FxkR Provides the Missing Link in the *fixL*-*fixK* Signal Transduction Cascade in *Rhizobium etli* CFN42

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Transcriptional control of the *fixK* gene in *Rhizobium etli* and *R. leguminosarum* bv. *viciae* is governed by a two-component signal transduction system that diverts from the conventional FixL-FixJ cascade that occurs in model rhizobia. Although a *fixL* gene, encoding a hybrid histidine kinase (hFixL), is present in *R. etli*, no *fixJ*, the cognate response regulator, has been identified. In this work, we present evidence that the pRet42f-located open reading frame RHE_PF00530 (*fxkR*) encodes a novel response regulator indispensable for *fixKf* activation under microaerobic growth. Moreover, results from complementation assays demonstrate that the activation of *fixKf* expression requires the presence of both hFixL and FxkR, and that the *fxkR* ortholog from *R. leguminosarum* bv. *viciae* is able to substitute for FxkR transcriptional control in *R. etli*. In addition, in these two organisms, hFixL- and FxkR-related proteins were identified in other bacteria, located in close proximity to a *fixK*-related gene. Using reporter fusions, site-directed mutagenesis, and electrophoretic mobility shift assays, we identified the FxkR binding site upstream from the transcriptional start site of *fixKf*. Similar to our previous observations for *fixL* and *fixKf* mutants, a null mutation in *fxkR* does not affect the symbiotic effectiveness of the strain. Thus, our findings reveal that FxkR is the long-standing missing key regulator that allows the transduction of the microaerobic signal for the activation of the FixKf regulon.

Nitrogen fixation by soil bacteria in association with leguminous plants requires the concerted expression of genes by both partners. Many regulatory circuits operate inside the nodule to carry out bacterial differentiation into nitrogen-fixing bacteroids and for the nitrogen fixation process per se. Research on the regulation of biological nitrogen fixation has gained interest during the last four decades. Forward and reverse genetic approaches have paved the way to describe key regulators and

their involvement in different regulatory cascades in rhizobia. Regulatory pathways of genes involved in nitrogen fixation are integrated in two different networks in a species-specific manner (Fischer 1994; Kaminski et al. 1998). Expression of *nif* and *fix* genes is under the control of several regulatory proteins, where low oxygen concentration operates as the most important regulatory signal. For most rhizobia, two regulatory cascades have been described. One includes the NifA and RpoN regulators, while FixL, FixJ, and FixK compose the other regulatory cascade, specific for symbiotic diazotrophs (Dixon and Khan 2004; Fischer 1994).

The regulatory control of nitrogen fixation genes involving FixLJ and FixK possess, as a common function, the regulation of *fixNOQP* and *fixGHIS* genes. FixL and FixJ are members of the two-component regulatory systems and FixK is a transcriptional regulator that belongs to the Crp/Fnr protein family. *fixK* and the cytochrome oxidase complex encoded by *fixNOQP* have been shown to participate in not only nitrogen fixation but also important physiological pathways such as photosynthesis, carbon, nitrogen, or iron metabolism, and hypoxia or anoxia responses in α -proteobacteria such as *Rhodospseudomonas palustris* and *Caulobacter crescentus* (Bose and Newman 2011; Britos et al. 2011; Crosson et al. 2005; Rey and Harwood 2010). The *fixL* and *fixJ* genes were described as a two-gene operon crucial for the activation of diverse nitrogen fixation genes, including *fixK* and *nifA*, under symbiotic and free-living microaerobic conditions in *Sinorhizobium meliloti* (David et al. 1988). At low oxygen concentration, 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). FixJ-P acts, in turn, as a positive regulator of the *fixK* gene necessary for the *fixLJ* regulatory function (Batut et al. 1989). To date, the presence of homologues of the *S. meliloti* *fixL*, *fixJ*, and *fixK* genes is a common feature among the different nitrogen fixers. However, the connectivity among the different cascades, the specific regulatory role of each element, and their target genes may vary (Anthamatten et al. 1991, 1992; Bauer et al. 1998; Fischer 1994; Kaminski et al. 1998).

Rhizobium etli CFN42 fixes nitrogen in association with bean plants and its genome contains a chromosome and six large plasmids (pRet42a to pRet42f) whose sizes range from 184.4 to 642.5 kb (González et al. 2006). Previously, we described that *R. etli* CFN42 employs a complex regulatory circuit for *fix* genes that integrates several novel characteristics

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Nucleotide sequence of the *Rhizobium leguminosarum* bv. *viciae* VF39 *fxkR* gene is available in the EMBL Nucleotide Sequence Database under accession number FR854234.

*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary figures and three supplementary tables are published online.

when compared with what are considered the conventional systems, *S. meliloti* and *Bradyrhizobium japonicum*. Interestingly, important regulatory elements, such as *fixL* and *fixK* that are found on the symbiotic plasmid of *S. meliloti*, appear on a different replicon in *R. etli*. *fixL* encodes an orphan histidine kinase located in a pRet42f region containing copies of the *fixK*, *fixNOQP*, and *fixGHIS* genes. *fixNOQP* reiterations are controlled by a novel *fixL*-*fixKf* cascade, where a *fixJ* gene is absent and the response regulator that activates transcription of *fixKf* remains to be described (Girard et al. 1996, 2000; González et al. 2003, 2006). The FixL protein of *R. etli* CFN42 is a hybrid histidine kinase (hHK) with no predicted transmembrane segments and, herein, we will differentiate the typical FixL from the hybrid one by denoting the latter group as hFixL. Also, no response regulator that could be proposed as the cognate response regulator is encoded in the vicinity of *hfixL*.

The genome of *R. etli* CFN42 codes six *fnr*-related genes: *fixKf*, *fnrNch*, *fnrNd*, *nnrR*, *stoRd*, and *stoRf* (González et al. 2006). The expression of these genes in *R. etli* depends on the FixKf regulator in microaerobic free-living conditions (Girard et al. 2000; Granados-Baeza et al. 2007; López et al. 2001). However, the complete regulatory pathway that controls expression of the FixK regulatory targets where an *hfixL* gene is involved has not yet been reported. The response regulator that activates *fixK* in coordination with hFixL might be a central distributor of the hypoxic response not only for nitrogen fixation in rhizobia but also for other important microaerobic traits in α -proteobacteria. The lack of sequences with a high percentage of identity to FixJ and the absence of the two reported consensus sequences for binding of FixJ (Ferrières and Kahn 2002) in the regulatory region of *fixKf* are suggestive of a novel two-component system operating in *R. etli*.

A similar distribution of *fix* genes is shared by *R. leguminosarum* bv. *viciae* VF39 (Patschkowski et al. 1996). The regulatory circuit of *fix* genes is also composed of an orphan hFixL histidine kinase and two *fnr* homologues (*fixK* and *fnrN*). Both *fnr* homologues seem to be necessary for optimal nitrogen fixation, although *fnrN* appears to be the central regulator of the *fixNOQP* reiterations (Schlüter et al. 1997).

hHK that share features of hFixL were found in different genomes, and all these proteins have unorthodox H boxes when compared with the classic HK domain in other FixL histidine kinases (Boesten and Priefer 2004). The His²⁰⁰ and Arg²²⁰ residues in *B. japonicum* FixL that are involved in heme coordination and oxygen binding (Dunham et al. 2003; Gong et al. 1998) are conserved in most described FixL and hFixL proteins (Boesten and Priefer 2004). It is important to remark that the only two models where an *hfixL* has been studied lack a *fixJ* gene, and the cognate response regulator of hFixL is waiting to be discovered (González et al. 2006; Young et al. 2006). Bacteria that encode hFixL-related proteins might share this novel response regulator.

