pLG5	<i>fixKf-fixL::uidA</i> expression vector in pBBMCS53	This work
pLG6	<i>fixL::uidA</i> expression vector (pLG5 derivative carrying a ΩTc ^r cassette in the <i>fixKf</i> gene).	This work
pLG7	pWM91 derivative <i>fixKf::ΩKm</i>	This work
pLG8	pSUP202 derivative <i>fixL::loxSp</i>	This work
pLG9	pWM91 derivative carrying <i>fixKd::ΩSp</i> allele	This work
pAD4	pSUP202 derivative <i>fixNf::loxSp</i>	This work
pOLF16	pJQ200SK ⁺ derivative <i>fixNd::ΩKm</i>	This work
pRK2073	Conjugation helper plasmid Sm-Sp ^r	Better and Helinski 1983
pRK2013	Conjugation helper plasmid Km ^r	Figurski and Helinski 1979

gene (data not shown), encoding a pseudoazurin that is possibly involved in electron transfer processes to dissimilatory nitrite reductases (Kakutani et al. 1981). Figure 3 shows the structural organization of the *fix*-reiterated genes in *R. etli* CFN42. The overall organization of the *fix*-reiterated region on pCFN42f displays a striking resemblance to the structural

organization reported for *R. leguminosarum* bv. *viciae* (Patschkowski et al. 1996).

Expression of *fix*-reiterated genes in *R. etli* CFN42.

To evaluate which factors govern the expression of the *fixK* reiterations, the promoter regions of each reiteration were fused with a promoterless β -glucuronidase gene. Plasmids containing the *fixKf::uidA* or the *fixKd::uidA* transcriptional fusion were introduced separately into *R. etli* strains CE3 (wild type), CFNX636 (*fixL::loxSp*), CFNX637 (*fixKf:: Ω Km*), CFNX638 (*fixKd:: Ω Sp*), and CFNX639 (*fixKf:: Ω Km*-*fixKd:: Ω Sp*). The presence of these plasmids and the absence of rearrangements in the native plasmids of the strains were verified by Eckhardt gels.

Transcriptional regulation of *fixK*-reiterated genes was studied under aerobic and microaerobic conditions. As shown in Table 2, expression of the *fixKf* reiteration was markedly induced (25-fold) under microaerobic conditions (line 1). In contrast, the *fixKd* reiteration displays a very low expression, unaffected by oxygen concentration (line 2) or the use of different mutant backgrounds (lines 4, 6, 8, and 10). Microaerobic expression of the *fixKf* reiteration is fully dependent on the presence of a wild-type *fixL* gene (Table 2, line 3). It is noteworthy that the induction level of the *fixKf* reiteration is increased in a *fixKf:: Ω Km* mutant background but remains at normal levels in a strain harboring a *fixKd:: Ω Sp* mutant allele (Table 2; compare lines 1, 5, and 7). Increased induction levels were also seen in a strain carrying the *fixKf:: Ω Km*-*fixKd:: Ω Sp* allelic combination (line 9). These results suggest that FixKf negatively regulates the expression of its own promoter.

Fig. 2. Shared sequence motifs between *Rhizobium etli* FixL and *R. meliloti* FixL and FixJ proteins. Corresponding sequence motifs are indicated by rectangles or ovals; H-T-H = helix-turn-helix motif on FixJ. Note the absence of a hydrophobic region in the amino terminus of *Rhizobium etli* FixL and the lack of a recognizable DNA-binding motif. Other critical residues are indicated on each protein.

Fig. 3. Structural organization of the *fix*-reiterated genes in *Rhizobium etli* CFN42. **A**, *fix* region in pCFN42f. **B**, *fix* region in pSym. Black rectangles or filled arrows = fragments used to construct transcriptional fusions with a promoterless *uidA* gene (empty arrows). Δ = mutagenized *fix* alleles. Small $\square$ = putative promoter elements. Arrowheads labeled A1, L1, or O2 = oligonucleotides used for construction. Restriction sites: P, *Pst*I; S, *Sal*I; Sc, *Sac*I.

