

Comprehensive Assessment of the Regulons Controlled by the FixLJ-FixK₂-FixK₁ Cascade in *Bradyrhizobium japonicum*^{∇†}

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Symbiotic N₂ fixation in *Bradyrhizobium japonicum* is controlled by a complex transcription factor network. Part of it is a hierarchically arranged cascade in which the two-component regulatory system FixLJ, in response to a moderate decrease in oxygen concentration, activates the *fixK₂* gene. The FixK₂ protein then activates not only a number of genes essential for microoxic respiration in symbiosis (*fixNOQP* and *fixGHIS*) but also further regulatory genes (*rpoN₁*, *nrrR*, and *fixK₁*). The results of transcriptome analyses described here have led to a comprehensive and expanded definition of the FixJ, FixK₂, and FixK₁ regulons, which, respectively, consist of 26, 204, and 29 genes specifically regulated in microoxically grown cells. Most of these genes are subject to positive control. Particular attention was addressed to the FixK₂-dependent genes, which included a bioinformatics search for putative FixK₂ binding sites on DNA (FixK₂ boxes). Using an in vitro transcription assay with RNA polymerase holoenzyme and purified FixK₂ as the activator, we validated as direct targets eight new genes. Interestingly, the adjacent but divergently oriented *fixK₁* and *cycS* genes shared the same FixK₂ box for the activation of transcription in both directions. This recognition site may also be a direct target for the FixK₁ protein, because activation of the *cycS* promoter required an intact *fixK₁* gene and either microoxic or anoxic, denitrifying conditions. We present evidence that *cycS* codes for a *c*-type cytochrome which is important, but not essential, for nitrate respiration. Two other, unexpected results emerged from this study: (i) specifically FixK₁ seemed to exert a negative control on genes that are normally activated by the N₂ fixation-specific transcription factor NifA, and (ii) a larger number of genes are expressed in a FixK₂-dependent manner in endosymbiotic bacteroids than in culture-grown cells, pointing to a possible symbiosis-specific control.

Members of several genera of the alphaproteobacteria, collectively named “the rhizobia,” are capable of living not only in soil or in laboratory culture (free-living) but also facultatively within the infected cells of legume root nodules (endosymbiotic). Bacteroids—as they are called in the symbiotic state—fix molecular nitrogen as a nitrogen source for the host plant. Within root nodules, the rhizobia encounter oxygen-limiting conditions (microoxia) which trigger the expression of specific genes. Microoxia has been clearly recognized as a key factor that drives the synthesis and activity of nitrogenase, the enzyme that converts N₂ to ammonium (6, 19, 24, 25).

In the soybean symbiont *Bradyrhizobium japonicum*, a sophisticated regulatory network consisting of two linked regulatory cascades coordinates the expression of genes required for microaerobic respiration (the FixLJ-FixK₂ cascade) and for nitrogen fixation (the RegSR-NifA cascade). In these two cascades, different oxygen-sensing mechanisms are responsible for a stepwise activation of downstream events (63). In the RegSR-NifA cascade, the low-oxygen-responsive NifA protein activates the tran-

scription of essential symbiotic nitrogen fixation genes at an oxygen concentration at or below 0.5% oxygen in the gas phase over a culture. In contrast, only a moderate decrease of the ambient oxygen concentration, to 5%, in the gas phase over a culture is already sufficient to trigger ATP-dependent autophosphorylation of the deoxygenated FixL hemoprotein in the FixLJ-FixK₂ cascade and subsequent transfer of the phosphoryl group to the cognate response regulator FixJ (31, 33). Phosphorylated FixJ then activates the expression of the *fixK₂* gene. The FixK₂ protein, in turn, plays a dual role in that it downregulates, directly or indirectly, the expression of its own gene (53) and acts as a transcriptional activator of genes for adaptation to microoxia, such as the *fixNOQP* genes for the *cbb₃*-type high-affinity terminal oxidase, an enzyme that allows bacteroid respiration inside root nodules (53, 57).

A comparison of the regulatory circuits operating in *B. japonicum* with those in other rhizobial species reveals differences in the connectivity and subordination of the regulatory players FixLJ, FixK, and NifA (19). In *Azorhizobium caulinodans*, the *nifA* gene is directly regulated by FixK, whereas in *Sinorhizobium meliloti*, FixJ is the master regulator that directly controls both *nifA* and *fixK* (25). In *B. japonicum*, the only known FixJ target is *fixK₂*, whose product in turn activates the regulatory protein genes *fixK₁*, *rpoN₁*, and *nrrR*, thus expanding the downstream end of the cascade (47, 49, 53) to compose, for instance, a FixLJ-FixK₂-NnrR cascade (47).

FixK₂ is one of the 16 cyclic AMP receptor protein/fumarate and nitrate reduction regulator (CRP/FNR)-type transcrip-

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tional regulators whose genes are present in the *B. japonicum* genome (for a review see references 40 and 48). FixK₂ recognizes a palindromic sequence motif (TTG-N₈-CAA, termed the FixK₂ box) (49) which is located around position -41 upstream of the transcription start site in the regulated promoters. Until now, the expression from their promoters of 14 genes or operons was known to be controlled either directly or indirectly by FixK₂. Microaerobically induced targets of FixK₂ include the operons *fixNOQP* (as mentioned above) and *fixGHIS* (58), both essential for microaerobic respiration; several heme biosynthesis genes (*hemA*, *hemB*, *hemN*₁, and *hemN*₂) (15, 27, 55); denitrification genes (*napEDABC*, *nirK*, *norCBQD*, and *nosRZDFYLX*) (18, 50, 60, 67, 68); and some hydrogen oxidation genes (*hup* genes) (21). In a cell-free transcription system (in vitro), RNA polymerase, together with purified FixK₂, was shown to directly activate transcription from the *fixNOQP*, *fixGHIS*, and *hemN*₂ promoters (49).

No target genes had been known so far for the FixK₁ protein. Although it is a FixK₂ homolog, FixK₁ differs from FixK₂ in its strong oxygen sensitivity (4). Hence, maximal FixK₁ activity in vivo is achieved only in anoxic conditions (nitrate respiration). Despite this difference, however, the FixK₂ and FixK₁ proteins share a certain functional similarity, because the phenotypic defects of a *fixK*₂ mutant could be partially restored by constitutive *fixK*₁ gene expression (4, 53). The oxygen sensitivity of FixK₁ is most likely due to the presence of a cysteine-rich N-terminal extension (missing in FixK₂) whereby the FixK₁ protein much more closely resembles the oxygen-sensitive *Escherichia coli* FNR protein in which a [4Fe-4S]²⁺ cluster is bound to that domain (44, 45; reviewed in reference 39).

In order to expand our knowledge of the regulation mediated by the FixLJ-FixK₂-FixK₁ cascade, we aimed in this work at a genome-wide transcription profiling of *B. japonicum* *fixJ*, *fixK*₂, and *fixK*₁ mutant strains (always in comparison with the wild type), which were grown in free-living microoxic conditions and, in the case of the *fixK*₁ mutant, also in an anoxic condition. The latter condition could not be applied to the *fixJ* and *fixK*₂ mutants because they are defective in anaerobic nitrate respiration (3, 53). Furthermore, the transcriptomes of Δ *fixJ* and Δ *fixK*₂ bacteroids from soybean nodules were investigated. Bioinformatics tools used in a FixK₂ binding site search, together with in vitro transcription studies of putative targets, have allowed us to identify eight new genes whose expression is directly activated by FixK₂. Moreover, novel regulatory interrelations were discovered that may help unravel new facets in the control of the symbiotic and free-living microoxic lifestyles of *B. japonicum*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. The bacterial strains and plasmids used in this study are listed in Table 1. A list of oligonucleotides used as primers is available from the authors on request.