This work uncovers a novel central response regulator of a widely studied regulatory circuit in *R. etli* CFN42 that provides the missing link in the *fixL*-*fixK* regulatory network for *fix* gene expression. Also, our findings permit us to propose that similar circuits could operate in different genera of rhizobia and probably also in non-nitrogen-fixing α -proteobacteria.

RESULTS

Probing for the genetic elements needed for *fixKf* activation in *R. etli*.

Total sequence analysis revealed that no sequences with a high percentage of identity to FixJ are present in *R. etli* CFN42. Nevertheless, because plasmids pRet42d and pRet42f

contain most of the *fix* genes (Girard et al. 1996, 2000; González et al. 2003, 2006), the participation of these two megaplasmids in *fixKf* microaerobic expression was evaluated. Plasmid pLG1 containing a *fixKf::uidA* transcriptional fusion (Girard et al. 2000) was introduced in different plasmid-containing derivatives of *R. etli* CFN42. The results show that the microaerobic expression of *fixKf* was observed in all tested genetic backgrounds, with the exception of strains lacking pRet42f (CFNX186, a CE3 derivative cured of Ret42f or CFNX218 carrying only a partially deleted pRet42e that retains the fragment that is indispensable for cell viability) (Fig. 1) (Brom et al. 1992). Expression of a gene expressed constitutively (*argC*) is not affected by the loss of this replicon (data not shown). Considering that the presence of hFixL is indispensable for *fixKf* microaerobic expression (Girard et al. 2000) and that this gene is located on pRet42f, loss of *fixKf* expression in strains lacking this plasmid (CFNX186 and CFNX218) was expected (Girard et al. 2000). Consequently we cannot affirm that the response regulator responsible for *fixKf* activation is encoded on this plasmid. Nevertheless, microaerobic expression of *fixKf* in a derivative carrying only pRet42f and the deleted pRet42e (CFNX218/pRet42f) proceeded at similar levels when compared with the wild-type strain (Fig. 1). Therefore, it can be concluded that the activator of *fixKf* expression could be located in pRet42f, the partially deleted pRet42e, or the chromosome. Preservation of *fixKf* expression in a CE3 derivative that was cured of pRet42d (strain CFNX89) (Brom et al. 1992) revealed that this plasmid was dispensable for the activation of this gene. The subtle induction of expression observed in this strain could be explained by the absence of StoRd, a negative transcriptional regulator in *R. etli* CFN42 (Granados-Baeza et al. 2007).

Identification of FxkR as the cognate response regulator for the hFixL sensor kinase in *R. etli*.

To identify a response regulator analogous to *fixJ*, we looked for the presence of response regulators in the *R. etli* CFN42 genome, using the database resource P2CS (Barakat et al. 2011). According to this database, 68 open reading frames (ORF) were predicted as response regulators; of those, 23 are

Fig. 1. Participation of *Rhizobium etli* pRet42d and pRet42f plasmids in the regulation of *fixKf* microaerobic expression. Expression of *fixKf* was analyzed under low oxygen concentration (1%) in different *R. etli* derivatives: CE3 (wild type), CFNX89 (cured of pRet42d), CFNX186 (cured of Ret42f), CFNX218 (carrying only a partially deleted pRet42e), and CFNX218 containing pRet42f. Cells were microaerobically induced for 10 h. β -glucuronidase specific activity is expressed as nanomoles of product per minute per milligram of protein. Data are the mean of two replicas from three independent experiments.

codified in different megaplasmids. In all, 14 response regulators were encoded in pRet42f while only 2 were found in the partially deleted pRet42e. Two proteins from plasmid pRet42f (RHE_PF00225 and RHE_PF00548) had significant sequence identity with FixJ from *S. meliloti*: 40 and 37%, respectively (genetic localization of the putative response regulators in pRet42f is illustrated in Supplementary Figure S1).

Following the rationale that pRet42f encodes the hFixL sensor and the FixKf regulator, in combination with the fact that pRet42f is able to complement strain CFNX218 for *fixKf* expression, we hypothesized that the activator of *fixKf* might also be located in this replicon. By vector-insertion mutagenesis (discussed below), we inactivated the two *fixJ*-related genes and six additional genes that encode putative response regulators in pRet42f. Genes RHE_PF00530 and RHE_PF00533, belonging to the OmpR/PhoB and NarL/FixJ superfamily, respectively, encode orphan response regulators. Genes RHE_PF00225, RHE_PF00479, and RHE_PF00548 belong to the NarL/FixJ superfamily and appear to be part of an operon that includes a histidine kinase. Finally, the three additional genes RHE_PF00072, RHE_PF00547, and RHE_PF00224 have no identifiable DNA-binding domain in the SMART database (Letunic et al. 2009), and the first one has an additional nucleotide-binding domain. These genes also appear to be part of a probable operon that includes a histidine kinase (Rhizobase Database).

The microaerobic expression of *fixKf* was studied by introducing plasmid pLG1 containing a *fixKf::uidA* transcriptional fusion (pBBMCS53::fixKf) (Girard et al. 2000) in the wild type and in various response regulator mutant backgrounds (SR72/pLG1, SR224/pLG1, SR225/pLG1, SR479/pLG1, SR530/pLG1, SR533/pLG1, SR547/pLG1, and SR548/pLG1). Our results clearly show that the microaerobic expression of *fixKf* is fully dependent on a functional protein encoded by ORF RHE_PF00530 (Table 1, line 6). No other response regulator mutant, including the FixJ-related proteins, had an effect on the microaerobic expression of *fixKf*. Gene RHE_PF00530 encodes a polypeptide of 241 amino acids and is located 19.5

kb downstream from the *hfixL* gene. Here, we designate this gene *fxkR* (for *fixK* regulator). It is noteworthy that FxkR is a response regulator that does not belong to the NarL/FixJ superfamily but to the OmpR/PhoB one.

To determine whether the regulatory activity of FxkR depends on an active hFixL, the expression pattern of *fixKf* in the wild-type strain and the *fixL::loxSp* (CFNX636) and *fxkR::loxSp* (CFN-LG1) mutants was monitored, making use of the reporter fusion in pLG1. The microaerobic expression of *fixKf* was completely dependent on the hFixL and FxkR regulators (Table 2, compare lines 1 to 3). Complementation analysis of the *fxkR* mutant strain with plasmid pfxkR-Ret, containing the wild-type *fxkR* gene under the control of the *lacZ* promoter, restored the maximal *fixKf* microaerobic expression (Table 2, compare lines 1 and 3 with line 5) but failed to restore *fixKf* expression to wild-type levels in the *hfixL* mutant (Table 2, compare lines 1 and 2 with line 4). The higher level of *fixKf* expression in the complemented *fxkR* mutant was probably due to multiple copies of the plasmid-borne activator. These results clearly demonstrate that both FxkR and hFixL are required for activation of *fixKf* expression.

