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Characterization of a *fixLJ*-Regulated *Bradyrhizobium japonicum* Gene Sharing Similarity with the *Escherichia coli fnr* and *Rhizobium meliloti fixK* Genes

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We describe the cloning, sequencing, regulation, and mutational analysis of a *Bradyrhizobium japonicum fixK*-like gene whose product belongs to the family of Fnr-Crp-related regulatory proteins. The predicted 237-amino-acid FixK protein was found to share between 28 and 38% sequence identity with the *Escherichia coli* Fnr protein, other bacterial Fnr-like proteins (FnrN, Anr, and HlyX), and two rhizobial FixK proteins. The *B. japonicum fixK*-like gene, when expressed from a *lac* promoter, could functionally complement an *fnr* mutant strain of *E. coli* and activate transcription from an *fnr*-dependent promoter in the *E. coli* background; this activation was sixfold higher in anaerobic cultures than in aerobically grown cells, a finding that suggested oxygen sensitivity of the FixK protein and was consistent with the presence of a cysteine-rich, putatively oxygen-responsive domain at its N-terminal end. Similar to the situation in *Rhizobium meliloti*, expression of the *fixK* gene in *B. japonicum* was shown to be induced at low O_2 tension and this induction was dependent on the two-component regulatory system FixLJ. Despite this dependency, however, a *B. japonicum fixK* mutant did not have the phenotypic characteristics of *B. japonicum fixL* and *fixJ* mutants: the *fixK* mutant was neither Fix$^-$ in symbiosis with soybean plants nor defective in anaerobic respiration with nitrate as the terminal electron acceptor. Also, the *fixK* mutant was unaffected in the expression of one of the two *B. japonicum* σ^{54} genes, *rpoN*$_1$, which was previously shown to be controlled by the *fixLJ* genes. When *fixK* was introduced into the *B. japonicum fixJ* mutant and expressed therein from a constitutive promoter (i.e., uncoupling it from regulation by FixJ), the FixK protein thus synthesized fully restored anaerobic nitrate respiration in that strain. We interpret this to mean that the *B. japonicum* wild type has two homologs of *fixLJ*-regulated *fixK* genes which can functionally substitute for each other.

In the facultative anaerobe *Escherichia coli*, the ability to perform anaerobic respiration depends on the action of a transcriptional regulator called Fnr (for reviews, see references 23, 54, and 60). This protein functions both negatively as a repressor (e.g., of its own synthesis) and positively as an activator of the expression of a large number of genes, including the fumarate, nitrate, and nitrite reductase genes. The oxygen-responsive Fnr protein is probably inactive under aerobic conditions, whereas it is converted to an active form when oxygen is removed. Although the mechanism of this conversion is not entirely understood, it has been suggested that cysteine residues in the N-terminal part of Fnr and a protein-bound iron ion are involved (19, 21, 32, 45, 53, 58). Fnr shows homology to Crp, the cyclic AMP receptor protein which mediates catabolite control in *E. coli* (48). There exists a growing family of Crp-related regulatory proteins. They all have a C-terminal DNA-binding domain, made up of a helix-turn-helix motif, and a characteristic β-roll structure in the N-terminal half of the protein which, in Crp, has been shown to be involved in nucleotide binding (61).

Members of the Crp-Fnr family have been described in a variety of bacteria. In *Actinobacillus pleuropneumoniae*, the HlyX protein was identified as a probable regulator of hemolysin synthesis (29). The *Pseudomonas aeruginosa* Anr protein is a regulator required for induction of nitrate reductase and arginine deiminase activities under anaerobic conditions (16, 43, 62). Three Fnr-like regulators were also found in rhizobia, in which they are involved in adaptation to symbiotic nitrogen-fixing conditions in nodules: FixK of *Rhizobium meliloti* (3, 4), FixK of *Azorhizobium caulinodans* (24), and the open reading frame 240 (ORF240) protein (lately termed FnrN) of *R. leguminosarum* biovar viciae (6).

The *fixK* gene has originally been identified in *R. meliloti*, the symbiont of alfalfa, as part of a complex regulatory cascade leading to the expression of genes (*nif* and *fix*) that are required for a nitrogen-fixing symbiosis (3). On top of the cascade is the two-component sensor-regulator system FixLJ. FixJ, the response regulator, is activated by FixL, the sensor, in response to conditions of low oxygen tension (17) and then activates expression of the genes for two other transcriptional regulators, NifA and FixK. In turn, the NifA protein leads to expression of *nif* and *fix* genes such as *nifHDKE* and *fixABCX* (8), whereas FixK is a regulatory protein that functions as an activator of expression of the *fixN* region and as a repressor of its own synthesis and that of NifA. Although the FixK protein of *R. meliloti* shows homology to *E. coli* Fnr, its activity does not depend on low oxygen concentrations. In fact, one major difference between *E. coli* Fnr and *R. meliloti* FixK is the lack of cysteine residues in the N terminus of FixK (3).

As in all rhizobia, expression of *nif* and *fix* genes in *Bradyrhizobium japonicum*, the root nodule symbiont of soybean plants, is controlled by oxygen. Under anaerobic conditions, the oxygen-responsive NifA protein is present in

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an active conformation and activates not only expression of the nitrogenase genes and many other *nif* and *fix* genes but also its own synthesis (14, 55). In contrast to *R. meliloti*, however, the *B. japonicum* NifA protein is synthesized aerobically at a certain basal level. Moreover, although *fixL*- and *fixJ*-like genes were also discovered in *B. japonicum*, these genes are not involved in regulation of *nifA* gene expression, as opposed to the situation in *R. meliloti* (2). Nevertheless, *B. japonicum fixLJ* mutants are defective in symbiotic nitrogen fixation, suggesting that the FixLJ system is part of a second regulatory circuit, besides that involving NifA, which is essential for a fully effective symbiosis. Apart from their involvement in symbiosis, the *fixLJ* genes were also found to be required for anaerobic respiration. While the *B. japonicum* wild type was able to grow anaerobically with nitrate as the terminal electron acceptor, *fixLJ* mutant strains failed to do so under such conditions, suggesting that the FixLJ system controls the anaerobic induction of genes concerned with nitrate respiration (2).