To ascertain the role of each *fixK* reiteration in the expression of *fixNd* and *fixNf*, the promoter region of each *fixN* reiteration was fused to a promoterless β -glucuronidase gene. Since a potential FixK-binding site (5'-TTGATGTAGATCA-A-3') is present in both *fixN* promoter regions, control by a FixK-like regulatory protein was expected. Expression of *fixN*-reiterated genes was analyzed under free-living conditions. As shown in Table 3 (lines 1 and 2), microaerobic conditions provoke a strong induction of *fixNd* (50-fold) and *fixNf* (140-fold), which is completely abolished in a *fixKf:: Ω Km* mutant background (Table 3, lines 3 and 4). In contrast, microaerobic expression remains unaltered in a strain carrying the *fixKd:: Ω Sp* mutant allele (Table 3, lines 5 and 6). As expected, neither *fixN* reiteration was expressed in a *fixKf:: Ω Km-*fixKd:: Ω Sp** double mutant (Table 3, lines 7 and 8). These results indicate a differential regulatory role of the *fixK* reiterations: the FixKf protein is needed as a positive element for the expression of both *fixN* reiterations, whereas FixKd appears to be dispensable. Notably, both *fixN* copies are regulated by a FixK protein encoded in a plasmid (pCFN-42f) other than the pSym.

Interestingly, expression of the *fixN* reiterations also shows a differential dependence for FixL. Microaerobic expression of *fixNf* is drastically reduced in a *fixL::loxSp* mutant back-

ground (Table 3, line 9), whereas expression of *fixNd* still shows microaerobic induction, albeit at a reduced level compared with the wild type (25-fold versus 48-fold; compare line 2 to 10 in Table 3).

fixL and *fixKf* do not form an operon.

Sequence data of the *fix* region in pCFN42f show that the start of the *fixL* coding region is located 24 bp downstream of the *fixKf* stop codon. This proximity allows the possibility that *fixL* and *fixKf* are organized as an operon. If that is the case, an insertional mutation within the coding region of *fixKf* should abort the expression of *fixL* as a result of a polar effect. To discern between these alternatives, the upstream region of *fixL* (harboring the intergenic region of *fixNf-fixKf*, the complete coding region of *fixKf*, and the 5' end of *fixL*) was cloned in front of a promoterless β -glucuronidase gene, thus generating a *fixL::uidA* transcriptional fusion; this construction was then modified by insertion of the Ω Tc interposon into the coding region of *fixKf*.

Measurements of β -glucuronidase activity in both constructs reveal a low expression of *fixL*, independent of oxygen concentration in the media (Table 4, line 1). Levels of β -glucuronidase activity remained unaltered, despite the introduction of a Ω Tc interposon within *fixKf* (Table 4, line 2).

Table 2. Expression of *fixK::uidA* gene fusions in *Rhizobium etli* wild-type and mutant backgrounds under free-living conditions

Strain	Strain genotype	Fusion genotype	Mean β -glucuronidase-specific activity ^a ($\pm$ SD)	
			Aerobic	Microaerobic
CE3/pLG1	Wild type	<i>fixKf::uidA</i>	8 $\pm$ 4	210 $\pm$ 21
CE3/pLG3	Wild type	<i>fixKd::uidA</i>	3 $\pm$ 3	Not detectable
CFNX636/pLG1	<i>fixL::loxSp</i>	<i>fixKf::uidA</i>	1 $\pm$ 2	2 $\pm$ 2
CFNX636/pLG3	<i>fixL::loxSp</i>	<i>fixKd::uidA</i>	1 $\pm$ 2	3 $\pm$ 5
CFNX637/pLG1	<i>fixKf::ΩKm</i>	<i>fixKf::uidA</i>	10 $\pm$ 9	948 $\pm$ 52
CFNX637/pLG3	<i>fixKf::ΩKm</i>	<i>fixKd::uidA</i>	1 $\pm$ 2	1 $\pm$ 1
CFNX638/pLG1	<i>fixKd::ΩSp</i>	<i>fixKf::uidA</i>	2	235 $\pm$ 15
CFNX638/pLG3	<i>fixKd::ΩSp</i>	<i>fixKd::uidA</i>	5	1 $\pm$ 2
CFNX639/pLG1	<i>fixKf::ΩKm-<i>fixKd::ΩSp</i></i>	<i>fixKf::uidA</i>	6	914 $\pm$ 42
CFNX639/pLG3	<i>fixKf::ΩKm-<i>fixKd::ΩSp</i></i>	<i>fixKd::uidA</i>	10	1 $\pm$ 1

^a In nm/min/mg of protein; means from at least two independent determinations.