These results also suggest that dependence for hFixL is not through the transcriptional control of the FxkR regulator. To corroborate this, the promoter region of *fxkR* was fused with a promoterless β -glucuronidase (GUS) gene (pARG2). This plasmid was introduced into *R. etli* strains CE3 (wild-type), CFNX636 (*fixL::loxSp*), and CFN-LG1 (*fxkR::loxSp*). Transcriptional regulation of *fxkR* was studied under aerobic and microaerobic conditions in the wild-type and in the *hfixL* and *fxkR* mutants. Measurements of GUS activity in the wild-type strain revealed that *fxkR* is expressed constitutively in both aerobic (552 ± 133) and microaerobic (642 ± 54) conditions. Similarly, levels of *fxkR* expression remained unaltered in the *hfixL* (aerobic 558 ± 138 and microaerobic 625 ± 138) and *fxkR* (aerobic 554 ± 155 and microaerobic 634 ± 80) mutants, regardless of the oxygen concentration in the culture. Therefore, it can be concluded that *fxkR* is expressed constitutively under the tested conditions, and that FxkR does not regulate

Table 1. Identification of the response regulator that controls *fixKf* microaerobic expression in *Rhizobium etli* CFN42

Strain	Gene mutated	Domain architecture and genetic organization	<i>fixKf</i> microaerobic expression (nmol min ⁻¹ mg ⁻¹ of protein) ^a
CE3		Wild type	381 ± 12
SR 72	RHE_PF00072	No HTH domain, with an additional nucleotide binding domain; orphan	330 ± 15
SR 224	RHE_PF00224	No HTH domain, probably in an operon that includes one histidine-kinase (HK) and two additional receiver domains	292 ± 22
SR 225	RHE_PF00225	NarL/FixJ superfamily, probably in the same operon as RHE_PF00224	282 ± 17
SR 479	RHE_PF00479	NarL/FixJ superfamily, probably in operon with RHE_PF00480 (HK)	289 ± 18
SR 530	RHE_PF00530	OmpR/PhoB superfamily; orphan	4 ± 4
SR 533	RHE_PF00533	NarL/FixJ superfamily; orphan	315 ± 17
SR 547	RHE_PF00547	No HTH domain, probably in operon with RHE_PF00548	299 ± 24
SR 548	RHE_PF00548	NarL/FixJ superfamily, probably in operon with RHE_PF00547	302 ± 17

^a β -Glucuronidase-specific activity was determined after 10 h at 30°C under microaerobic conditions. Data are the mean of two replicas from three independent experiments.

Table 2. Microaerobic expression analysis of the *Rhizobium etli* *fixKf::uidA* transcriptional fusion in different genetic backgrounds

Strain	Strain genotype	Plasmids	Expression level (nmol min ⁻¹ mg ⁻¹ of protein) ^a
CE3/pLG1	Wild type	pBBMCS53::fixKf	367 ± 20
CFNX636/pLG1	<i>fixL::loxP</i> Sp	pBBMCS53::fixKf	2 ± 2
CFN-LG1/pLG1	<i>fxkR::loxP</i> Sp	pBBMCS53::fixKf	1 ± 4
CFNX636/pLG1/pfxkR-Ret	<i>fixL::loxP</i> Sp	pBBMCS53::fixKf pRK415::fxkR-Ret	60 ± 12
CFN-LG1/pLG1/pfxkR-Ret	<i>fxkR::loxP</i> Sp	pBBMCS53::fixKf pRK415::fxkR-Ret	697 ± 70
CFN-LG1/pLG1/pfxkR-VF	<i>fxkR::loxP</i> Sp	pBBMCS53::fixKf pRK415::fxkR-Rleg	510 ± 71
CFN-LG1/pLG1/pfixJ-Sm	<i>fxkR::loxP</i> Sp	<i>fixKf::uidA</i> pRK415::fixJ-Smel	6 ± 5
CFN-LG1/pLG1/pRK415	<i>fxkR::loxP</i> Sp	pBBMCS53::fixKf pRK415	2 ± 2

^a β -Glucuronidase-specific activity was determined after 10 h at 30°C under microaerobic conditions. Data are the mean of two replicas from two independent experiments.

the expression of its own promoter. Considering that *fixKf* expression is highly induced under microaerobiosis (25-fold) (Girard et al. 2000), we can propose that hFixL activates FxkR post-translationally in this condition.

Plasmid pRL9 of *R. leguminosarum* bv. *viciae* 3841 harbors an *fxkR*-related gene.

As mentioned earlier, *R. etli* CFN42 and *R. leguminosarum* bv. *viciae* share a novel hFixL and the absence of an annotated *fixJ* gene (Girard et al. 2000; Patschkowski et al. 1996). Given these similarities, it was considered probable that an *fxkR*-like gene would be present in *R. leguminosarum* bv. *viciae*. Sequence analysis by BLASTP (National Center for Biotechnology Information [NCBI] GenBank Database) revealed that gene pRL90026 of *R. leguminosarum* bv. *viciae* 3841 encodes a 220-amino-acid polypeptide with high similarity to FxkR of *R. etli*. Based on the pRL90026 nucleotide sequence, a 1,230-bp-long polymerase chain reaction (PCR) fragment was synthesized from both *R. leguminosarum* bv. *viciae* 3841 and VF39 strains. Complete sequence analysis of the PCR product from strain VF39 revealed a complete ORF (accession number FR854234) with high identity to pRL90026 and RHE_PF530 (98 and 88%, respectively). To determine whether this gene encodes a functional homolog of *R. etli* FxkR, a constitutive expression plasmid was generated by cloning the putative *R. leguminosarum* bv. *viciae* VF39 *fxkR* gene under the control of the *lacZ* promoter in the pRK415 plasmid vector (pfxkR-VF). This plasmid was introduced into the *R. etli* *fxkR* mutant strain (CFN-LG1) containing the *fixKf::uidA* fusion. Complementation of the CFN-LG1 (*fxkR::loxSp*) mutant strain with plasmid pfxkR-VF restored *fixKf* microaerobic expression to levels similar to those observed when the *fxkR::loxSp* mutant was complemented with its own *fxkR* gene (Table 2, compare lines 1 and 3 with 5 and 6). These results indicate that *fxkR* is a novel regulatory element, present in both *R. etli* CFN42 and *R. leguminosarum* bv. *viciae*, and is indispensable in activating *fixKf* transcription. We also tested the ability of *fixJ* from *S. meliloti* to restore expression of *fixKf* in the absence of FxkR in *R. etli*. As expected, this gene was unable to activate expression of *R. etli* *fixKf* (Table 2, line 7). This result is in agreement with the observation that the consensus binding sites of FixJ (Ferrières and Kahn 2002) are not conserved in the regulatory region of *fixKf* (data not shown). It is noteworthy that, even if there were less-effective binding sites for FixJ in the regulatory region of *fixKf*, hFixL might not be able to use FixJ from *S. meliloti* as its cognate response regulator.

FxkR-related proteins are widely distributed in α -proteobacteria.