To analyze the regulatory circuits present in *B. japonicum* further, we searched for a *fixK*-like gene in *B. japonicum*. The incentives for doing this were twofold. (i) We wished to test whether the FixLJ system regulates certain nitrogen fixation genes (see above) directly or whether this occurs via a FixK-like protein, as in one of the regulatory cascades that exist in *R. meliloti*. (ii) By analogy with the regulation known in *E. coli* and *P. aeruginosa*, we wanted to see whether the process of nitrate respiration in *B. japonicum* is controlled by an Fnr-like protein.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used are listed in Table 1. *B. japonicum* 110*spc*4 was used as the wild-type strain. *E. coli* RR28 was used as the host for plasmid construction, and *E. coli* JM101 served as the recipient for transfection with M13 derivatives. *E. coli* S17-1 and HB101 (the latter carrying pRK2013 as a helper plasmid) were used for mobilization of pSUP202 and pRK290 derivatives, respectively. *E. coli* 294*cys*::Tn*5* was used for random Tn*5* mutagenesis of recombinant plasmids. The use of other strains is given at appropriate places in the text.

Media and growth of cells. *B. japonicum* strains were routinely grown aerobically in peptone-salts-yeast extract medium (40). In certain experiments, cultures were grown either aerobically or anaerobically in yeast extract-mannitol medium (7) containing 10 mM KNO_3. Antibiotics were added at the following final concentrations: spectinomycin, 100 μg ml^{-1}; kanamycin, 200 μg ml^{-1}; tetracycline, 100 μg ml^{-1}. Maintenance of pRK290 plasmid derivatives in *B. japonicum* was achieved by adding 50 μg of tetracycline ml^{-1} to liquid cultures. *E. coli* strains were grown in Luria-Bertani (LB) medium (34); for plasmid selection, it contained the following concentrations of antibiotics: ampicillin, 200 μg ml^{-1}; kanamycin, 30 μg ml^{-1}; tetracycline, 10 μg ml^{-1}. After Tn*5* mutagenesis, recombinant plasmids with Tn*5* insertions were selected by applying kanamycin at a concentration of 300 μg ml^{-1} in plates. Complementation of *E. coli fnr* mutant strains was tested either by the ability to restore anaerobic growth on minimal plates containing glycerol and nitrate (51) or by formation of gas from glucose (38). Gas formation was also recorded after microaerobic growth of the cultures in LB medium with 1.4% (wt/vol) glucose, 1 μM Na_2MoO_4, and 1 μM Na_2SeO_3. Antibiotics were added to select for recombinant plasmids.

DNA manipulations. Recombinant DNA work was done by using standard protocols (30). Isolation of chromosomal DNA from *B. japonicum* strains has been described previously (20). Heterologous Southern blot hybridizations were performed at 58°C by using randomly primed, ^{32}P-labelled DNA fragments as probes. Homologous Southern blot hybridizations were done at 68°C. Probes for homologous hybridizations were labelled by using the nonradioactive labelling kit from Boehringer GmbH, Mannheim, Germany.

DNA sequence analysis. Defined restriction fragments from plasmids pRJ7422 and pRJ7440 (see Fig. 2) were cloned into vectors M13mp18 and M13mp19. The DNA was sequenced on both strands from overlapping M13 subclones by using M13-specific fluorescent dye primers and the chain termination method (42). The sequencing reactions were analyzed on a DNA sequencer (model 370 and dye primers from Applied Biosystems, Foster City, Calif.). Computer-assisted sequence analyses and protein alignments were done by using programs of the University of Wisconsin Genetics Computer Group, Madison.

Localized mutagenesis. To construct *fixK* deletion mutants, plasmid pRJ7440 was digested with *Bam*HI. After a fill-in reaction and *Hin*dIII linker ligation, a shortened 6.8-kb *Hin*dIII vector fragment was ligated with a 1.3-kb *Hin*dIII fragment from pRJ1035 (containing the kanamycin resistance gene) in both orientations. The resulting plasmids were linearized by partial digestion with *Hin*dIII and ligated with a 1.7-kb *Hin*dIII fragment from pRJ7422 (carrying 87 bp of the 3′ end of the *fixK* coding region plus DNA downstream of *fixK*). The 3.6-kb *Xho*I fragments from the resulting plasmids, in which the 1.7-kb *Hin*dIII fragment was inserted between the *aph* gene and the vector, were cloned into the *Xho*I site of pSUP202X. The pSUP202X derivatives were named pRJ7453 (with the kanamycin resistance gene reading in the opposite direction of *fixK*) and pRJ7454 (in which the *aph* gene has the same orientation as *fixK*). To mutagenize the DNA region downstream of *fixK*, we made an analog of pRJ7422 in which the *Sal*I site was converted to an *Eco*RI site after a fill-in reaction and *Eco*RI linker ligation. Random Tn*5* mutagenesis of this plasmid and pRJ7440 (with *fixK* upstream DNA) in *E. coli* 294*cys*::Tn*5* was done as described previously (15, 20). The *Eco*RI fragments from plasmids with appropriate Tn*5* insertions were then cloned into pSUP202. All of the pSUP202 derivatives described before were mobilized from *E. coli* S17-1 to *B. japonicum* 110*spc*4 for marker exchange mutagenesis (20). For the physical map of the resulting *fixK* deletion strains, 7453 and 7454, and the locations of the Tn*5* insertions, see Fig. 2. The correct genomic structures of the corresponding mutant strains were confirmed by appropriate Southern blot analyses of total DNAs.

Construction of a translational *fixK′-′lacZ* fusion. A 3.3-kb *Pvu*II-*Pst*I fragment from plasmid pRJ7440 (which encodes the first 15 amino acids of FixK) was cloned into a pNM480X vector that had been linearized with *Sma*I and *Pst*I. The reading frame of the resulting in-frame *fixK′-′lacZ* fusion was confirmed by sequence analysis. A 6.2-kb *Xho*I-*Xba*I fragment of this plasmid construct, carrying the *fixK′-′lacZ* fusion with 1.0 kb of *B. japonicum fixK* upstream DNA, was ligated with pRKPOL2, which had been linearized with *Xho*I and *Xba*I. This led to plasmid pRJ9024 (see Fig. 2).