Media and growth conditions. *Escherichia coli* cells were routinely grown in Luria-Bertani (LB) medium (51) at 37°C. Where appropriate, antibiotics were used at the following concentrations (in μ g per ml): ampicillin, 200; kanamycin, 30; and tetracycline, 10. *B. japonicum* was grown aerobically and microaerobically (0.5% O₂ in the gas phase) in a modified peptone-salts-yeast extract medium (59) that contained the following ingredients (per liter): KH₂PO₄, 300 mg; Na₂HPO₄, 300 mg; CaCl₂ · 2H₂O, 5 mg; MgSO₄ · 7H₂O, 100 mg; peptone, 3 g; yeast extract, 1 g; H₃BO₃, 10 mg; ZnSO₄ · 7H₂O, 1 mg; CuSO₄ · 5H₂O, 0.5 mg;

Na₂MoO₄ · 2H₂O, 0.1 mg; MnCl₂ · 4 H₂O, 0.1 mg; FeCl₃, 0.19 mg; thiamine-HCl, 1 mg; biotin, 1 mg; Na-panthothenate, 1 mg; L-arabinose, 1 g. Yeast extract-mannitol medium (17) supplemented with 10 mM KNO₃ was used for *B. japonicum* growth under anoxic conditions (N₂ in the gas phase). Further details relevant for the growth of microaerobic and anaerobic cultures in transcriptome analyses have been described elsewhere (37, 56). The concentrations of antibiotics in *B. japonicum* cultures were as follows (in μ g per ml): chloramphenicol, 20; spectinomycin, 100; kanamycin, 100; streptomycin, 50; and tetracycline, 50 (solid medium) or 25 (liquid medium).

Plant growth. Seeds of soybean (*Glycine max* [L.] Merr. cv. Williams) were surface sterilized (5 min with ethanol and 15 min with 30% H₂O₂), rinsed several times with abundant sterile water, and incubated in darkness for 48 h on water-agar plates (1.5% agar). The inoculation and growth of the plants were carried out as described previously (34, 36). For transcriptome analyses, nodules were harvested 21 days postinoculation. They were then immediately frozen in liquid nitrogen and stored at -80°C for later RNA isolation. The in-nodule nitrogenase activity of *B. japonicum* strains was determined with an acetylene reduction assay (34, 36).

RNA isolation, cDNA synthesis, and microarray analysis. *B. japonicum* cultures were grown to mid-exponential phase, which corresponded to an optical density at 600 nm of 0.4 to 0.5 in microoxic cultures (peptone-salts-yeast extract-arabinose medium) and an optical density at 600 nm of 0.175 to 0.225 in anoxic cultures (yeast extract-mannitol-nitrate medium). Cell harvest, isolation of total RNA, cDNA synthesis, fragmentation, labeling, and conditions for microarray hybridization were done as described recently (37, 46, 56). A description of the custom-designed *B. japonicum* gene chip BJAPETHa520090 (Affymetrix, Santa Clara, CA) is given elsewhere (37).

For transcriptome profiling of bacteroids, all nodules from five plants infected with either the wild type or the *fixK*₂ or *fixJ* mutants were collected for each RNA extraction and hybridization experiment. The RNA was isolated by using a protocol of Pessi et al. (56). Amounts of 2.2 μ g and 5.5 μ g cDNA generated from RNA of culture-grown bacteria and nodules, respectively, were hybridized to the arrays. The amount of bacteroid-derived cDNA was estimated from the proportion of bacterial-to-plant rRNA in nodules (Bioanalyzer; Agilent Technologies, Palo Alto, CA). A minimum of six or three independent biological samples of each strain grown under free-living or symbiotic conditions, respectively, were analyzed. The primary data analysis was done with Affymetrix GeneChip Operating Software (GCOS) version 1.2. GeneSpring GX 7.3.1 software (Agilent Technologies) was used for comparative analyses. Only those probe sets that were called "present" or "marginal" in $\geq 69\%$ of the replicates of each experiment were considered for further analysis. The details of data processing, normalization, and further analyses are described elsewhere (56). Genes were considered to be differentially expressed only when they had passed the statistical tests and when the change in expression (measured as *n*-fold change [FC]) was ≥ 2 or ≤ -2 in comparisons between two strains or two different conditions.

Operon prediction and genome-wide FixK₂ binding site search. Operon prediction was done essentially by applying previously described criteria (37, 52). Genes were considered to be in an operon-like organization if they were oriented in the same direction and separated by less than 32 bp. This distance was enlarged to 100 bp if the first three letters in the gene names were identical. Additionally helpful was a tiling analysis of all probe sets within and around a gene of interest (37). For the identification of potential FixK₂ binding sites, we used a position-specific frequency matrix (PSFM) consisting of experimentally verified FixK₂ binding sites (see Table S1 in the supplemental material) in combination with a motif prediction algorithm (28). A similar strategy has previously been applied successfully for the identification of NifA + σ ⁵⁴- and RegR-dependent targets (37, 46). Putative promoter regions of 500 bp in length were searched for the PSFM motif. Sites considered to be putative FixK₂ binding sites were only those that had a higher score than that of the lowest-scoring motif in the set of already validated FixK₂ binding sites which had been used for the generation of the PSFM. For the identification of putative FixJ binding sites, a previously described de novo transcription factor binding site prediction was applied (29). This bioinformatics tool was applied for genes that showed decreased expression in a Δ *fixJ* strain (FC, ≤ -2) but did not depend on FixK₂.