To identify the presence of FxkR- and hFixL-related proteins in other bacteria, a BLASTP search was performed using the *R. etli* CFN42 hFixL and FxkR proteins as query sequences. The proteins with the highest percentage of identity to *R. etli* CFN42 FxkR and hFixL belonged to bacteria from the rhizobial group (a list containing the complete BLASTP hits is reported in Supplementary Table S2). For further comparison analysis, we selected those bacteria that harbored both highly conserved hFixL and FxkR proteins and whose genomes were completely sequenced. To this end, hFixL proteins were selected on the basis of the presence of a C-terminal receiver domain, the conserved His²⁰⁰ and Arg²²⁰ that are important for oxygen binding in FixL from *B. japonicum*, and the presence of the unorthodox H box sequence HDFNNLL (Boesten and Priefer 2004; Dunham et al. 2003; Gong et al. 1998). Multiple sequence analysis of hFixL-related proteins is presented in Supplementary Figure S2. The FxkR-related proteins were selected considering a score alignment of ≥ 48 defined by ClustalW2, the

presence of key amino acids for the function of the OmpR regulator (Supplementary Fig. S3), and their genomic context. The schematic representation of the genomic context and the amino acid identity between the hFixL- and FxkR-related proteins when compared with the *R. etli* counterparts is shown in Figure 2. Interestingly, genomic context analysis revealed the presence of encoded FixKf-related proteins in all the organisms studied. Three types of arrangement defined by gene conservation or gene orientation were observed when the genomic context of *hfixL*- and *fxkR*-related genes was analyzed (Fig. 2A). The type 1 arrangement is characterized by the presence of known targets of the *R. etli* FixKf regulator; namely, the *fixNOQP* operon (Girard et al. 2000) and the *azuPf* gene (L. Girard, unpublished data). Also, it is noteworthy that *fxkR* is not contiguous to *hfixL*. The type 2 and 3 arrangements both have contiguous *fxkR*, *fixK*, and *hfixL* genes and differ only with respect to gene orientation (Fig. 2A). Remarkable aspects of this set of genes are further addressed in the discussion.

The regulatory region of *fixKf*-related genes shares a common regulatory box.

The conserved genomic context of *fixKf*-related genes led us to propose that they might be similarly regulated. To identify common regulatory sequence-elements upstream of the structural genes, we used the web server MEME (Bailey and Elkan 1994). A conserved 20-bp motif with an E value of $1.6(10)^{-29}$ was discovered in the upstream region of all 11 *fixKf*-related genes. A graphic representation of the motif alignment, its position with respect to the translational start site, and the representation of the consensus sequence (Logo) are shown in Figure 3. The conserved motif is composed of two repetitive 6-bp subsites separated by four variable nucleotides (GTTACA-N₄-GTTACA). The third position of the first subsite is the least conserved and the motifs found in the *fixKf*-related genes of *Acidiphilum cryptum* JF5 and *Acidiphilum multivorum* AIU301 present considerable variations with respect to the GTTACA-N₄-GTTACA consensus (Fig. 3A). This sequence highly resembles the F1 binding motif found in *ompF*, which is bound with high affinity by the OmpR regulator (Yoshida et al. 2006). We named this conserved motif the K box (for *fixKf* regulatory region) and the two subsites Ka and Kb, respectively.

To determine the relevance of the K box for the activation of *fixKf* microaerobic expression, we designed an expression analysis. For this purpose, the transcriptional start site of *R. etli* *fixKf* was identified, by rapid amplification of cDNA (RACE) 5' ends assays, using total RNA, purified from wild-type cells cultured under microaerobic conditions. Our results show that the *fixKf* transcriptional start site is an A located 21 nucleotides upstream from the putative translational start codon. Analysis of the DNA region upstream of the *fixKf* gene revealed the presence of a purine-rich Shine-Dalgarno-like sequence (AGGGGA) seven bases in front of the *fixKf* ATG codon. Thus, the conserved motif (GTTACA-N₄-GTTACA) identified in this work is centered at position -69 bp from the *fixKf* transcriptional start site (Fig. 4A). Different transcriptional fusions were constructed using DNA fragments containing deletions of the *fixKf* upstream sequence, synthesized by PCR, and fused with a promoterless GUS gene (Fig. 4A). The plasmid pDZS1 (*fixKf::uidA* K box⁻) carries a PCR fragment of 528 bp that does not include the K box; plasmid pDZS2 (*fixKf::uidA* K box) contains a shortened regulatory region (560 bp long) that begins with the K box; in pDZS3 (*fixKf::uidA* Ka-GTT), the first three nucleotides of the Ka subsite were deleted whereas, in pDZS4 (*fixKf::uidA* Ka-GTTACA⁻), the GTTACA sequence in Ka was deleted. Plasmids containing transcriptional fusions were introduced separately into the *R. etli* CE3 wild-type strain and their transcriptional activity was moni-

tored under microaerobic conditions and compared with the expression pattern given by plasmid pLG1 (which contains the complete *fixNf*–*fixKf* intergenic region) (Fig. 4A).

The microaerobic expression of *fixKf* was drastically affected when the K box was deleted, indicating that this sequence is required (Fig. 4B compare the level of expression of pLG1 versus pDZS1). The level of microaerobic expression observed in the wild-type strain (281 ± 7) remained unaffected in pDZS2 (292 ± 8), suggesting that the regulatory region present in this plasmid is sufficient for *fixKf* expression. A nearly 50% decrease in the expression of *fixKf* was observed when the first three nucleotides of the Ka subsite were deleted (pDZS3, 136 ± 6), whereas deletion of the GTTACA sequence completely abolished transcription (pDZS4, 17 ± 6) (Fig. 4B). This result strongly suggests that the complete GTTACA region is an essential target site for *fixKf* activation. To evaluate the regula-

tory role of each Ka and Kb subsite for the expression of *fixKf*, site-specific mutations were introduced in the regulatory region of *fixKf* present in the reporter fusion pLG1. The GTTACA motif of each subsite was substituted for an *EcoRI* restriction site individually in pKa* or pKb* (from GTTACA to GAATTC) (Fig. 4A). It was found that microaerobic expression of *fixKf* was drastically affected when either Ka or Kb motifs were mutated (17 ± 8 and 11 ± 2 , respectively) (Fig. 4B), confirming that the K box is crucial for *fixKf* activation and that both subsites are necessary for its correct function.

FxkR binds the *fixNf*–*fixKf* intergenic region in vitro.

To ascertain whether FxkR could directly interact with the *fixKf* regulatory region, gel shift assays were performed with an *R. etli*-purified recombinant His₆-FxkR protein and a 300-bp DNA fragment containing either the wild type or the mu-

tated pKa* and pKb* subsites (Fig. 5A). Incubation of the labeled wild-type probe with increasing concentrations of His₆-FxxR resulted in a higher amount of DNA-protein complex (Fig. 5B). Binding of this recombinant protein was specific, as judged by the fact that a 100-fold excess of unspecific unlabeled DNA (salmon sperm DNA) did not disrupt the FxxR-template complex whereas a 100-fold excess of unlabeled template displaced the labeled DNA in the complex (Fig. 5C, compare lanes 1 and 2). Additionally, competition assays revealed that the unlabeled mutated regions competed considerably less than the wild-type template, suggesting that FxxR is unable to bind to a region containing specific mutations in the Ka or Kb subsite (Ka* or Kb*, respectively) (Fig. 5C, lanes 3 and 4). Furthermore, substitution of the Ka or Kb subsites with an *Eco*R1 restriction site (Ka* and Kb*) severely compromised binding of the His₆-FxxR protein (Fig. 5D). These results permit us to conclude that FxxR is the direct activator of *fixKf* expression, that the GTTACA sequence is the binding motif recognized by FxxR, and that both subsites are necessary for the correct binding of the regulator.