Table 3. Role of FixL and FixK in regulation of *fixN* gene reiterations

Strain	Strain genotype	Fusion genotype	Mean β -glucuronidase specific activity ^a ($\pm$ SD)	
			Aerobic	Microaerobic
CE3/pLG2	Wild type	<i>fixNf::uidA</i>	3 $\pm$ 3	422 $\pm$ 30
CE3/pLG4	Wild type	<i>fixNd::uidA</i>	14 $\pm$ 3	670 $\pm$ 43
CFNX637/pLG2	<i>fixKf::ΩKm</i>	<i>fixNf::uidA</i>	Not detectable	10 $\pm$ 7
CFNX637/pLG4	<i>fixKf::ΩKm</i>	<i>fixNd::uidA</i>	Not detectable	13 $\pm$ 4
CFNX638/pLG2	<i>fixKd::ΩSp</i>	<i>fixNf::uidA</i>	0.6	482 $\pm$ 3
CFNX638/pLG4	<i>fixKd::ΩSp</i>	<i>fixNd::uidA</i>	3	684 $\pm$ 2
CFNX639/pLG2	<i>fixKf::ΩKm-<i>fixKd::ΩSp</i></i>	<i>fixNf::uidA</i>	Not detectable	36 $\pm$ 0.2
CFNX639/pLG4	<i>fixKf::ΩKm-<i>fixKd::ΩSp</i></i>	<i>fixNd::uidA</i>	Not detectable	17 $\pm$ 0.4
CFNX636/pLG2	<i>fixL::loxSp</i>	<i>fixNf::uidA</i>	3 $\pm$ 2	38 $\pm$ 11
CFNX636/pLG4	<i>fixL::loxSp</i>	<i>fixNd::uidA</i>	14 $\pm$ 5	351 $\pm$ 14

^a In nm/min/mg of protein; means from at least two independent determinations.

Table 4. Analysis of *Rhizobium etli* *fixL* expression under free-living conditions

Strain	Strain genotype	Fusion genotype	Mean β -glucuronidase specific activity ^a ($\pm$ SD) under	
			Aerobic	Microaerobic
CE3/pLG5	Wild type	<i>fixKf-fixL::uidA</i>	73 $\pm$ 14	80 $\pm$ 14
CE3/pLG6	Wild type	<i>fixKf::ΩTc-<i>fixL::uidA</i></i>	64 $\pm$ 13	72 $\pm$ 20

^a Values are expressed as nm/min/mg of protein from three independent experiments.

These data contradict the possibility that *fixKf*–*fixL* constitute an operon, suggesting that a separate promoter region for *fixL* is located inside the *fixKf* coding region. The differential phenotype observed for mutations in *fixKf* and *fixL* on *fixKf* expression (loss of negative autoregulation and lack of expression, respectively) also militate against the possibility of *fixKf*–*fixL* being part of an operon.

Phenotypic analysis of *R. etli* *fix* mutants.

To evaluate the relative participation of each *fix* gene on nitrogen fixation, bean plants were inoculated with the whole set of mutants and evaluated afterward for nitrogenase activity (by acetylene reduction) and shoot dry weight (Table 5).

On the basis of the microaerobic expression data, the different mutants should be arranged in a hierarchical order (regarding *fixN* expression) of *fixKf*:: Ω Km < *fixL*::*loxSp* < *fixKd*:: Ω Sp, where the last mutant does not present any effect on the expression of either *fixN* copy. Interestingly, none of these mutants shows a significant reduction in any of the symbiotic parameters analyzed when tested with single mutants or the *fixKf*:: Ω Km–*fixKd*:: Ω Sp double mutant (Table 5).

Fig. 4. Regulatory model for *fix* gene expression in *Rhizobium etli* CFN42 under microaerobic conditions. + = positive regulation; – = negative regulation. X and ? = hypothetical DNA-binding proteins.

In contrast, the *fixNd*:: Ω Km mutant provokes a strong reduction in symbiotic efficiency, whereas a *fixNf*::*loxSp* mutation does not alter nitrogen fixation. Thus, contrary to our expectation, the symbiotic behavior of these mutants does not mirror the effect observed in free-living expression.