Construction of *B. japonicum fixK* expression plasmids. In a first step, we wished to combine the cloned regions of pRJ7422 and pRJ7440. A 1.7-kb *Hin*dIII fragment from plasmid pRJ7422 (containing the 3′ end of the *fixK* gene) was cloned into pRJ7440, which had been linearized with

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or origin
E. coli strains		
RR28	*hsdR hsdM recA pheS12 thi leu pro lac gal ara mtl xyl supE44 endA*	22
S17-1	*hsdR*/RP4-2 *kan*::Tn*5 tet*::Mu, integrated in the chromosome	49
HB101	*hsdR hsdM recA13* Str^r	10
JM101	Δ*lac-proAB thi supE* F'(*traD36 proAB*$^+$ *lacI*^{q} *Z* ΔM15)	33
MC1000	Δ(*lacIZYA*) X74 *galU galK* Δ(*ara-leu*) *rpsL*	11
JRG1728	MC1000 Δ(*tyrR-fnr-trg*)	52
294*cys*::Tn*5*	*pro thi hsdR hsdM recA cys*::Tn*5*	15
B. japonicum strains		
110*spc*4	Sp^r (wild type)	40
7403	Sp^r Km^r; *fixL*::*aph*	2
7360/7361	Sp^r Km^r; *fixJ*::*aph*	2
7453[a]	Sp^r Km^r; *fixK*::*aph*	This work
7454[a]	Sp^r Km^r; *fixK*::*aph*	This work
Plasmids		
pSUP202	Ap^r Tc^r Cm^r; *oriT* from RP4	49
pSUP202X	Ap^r Tc^r Cm^r; *oriT* from RP4; *Xho*I linker insertion in *Eco*RI site	C. Kündig
pRKPOL2	Tc^r (pRK290); pBLSII polylinker in *Eco*RI site	P. Grob
pSF6	Sp^r Sm^r; *oriT* from RP4	44
pPG3	Sp^r Sm^r Km^r (pSF6); *oriT* from RP4	P. Grob
pRK2013	Km^r; *tra*$^+$	12
pNM480	Ap^r; *'lacZ*	35
pNM480X	Ap^r (pNM480); *'lacZ*; *Xba*I linker insertion in *Stu*I site	M. Goettfert
pUC-4-KIXX	Ap^r; *lacZ*::*aph*	Pharmacia LKB Biotechnology, Uppsala, Sweden
pGem-1, pGem-2	Ap^r; SP6 and T7 promoters	Promega Biotec, Madison, Wis.
pBLSII (KS+)	Ap^r; f1 (+) *ori*	Stratagene, La Jolla, Calif.
M13mp18, M13mp19	Vectors for sequencing	37
pDD5	Ap^r Tc^r (pBR322); *fixK* (*R. meliloti*)	9
pRW2FF	Tc^r; *lacZ* (Fnr-dependent promoter)	28
pGS24	Ap^r (pBR322); *fnr* (*E. coli*)	46
pRJ1035	Km^r Tc^r (pSUP202); Rsα9 (*B. japonicum*)::*aph*::Rsβ3 (*B. japonicum*)	1
pRJ7422[b]	Ap^r (pGem-2); 2.3-kb *Eco*RI-*Sal*I fragment	This work
pRJ7440[b]	Ap^r (pGem-1); 4.8-kb *Hin*dIII-*Bgl*II fragment	This work
pRJ7453	Ap^r Tc^r (pSUP202); *fixK*::*aph*	This work
pRJ7454	Ap^r Tc^r (pSUP202); *fixK*::*aph*	This work
pRJ7464[b]	Ap^r (pGem-1); 6.6-kb *Sal*I-*Bgl*II fragment	This work
pRJ8002	Tc^r (pRK290X); *rpoN1'-'lacZ*	26
pRJ9020[b]	Ap^r (pBLS [KS+]); 0.95-kb *Bam*HI-*Xba*I (*Sma*I) fragment	This work
pRJ9024[b]	Tc^r (pRKPOL2); in-frame *fixK'-'lacZ*	This work
pRJ9031[b]	Sp^r Sm^r Km^r (pPG3); 0.97-kb *Sma*I-*Xba*I (*Sma*I) fragment	This work

[a] The genomic structures of these mutants are shown in Fig. 2.
[b] The inserts of these plasmids are shown in Fig. 2.

*Hin*dIII. This led to plasmid pRJ7464 (see Fig. 2). The correctly fused DNA sequence around the *Hin*dIII site was confirmed by sequence analysis. pRJ7464 was then digested with *Sma*I, and after *Xba*I linker ligation, a 0.95-kb *Bam*HI-*Xba*I fragment (containing the entire *fixK* gene and 150 bp of upstream DNA) was ligated with a *Bam*HI- and *Xba*I-linearized pBLSII (KS+) vector. The resulting plasmid, pRJ9020, expresses the *B. japonicum fixK* gene under control of the *lac* promoter of the pBLS vector (see Fig. 2). To construct a broad-host-range *fixK* expression plasmid useful for complementation of a *B. japonicum fixJ* mutant strain, a 0.95-kb *Sma*I-*Xba*I fragment of pRJ9020 was cloned into plasmid pPG3. This led to plasmid pRJ9031, which expresses the *fixK* gene constitutively from the promoter of Tn*5*-derived kanamycin resistance gene *aph* (see Fig. 2).

β-Galactosidase assays. *E. coli* strains harboring *lacZ* fusion plasmids were grown either aerobically in 100-ml Erlenmeyer flasks containing 10 ml of LB medium for 6 h at 37°C or under oxygen limitation in 8-ml airtight screw-cap plastic vials containing 7.5 ml of LB medium overnight at 28°C. To monitor β-galactosidase activity in *B. japonicum* strains that carried *lacZ* fusions, the cultures were grown aerobically for 3 days either in peptone-salts-yeast extract medium or in yeast extract-mannitol medium with 10 mM KNO_3. Anaerobic cultures were grown for 5 days in yeast extract-mannitol medium with 10 mM KNO_3 before assay of β-galactosidase activity. Microaerobic conditions were achieved by growing the cultures in 8-ml airtight plastic bottles containing 7 ml of peptone-salts-yeast extract medium for 3 days without shaking. β-Galactosidase assays were performed with 50-μl samples of at least 10 independent cultures as described by Miller (34).