DISCUSSION

We have shown here that a response regulator that belongs to the OmpR/PhoB superfamily, herein named *fxxR*, is a key regulatory element that completes the hFixL-dependent regula-

tory cascade for *fix* gene regulation. Our results demonstrate that FxxR is strictly necessary to activate *fixKf* expression in response to low oxygen concentration in *R. etli* CFN42, by binding to a specific sequence (GTTACA-N₄-GTTACA) in the *fixKf* regulatory region (K box) upon its activation. Because FxxR is the first transcriptional regulator reported to activate microaerobic expression of *fixKf*, this work casts light on a longstanding unanswered question of relevance in the field of nitrogen-fixation gene regulation.

Co-evolutionary adaptation has shaped the genetic circuits that control symbiosis and nitrogen fixation, leading to appreciable differences between unrelated symbiotic partners. The *R. etli* intricate regulatory cascade revealed a novel player, the response regulator FxxR. Notably, this response regulator is not related to the family of FixJ regulators that are widely accepted as cognate proteins of FixL sensors and the main distributors of the oxygen signal in several α -proteobacteria.

It has been previously reported that mutations in either *fixL* or *fixKf* do not affect nitrogen fixation. For that reason, it was not unexpected that the *R. etli* FxxR mutant showed an Fix⁺ phenotype (data not shown). Under symbiotic conditions, FnrN proteins are able to substitute for FixK to maintain nitrogen fixation in the bacteroids. The absence of a K box in the regulatory region of the *fnrN* genes strongly suggests that these regulators are not under the control of FxxR in the nodule environment. The fact that the FnrN regulators were char-

Fig. 3. Identification of the K box in the regulatory region of the *fixKf*-related genes. **A**, Sequence pattern and *P* values found by MEME analyzing 200 bp upstream of *fixKf*-related genes. Nucleotides in black boxes are the most conserved. **B**, Graphic representation of the conserved motif location with respect to the translational start of *fixK*. **C**, Logo representation that depicts the degree of conservation of the nucleotides along the discovered motif.

acterized as oxygen sensors with the capability of autoactivation may explain their role as an independent branch in the control of nitrogen-fixing genes.

One of the aims of this work was to set the foundations for future work in other bacteria, which could share this novel regulatory element. For this purpose, we made an *in silico* search to find some conserved genetic tracts that include an *fxkR* gene in related bacteria. *hfixL* and *fxkR* genes were found in plasmids as well as in the chromosome of nitrogen-fixing and non-nitrogen-fixing α -proteobacteria, not restricted to organisms that lack *fixL* and *fixJ* genes or have a *fixNOQP* operon. An open question would be whether these related genes have diverted to accomplish different physiological tasks. A closely related regulatory cascade was previously reported in *R. leguminosarum* bv. *viciae* VF39, which induces efficient symbiosis with legume plants belonging to the tribe *Fabeae* (Patschkowski et al. 1996). Here, we identified the presence of a highly conserved *fxkR* gene in *R. leguminosarum* bv. *viciae* VF39 and demonstrated that this gene encoded for an FxkR protein able to restore the *R. etli* CFN42 *fixKf* gene expression in an *fxkR* mutant. A promising perspective is to test whether this regulator controls the expression of *fixK* or *fmrN* in its own genetic background. Genome analysis of *R. leguminosarum* bv. *viciae* 3841 revealed the presence of a K box in the regulatory region of the three *fixKf*-related genes but not in that of *fmrN*. Therefore, one or all of these three genes might control expression of the symbiotic terminal oxidases directly or indirectly via *fmrN*. The regulatory scenario becomes more complex if we integrate the hFixL- and FxkR-related proteins encoded in the

chromosome of this bacterium. Demonstration of the specificity of the FxkR regulators for the K boxes found upstream from different *fixKf*-related genes and the specificity of the hFixL kinases for the different FxkR-related regulators could reveal whether these two-component systems are part of sure-fire or cross-talk circuits.

Using the available genomic information, we identified closely related hFixL, FxkR, and FixKf proteins in both nitrogen-fixing and non-fixing α -proteobacteria (Fig. 2). *Methylobacterium nodulans* ORS2060 and *S. meliloti* SM11 have perhaps the most interesting gene set with respect to microaerobic response, due to the fact that, in addition to an FixL-FixJ-FixK regulatory cascade that controls expression of *fixNOQP* and *fixGHIS* operons, an hFixL-FxkR-FixK-related genetic locus is also present. It would be worth determining the participation of each regulator in the control of the microoxic-dependent expression and to learn which of these cascades control the expression of the different FNR/CRP regulators. Moreover, the fact that both types of FixL proteins (canonical and hybrid) coexists in the same cell raises the question of whether they detect the same stimulus and, if that is the case, what differences in the detecting threshold may exist. All these concerns should be addressed in order to better understand how similar regulatory pathways involved in signal transduction are orchestrated within the cell.

The presence of FixL-FixJ and hFixL-FxkR regulatory systems in non-nitrogen-fixing α -proteobacteria implies a conserved modular design for oxygen sensing. Previous analyzes have described a correlation between the presence of this sen-

Fig. 4. Genetic analysis of the putative FxkR-binding site in the expression of *Rhizobium etli* *fixKf*. **A**, Schematic representation of the *fixKf* promoter region and fragments cloned in plasmid pBBMCS53 to generate *fixKf*-GUS transcriptional fusions. The *fixNf* and *fixKf* structural genes are represented by arrows that indicate transcription direction. The transcriptional start site of each gene is indicated by a +1 and by bent arrows. The K box is represented by a black or white box. The *fixNf* anaerobox is represented by an open box named ANB. Relative positions of the regulatory elements identified and the size of fragments cloned in plasmid pBBMCS53 are indicated. A scale representation of the length of the fusions is shown by dotted lines. Arrows at the end of each transcriptional fusion represent the structural *uidA* gene. The pKa* and pKb* legends denote the presence of site-directed mutations in the Ka and Kb subsites, respectively. Nucleotides underlined indicate introduced *EcoRI* site. **B**, Microaerobic expression analysis. Each bar of activity is positioned in accordance to the corresponding transcriptional fusion with respect to the graphical representation in A. GUS-specific activity is expressed as nanomoles of product per minute per milligram of protein. Data are the mean of two replicas from three independent experiments.

sory systems and a *ccoNOQP* operon. In *C. crescentus*, the FixL-FixJ system also controls the expression of alternative cytochrome oxidases (Cosseau and Batut 2004; Crosson et al. 2005). Here, we report at least three different α -proteobacteria that lack *cbb₃* and possess an hFixL-FxkR sensory system (*Acidiphilum cryptum*, *A. multivorum*, and *Agrobacterium radiobacter* K84). These findings suggest to us that the utilization of this oxygen-responsive sensory system is not restricted to bacteria that have evolutionarily adapted to microaerobic environments by the presence of a high-affinity *cbb₃* complex. Interestingly, in *Acidiphilum cryptum* and *A. multivorum*, we identified FixK-binding motifs (TTGAT-N₄-ATCAA) in the regulatory region of genes implicated in different types of respiration processes such as *cydA* (involved in aerobic respiration), *narG* (important for nitrate-dependent respiration), or *hemN* and *bchE* genes that form part of the tetrapyrrole pathway. These last two genes are involved in microaerobic respiration and photosynthesis in *Rubrivivax gelatinosus* (Ouchane et al. 2007). It is also significant that, in addition to FixK (Acry_0576 or ACMV_06500), a regulator of the FNR/CRP family with this conserved box is present. Both regulators may function in coordination to control the mentioned genes. In order to better understand oxygen-dependent adaptation among bacteria, it will be necessary to compare genes and proteins that are controlled in response to this signal.