In vitro transcription experiments. The plasmids used as transcription templates were based on pRJ9519 and pRJ8870 (Table 1), which contain one and two *B. japonicum* *rrn* transcription terminators, respectively. Plasmid pRJ8870 was particularly useful in the analysis of a promoter region of two adjacent but divergently transcribed genes, as it harbors two transcriptional terminators located at different positions. Simultaneous transcription from divergently oriented promoters then yields transcripts differing by about 50 nucleotides. Multiple-round in vitro transcription assays were carried out at 37°C with RNA polymer-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
<i>E. coli</i> strains		
DH5 α	<i>supE44 ΔlacU169</i> (ϕ 80 <i>lacZ</i> ΔM15) <i>hsdR17 recA1 gyrA96 thi-1 relA1</i>	BRL, Gaithersburg, MD
S17-1	Sm ^r Sp ^r <i>hsdR</i> (RP4-2 <i>kan::Tn7 tet::Mu</i> ; integrated into the chromosome)	64
<i>B. japonicum</i> strains		
110 <i>spc4</i>	Sp ^r ; wild type	59
7360	Km ^r <i>fixJ::aphII</i> (same orientation)	3
9043	Sp ^r Sm ^r <i>fixK₂::Ω</i>	53
7454	Km ^r <i>fixK₁::aphII</i> (same orientation)	4
9039K ₂	Sp ^r Sm ^r Km ^r <i>fixK₂::Ω fixJ::aphII fixK₁^c</i> (constitutive expression of <i>fixK₁</i>)	53
8882	Km ^r <i>cycS::aphII</i> (same orientation)	This work
8883	Km ^r <i>cycS::aphII</i> (opposite orientation)	This work
8884	Sp ^r Tc ^r <i>cycS-lacZ</i> chromosomally integrated into 110 <i>spc4</i>	This work
8884K ₂	Sp ^r Sm ^r Tc ^r <i>cycS-lacZ</i> chromosomally integrated into 9043	This work
8884JK ₂	Sp ^r Sm ^r Km ^r Tc ^r <i>cycS-lacZ</i> chromosomally integrated in 9039K ₂	This work
Plasmids		
pBluescript SK(+)	Ap ^r cloning vector	Stratagene, La Jolla, CA
pGEM-T Easy	Ap ^r cloning vector	Promega, Madison, CA
pBSL86	Ap ^r Km ^r	1
pSUP202pol4	Tc ^r (pSUP202) part of the polylinker from pBluescript II KS(+) between EcoRI and PstI	26
pSUP202pol6K	Tc ^r (pSUP202pol4) KpnI linker into SmaI site	69
pSUP3535	Tc ^r (pSUP202pol4) 3.2-kb EcoRI-DraI fragment from pME3535, transcriptional <i>lacZ</i> fusion vector	Laboratory collection
pRJ9519	Ap ^r [(pBluescript SK(+)) 308-bp BstXI-KpnI fragment containing the <i>B. japonicum</i> <i>rrn</i> terminator cloned into the HincII and KpnI sites	8
pRJ8870	Ap ^r (pRJ9519) 210-bp SacII-XbaI fragment containing a second <i>B. japonicum</i> <i>rrn</i> terminator cloned into the SacII and XbaI sites	This work
pRJ9601	Ap ^r [pBluescript SK(+)] <i>B. japonicum</i> <i>rrn</i> promoter and <i>rrn</i> terminator on 468-bp SacI-SmaI fragment	8
pRJ8817	Ap ^r (pRJ9519) <i>fixGHIS</i> promoter on 524-bp XbaI-EcoRI fragment	49
pRJ8860	Ap ^r (pRJ9519) blr6062 (<i>cycS</i>) promoter on 409-bp XbaI-SpeI fragment	This work
pRJ8861	Ap ^r (pRJ9519) blr6070 promoter on 455-bp BamHI-EcoRI fragment	This work
pRJ8862	Ap ^r (pRJ9519) bsr7087 promoter on 316-bp BamHI-EcoRI fragment	This work
pRJ8863	Ap ^r (pRJ9519) blI2388 promoter on 336-bp BamHI-EcoRI fragment	This work
pRJ8864	Ap ^r (pRJ9519) blI6073 promoter on 509-bp XbaI-SpeI fragment	This work
pRJ8865	Ap ^r (pRJ9519) blr4637 promoter on 407-bp BamHI-EcoRI fragment	This work
pRJ8867	Ap ^r (pRJ9519) blI3998 promoter on 641-bp BamHI-EcoRI fragment	This work
pRJ8869	Ap ^r (pRJ9519) blr4655 promoter on 428-bp BamHI-EcoRI fragment	This work
pRJ8871	Ap ^r (pRJ8870) <i>fixK₁-cycS</i> intergenic region on 171-bp XbaI-EcoRI fragment	This work
pRJ8872	Ap ^r [(pBluescript SK(+)) 3'-flanking sequence of <i>cycS</i> on 554-bp HindIII-XbaI fragment	This work
pRJ8875	Ap ^r [(pBluescript SK(+)) 5'-flanking sequence of <i>cycS</i> on 561-bp Acc65I-HindIII fragment	This work
pRJ8879	Ap ^r (pRJ8872) 561-bp Acc65I-HindIII fragment from pRJ8875	This work
pRJ8880	Ap ^r Km ^r (pRJ8879) <i>cycS::aphII</i> , 1,182-bp HindIII from pBSL86 (same orientation)	This work
pRJ8881	Ap ^r Km ^r (pRJ8879) <i>cycS::aphII</i> , 1,182-bp HindIII from pBSL86 (opposite orientation)	This work
pRJ8882	Km ^r Tet ^r (pSUP202pol6K) 2,297-bp Acc65I-XbaI fragment from pRJ8880	This work
pRJ8883	Km ^r Tet ^r (pSUP202pol6K) 2,297-bp Acc65I-XbaI fragment from pRJ8881	This work
pRJ8884	Tc ^r (pSUP3535) <i>cycS-lacZ</i> , <i>cycS</i> promoter on 541-bp SmaI fragment	This work
pRJ8886	Ap ^r [(pBluescript SK(+)) 5'-flanking sequence of <i>cycS</i> on 741-bp SmaI-XbaI fragment	This work

ase holoenzyme purified from *B. japonicum* at 37°C as described previously (49). Different amounts (0 to 2.5 μM dimer) of FixK₂ protein purified as described earlier (49) were added to the reaction mixture. Suitable RNA size markers were prepared in vitro with T3 RNA polymerase (49). Electrophoresis of radioactive

transcription products was done in a denaturing 6% polyacrylamide gel, and the reaction products were visualized with a phosphorimager. The quantification of signal intensities was performed with Bio-Rad Quantity One software, version 4.6.1 (Bio-Rad, Reinach, Switzerland).

Primer extension experiments. The *in vivo* transcription start site of *cycS* was mapped in a primer extension experiment using *cycS*-specific oligonucleotides according to previously described protocols (5, 54). RNA was isolated as described above from the wild-type *B. japonicum* strain and from *fixK₂* mutant cells grown in microoxic or anoxic (applicable only to the wild type) conditions. Determination of the transcription start site of the *in vitro*-synthesized *cycS* transcript was carried out according to the method of Mesa et al. (49), using primer 9519-1. The extension products were analyzed on denaturing 6% polyacrylamide gels adjacent to sequencing ladders generated with the same oligonucleotides and plasmids pRJ8886 and pRJ8860 (for the *in vivo* and *in vitro* start sites, respectively).

Construction of *cycS* mutant strains. *B. japonicum cycS* mutant strains 8882 and 8883 were constructed by marker exchange mutagenesis. To do so, 5' and 3' flanking regions of the *cycS* gene were amplified by PCR and cloned into pSUP202pol6K (Table 1). A kanamycin resistance cassette from pBSL86 was inserted in both orientations between the two *B. japonicum* DNA fragments. The resulting plasmids, pRJ8882 and pRJ8883, were transferred via conjugation into *B. japonicum* 110*spc4* by using *E. coli* S17-1 as donor. The correct genomic structures of the *cycS* mutations were confirmed by PCR. The mutant strains are listed in Table 1.

Construction of a chromosomally integrated *cycS-lacZ* fusion, and β -galactosidase activity testing. A transcriptional *cycS-lacZ* fusion was obtained by PCR amplification of a 541-bp *Sma*I fragment with the *cycS* promoter region which was then cloned into pSUP3535, yielding plasmid pRJ8884. Plasmid pRJ8884 was mobilized by conjugation into *B. japonicum* strains 110*spc4*, 9043, and 9039K₂. The correct genomic integration was verified by PCR. The determination of β -galactosidase activities was carried out as described previously (27).