DISCUSSION

In this study, we used a combination of structural and gene fusion analyses to gain insight into the mechanisms employed in *R. etli* for the regulation of nitrogen fixation genes. Figure 4 shows our current view of the circuitry used for regulation of the *fixNOQP* genes under microaerobic conditions. This model integrates the participation of genes located in two different plasmids, thus supporting our contention that plasmids in *Rhizobium* constitute a fully integrated genomic complex (García-de los Santos et al. 1996). Moreover, this regulatory scheme shows several novel characteristics compared with the conventional systems, *R. meliloti* and *B. japonicum*.

Among these differences is that *R. etli* starts the regulatory cascade with a novel FixL product. This predicted polypeptide displays an arrangement that is suggestive of the fusion of the receiver and transmitter modules of a two-component regulatory system, yet lacks any recognizable motif for DNA binding (Fig. 2). This unusual array of characteristics is, to our knowledge, shared only with the FixL product of *R. leguminosarum* bv. *viciae* VF39 (Patschkowski et al. 1996).

Our results show that this novel FixL achieves transcriptional activation under microaerobic conditions of one of the *fixK*-reiterated copies, the *fixKf* gene. Because of the absence of a recognizable DNA binding motif, however, it is unlikely that FixL acts as a direct transcriptional activator. In other *Rhizobium* species, FixL acts in concert with FixJ, which is the direct transcriptional activator (Fischer 1994). We tried a variety of experimental approaches in our unsuccessful search for a *fixJ* homolog in *R. etli*, including low-stringency Southern hybridization with *fixJ* probes from different *Rhizobium* species and polymerase chain reaction (PCR) amplification primed with degenerate oligonucleotides designed as consensus from different *fixJ* reported sequences (data not shown). These data suggest that *R. etli* CFN42 lacks the *fixJ* structural homolog is as in *R. leguminosarum* bv. *viciae* VF39 (Patschkowski et al. 1996). Current efforts in our laboratory are aimed at clarifying which protein acts with FixL to activate the expression of *fixKf*.

In *R. etli* CNPAF512, however, there is clear evidence of a conventional *fixJ* homolog and a variant *fixL* gene lacking the

Table 5. Participation of the reiterated *fix* genes in nitrogen fixation^a

Strain	Relevant genotype	Total nitrogenase activity ^b	Specific nitrogenase activity ^c	Shoot dry weight ^d
CE3	Wild type	2.31 ± 0.54	26.3 ± 8.2	0.67 ± 0.05
CFNX636	<i>fixL</i> :: <i>loxSp</i>	2.54 ± 1.07	32.5 ± 17.4	0.59 ± 0.12
CFNX637	<i>fixKf</i> :: <i>loxSp</i>	2.56 ± 1.05	29.7 ± 5.1	0.56 ± 0.17
CFNX638	<i>fixKd</i> :: Ω Sp	2.72 ± 1.61	32.7 ± 23.3	0.69 ± 0.11
CFNX639	<i>fixKf</i> :: Ω Km– <i>fixKd</i> :: Ω Sp	2.48 ± 0.53	27.2 ± 10.5	0.63 ± 0.12
CFNX640	<i>fixNf</i> :: <i>loxSp</i>	2.47 ± 0.46	26.5 ± 4.6	0.66 ± 0.11
IBTmfixNd	<i>fixNd</i> :: Ω Km	0.05 ± 0.01	1.8 ± 0.7	0.27 ± 0.04
CFNX641	<i>fixNf</i> :: <i>loxSp</i> – <i>fixNd</i> :: Ω Km	0.05 ± 0.01	1.4 ± 0.7	0.26 ± 0.05

^a Analyzed 32 days after inoculation.

^b μ M ethylene per plant per h.

^c μ M ethylene per g nod per h.

^d Grams per plant.

central heme-binding domain (D'hooghe et al. 1995; D'hooghe et al. 1998). This suggests appreciable intraspecies diversity in regard to the presence of important elements for regulation of *fix* genes. To clarify which arrangement is more prevalent, we screened a collection of 27 *R. etli* isolates from different geographic locations. More than two-thirds shows a CFN 42-like *fixL* homolog and the absence of a *fixJ* homolog (L. Girard, S. Brom, and J. Sanjuan, *unpublished*). The arrangement reported here thus appears to be predominant to the species.