Symbiotic nitrogen fixation. Plant infection tests with soybean (*Glycine max* L. Merr. cv. Williams) seedlings have

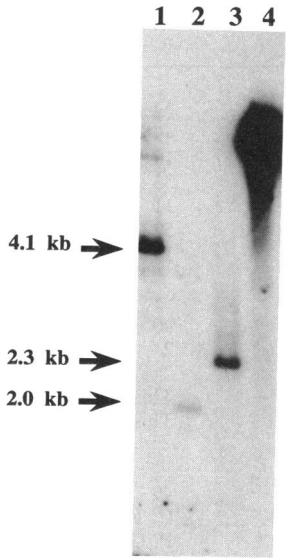

FIG. 1. Southern blot hybridization analysis of chromosomal DNAs of the *B. japonicum* wild type (lanes 1 to 3) and *R. meliloti* 2011 (lane 4). Genomic DNA was cut with *Eco*RI-*Bam*HI (lane 1), *Eco*RI-*Xho*I (lane 2), *Eco*RI-*Sal*I (lane 3), or *Eco*RI (lane 4) and hybridized with an *R. meliloti fixK* probe (see text).

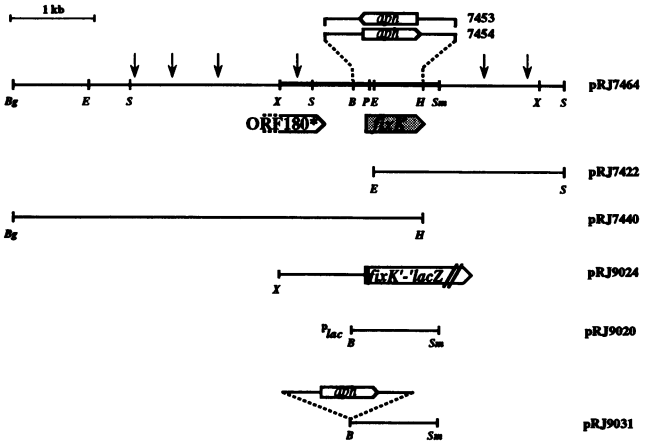

FIG. 2. Physical map of the *B. japonicum fixK*-like DNA region. The bold line indicates the region sequenced. The structures of *fixK* mutant strains 7453 and 7454, carrying an *aph* insertion, are shown on top. The positions of Tn*5* insertions are marked with vertical arrows. The numbers of the three clones covering the entire cloned region are given in the right margin. The structure of the translational *fixK-lacZ* fusion is shown below (pRJ9024). At the bottom, the clones carrying the *fixK* gene under control of the *lac* promoter (pRJ9020) and the *aph* promoter (pRJ9031) are shown. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; X, *Xho*I.

been described previously (18, 20). Symbiotic nitrogen fixation activity of whole root nodules was determined 20 days after inoculation by the acetylene reduction assay (59).

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to the GenBank data library and assigned accession no. M86805.

RESULTS

Cloning and DNA sequence of a *B. japonicum fixK*-like DNA region. Total genomic DNA of *B. japonicum* was examined in Southern blot hybridizations for the presence of *fixK*-homologous DNA regions by using the *fixK* gene of *R. meliloti* as a probe. A specific *fixK*-internal *R. meliloti* probe was obtained by subcloning of a 670-bp *Bgl*II-fragment from plasmid pDD5 (9) into *Bam*HI-linearized vector pGem-1 and subsequent isolation of a 485-bp *Eco*RI-*Pvu*I fragment thereof. Hybridization of this heterologous probe with *B. japonicum* total DNA revealed the presence of only one strongly hybridizing region in the genome (Fig. 1). A hybridizing 2.3-kb *Eco*RI-*Sal*I fragment from *B. japonicum* total DNA (Fig. 1, lane 3) was cloned into vector pGem-2, leading to plasmid pRJ7422 (Fig. 2). To obtain additional, adjacent DNA, an overlapping 4.8-kb *Hin*dIII-*Bgl*II fragment from *B. japonicum* total DNA was cloned into *Hin*dIII- and *Bam*HI-linearized vector pGem-1, leading to plasmid pRJ7440 (Fig. 2).

Nucleotide sequence analysis of a 1.82-kb *Xho*I-*Sma*I fragment showed the presence of one major ORF with high coding probability (Fig. 3). On the basis of its homology to the *fixK* sequence of *R. meliloti* (3), this ORF was named *fixK*. It was 711 bp long, predicting a protein of 237 amino acids. In front of the first possible ATG start codon at position 1026, a potential purine-rich Shine-Dalgarno sequencelike sequence was identified (Fig. 3). At positions 925 to 939, a sequence motif homologous to the *E. coli* Fnr consensus binding site (11) was found (Fig. 3). Upstream of the *fixK*-like gene, another ORF with high coding probability was identified, i.e., ORF180*, whose start extended beyond the beginning of the sequenced region. However, no homologies of this ORF to other known sequences were found in data bank searches.

Amino acid sequence similarities to *R. meliloti* FixK and other FixK- and Fnr-like proteins. The deduced amino acid sequence of the *B. japonicum* FixK-like protein showed a high degree of similarity to FixK- and Fnr-like proteins identified in other bacteria. It showed 38% identity to *R. meliloti* FixK (3), 35% identity to *R. leguminosarum* ORF240 (FnrN; 6) and *A. caulinodans* FixK (24), 29% identity to *P. aeruginosa* Anr (43, 62), and 28% identity to *E. coli* Fnr (47, 57) and *A. pleuropneumoniae* HlyX (29). The highest homologies among these proteins were found in the helix-turn-helix motif in the C-terminal region (Fig. 4), which is probably involved in DNA binding (5, 48). Additionally, we found a protein stretch containing several conserved glycine residues, of which four were present in *B. japonicum* FixK (between amino acids 60 and 110 of the alignment shown in Fig. 4). This sequence is believed to be an important determinant for the three-dimensional structure of a domain consisting of a β-roll structure in the first third of the proteins (48, 61). The N-terminal region of the *E. coli* Fnr protein was shown to contain four cysteine residues, three of which are crucial for the function of the protein, namely, Cys-20, Cys-23, and Cys-29; a fourth essential cysteine residue, Cys-122, was located in the central part of the protein (32, 45, 53). A similar motif with slightly different spacing between the cysteines (Cys-X_2-Cys-X_7-Cys-X_{87}-Cys) was found to be present in the *B. japonicum* FixK protein (Fig. 4), as well as in the ORF240 (FnrN) protein of *R. leguminosarum* (6), whereas the N-terminal regions of the *R. meliloti* and *A. caulinodans* FixK proteins contained no cysteine residues. In *A. pleuropneumoniae* HlyX and *P. aeruginosa* Anr, the spacing between the cysteine residues was the same as in the Fnr protein (29, 43, 62).