Cell fractionation, SDS-polyacrylamide gel electrophoresis, and cytochrome *c* staining. *B. japonicum* cells were grown anoxically and harvested at stationary phase. Cell fractionation was carried out as indicated earlier (27). Soluble fractions were loaded without boiling onto sodium dodecyl sulfate (SDS)-18% polyacrylamide gels (42). The proteins were stained for heme-dependent peroxidase activity by using a "Supersignal West pico chemiluminescent substrate" chemiluminescence detection kit (Perbio Science, Lausanne, Switzerland). The protein concentration was estimated by using a Bio-Rad assay (Bio-Rad, Reinach, Switzerland) with bovine serum albumin as the standard.

Microarray data accession number. The microarray data are available in the NCBI Gene Expression Omnibus database (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) under GEO Series accession number GSE12491.

RESULTS

Global assessment of genes controlled by the FixLJ-FixK₂-FixK₁ cascade in microoxically cultured cells. Microarray analysis has led to a registry of *B. japonicum* genes that are induced (FC, ≥ 2) in microoxically grown cells (0.5% O₂ in the gas phase) in comparison to their expression in oxically grown cells (21% O₂ in the gas phase) (56). Which and how many of these genes are subject to regulation by the hierarchically organized transcription factors FixJ, FixK₂, and FixK₁ was assessed by comparing the transcriptomes of wild-type cells and *fixJ*, *fixK₂*, and *fixK₁* mutants, all grown microoxically. The total number of genes that are upregulated in microoxic conditions and, at the same time, regulated exclusively by FixJ, FixK₂, or FixK₁ is shown in Fig. 1, and the corresponding genes are listed in Tables S2 and S3 in the supplemental material.

It becomes evident that the regulon size of FixK₂ is much larger than those of FixJ and FixK₁ (Fig. 1). The FixJ and FixK₂ regulons consist almost solely of positively controlled genes, whereas a substantial portion of the FixK₁-regulated genes are negatively controlled (12 out of 29).

Prior to this work, the only gene known to be controlled by the FixJ response regulator was *fixK₂* (53). An additional 25 genes have now been found to be specifically regulated by FixJ (Fig. 1). However, a more-detailed examination of these genes was not pursued for three reasons. (i) The majority have no predicted function (see Tables S2 and S3 in the supplemental

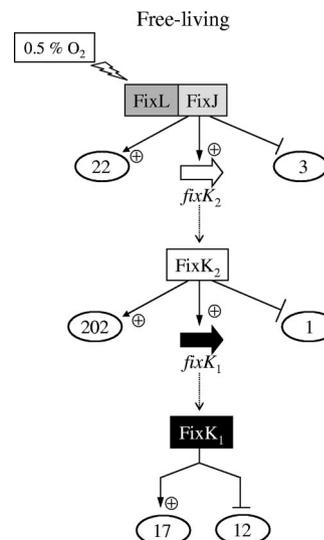


FIG. 1. Schematic representation of microoxically induced *B. japonicum* genes that are regulated by FixJ, FixK₂, or FixK₁. The numbers of genes controlled by these regulators are circled by ovals. The *fixK₁* gene and its product are highlighted with a black arrow and white letters in a solid rectangle, respectively. Details are explained in the text. Note that although the model suggests a direct hierarchical organization, the existence of additional control levels in between FixLJ and FixK₂ and in between FixK₂ and FixK₁ cannot be excluded. Therefore, the regulation of target genes by FixJ, FixK₂, or FixK₁ may be direct or indirect. +, positive regulation; -, negative regulation.

material). (ii) With few exceptions, their levels of up- or down-regulation are comparatively small, suggesting that they are not strongly activated or repressed by FixJ. (iii) The use of bioinformatics search tools (see Materials and Methods) has not led to the identification of a conserved nucleotide sequence motif that might serve as a FixJ binding site in the DNA regions upstream of FixJ-regulated genes. Such a putative "FixJ box" would have been helpful in a first approximation to possibly distinguish directly from indirectly controlled genes.

Our primary attention in this work was, therefore, addressed to the genes positively controlled by FixK₂. Apart from previously identified FixK₂-dependent genes (*fixK₁*, *rpoN₁*, *nrrR*, *nirK*, *hemN₁*, and *hemN₂*) and operons (*fixNOQP*, *fixGHIS*, and *napEDABC*), new genes have now been identified that must be regarded as promising candidates for being FixK₂ targets (see Table S2 in the supplemental material). Comments on a few examples follow. (i) There are cytochrome genes, such as *blr4955* (cytochrome *b₅₆₁*) or *blr6128* (*cycB*, encoding cytochrome *c₅₅₂*), which suggests that these genes are possibly important for life under conditions of oxygen deprivation. (ii) There are genes such as *blr3998*, coding for a succinate-semialdehyde dehydrogenase (a tricarboxylic acid cycle bypass enzyme); *blr4655*, coding for a phosphoenolpyruvate synthase (gluconeogenesis enzyme); and *blr6073* (*phbC*), coding for a poly- β -hydroxybutyrate (PHB) polymerase, that indicate an involvement of FixK₂ in regulating carbon and energy metabolism. (iii) There are genes for general stress response, such as *blr4635* (*groEL₅*) and *blr4653* (*dnaJ*), and a heat shock-related gene (*blr4637*), an observation that has already been made by Bobik et al. (10) when these authors examined the *S. meliloti* FixJ regulon. (iv) Like *fixK₁* and *nrrR* (4, 47, 53), FixK₂ ap-

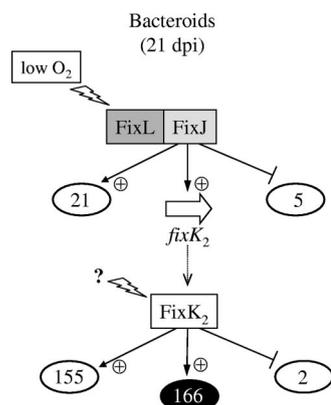


FIG. 2. Schematic representation of soybean bacteroid-induced genes that are controlled by FixJ or FixK₂. For details, see the text and the Fig. 1 legend. A set of 166 FixK₂-activated genes is not at the same time dependent on FixJ (indicated in white letters on a black background). An unknown regulatory signal might be sensed at the level of FixK₂ (directly or indirectly). dpi, days postinoculation; +, positively regulated; -, negatively regulated.

pears to control other regulatory genes, e.g., blI2109 and blI3466, both coding for CRP-type regulators, which could imply a further expansion of the regulatory cascade.

No FixK₁-controlled gene had been identified so far. To find out how many genes are exclusively controlled by FixK₁, the transcription profiling of a $\Delta fixK_1$ strain was compared with that of the wild type and the $\Delta fixK_2$ strain, all grown in microoxic conditions. A relatively small number of genes showed differential expression in the $\Delta fixK_1$ strain (17 positively and 12 negatively controlled genes) (Fig. 1; see Tables S2 and S3 in the supplemental material). Among the positively regulated genes is *hemN₁*, whose expression was previously shown to depend on FixK₂ (27). This shows that the FixK₂ dependency of *hemN₁* expression proceeds indirectly via FixK₁, although a direct contribution by FixK₂ cannot be excluded. Interesting new FixK₁ targets are two cytochrome genes (blI2388 [cytochrome *c*₂] and blr6062 [cytochrome *c*₆]), which will be the subject of further transcriptional studies (see below).