Previous studies on the distribution of plasmid genetic markers in bean-nodulating strains revealed appreciable intra-species diversity. Most of the *Rhizobium etli* strains tested showed the presence of a CFN42 *hfixL* homolog encoded in a plasmid different from the symbiotic one, and the absence of a *fixJ* gene (Brom et al. 2002). Using CFN42 *fxkR*- and *hfixL*-specific primers, we obtained the expected PCR products from 18 *R. etli* strains. Partial sequence analysis from 12 *fxkR* products, including those from CFN42 and *R. leguminosarum* bv. *viciae* as controls, demonstrated that the products obtained correspond to *fxkR* (L. Girard, unpublished data). This result supports the idea that the presence of hFixL and FxkR is predominant in *R. etli* strains and suggests that this regulatory circuit is common among these closely related bacteria. However, most of these strains were isolated from Spain or Mexico, and it would be interesting to know how conserved the *hfixL* and *fxkR* genes are in a richer collection of *R. etli* strains isolated from different soil sources.

Despite the evident variability between the intergenic regions of *fixKf* genes, the regulatory box was highly but not completely conserved among them. The least-conserved K boxes with the lowest position *P* value (Fig. 3A) were those of *Acidiphilum cryptum*, *Acidiphilum multivorum*, and *Oceanicola batsensis* HTCC2597. FxkR proteins from these bacteria also possessed an HTH domain with the lowest ClustalW2 score when compared with the domain of FxkR from *R. etli*. It is probable that the FxkR-related proteins in these bacteria bind a different kind of regulatory box. PhoB-binding sites (TGTCa and TTACA) have been identified in the *pstS* promoter in *Escherichia coli* (Makino et al. 1996). Interestingly, the TTACA box is also included in the K box but the TGTCa region is not, although it can be found in the intergenic regions of *fixKf*-related genes from *Agrobacterium radiobacter*, *Methylobacterium nodulans*, *Acidiphilum cryptum*, and *Acidiphilum multivorum* and in the *fixKf*-related gene encoded in the chromosome of *R. leguminosarum* (data not shown). The importance of these nucleotides remains to be shown.

From the evidence presented in this work, we propose that hFixL and FxkR are part of a novel phosphorelay system that activates transcription of *fixKf*-related genes in response to oxygen, not only in the *Rhizobium* genus but also in other

groups of α -proteobacteria. To better understand the role of phosphorylation in the regulatory mechanisms of this activator, further biochemical and genetic characterization will be crucial. In this direction, it will be necessary to know the role of the conserved aspartate target of phosphorylation in the ability to control binding affinity or the oligomeric state of the regulator. Because the two-component systems that involve hHK are characterized by a cascade of phosphate transfer reactions, where the activities of the histidine-kinase (HK), histidine phosphotransferase, and receiver domains (RR) are essential, experiments are underway in our laboratory to determine whether additional elements are necessary for the functionality of the hFixL-FxkR regulatory cascade.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used are listed in Table 3. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium and the antibiotics were added at the following concentrations: carbenicillin, 100 μ g ml⁻¹; gentamicin (Gm), 15 μ g

Fig. 5. *Rhizobium etli* FxkR fusion protein binds specifically to the K box. **A**, Graphic representation of the region used as probe for the electrophoretic mobility shift assays (EMSA). Wild-type probe and the substitution mutations indicated beneath (WT, Ka*, and Kb*, respectively). **B**, EMSA was performed without His₆-FxkR protein (lane 1) or with 0.3, 0.7, 1.4, and 2.9 nM of His₆-FxkR protein (lanes 2,3,4, and 5 respectively) plus end-labeled WT probe. **C**, Competition assay was performed with 2.9 nM of His₆-FxkR plus end-labeled WT probe without specific unlabeled competitor (lane 1) or with 100-fold excess of specific cold competitor: WT (lane 2), Ka* (lane 3), and Kb* (lane 4). **D**, Binding assay using as probes fragments with mutations in either Ka (lanes 1 and 2) or Kb (lanes 3 and 4) subsites without or with 2.9 nM His₆-FxkR, as indicated. The FxkR-DNA complex (C→) and free DNA (F→) are indicated.

ml⁻¹; kanamycin (Km), 30 µg ml⁻¹; and spectinomycin (Sp), 100 µg ml⁻¹. *Rhizobium* strains were grown at 30°C in peptone-yeast (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). Antibiotics were used at the following concentrations: streptomycin (Sm), 200 µg ml⁻¹; Gm, 15 µg ml⁻¹; Km, 30 µg ml⁻¹; nalidixic acid, 20 µg ml⁻¹; neomycin, 60 µg ml⁻¹, and Sp, 100 µg ml⁻¹. When required, sucrose was added at 12.5% (wt/vol). For detection of β-galactosidase and GUS activity, 5-bromo-4-chloro-3-indolyl-β-D-galactoside and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, respectively, were used in plates at 20 µg ml⁻¹. For growth under microaerobic conditions, cultures were grown to mid-exponential phase in PY medium. Cells were collected by centrifugation, washed with sterile minimal medium (MMY), and concentrated 100-fold. MMY (20 ml) containing 10 mM succinate and 10 mM ammonium chloride as carbon and nitrogen sources were inoculated with these suspensions at an initial optical density at 540 nm (OD₅₄₀) of 0.05. 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 (Granados-Baeza et al. 2007). In experiments that included the CE3 deriva-

tives cured of pRet42f (CFNX186 or CFNX218), cultures were inoculated at OD₅₄₀ of 0.25 and the bottles, flushed with an oxygen/argon (1:99, vol/vol) mixture, were incubated for 10 h at 30°C for microaerobic induction.

Measurement of GUS activity.

Aerobic and microaerobic cultures of *R. etli* strains harboring transcriptional fusions were grown as described previously (Girard et al. 1996). Quantitative GUS activity was determined on 1.0-ml culture samples using 4-nitrophenyl β-D-glucuronide as substrate, as described previously (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. Plasmid profiles of the samples used for GUS determinations were analyzed using a modified Eckhardt procedure (Hynes and McGregor 1990).

PCR amplification.

Specific PCR primers were designed using the Oligo 6.0 software and were purchased from Unidad de Síntesis Química IBT-UNAM. Accuprime *Taq* DNA polymerase high fidelity (Invitrogen, Carlsbad, CA, U.S.A.) was used in PCR reactions with a cycling regime that includes a denaturing step at 94°C for 1 min