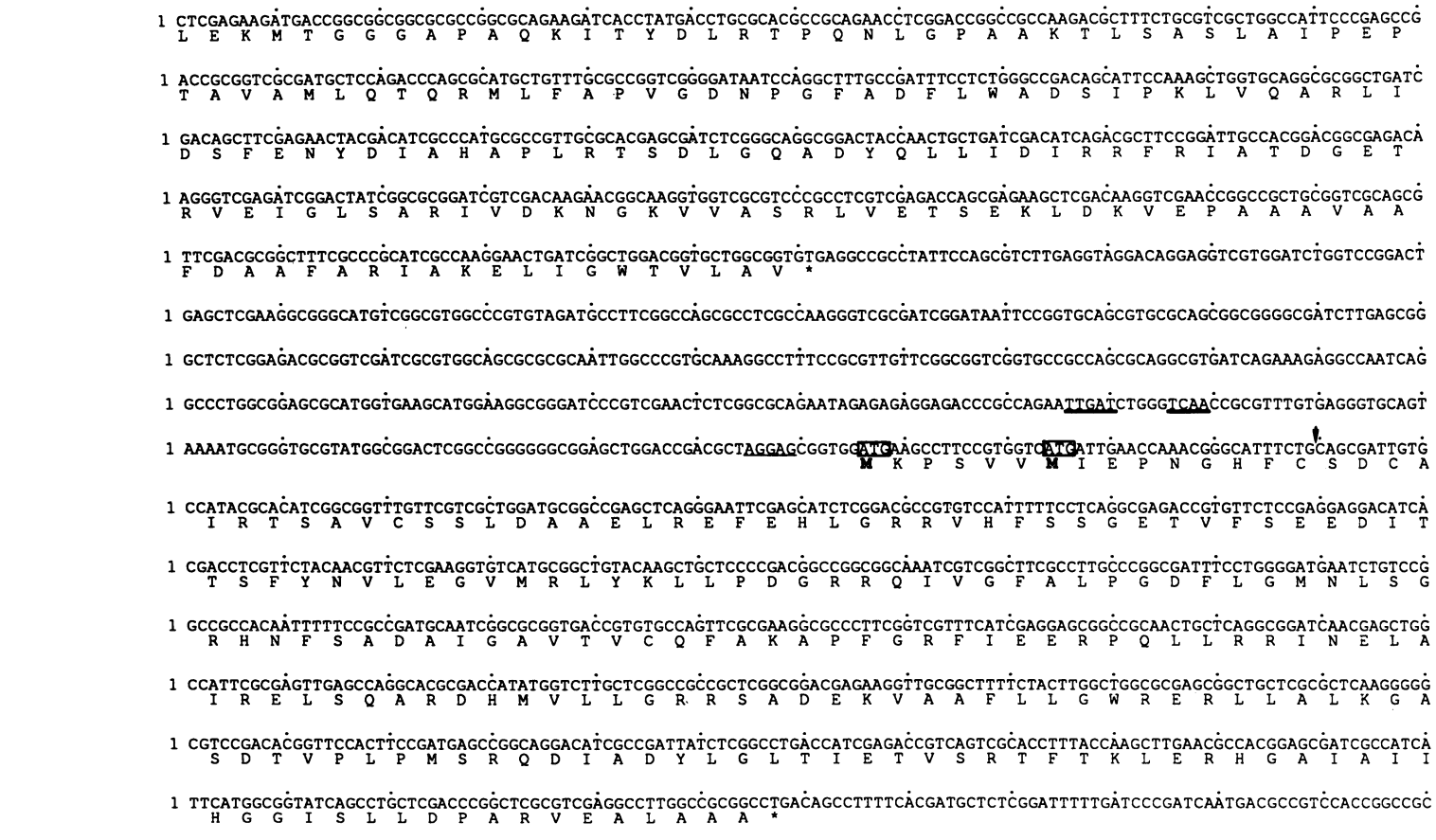

FIG. 3. Nucleotide sequence of the *fixK*-like DNA region. The deduced amino acid sequences of the two potential ORFs, *ORF180** and *fixK*, are shown below the DNA sequence. Putative start codons of *fixK* are boxed, and the corresponding amino acids are in boldface. A potential Shine-Dalgarno sequencelike sequence is underscored, and the sequence motif resembling an *E. coli* Fnr box is underlined by black bars. The arrow indicates the site of the translational *fixK'-'lacZ* fusion.

Expression of *B. japonicum fixK* is controlled by the FixLJ system. Recently, the *fixLJ* genes were identified in *B. japonicum* (2). To test whether expression of the *fixK*-like gene of *B. japonicum* depends on the oxygen conditions and is regulated by the FixLJ system, a translational *fixK'-'lacZ* fusion was constructed (see Materials and Methods). Plasmid pRJ9024, harboring the *fixK'-'lacZ* fusion with 1 kb of upstream DNA, was introduced into the *B. japonicum* wild type and into different mutant strains. In the wild type, the *fixK'-'lacZ* fusion was expressed at a low level under aerobic conditions (Table 2). In microaerobic cultures, this expression was enhanced by a factor of 4. In *fixL* and *fixJ* mutant strains 7403 and 7360, expression of the *fixK'-'lacZ* fusion was reduced to background levels. Thus, the *B. japonicum fixK* gene was maximally expressed in microaerobiosis and this expression was under control of the *fixLJ* gene products. A translational *fixK'-'lacZ* fusion carrying only 150 bp of *fixK*-upstream DNA showed the same regulation as pRJ9024 (data not shown), indicating that the DNA region essential for regulated expression of *fixK* was located within these 150 bp. FixK did not appear to regulate its own synthesis, because expression of the *fixK'-'lacZ* fusion was not significantly affected in *fixK* mutant strains 7453 and 7454 (Table 2).