When we looked at the genes that are negatively regulated by FixK₁, an intriguing observation was made. In addition to the 12 genes shown in Fig. 1, we noticed another 34 genes whose expression was not at the same time increased in the $\Delta fixK_2$ mutant. These are listed at the bottom of Table S3 in the supplemental material. This makes a total of 46 genes that appear to be repressed, directly or indirectly, by FixK₁. Surprisingly, almost all of these negatively regulated genes (45 out of 46) are known to be under positive control by the transcriptional activator NifA (37). Similarly, when we compared the transcription profile of $\Delta fixK_1$ cells with that of wild-type cells, both grown under anoxic conditions (nitrate respiration), a substantial number of genes overlapped with the NifA regulon (32 out of 44) (data not shown). Taken together, these results indicate that a hitherto unrecognized regulatory interference might exist between the FixLJ-FixK₂-FixK₁ cascade and the RegSR-NifA cascade (see Discussion).

Transcription profiling of the *B. japonicum* *fixJ* and *fixK₂* mutants in symbiosis. A regulatory pattern that was similar though not completely identical to that found in microoxically

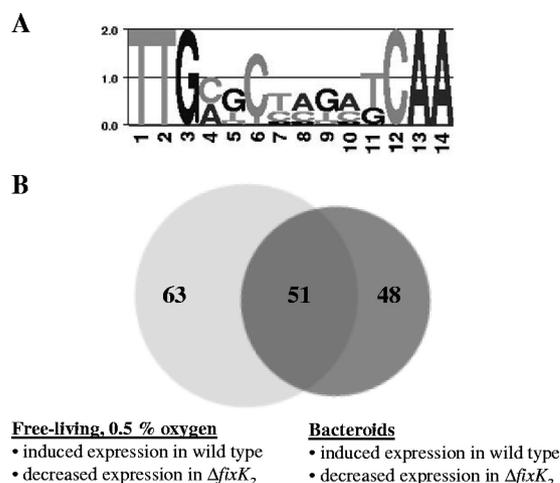


FIG. 3. Strategies for the identification of direct FixK₂ targets. (A) Sequence logo for the FixK₂ binding site created with “WebLogo” (16). The consensus motif is based on the sequences listed in Table S1 in the supplemental material (see also Materials and Methods). (B) Venn diagram representing FixK₂-dependent mono-, di-, or polycistronic transcription units which contain putative FixK₂ boxes in their upstream promoter regions. The left circle contains 114 transcription units induced in free-living, microoxic culture, whereas the right circle contains 99 transcription units induced in bacteroids. For further details, see the text and Table 2.

cultivated cells was seen for genes expressed in endosymbiotic bacteroids (Fig. 2; see Tables S4 and S5 in the supplemental material). In this case, we analyzed the transcriptomes of the wild type and of $\Delta fixJ$ and $\Delta fixK_2$ mutant bacteroids, but not of $\Delta fixK_1$ bacteroids, because in contrast to *fixJ* and *fixK₂*, the *fixK₁* gene is not essential for symbiotic nitrogen fixation (4, 53).

In a previous investigation, Pessi et al. (56) reported 692 *B. japonicum* genes to be induced in soybean bacteroids (21 days postinoculation). In this work, we noticed that a substantial proportion of these belong to the regulons of the FixLJ-FixK₂ cascade (i.e., 183 genes). The expression of the majority (155 genes) is decreased at the same time in $\Delta fixK_2$ bacteroids and in $\Delta fixJ$ bacteroids, which demonstrates again that FixJ is the hierarchically superimposed regulator of *fixK₂* (Fig. 2; see Table S4 in the supplemental material). The small number of negatively controlled genes is listed in Table S5 in the supplemental material.

Unexpectedly, bacteroids were found to express 166 FixK₂-activated genes (Fig. 2) which are not dependent at the same time on FixJ. A possible implication of this finding is that another type of regulatory signal or protein, uncoupled from FixJ control, acts at the level of the FixK₂ protein. Interestingly, among this set of 166 genes are putative regulator and sigma factor genes. Examples are blr1880 (LuxR-like) and blr3042 (ECF-type sigma factor).

Identification of direct FixK₂ targets. In order to find out which genes are directly controlled by FixK₂, we first used a bioinformatics approach and subsequently a biochemical approach. The purpose of the bioinformatics approach was to identify genes that carry a putative FixK₂ binding site (FixK₂ box) (49) in their promoter regions (see Materials and Methods and Fig. 3A) (28). Relevant for this analysis were the 220

TABLE 2. List of the 51 FixK₂ box-associated promoter regions and genes whose expression is decreased in the *fixK₂* mutant in microoxic free-living conditions and in bacteroids compared to their expression in the wild type