Table 3. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics ^a	Reference
Rhizobiales		
CE3	Sm ^r derivative of CFN42	Noel et al. 1984
CFNX89	CE3 derivative pRet42d	Brom et al. 1992
CFNX186	CE3 derivative pRet42f	Brom et al. 1992
CFNX218	CE3 derivative pRet42a ⁻ ,b ⁻ ,c ⁻ ,d ⁻ , e _Δ ,f	Brom et al. 2000
CFNX636	CE3 derivative, <i>fixL::loxP</i> Sp	Girard et al. 2000
<i>R. leguminosarum</i> bv. <i>viciae</i> VF39	Wild type, Sm ^r	Hynes et al. 1988
<i>R. leguminosarum</i> bv. <i>viciae</i> 3841	Wild type	Johnston and Beringer 1975
<i>Sinorhizobium meliloti</i> 1021	Wild type	Meade et al. 1982
CFN-LG1	CE3 derivative, <i>fixK::loxP</i> Sp	This work
Escherichia coli		
DH5α	<i>supE44ΔlacU169(φ80lacZΔM15) hsdR17recA1endA1gyrA96 thi-1relA1</i>	Sambrook et al. 1989
BL21 (DE3)	<i>F-ompT hsdS_B(r_B-m_B-) gal dcm</i> (DE3)	Invitrogen
Plasmids		
pCR2.1 TOPO	Cloning vector for PCR products, Ap ^r Km ^r	Invitrogen
pK [*] <i>mobsacB</i>	Suicide cloning vector, Km ^r	Schäfer et al. 1994
pRK415	Broad host range plasmid, Tc ^r	Keen et al. 1988
pET28a	Expression vector, His-Tag, Km ^r	Novagen
pBBMCS53	Δ <i>lacZ</i> pBBR1MCS-5 derivative, carrying the promoterless <i>uidA</i> gene, Gm ^r	Girard et al. 2000
pRK2073	Conjugation helper plasmid, Sm ^r /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
pARG1	pK [*] <i>mobsacB</i> derivative, <i>fixK::loxP</i> Sp	This work
p <i>fixK</i> R-Ret	pRK415 derivative carrying the <i>R. etli</i> <i>fixK</i> R gene under the <i>lacZ</i> promoter	This work
p <i>fixK</i> R-VF	pRK415 derivative carrying the <i>R. leguminosarum</i> bv. <i>viciae</i> VF39 <i>fixK</i> R ortholog under the <i>lacZ</i> promoter	This work
p <i>fixJ</i> -Sm	pRK415 derivative carrying the <i>S. meliloti</i> <i>fixJ</i> gene under the <i>lacZ</i> promoter	This work
pARG2	pBBMCS53 derivative <i>fixK::uidA</i>	This work
pDZS1	pBBMCS53 derivative <i>fixKf::uidA</i> transcriptional fusion containing 459 bp of the <i>fixKf</i> CDS and 67 bp upstream <i>FixKf</i> ATG, without K box	This work
pDZS2	pBBMCS53 derivative <i>fixKf::uidA</i> transcriptional fusion containing 459 bp of the <i>fixKf</i> CDS and 100 bp upstream <i>FixKf</i> ATG, including the K box complete	This work
pDZS3	pBBMCS53 derivative <i>fixKf::uidA</i> transcriptional fusion containing 459 bp of the <i>fixKf</i> CDS and 97 bp upstream <i>FixKf</i> ATG with a deletion of the GTT in the Ka subsite	This work
pDZS4	pBBMCS53 derivative <i>fixKf::uidA</i> transcriptional fusion containing 459 bp of the <i>fixKf</i> CDS and 94 bp upstream <i>FixKf</i> ATG without the Ka subsite	This work
pDZS5	pBBMCS53 derivative <i>fixKf</i> - pKa [*] :: <i>uidA</i> transcriptional fusion with specific mutations in subsite Ka	This work
pDZS6	pBBMCS53 derivative <i>fixKf</i> -Kb [*] :: <i>uidA</i> transcriptional fusion with specific mutations in subsite Kb	This work
pARG3	pET28a derivative carrying the <i>R. etli</i> <i>fixK</i> R gene	This work

^a Sm^r, Ap^r, Km^r, Tc^r, Gm^r, and Sp^r indicate resistant to streptomycin, ampicillin, kanamycin, tetracycline, gentamicin, and spectinomycin, respectively; PCR = polymerase chain reaction.

followed by 30 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.

5' RACE.

Transcriptional start sites for the *fixKf* and *fixNf* genes were identified in assays using the 5' RACE system for rapid amplification of cDNA ends (version 2.0; Invitrogen), as described previously (Gómez-Hernández et al. 2011). scDNA was synthesized from 2 µg of total RNA purified from microaerobic cultures of the wild-type strain derivatives carrying *fixKf* and *fixNf* transcriptional fusions in plasmid pBBMCS53 (pLG1 and pLG2, respectively) (Girard et al. 2000), using complementary primer *gusLw*. After first-strand cDNA synthesis, the mRNA was removed by treatment with RNase and the scDNA was purified using a SNAP column (Invitrogen). Terminal deoxynucleotidyl transferase and dCTP were used to add homopolymeric tails to the 3' ends of the cDNA. Nested PCR reactions were done using gene specific primers *fixKf*-RT (5'-GGCGAAATGAAATGCGGTTAT-3', in positions 214 to 234) and *fixNf*-RT (5'-CGCTGCACCGTAGAAC-3', in positions 403 to 420) and the abridged anchor primer (GGCCACGCGT CGACTAGTACGGGIIIGGGIIGGGIIG, provided in the kit). The products were cloned into the pCR2.1 TOPO vector for sequencing.

Plasmid construction.

To introduce a mutation into the *fixK* gene, a 1,578-bp product was obtained by PCR using total DNA from strain CE3 and the specific primers UpDR530 and LwDR530. This PCR product was cloned by T-A annealing into pCR 2.1-TOPO (Invitrogen) and analyzed by sequencing. To construct a suicide plasmid useful for homogenotization (pARG-1), the 1,517-bp *Bgl*II-*Hind*III fragment containing the *fixK* allele was cloned into the *Bam*HI-*Hind*III sites in pK^{*}mobsacB (Schäfer et al. 1994). Mutagenesis of the *fixK* gene was achieved by introducing into the *Sal*I site (nucleotide 274) a *loxP*Sp interposon (Martínez-Salazar and Romero 2000). Plasmids for mutagenesis by vector-insertion (VIM) were constructed cloning a specific intragenic PCR product of the desired gene into the pK^{*}mobsacB vector, a *Rhizobium* suicide plasmid. Intragenic regions were obtained by PCR using total DNA from strain CE3 and specific primers (primers and specific intragenic regions used for VIM are shown in Supplementary Table S1). These PCR products were cloned by T-A annealing into pCR 2.1-TOPO (Invitrogen) and analyzed by sequencing. To construct a suicide plasmid useful for VIM, the intragenic regions were purified using the *Eco*RI sites of the vector, with the exception of fragments for pPF530 and pPF72 that contain an internal *Eco*RI site, and cloned into the pK^{*}mobsacB (Schäfer et al. 1994).

Plasmids carrying the *R. etli* CFN42 *fixK*R (*pfixK*R-Ret), *R. leguminosarum* bv. *viciae* VF39 *fixK*R (*pfixK*R-VF), and *S. meliloti* 1021 *fixJ* (*pfixJ*-Sm) genes constitutively expressed from the *lacZ* promoter were obtained by cloning the respective coding region in the conjugative plasmid pRK415 (Keen et al. 1988). To construct *pfixK*R-Ret, a 981-bp product was obtained by PCR using total DNA from strain CE3 and the specific primers Up530-*Hind*III and Lw530-*Xho*I. To generate plasmid *pfixK*R-VF, a 1,230-bp PCR product was obtained by PCR using total DNA from strain VF39 and the specific primers, based on the pRL90026 nucleotide sequence, UpRL9-26, and LwRL9-26. The *fixJ* gene of *S. meliloti* 1021 was obtained by PCR amplification using specific primers Up-fxJSm and Lw-fxJSm. To generate a plasmid for expression analysis of the *R. etli* *fixK*R gene, the corresponding regulatory region was synthesized by PCR using primers UP-530gus and Lw-PF530. An *Eco*RI frag-

ment of 767 bp (containing 262 bp upstream from the *Fxk*R start codon) was cloned in the broad-host plasmid pBBMCS53 that carries a promoterless *uidA* gene (Girard et al. 2000), giving plasmid pARG-2. The correct orientation of the fragments in the plasmids was verified by PCR using primer *gusLw* and the corresponding upper primer in each case.