Two *fixK* genes were found in *R. etli* CFN42. One of these, *fixKf* (located in pCFN42f), shows *fixL*-dependent microaerobic expression. Moreover, the expression pattern of a *fixKf::uidA* fusion in different mutant backgrounds is consistent with negative autoregulation for this gene. The other gene (*fixKd*, located on the pSym) appears to be constitutive, displaying marginal expression under all conditions tested.

Both *fixN* copies detected in this work are positively regulated by the FixKf gene product, whereas the FixKd product is apparently dispensable (Fig. 4). This situation is clearly different from the one reported for *R. leguminosarum* bv. *viciae* VF39, where expression of both *fixNOQP* reiterations is independent of FixK, depending instead on *fmrN* (Schlüter et al. 1997). In this regard, the architecture of the regulatory mechanism in *R. etli* is more similar to that of *B. japonicum*, where only one of the two *fixK* reiterations (*fixK₂*) regulates the *fixNOQP* operon (Fischer 1994).

A novel aspect of our work is related to differential regulation of the *fixN* reiterations by FixL. In most systems, including *R. meliloti* (David et al. 1988), *B. japonicum* (Preisig et al. 1993), and *A. caulinodans* (Mandon et al. 1994), expression of *fixN* requires FixL, FixJ, and FixK. In *R. leguminosarum* bv. *viciae* VF39, expression of *fixN* is independent of FixK, but it still requires FixL (Schlüter et al. 1997). In *R. etli* CFN42, these genes exhibit a differential dependence for FixL, although expression of *fixNf* and *fixNd* requires FixKf. Expression of *fixNf* is fully dependent on FixL, but transcription of *fixNd* can proceed at adequate levels (50% of the wild-type levels, Table 3) in the absence of FixL. These data are even more striking when it is considered that the absence of FixL halts the production of FixKf. This evidence strongly suggests the existence of an additional regulatory protein that participates in the activation of the *fixNd* promoter in *R. etli* (Fig. 4). Interestingly, the action of this hypothetical regulatory protein is detected only in the absence of FixL, suggesting that FixL negatively regulates (directly or indirectly) the expression of this protein.

A plausible alternative to achieve FixL-independent microaerobic induction of *fixNd* could be an *fmr*-like regulatory protein, as described for *R. leguminosarum* bv. *viciae* strains VF39 (Schlüter et al. 1997) and UMP791 (Gutiérrez et al. 1997). According to this hypothesis, this type of protein should be able to bind preferentially to *fixNd* but not, or only weakly, to *fixNf*. The situation is further complicated by the existence of two *fmr* homologs in *R. etli* CFN42 (Soberón et al. 1999a). If either of the *fmr* homologs participates in the regulation of *fixNd*, our data predict that its expression must be negatively regulated by FixL. Preliminary data indicate that this is indeed the case for one of the *fmr* homologs (M. Soberón and L. Girard, *unpublished*).

Perhaps the more puzzling aspect of our results is the dissimilarity between the behavior observed under microaerobic

conditions and the symbiotic phenotype of the different mutants analyzed. On the basis of free-living results, the more extreme phenotype regarding symbiotic effectiveness should have been observed for the strain carrying a *fixKf:: Ω Km* allele, where microaerobic expression of both *fixN* reiterations is completely blocked. Surprisingly, the symbiotic effectiveness of this mutant was unaffected. Wild-type symbiotic effectiveness was also observed for strains carrying the *fixKd:: Ω Sp*, *fixL::loxSp*, *fixNf::loxSp*, or the *fixKf:: Ω Km*–*fixKd:: Ω Sp* allelic combination. A significant reduction in nitrogen fixation ability, to almost zero levels, was observed only in strains harboring the *fixNd:: Ω Km* allele.

Taken together, these results indicate that the *fixNd* reiteration is the main contributor to nitrogen fixation ability in *R. etli*, with *fixNf* reiteration having an ancillary role. Moreover, the discrepancy between the microaerobic and in planta results casts some doubt on the applicability of the regulatory microaerobic system hereby described to symbiotic conditions. Expression analysis in symbiotic conditions is clearly needed for a full interpretation of symbiotic mutant phenotypes. Although it is still possible that this regulatory scheme is acting during symbiosis, our results raise the intriguing possibility that there are other regulatory inputs superimposed, or even overriding, the inputs exerted by oxygen concentration that act to determine gene expression during symbiosis. This is an important question to be addressed in the near future.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions.