Locus tag ^a	Gene ^b	FC value in microoxic conditions ^c	FC value for bacteroids ^d	Description ^e	Predicted operon structure ^f	Position ^g	Motif ^h
blr0497		-27.4	-21.6	Hypothetical protein		-31	TTGATCCAGCGCAA
blI0818		-9.3	-7.5	Unknown protein		-66	TTGATCCCAGGTCAA
blr1289		-23.1	-2.2	Hypothetical protein		-37	TTGATCCAGCGCAA
blr1311		-69.9	-10.0	Outer membrane protein		-60	TTGATCGGCGTCAA
blI1766		-4.9	-3.8	Outer membrane protein		-228	TTGATTTGGTATCAA
blr1883	<i>rpoN₁</i>	-3.5	-3.7	RNA polymerase σ^{54} subunit		-81	TTGCGCGCATCAA
blI2007	<i>hemN₁</i>	-16.8	-15.3	Coproporphyrinogen III dehydrogenase		-138	TTGACATAACGCAA
blI2109		-2.5	-4.8	Transcriptional regulatory protein CRP family		-38	TTGCGTCACTCAA
blI2330		-18.9	-4.6	Hypothetical protein	blI2330-bsI2328	-73	TTGATCCAGATCAA
blI2388	<i>cy₂</i>	-5.3	-10.4	Cytochrome <i>c₂</i>		-140	TTGATGCAGGACAA
blI2471		-35.5	-10.1	Hypothetical protein		-81	TTGATCTAGCGCAA
blr2659		-16.3	-4.0	Hypothetical protein		-79	TTGCCTGGCATCAA
blI2662		-14.6	-5.2	Hypothetical protein		-27	TTGATCTGCATCAA
bsr2670		-5.6	-14.5	Unknown protein		-141	TTGAAGGAGGTCAA
blr2761	<i>orf277</i>	-26.7	-12.7	Hypothetical protein		-71	TTGATCTATCTCAT
blr2763	<i>fixN</i>	-101.9	-18.8	Cytochrome- <i>c</i> oxidase	<i>fixNOQP</i>	-70	TTGATTTCAATCAA
blr2767	<i>fixG</i>	-63.3	-53.2	Iron-sulfur cluster-binding protein	<i>fixGHIS</i>	-71	TTGAGCTGGATCAA
blr2987		-10.1	-9.7	Hypothetical protein		-77	TTGATTTGCGTCAA
bsr3073		-10.0	-32.5	Hypothetical protein		-101	TTGACGCGATCAA
blI3835		-8.7	-8.3	Hypothetical protein		-93	TTGCTGCAAATCAA
blI3998		-56.5	-10.9	Probable succinate-semialdehyde dehydrogenase		-120	TTGACCTGTCTCAA
blr4114		-62.5	-25.6	Hypothetical protein	blr4114-blr4115	-113	TTGATCTGGATCAT
blr4174		-46.7	-6.5	Hypothetical protein		-71	TTGATCGAGCGCAA
bsr4175		-5.0	-5.7	Hypothetical protein		-324	CTGCGCCAGTCAA
blr4637		-111.5	-20.7	Probable HspC2 heat shock protein		-86	TTGACAAAATCAA
bsI4650		-20.9	-6.6	Unknown protein		-165	CTGATCTAGCGCAA
blI4651		-31.3	-7.1	Hypothetical protein		-98	TTGATGTCCATCAA
blr4652		-95.2	-6.9	Hypothetical protein	blr4652-blr4654	-48	TTGATCGACATCAA
blr4955		-16.1	-8.1	Putative cytochrome <i>b₅₆₁</i>		-161	ATGAGGTGGATCAA
bsI5002		-5.5	-5.6	Unknown protein		-31	TTGATCTGCATCAT
bsr5273		-102.6	-68.5	Unknown protein		-59	TTGCGGTGCATCAA
blI5315		-34.4	-10.2	Hypothetical protein		-49	TTGATCCTGCGCAA
bsr5316		-22.5	-9.7	Hypothetical protein		-48	CTGATCTAGATCAA
blI5655		-36.2	-3.6	Alcohol dehydrogenase		-69	TTGACTCCAATCAA
blI6061	<i>fixK₁</i>	-19.1	-3.9	Transcriptional regulatory protein CRP family		-100	TTGATCTGGGTCAA
blI6069		-29.4	-14.6	Hypothetical protein		-72	TTGACCTCCCTCAA
blr6070		-7.3	-2.4	Putative alcohol dehydrogenase	blr6070-blr6072	-78	TTGAGGGAGGTCAA
blI6073	<i>phbC</i>	-27.9	-6.0	Probable poly- β -hydroxybutyrate polymerase		-81	TTGATGCAGCTCAA
blr6074		-90.9	-5.6	Hypothetical protein		-143	TTGAGCTGCATCAA
blr6128	<i>cycB</i>	-16.0	-13.7	Cytochrome <i>c₅₅₂</i>		-266	TTGCGGCGATCAA
blI6987		-2.0	-4.0	Hypothetical signal peptide protein		-466	TTGACATCGATCAA
bsr7036	<i>napE</i>	-64.1	-6.8	Periplasmic nitrate reductase protein	<i>napEDABC</i>	-101	TTGATCCAGATCAA
blI7086	<i>hemN₂</i>	-97.1	-4.4	Anaerobic coproporphyrinogen III oxidase		-140	TTGCGCGAGCGCAA
bsr7087		-53.8	-30.7	Unknown protein	bsr7087-blr7088	-115	TTGCGCTCGCGCAA
blr7345		-16.8	-4.0	Unknown protein		-76	TTGATCCGCATCAA
bsI7372		-35.1	-2.9	Hypothetical protein		-67	TTGACGGAGATCAA
blI7553		-11.4	-4.3	Unknown protein		-73	TTGATATGCGTCAA
bsI7781		-3.0	-4.2	Unknown protein		-102	TTGATTCGGCGCAA
blI7787		-19.1	-15.3	Unknown protein		-118	TTGACCCAGATCAA
blr7961		-41.7	-28.3	Probable HspC2 heat shock protein		-82	TTGAGACAAATCAA
blI7982		-13.5	-8.3	Hypothetical protein	blI7982-blI7981	-96	TTGATCTGAAACAA

^a Nomenclature according to Kaneko and coworkers (38).

^b Gene name as indicated in the EMBL-EBI database.

^c Values from a comparison of $\Delta fixK_2$ cells with wild-type cells grown in microoxic conditions. Note that the expression of these genes under these conditions is induced in the wild-type strain (compared to the expression in free-living oxic cells [56]).

^d Values from a comparison of $\Delta fixK_2$ bacteroids with wild-type bacteroids. Note that the expression of these genes under these conditions is induced in the wild-type strain (compared to the expression in free-living oxic cells [56]).

^e Protein description according to Kaneko and coworkers (38).

^f Operons were predicted as described in Materials and Methods.

^g Position of the 5'-end nucleotide of the motif relative to the annotated translational start site of the associated gene.

^h Predicted FixK₂ binding site.

FixK₂-dependent genes induced in free-living bacteria, as shown in Fig. 1 (i.e., 202 + *fixK₁* + 17), and the 321 FixK₂-dependent genes induced in bacteroids, as shown in Fig. 2 (i.e., 155 + 166). These two sets respectively contained 114 and 99 putative FixK₂ boxes in promoter regions upstream of genes organized in mono-, di-, or polycistronic transcription units (Fig. 3B). The overlap resulted in 51 FixK₂ box-associated

transcription units (Fig. 3B and Table 2). These 51 cases provided the basis for the selection of seven promoter regions that were tested for direct FixK₂-dependent activation of transcription in vitro. These are blI2388, blI3998, blr4637, blI6061 (*fixK₁*), blr6070, blI6073 (*phbC*), and bsr7087. Included in this study also were two genes induced in free-living bacteria, but not in bacteroids, in a FixK₂-dependent manner (blr4655

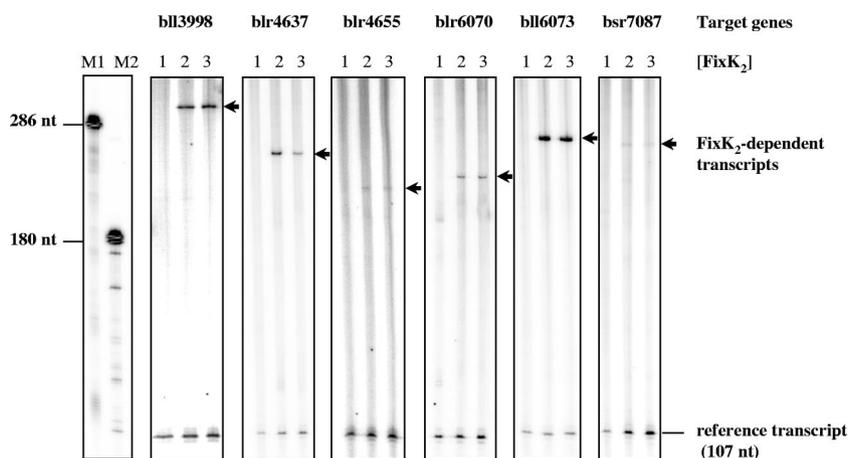


FIG. 4. In vitro transcription activation mediated by purified FixK₂. Supercoiled template plasmids comprising the promoter regions of target genes (shown at top) and a strong transcriptional terminator were used for multiple-round in vitro transcription assays with FixK₂ protein and RNA polymerase from *B. japonicum* cells. FixK₂ dimer concentrations were as follows: no protein (lane 1), 1.25 μM (lane 2), and 2.5 μM (lane 3). Transcripts synthesized in vitro in the presence of [α -³²P]UTP were separated on a 6% denaturing polyacrylamide gel and visualized by phosphorimager analysis of the dried gel. RNA size markers (M1 and M2) were generated as described earlier (49). The positions of the FixK₂-dependent transcripts are marked by arrows. Also shown is a FixK₂-independent reference transcript that is encoded on the vector portion of the template plasmids. nt, nucleotides.