Phenotypic analysis of *B. japonicum fixK* mutants. After we found *fixLJ* dependency of *B. japonicum fixK* expression, it was of interest to see whether *fixK* mutants would exhibit phenotypes similar to those known for *B. japonicum fixL* and *fixJ* mutants. In a previous publication (2), we reported that *fixLJ* mutants had a strongly reduced Fix phenotype in root nodule symbiosis with soybean plants and were deficient in growing anaerobically with nitrate as the terminal electron acceptor. In the present work, we constructed a *fixK* deletion mutant via marker exchange mutagenesis (see Materials and Methods). To exclude any effect of a possible outreading promoter activity of the inserted kanamycin resistance gene in the mutant strains, the *aph* gene cartridge was inserted in both orientations (Fig. 2). The Nod and Fix phenotypes of *fixK* mutant strains 7453 and 7454 were tested on soybean plants; both showed the same nodulation behavior and unaltered symbiotic nitrogen fixation activity compared with the *B. japonicum* wild type (data not shown). In another set of experiments, strains 7453 and 7454 were tested for anaerobic growth with nitrate. Both mutants were able to grow under such growth conditions. All of these experiments showed that the *fixK* gene characterized here was apparently not essential for the ability of *B. japonicum* to establish an effective symbiosis and perform nitrate respiration.

To analyze the DNA regions up- and downstream of *fixK*

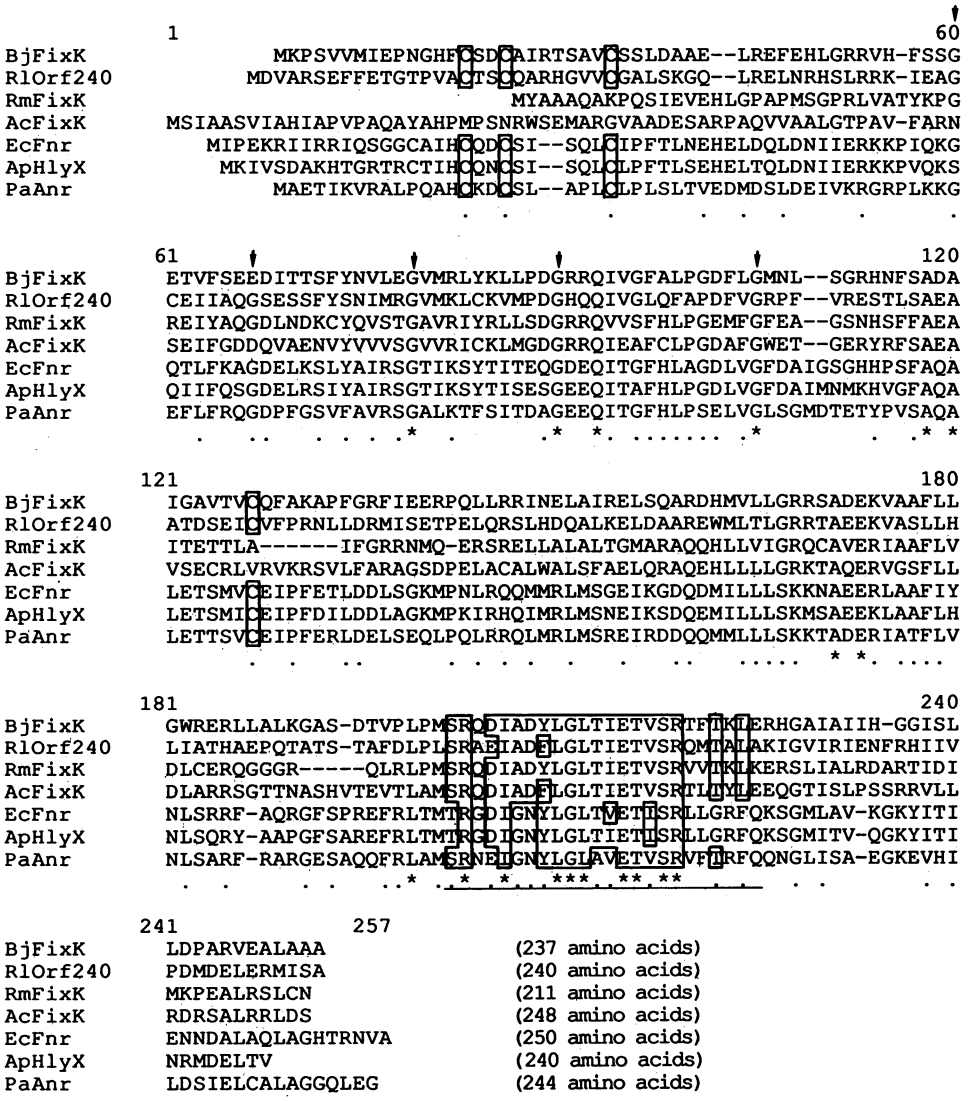

FIG. 4. Amino acid sequence alignment of the FixK proteins of *B. japonicum* (Bj), *R. meliloti* (Rm), and *A. caulinodans* (Ac); the ORF240 protein of *R. leguminosarum* biovar viciae (Rl); the Fnr protein of *E. coli* (Ec); the HlyX protein of *A. pleuropneumoniae* (Ap); and the Anr protein of *P. aeruginosa* (Pa). Asterisks indicate amino acids that are identical in all six sequences; amino acids identical in four or more sequences are shown by dots. Conserved cysteine residues that were shown to be essential for *E. coli* Fnr activity and amino acid residues identical in four or more sequences in the helix-turn-helix motif (underlined) are boxed. Conserved glycine residues are marked by arrows. See the text for references to the sources of the sequences shown.

for their possible function in symbiosis, plasmids pRJ7422 and pRJ7440 were mutagenized by Tn*5* insertions (see Materials and Methods). Six Tn*5* insertions were chosen and introduced into the *B. japonicum* genome (Fig. 2). One of the insertions was shown by restriction analysis to be located within ORF180*. All of the mutant strains were Nod$^+$ Fix$^+$ on soybean plants, which implies that the affected DNA regions do not contain symbiotically essential genes.