The bacterial strains and plasmids used are listed in Table 1. *Rhizobium* strains were grown at 30°C in PY-rich medium (Noel et al. 1984) or in Y-minimal medium (MMY) with 10 mM of succinate and 10 mM of ammonium chloride as carbon and nitrogen sources, respectively (Bravo and Mora 1988). *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium. The following antibiotics were added per milliliter: 100 μ g of carbenicillin (*E. coli*); 15 μ g of chloramphenicol (*E. coli*); 10 or 15 μ g of gentamycin (*R. etli* and *E. coli*, respectively); 30 μ g of kanamycin (*E. coli* and *R. etli*); 20 μ g of nalidixic acid (*R. etli*); 100 μ g of spectinomycin (*E. coli* or *R. etli*); and 4 or 10 μ g of tetracycline (*R. etli* and *E. coli*, respectively). Sucrose was added when required at 12.5% (wt/vol). For detection of β -galactosidase and β -glucuronidase activity, 30 μ g of 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) per milliliter and 30 μ g of 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-gluc) per milliliter were used, respectively, in plates. Cells for fusion analysis were grown until the midexponential phase (Girard et al. 1996) in MMY with succinate and ammonium chloride as carbon and nitrogen sources, respectively. Microaerobic conditions during growth were set up by putting the cultures in 150-ml bottles that were flushed with several volumes of a 1% oxygen–99% argon mixture and closed with an airtight stopper.

Microbiological and recombinant DNA methods.

Plasmid DNA was isolated by an alkaline–sodium dodecyl sulfate lysis method and transformed with standard protocols into CaCl₂-treated *E. coli* cells (Sambrook et al. 1989). Conjugative transfer of plasmids from *E. coli* to *Rhizobium* routinely was done by triparental matings with the use of

pRK2013 or pRK2073 as helpers. Some constructions were done by biparental mating with S17.1 as a donor strain (Simon 1984). A modified Eckhardt procedure was used to determine plasmid profiles (Hynes and McGregor 1990).

All restriction endonucleases, T4 polynucleotide kinase, T4 DNA ligase, α^{32} P-CTP and γ^{32} P-ATP, hybridization buffer, and labeling systems were purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, England). For Southern hybridizations, DNA was digested with appropriate restriction enzymes, electrophoresed in 1% (wt/vol) agarose gels, and blotted onto nylon (Hybond N+, Amersham, Arlington Heights, IL, U.S.A.). Hybridization under high-stringency conditions was carried out with Rapid-Hyb (Amersham) buffer; low-stringency conditions were established with 30% formamide at 42°C (Girard et al. 1991). Probes were linearized and labeled with α^{32} P-CTP by random priming with the Rediprime system (Amersham).

To isolate the pCFN42f *fix* homologous region, a cosmid library from *R. etli* CFN42 (constructed with *Eco*RI fragments in pLAFR1) was screened by Southern blot hybridization at high stringency with intragenic fragments from pCFN42d *fixK* and *fixP* genes used as probes.

Sequence analysis and PCR amplification.

Double-stranded DNA was sequenced with a thermosequencing cycle sequencing kit (Amersham). Oligonucleotides were labeled by kinasing with [γ - 32 P] ATP (Amersham). Sequencing reactions were electrophoresed in 6% polyacrylamide–8M urea gels. Initial stages of sequencing were done by subcloning the cosmid clones into pSK⁺ with commercial oligonucleotides (–21 and reverse) as primers. The double-stranded sequence from the *fixL*, *fixKf*, and *fixNf* genes was obtained by primer walking with custom-made oligonucleotides (Biosynthesis Inc., Lewisville, TX, U.S.A.). The GenBank accession number for the sequence reported here is AF273679.

PCR amplifications and cycle sequencing reactions were performed in an Eppendorf Mastercycler 5330 (Hamburg, Germany). PCR amplifications were carried out by 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 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.

Plasmid construction.

To generate *uidA* transcriptional fusions with the desired promoters, four intermediate vectors carrying the appropriate intergenic regions were constructed in the broad-host-range plasmid pBBR1MCS-5 (Kovach et al. 1995). Since each intergenic region contains two promoters that transcribe in divergent orientations, each construction was aimed to generate alternative orientations for each region.