[*ppsA*] and blr6062 [cytochrome *c*₆]). All nine genes were used as templates for transcription activation in vitro with purified RNA polymerase and purified FixK₂ protein from *B. japonicum*. One of them (bll2388) did not result in the synthesis of a detectable transcript. The results depicted in Fig. 4 show that six examples yielded clearly identifiable transcripts of meaningful sizes (i.e., their transcription start sites were within the canonical distance downstream of the putative FixK₂ box). Although transcript formation was weak in two cases (blr4655 and bsr7087), the transcripts were synthesized only when FixK₂ protein was present in the assay. These six genes are now considered to be new direct targets of FixK₂. The transcription of the other two genes (bll6061 and blr6062) will be described in the next paragraph.

Divergent transcription of *fixK*₁ and *cycS*. A comparison between the FixK₂ and FixK₁ regulons in cells grown under microoxic conditions revealed 17 genes whose expression specifically depended on FixK₁ (see Table S2 in the supplemental material). Among those we found blr6062, which had been annotated as a putative cytochrome *c* gene (cytochrome *c*₆-like) (2, 38). Therefore, blr6062 will be named *cycS* hereafter. Incidentally, *cycS* is located directly adjacent but in the opposite orientation to the *fixK*₁ gene (bll6061) (Fig. 5A). Only one FixK₂ box was identified between the two genes, suggesting that it serves for the transcription activation not only of *fixK*₁ but also of *cycS*. This inference was tested by inducing FixK₂-dependent transcription activation in vitro, using as DNA template the *fixK*₁-*cycS*-spanning fragment illustrated in Fig. 5B. Indeed, two transcripts were detected, one representing *fixK*₁ mRNA and the other *cycS* mRNA (Fig. 5C).

In order to determine the 5' end of the *cycS* RNA, primer extension experiments were performed by using RNA isolated from the wild-type *B. japonicum* strain and from the Δ *fixK*₂ strain, grown under different conditions (Fig. 6). The results of reverse transcription revealed a *cycS* transcription start point at a G located 25 nucleotides upstream of the annotated *cycS*

start codon (Fig. 6). The same transcription start site was identified when the in vitro-synthesized *cycS* mRNA was used for primer extension (data not shown). The results of densitometric analysis presented in Fig. 6 showed that the amount of cDNA derived from RNA in anoxically grown cells (conditions of nitrate respiration) was sixfold higher than the amount from microoxically grown cells. That the transcription of *cycS* depends on FixK₂ was confirmed, as deduced from the absence of the corresponding extension product in the microoxically grown *fixK*₂ mutant. Taken together, the results of these experiments allowed us

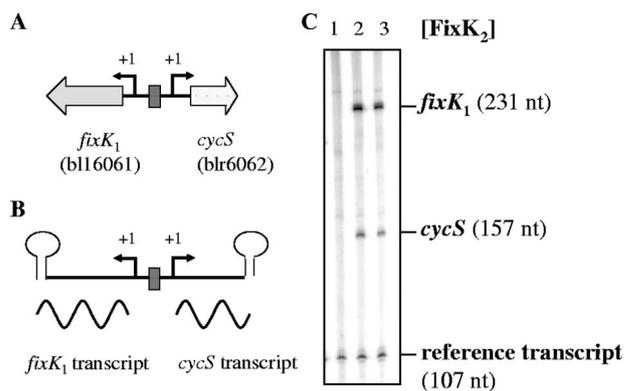


FIG. 5. FixK₂-dependent transcription from the divergently oriented *fixK*₁ and *cycS* promoters. Shown are a simplified map of the *B. japonicum* *fixK*₁ and *cycS* genes (A), a schematic of the relevant template (B), and the results of its use for in vitro transcription (C). The single FixK₂ binding site is symbolized by a dark box. The transcription start sites of *fixK*₁ and *cycS* are marked as “+1.” The stem-loops symbolize the transcription terminators. Transcripts from the template plasmid pRJ8871 were generated by multiple-round in vitro transcription using *B. japonicum* RNA polymerase and purified FixK₂ (no protein, lane 1; 1.25 μM, lane 2; and 2.5 μM, lane 3). The positions and sizes of the *fixK*₁ transcript, the *cycS* transcript, and the vector-encoded reference transcript are indicated. nt, nucleotides.

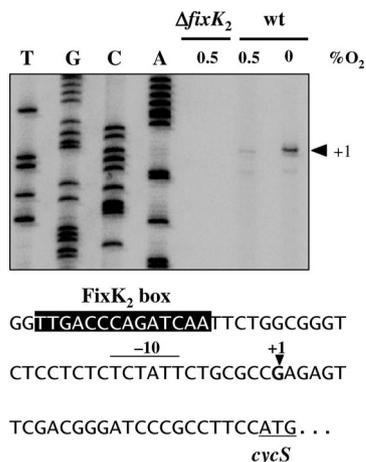


FIG. 6. Mapping of the transcription start site of *cycS*. Total RNA was isolated from microoxic (0.5% O₂) or anoxic (nitrate respiring) cells of the wild-type (wt) and the Δ *fixK*₂ strain and used for primer extension experiments with two *cycS*-specific primers (results are shown for only one of the primers). The sequencing ladder on the left was generated with plasmid pRJ8886 and the same primer used for transcript mapping. The relevant nucleotide sequence of the *cycS* promoter is shown at the bottom. The putative -10 element is overscored, the FixK₂ box is highlighted by white letters on a black background, the transcription start site is marked by “+1,” and the start codon of the *cycS* gene is underlined.

to precisely locate the axis of symmetry of the FixK₂ box at position -39.5 (TTGACCCAGATCAA) upstream of the *cycS* transcription start site and the reverse complementary box (TTGATCTGGGTCAA) at position -40.5 in the *fixK*₁ promoter region (53).

Despite the aforementioned activation of *cycS* by FixK₂, our microarray experiments had initially classified *cycS* as a member of the FixK₁ regulon. In fact, among the 17 positively controlled FixK₁ regulon members, the *cycS* gene was the one whose expression depended most strongly on FixK₁ (FC, -24.9) (see Table S2 in the supplemental material). From this observation and from the fact that FixK₁ is a FixK₂-homologous transcription factor, we reasoned that, apart from FixK₂, FixK₁ might also have the capacity to activate transcription from the unique *cycS*-*fixK*₁ intergenic FixK₂ box. Therefore, we tested the microaerobic expression of a transcriptional *cycS*-*lacZ* fusion in a *B. japonicum* *fixJ*-*fixK*₂ double mutant in which the *fixK*₁ gene was constitutively expressed from a foreign promoter (*aphII* promoter in strain 8884JK₂) (Table 1). The controls showed good *cycS* expression in the wild type (85 ± 16 [mean \pm standard deviation] Miller units) and only background levels in a *fixK*₂ null mutant (2 ± 0.9 Miller units). Up to 32% of wild-type *cycS* expression was restored in strain 8884JK₂ (27 ± 5 Miller units). This suggests that FixK₁, in concert with FixK₂, contributes to maximal *cycS* expression.