Plasmids pDZS1, pDZS2, pDZS3, and pDZS4 are shortened variants of plasmid pLG-1. Four different PCR products were obtained using plasmid pLG-1 as template and the specific forward primers Up-NA, Up-GTT, Up-ACA, and Up-CAC and the same reverse primer Lw-RRKf. The amplification products were cloned by T-A annealing into pCR 2.1-TOPO and sequenced. Fragments of 526 bp (containing 67 bp upstream from the *FixKf* start codon), 559 bp (containing 100 bp upstream from the *FixKf* start codon), 556 bp (containing 97 bp upstream from the *FixKf* start codon), and 553 bp (containing 94 bp upstream from the *FixKf* start codon) were cloned separately in pBBMCS53, giving plasmids pDZS1, pDZS2, pDZS3, and pDZS4. The correct orientation of the fragments in the plasmids was verified by PCR using primer *gusLw* and the corresponding upper primer in each case. Mutations of the putative K box in *fixKf* promoter region were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). Each subsite was modified by introducing an *Eco*RI site instead of the wild-type sequence (GTTACA). The primers used to mutate the Ka subsite were UpKa-mut and LwKa-mut. To mutate the Kb subsite, we used primers UpKb-mut and LwKb-mut. Underlined nucleotides in each primer denote the *Eco*RI site introduced. Both modified *fixKf* regulatory regions were cloned in plasmid pBBMCS53 to obtain plasmids pDZS5 and pDZS6.

To generate a recombinant *Fxk*R protein with a 6-histidine tag, the structural *fixK* region was amplified by PCR using total DNA from strain CE3 as template with the oligonucleotides Up*Fxk*R-*Nde*I and Lw530-*Xho*I. The *Nde*I and *Xho*I sites introduced by the primers were used to clone the fragment into the pET28a vector to obtain pARG3. Primers used are described in the Supplementary Table S3.

R. etli mutant derivatives.

Mutagenesis by vector insertion was done using plasmids pPF72, pPF224, pPF225, pPF479, pPF530, pPF533, pPF547 and pPF547. Each plasmid, transferred by conjugation to *Rhizobium* spp., was co-integrated by single recombination with the homologous target, producing a knockout of the gene of interest (Landeta et al. 2011). Mutants were selected as resistant to Sm, Sp, and Km and the correct integration of the plasmid was verified by Southern blot hybridization.

Replacement of the *R. etli* CFN42 *fixK*R wild-type allele by the *fixK*R::*loxP*Sp mutant allele in plasmid pARG1 was carried out by homogenotization using the *sacB* marker present in the pK^{*}mobsacB vector; this marker allows an easy selection for homogenotization events, by scoring for resistance to sucrose. Double recombinants were selected as resistant to Sm and Sp and susceptible to Km. To verify that the gene replacement had occurred, both plasmid profiles as well as total DNA blots of the derivatives were analyzed by Southern hybridization against the appropriate gene and cassette probes.

Purification of His₆-*Fxk*R.

Plasmid pARG3 was used to overexpress the recombinant His₆-*Fxk*R in the genetic background of *E. coli* BL21 (DE3). Overexpression was achieved by induction of cultures grown in LB media (OD₅₄₀ = 0.6) with 0.3 mM isopropyl-thio-galactopyranoside for 2 h at 30°C. Cells were collected by centrifugation at 4°C and lysed in native lysis buffer (50 mM NaH₂PO₄ and 300 mM NaCl, pH 8.0) after at least two passes through a

French press. Separation of the soluble fraction was done after centrifugation at $10,000 \times g$ for 30 min at 4°C. Samples of uninduced and induced cultures as well as of the soluble and insoluble fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). His₆-FxxR was purified from 5 ml of the soluble fraction using Ni-NTA agarose (Invitrogen) in plastic columns (Bio-Rad, Hercules, CA, U.S.A.) following the instructions of the manufacturer. Briefly, the column was washed three times with washing buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 40 mM imidazole; pH 8.0), and 16 fractions were collected after addition of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 500 mM imidazole; pH 8.0). Samples of each purification step were analyzed by SDS-PAGE.

Electrophoretic mobility shift assays.

A 300-bp region encompassing the intergenic region between *fixNF* and *fixKF* was amplified by PCR using, as template, pLG-1 or pDSZ-5 or pDSZ-6 with primers Up-RRKf and Lw2-RRKf. The PCR products were purified with the PureLink PCR purification kit (Invitrogen) and quantified with a Nanodrop spectrophotometer. DNA (100 ng) was used in the labeling reaction with 3 µl of [γ -³²P]-ATP at 6,000 Ci/mmol in a final volume of 20 µl. Unincorporated label was removed with Illustra Microspin G-25 columns (Amersham, Bucks, U.K.) following the instructions of the manufacturer.

For the binding reactions, 1 ng of labeled DNA was incubated with an increasing concentration of pure protein (0.3 to 1.4 nM) in a binding buffer containing 100 mM Tris-HCl (pH 7.4), 100 mM KCl, 10 mM MgCl₂, 10% glycerol, 2 mM dithiothreitol, and 1 mM lithium potassium acetyl phosphate (Sigma-Aldrich, St. Louis) in a final volume of 20 µl for 20 min at room temperature. When indicated, 100 ng of salmon sperm DNA or 100 ng of unlabeled DNA was used as nonspecific or specific competitors. The electrophoresis run was carried on a 5% polyacrylamide (79:1) gel in 0.5× Tris-borate-EDTA buffer for 1 h at 300 V at room temperature.

Bioinformatics resources.

BLAST (blastp) was used to identify similar FxxR proteins in related organisms. The genomic context of the *fixKR* genes was analyzed with the gene information viewer of the NCBI web page. Sequence alignment for the analysis of conserved residues and percentage of identity was performed with default parameters of the web server CLUSTAL2 of EMBL-EBI bioinformatic tools (Larkin et al. 2007). Domain conservation was analyzed using different domain libraries such as INTERPRO, PFAM, PROSITE, SMART, and SUPERFAMILY (Finn et al. 2010; Gasteiger et al. 2003; Letunic et al. 2009; Sigrist et al. 2010).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Basic NCBI/BLAST database: blast.ncbi.nlm.nih.gov/Blast.cgi
 ClustalW2, Multiple Sequence Alignment database: www.ebi.ac.uk/Tools/msa/clustalw2
 European Molecular Biology Laboratory SMART database: smart.embl-heidelberg.de/
 ExPASy Prosite database: prosite.expasy.org/
 Kazusa DNA Research Institute Rhizobase database: <http://genome.kazusa.or.jp/rhizobase/Etli>
 MEME Multiple Em for motif elicitation database: meme.sdsc.edu/meme/cgi-bin/meme.cgi
 Prokaryotic 2-Component Systems (P2CS): www.p2cs.org
 Protein-Domain Sequence Analyzers database: www.ebi.ac.uk/interpro
 SUPERFAMILY database: supfam.cs.bris.ac.uk/SUPERFAMILY
 Wellcome Trust Sanger Institute Pfam database: pfam.sanger.ac.uk