For the *fixNd*–*fixKd* intergenic region, a 978 bp *Pst*I fragment from plasmid pOLf02 (O. López and M. Soberón, unpublished) was cloned in both orientations into *Pst*I-digested pBBR1MCS-5. Plasmids containing each orientation for this region (clockwise-P*fixKd* or clockwise-P*fixNd*) were modified by substituting a *Kpn*I–*Sph*I fragment containing the *lacZ* promoter in both plasmids with a 2,204-bp *Kpn*I–*Sph*I fragment from pWM5 (Metcalfe and Wanner 1993), thus fusing the

corresponding promoter with the promoterless structural gene for β -glucuronidase (plasmids pLG3 and pLG4).

A 1,838-bp *Sal*I–*Pst*I fragment from the *fixNf*–*fixKf* intergenic region was filled in with Klenow polymerase and cloned into *Sma*I-digested pBBR1MCS-5 (clockwise-P*fixKf*) or into *Sal*I–*Pst*I-digested pBBR1MCS-5 (clockwise-P*fixNf*). Transcriptional fusions with the corresponding promoter were then generated, as described previously (plasmids pLG1 and pLG2).

In order to construct a fusion of *fixL* with the β -glucuronidase structural gene, plasmid pBBR1MCS-5 first was modified by substituting a *Kpn*I–*Sph*I fragment with a 2,204-bp *Kpn*I–*Sph*I fragment from pWM5, thus generating pBBMCS53. A 3,217-bp *Sal*I fragment (including the 5' end of *fixNf*, the complete *fixKf* coding region, and the 5' end of *fixL*) was cloned in the appropriate orientation into *Sal*I-digested pBBMCS53, thus generating a *fixKf*–*fixL*::*uidA* transcriptional fusion (pLG5). This plasmid was further modified by inserting a Ω Tc cassette (Prentki and Krisch, 1984) into the *Stu*I site of *fixKf*, giving rise to pLG6.

To introduce a mutation into *fixL*, total DNA from strain CFNX89 (lacking the pSym) was used as a template in PCR amplifications employing primers L1 (5'-TGCTGGCGACG-AAGTTTCAC-3') and A1 (5'-CGCTGCCGAGACAATCAAGG-3'). The resulting 2,115-bp PCR product (containing most of the *fixL* gene and the 5' end of the *azu* gene) was cloned by TA annealing into the pMOSBlue vector (Amersham). In the resulting plasmid, a *fixL*::*loxSp* deletion–substitution allele was generated by deleting a 37-bp *Sal*I fragment of *fixL* (codons 363 to 375), inserting in its place a *loxSp* interposon (Martínez-Salazar and Romero 2000). Construction of a suicide derivative useful for homogenotization was done by cloning a *Pst*I–*Eco*RI fragment containing this mutation into the mobilizable plasmid pSUP202 (plasmid pLG8).

Insertional mutagenesis of *fixKf* was done in a similar manner. The corresponding genomic region was PCR-amplified from strain CFNX89 with the specific primers O2 (5'-GCCG-CATACCTTCCACTTTCTC-3') and A1. The 5,081-bp PCR product (containing the 5' end of *fixOf*, the complete coding regions for *fixNf*, *fixKf* and *fixL*; and the 5' end of the *azu* gene) was cloned into pMOSBlue. The resulting plasmid was modified by inserting a blunt-ended Ω Km cassette (Prentki and Krisch 1984) into the *Stu*I site of *fixKf* (codon 33). For homogenotization, a *Spe*I–*Sac*I fragment containing the *fixKf*:: Ω Km allele was cloned into the suicide pWM91 (Metcalfe et al. 1996), giving rise to pLG7.

To facilitate the cloning steps needed to generate a *fixKd* mutation, the plasmid vector pSK⁺ was digested with *Sma*I–*Eco*RV and religated by removing a segment that carried *Sma*I, *Pst*I, *Eco*RI, and *Eco*RV from the polylinker. A 2,832-bp *Sac*I fragment from pOLf02 carrying part of the *fix*-reiterated region on pCFN42d (O. López and M. Soberón, unpublished) was subcloned into this modified pSK⁺ vector. Mutagenesis of the *fixKd* gene was achieved by introducing a *Sma*I–*Sma*I Ω Sp cassette into the *Eco*RV site (codon 121). From this derivative, the *Sac*I fragment carrying the *fixKd*:: Ω Sp allele was subcloned into pWM91, giving rise to pLG9.