Expression of $rpoN_1$ in the *fixK* mutant. Transcription of *nif* genes and some of the *fix* genes is initiated at −24/−12-type promoters that are recognized by a specific sigma factor, σ^{54} (for reviews, see references 27 and 56). In a recent study, two functional homologs of σ^{54}-encoding genes were identified in *B. japonicum*, $rpoN_1$ and $rpoN_2$ (26), and expression of one of these genes ($rpoN_1$) was found to be regulated by FixL and FixJ. To determine whether this regulation was

TABLE 2. Expression of a plasmid-borne *fixK'-'lacZ* translational fusion in the *B. japonicum* wild type and various mutant strains grown under different oxygen conditions

Strains	Relevant genotype	β-Galactosidase activity (U)[a]	
		Aerobic	Microaerobic
110*spc*4	Wild type	23 (16)	82 (22)
7403	*fixL*::*aph*	6 (2)	11 (3)
7360	*fixJ*::*aph*	5 (3)	10 (5)
7453	*fixK*::*aph* (→)[b]	26 (17)	112 (24)
7454	*fixK*::*aph* (←)[b]	26 (13)	109 (20)

[a] Cultures were grown in peptone-salts-yeast extract medium as described in Materials and Methods. The background β-galactosidase activity of the *B. japonicum* wild type without any *lacZ* fusion was less than 3 Miller units (U). Standard deviations are in parentheses.

[b] The arrows denote the orientations of the *aph* genes inserted in the mutant strains with respect to the *fixK* reading frame as shown in Fig. 2.

TABLE 3. Effect of *B. japonicum fixK* on expression of an Fnr-dependent *E. coli* promoter

Strain[a] (plasmids)	Relevant genetic background	β-Galactosidase activity (U)[b]	
		Aerobic	Anaerobic
MC1000(pBLS, pRW2FF)	Wild type	32 (11)	1,580 (436)
JRG1728(pBLS, pRW2FF)	*fnr*	12 (5)	39 (12)
JRG1728(pGS24, pRW2FF)	*fnr*$^+$	363 (64)	1,816 (149)
JRG1728(pRJ9020, pRW2FF)	*fnr fixK*$^+$	38 (7)	221 (12)

[a] Strain JRG1728 was derived from *E. coli* MC1000 (52).
[b] Cultures were grown in LB medium as described in Materials and Methods. Standard deviations are in parentheses. At least eight cultures were assayed twice. β-Galactosidase activity is expressed in Miller units (U).

mediated via FixK, a translational *rpoN*$_1$'-'*lacZ* fusion (on plasmid pRJ8002) was introduced into the *fixK* mutant strains. However, expression of the *rpoN*$_1$'-'*lacZ* fusion was not affected by mutations in *fixK* (data not shown).

Complementation of an *E. coli fnr* mutant by the *B. japonicum fixK* gene. To obtain further information about the possible function of the *B. japonicum* FixK protein, we tested its ability to substitute for Fnr in *E. coli*. Expression of the *B. japonicum fixK* gene in *E. coli* was achieved by constructing pRJ9020, a plasmid in which *fixK* was expressed from the *lac* promoter of the vector (see Materials and Methods). *E. coli fnr* mutant strain JRG1728 cannot grow in nitrate medium under anaerobic conditions (52). Plasmid pRJ9020 restored the ability of this mutant to grow anaerobically on glycerol-nitrate minimal plates. Additionally, gas formation was observed in JRG1728 harboring pRJ9020 whereas the plasmid-free strain did not produce gas. These results indicate that FixK can function as a homolog of Fnr in *E. coli*.

To analyze more directly the function of FixK as a transcriptional activator, we tested its effect on transcription from an Fnr-dependent *E. coli* promoter. We used plasmid pRW2FF, which carried a *lacZ* gene with an Fnr-dependent promoter in front of it. In the *E. coli* MC1000 wild-type strain, this fusion was expressed at background levels under aerobic conditions whereas expression was enhanced about 50 times when cells grew anaerobically (Table 3). Anaerobic induction was abolished in JRG1728, the *fnr* mutant strain. By complementing JRG1728 with *fnr* in *trans* on plasmid pGS24 (46), regulation of the *lacZ* fusion was restored. Strain JRG1728, harboring pRJ9020, showed very low, FixK-dependent expression of the *lacZ* fusion under aerobic conditions, whereas anaerobically this expression was enhanced sixfold (Table 3). These findings indicate that FixK can act as a transcriptional activator in *E. coli* and that its optimal function depends on anaerobic conditions.

Complementation of a *B. japonicum fixJ* mutant by the constitutively expressed *fixK* gene. Although expression of the *B. japonicum fixK* gene was regulated by the FixLJ system, *fixK* mutant strains did not show any of the phenotypes known for *fixLJ* mutants (see above). To test whether this was perhaps due to a second homolog of a *fixK*-like gene in *B. japonicum* that was able to replace functionally the *fixK* gene described in this study, we tried to complement *fixJ* mutant strain 7361 with a constitutively expressed *fixK* gene. We constructed plasmid pRJ9031 (see Materials and Methods), in which *fixK* was expressed from the promoter of the kanamycin resistance gene (Fig. 2). *B. japonicum fixJ* mutant strain 7361 carrying solely vector pPG3 (control) was not able to grow anaerobically with nitrate (Fig. 5). Plasmid

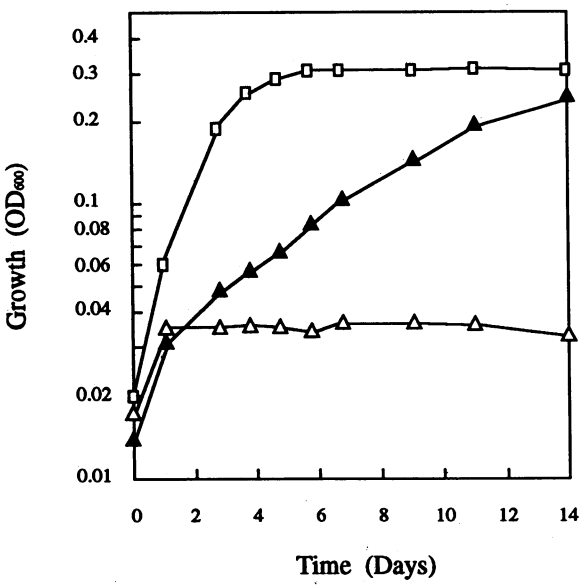

FIG. 5. Growth of the *B. japonicum* wild type and a *fixJ* mutant complemented with the *B. japonicum fixK* gene. The strains were grown anaerobically in selective yeast extract-mannitol medium with nitrate. Growth was determined by measuring the optical densities of the cultures at 600 nm (OD_{600}). Symbols: □, wild type carrying vector pPG3; △, *fixJ* mutant 7361 with pPG3; ▲, strain 7361 with pRJ9031.

pRJ9031, however, restored the ability of *fixJ* mutant strain 7361 to grow under such conditions (Fig. 5). This result supports the hypothesis of the existence of a second *fixK*-like gene in *B. japonicum* and indicates that at least the induction of genes involved in nitrate respiration by FixLJ occurs via FixK.

DISCUSSION

We report the discovery of a *B. japonicum* gene whose product is a new member of the Fnr-Crp family of bacterial gene regulators. Although this gene is neither structurally nor functionally fully analogous to any one of the other members of that family (see Introduction), we have termed it *fixK*, mainly for two reasons. (i) Its expression is maximal under reduced oxygen conditions and depends on the two-component regulatory system FixLJ. Exactly the same type of regulation was also reported for the *R. meliloti* and *A. caulinodans fixK* genes (3, 24). (ii) It shares the strongest overall amino acid sequence similarity with the rhizobial FixK proteins, especially with the one of *R. meliloti*. The homology is particularly striking in the C-terminal, putatively DNA-binding domain, in which 17 of 24 amino acids are identical in all four rhizobial homologs (Fig. 4). This may imply that they all recognize the same, *Rhizobium*-specific nucleotide sequence in front of the regulated target genes, a sequence that is very similar, but perhaps not identical, to the so-called Fnr box in *E. coli* (A_3-N-TTGAT-N_4-ATCAA-N-T_3; 11). In fact, on the basis of comparisons between similar DNA sequences in front of different rhizobial genes, of which some were known to be regulated by *R. meliloti* FixK, Colonna-Romano et al. (6) proposed a slightly modified consensus for a *Rhizobium* "anaerobox" (ANTTGATC-N_2-GATCAA).

There is one structural feature that is not common to all of

the rhizobial FixK-like proteins: the cysteine-rich amino terminus. This domain is present in the *B. japonicum* FixK protein and in the ORF240 (FnrN) protein of *R. leguminosarum* biovar viciae but absent in the *R. meliloti* and *A. caulinodans* FixK proteins (Fig. 4). A similar cysteine motif has been shown to be important for the activity of *E. coli* Fnr and might be involved in oxygen sensing, probably via interaction with an Fe ion (19). We have shown here (Table 3) that maximal activation of an Fnr-dependent promoter by FixK of *B. japonicum* occurs only under conditions of low oxygen concentration. The *B. japonicum* FixK protein thus appears to represent another oxygen-responsive regulator, possibly involving a mechanism of oxygen sensing similar to that proposed for the *E. coli* Fnr protein (19) and the *B. japonicum* NifA protein (13, 21). Oxygen control of FixK activity in *B. japonicum* is therefore exerted at two different levels, i.e., (i) the level of transcription of the *fixK* gene, which is subject to control by the oxygen-responsive FixLJ system, and (ii) the FixK protein level. The question of whether or not the same types of control also apply to the ORF240 (FnrN) protein of *R. leguminosarum* biovar viciae was not addressed in the work of Colonna-Romano et al. (6).

Initially, a surprising result in our work was that we did not come across a discernible phenotype for a *B. japonicum fixK* mutant. Likewise, an *R. leguminosarum* biovar viciae ORF240 (*fnrN*) mutant had no phenotypes other than a marginally affected symbiotic nitrogen fixation activity (6). In *B. japonicum*, the lack of a phenotype was particularly puzzling in view of the observed regulation of the *fixK* gene by the FixLJ system (Table 2) and our previous reports that *fixLJ* genes are required for efficient symbiotic nitrogen fixation, nitrate respiration, and $rpoN_1$ gene expression (2, 26). We can think of only two different options to explain this apparent discrepancy.

(i) FixK may regulate genes that are concerned with certain microaerobic or anaerobic processes other than nitrogen fixation and nitrate respiration. If this assumption is correct, it implies that the FixLJ system regulates $rpoN_1$ and some nitrogen fixation and nitrate respiration genes directly, whereas other genes (yet to be detected) are controlled by FixLJ via the *fixK* gene. A good example for a gene activated by FixJ, but not by FixK, is the *R. meliloti nifA* gene (3). Among possible candidates for *fixK*-regulated *B. japonicum* genes could be the hydrogen uptake (*hup*) and δ-aminolevulinate synthase (*hemA*) genes because both were shown to be derepressed at low oxygen tensions (25, 50). Interestingly, the *hemA* gene even has an anaerobox in close proximity to the promoter region (31). It will therefore be of interest to test whether these genes are regulated by *fixK*.

(ii) Alternatively, it is possible that there is a second homolog of a *fixK*-like gene in the *B. japonicum* genome which must also be regulated by FixLJ and whose product must have the required ability to replace functionally the product of the other *fixK* gene that was mutated in this work. Duplication of genes involved in symbiotic nitrogen fixation is not uncommon in rhizobia (26, 36, 39), and even the *fixK* gene itself is reiterated in the symbiotic plasmid of *R. meliloti* (41). In all of these cases, the gene repeats were easily identifiable by hybridization. By contrast, from several hybridization experiments done with *B. japonicum* total DNA (e.g., Fig. 1) we could not convince ourselves that there is a second *fixK*-homologous DNA region in *B. japonicum*. Such a second gene is obviously not so well conserved by DNA sequence, which will make it quite difficult to find. Strong evidence in favor of the existence of a second *fixK*-like gene in *B. japonicum* finally arose from the observation that the *fixK* gene characterized here, when expressed constitutively from an unregulated foreign promoter, was capable of complementing the nitrate respiration defectiveness phenotype of a *B. japonicum fixJ* mutant (cf. Fig. 5). This result makes sense if one assumes that the *B. japonicum* wild type possesses two functionally similar *fixK* homologs, both of which are regulated by the FixLJ system (as shown for one *fixK*-like gene in Table 2). In a *fixJ* mutant, none of these two homologs and, hence, none of the genes regulated by them would be expressed, and restoration of a wild-type phenotype would appear to become possible by providing a constitutively expressed *fixK* gene in *trans*. This was the underlying rationale of the experiment of Fig. 5, and as far as the nitrate respiration phenotype is concerned, the results lived up to expectations. In conclusion, our current hypothesis is that *B. japonicum*, like *R. meliloti*, has two *fixLJ*-dependent *fixK* genes whose products, in turn, are activators of the transcription of a group of genes involved in anaerobic processes such as denitrification (*B. japonicum*) and nitrogen fixation (*R. meliloti* and possibly *B. japonicum*).

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