A 2,447-bp *Pst*I fragment from cos772 (containing the 5' end of *fixOf*, the complete *fixNf* gene, and the 5' end of *fixKf*) was cloned into a pSK⁺ derivative lacking *Sal*I and *Xho*I restriction sites in the polylinker. A *Sal*I–*Sal*I *loxSp* cassette

(Martínez-Salazar and Romero 2000) was inserted into the unique *SalI* site of *fixNf* (codon 379). The *PstI* fragment carrying the *fixNf::loxSp* allele was cloned into the *PstI* site of pSUP202, generating plasmid pAD4.

To mutagenize *fixNd*, a 2,832-bp *SacI* fragment from pOLF02 carrying part of the *fix*-reiterated region on pCFN42d was subcloned into a modified pSK⁺ derivative lacking a *SalI* restriction site in the polylinker. In this plasmid, a *fixNd::ΩKm* deletion–substitution allele was generated by deleting a 575-bp *SalI* fragment of *fixNd* (codons 186 to 378). Insertion of a *HindIII–HindIII ΩKm* cartridge (Prentki and Krisch 1984) was achieved by rendering both fragments blunt by end-filling with Klenow followed by ligation. The fragment containing the *fixNd::ΩKm* deletion–substitution allele was then removed by *SacI* digestion, blunted, and inserted into *SmaI*-restricted pJQ200SK⁺ (Quandt and Hynes 1993), giving rise to plasmid pOLF16.

Construction of *fix* mutants.

Introduction of all the *fix* mutant alleles into *R. etli* was carried out by homologotization. To introduce the *fixL::loxSp* deletion–substitution allele, *E. coli* HB101/pLG8 was mated in a triparental cross with *R. etli* CE3 as recipient and HB-101/pRK2013 as helper. Double recombinants were selected as Nal^r Sp^r Tc^s transconjugants. This procedure yielded strain CFNX636. Introduction of the *fixNf::loxSp* allele was done in the same way, but pAD4 was used as donor plasmid (strain CFNX640).

Homogenization of some mutant alleles was greatly aided by the existence of the *sacB* marker in some of the donor plasmids. This marker allows an easy selection for homologotization events by scoring for resistance to sucrose. *R. etli* strains containing *fixKf::ΩKm* (strain CFNX-637), *fixKd::ΩSp* (strain CFNX638), or *fixNd::ΩKm* (strain IBTmfixNd[−]) alleles were constructed in this way with plasmids pLG7, pLG9, or pOLF16 as donors, respectively.

To construct a derivative harboring the *fixKf::ΩKm–fixKd::ΩSp* allelic combination, *E. coli* BW21038/pLG9 was mated in a triparental cross with *R. etli* CFNX637 (*fixKf::ΩKm*) as recipient and *E. coli* HB101/pRK2013 as conjugation helper. Double recombinants were selected as Nal^r Km^r Sp^r and sucrose-resistant transconjugants. This procedure yielded strain CFNX639. Construction of a derivative carrying the *fixNf::loxSp–fixNd::ΩKm* allelic combination (strain CFNX641) was done in a biparental cross with *E. coli* S17-1/pAD4 as donor and *R. etli* strain IBTmfixNd[−] (*fixNd::ΩKm*) as recipient. Double recombinants were selected as Nal^r Kmr Spr Tcs transconjugants. To verify that the desired gene replacement had occurred, the plasmid profiles and total DNA blots of all the derivatives were analyzed by Southern hybridization against the appropriate *fix* and cassette probes.

Measurement of β-glucuronidase activity.

Aerobic and microaerobic cultures of *R. etli* strains harboring transcriptional fusions were grown (Girard et al. 1996). Quantitative *uidA* activity was performed on 1-ml culture samples with *p*-nitrophenyl glucuronide as substrate (Corvera et al. 1999). Data were normalized to total cell protein concentration by the Lowry method over a second set of 1-ml samples.

Plant assays.

Phaseolus vulgaris cv. Negro Jamapa plants were inoculated with the desired strain and grown in a greenhouse (Girard et al. 1996). After 32 days, acetylene reduction assays for nitrogenase activity were carried out, as described by Romero et al. (1988).

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