The *cycS* gene codes for a soluble c-type cytochrome expressed in anaerobic, nitrate-grown cells. Owing to the presence of characteristic amino acid sequence motifs (signal sequence and heme-binding site [C₃₇ARCH₄₁]), the *cycS* gene product was predicted to be a soluble, periplasmic c-type cytochrome (2, 38). We sought to obtain experimental support for this assumption. Two Δ *cycS* mutant strains (8882 and 8883) (Table 1) were constructed by marker exchange mutagenesis.

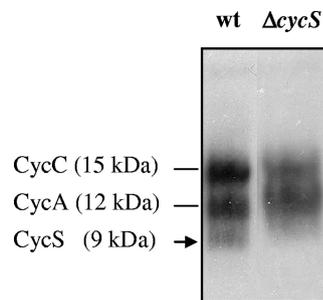


FIG. 7. Heme staining of soluble proteins expressed in nitrate-respiring cells. Wild-type *B. japonicum* (wt) and Δ *cycS* strains were grown in anoxic conditions (see Materials and Methods). Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and stained for having covalently bound heme. Cytochromes CycC (15 kDa) and CycA (12 kDa), which were identified previously, are specified on the left. Each lane was loaded with a sample of cell extract containing 80 μ g soluble proteins.

Both *cycS* mutants showed no differences with regard to aerobic growth and symbiotic nitrogen fixation phenotypes compared with the wild type (data not shown). In anoxic culture with nitrate as the terminal electron acceptor, however, a longer lag phase and a decreased growth rate were observed for the Δ *cycS* strains (see Fig. S1 in the supplemental material, showing the results with strain 8882). This was interpreted to mean that *cycS* is important but not essential for anaerobic nitrate respiration. Soluble and membrane proteins were extracted from the wild type and the *cycS* mutant (strain 8882), separated by SDS-polyacrylamide gel electrophoresis, and stained for covalently bound heme. In contrast to the membrane fraction, where no differences were found (data not shown), the soluble fraction of the mutant lacked one of the three stained bands detectable in the wild type (Fig. 7). The 15-kDa and 12-kDa proteins present in both cases had previously been identified as the *B. japonicum* soluble c-type cytochromes CycC and CycA (11, 66), whereas the 9-kDa cytochrome missing in strain 8882 obviously corresponds to the processed (i.e., secreted) CycS holocytochrome (predicted molecular mass of 9,825 Da, including covalently bound heme).

DISCUSSION

This work has led to a substantial expansion of knowledge about the regulon sizes, target genes, and regulatory ramifications associated with the hierarchically organized transcription factors in the *B. japonicum* FixLJ-FixK₂-FixK₁ cascade.

The FixJ regulon. If one subtracts the large number of FixK₂-controlled genes from the initially observed FixJ regulon, there are comparatively few genes left that might be specifically controlled solely by FixJ. A similar situation was previously noticed for *S. meliloti*, in which FixJ directly regulates only five genes and exerts its control via the regulatory genes *nifA* and *fixK* (10). The other three FixJ-dependent *S. meliloti* genes (*proB*₂, *Smc03253*, and *fixT*₃) (10) do not have orthologues in *B. japonicum* (National Center for Biotechnology Information [NCBI] genome comparison website, <http://www.ncbi.nlm.nih.gov/sutils/geneplot.cgi>). Hence, the physiological context of FixJ control—apart from that for *fixK*₂ in *B. japonicum* and for *nifA* plus *fixK* in *S. meliloti*—remains enigmatic.

Nevertheless, we have tried to further analyze those few *B. japonicum* genes on which FixJ appeared to exert a marginal positive control. A biocomputing approach was applied in order to make a de novo prediction of a FixJ binding site within the promoter regions of the putative FixJ targets. This attempt, however, did not result in the identification of a consensus "FixJ box," which made it impossible to distinguish putative direct FixJ targets from genes that are only indirectly regulated by FixJ. Likewise, the previous search for an "FixJ box" in *S. meliloti* had been inconclusive (23, 41).

The FixK₂ regulon. The analysis of the FixK₂ regulon proved to be more straightforward, despite its large size, and was greatly aided by the genome-wide prediction of FixK₂ boxes in the promoter regions of putative target genes. Not less than 51 promoter regions were identified from which FixK₂-activated transcription might occur not only in free-living, microoxically grown cells but also in endosymbiotic soybean bacteroids. These and other promoter candidates allowed us to zoom in on several of them as DNA templates in cell-free transcription activation assays, using purified RNA polymerase and FixK₂ protein. We thus ascertained the direct activation of transcription by FixK₂ from eight novel promoter regions into their adjacent genes: bll3998, blr4637, blr4655 (*ppsA*), bll6061 (*fixK₁*), blr6062 (*cycS*), blr6070, bll6073 (*phbC*), and bsr7087. This increases to 11 the total number of promoter regions for which FixK₂-activated transcription was shown in vitro. Prior to this study, this feature applied to the promoters of the *hemN₂* gene and the *fixNOQP* and *fixGHIS* operons (49).

The newly discovered direct FixK₂ targets are of interest also because the products of the respective genes might help uncover new facets in the physiology of *B. japonicum* in either the symbiotic or the free-living state. For example, three of these genes code for enzymes involved in carbon metabolism: bll3998, blr4655 (*ppsA*), and bll6073 (*phbC*). The bll3998 gene encodes a succinate semialdehyde dehydrogenase, implying that the tricarboxylate cycle bypass proposed by Green and collaborators (35) is under FixK₂ control and operates in symbiosis. The *ppsA* gene codes for a phosphoenolpyruvate synthase, an anaplerotic and gluconeogenic enzyme that becomes important when C₄-dicarboxylates are the carbon sources, as when they are provided by the host plant to bacteroids (20). More difficult to rationalize is why the last biosynthetic step for the carbon storage compound PHB, catalyzed by the bll6073 (*phbC*)-encoded PHB polymerase, is under FixK₂ control. Intriguingly, while *B. japonicum* contains five *phbC* homologs (65), only this FixK₂-dependent copy (bll6073) is induced in bacteroids (56). Moreover, the bll6073-encoded protein is expressed in bacteroids, suggesting that it has a function in symbiosis (62). Taken together, these are cumulative indications that hint at a hitherto unanticipated role of FixK₂ in regulating certain pathways of carbon metabolism in *B. japonicum* which is worthy of exploration in future research.

Three other cases of genes directly activated by FixK₂ are blr4637, which codes for a small heat shock protein (HspC2); the unknown-function gene bsr7087, which probably forms an operon together with the downstream gene blr7088 that codes for a putative periplasmic copper-binding protein (CopC) (13); and blr6070, encoding a putative Zn-containing alcohol dehydrogenase (38). For lack of functional data, speculations about the possible role of these gene products in the context of

symbiotic nitrogen fixation or microoxic lifestyle, though enticing, go beyond the scope of this paper. In contrast, a little more information is available on the remaining two of the eight new direct FixK₂ targets, bll6061 (*fixK₁*) and bll6062 (*cycS*), which will be treated in a separate paragraph (see below).

Out of the 203 microoxically induced *B. japonicum* genes that exclusively depend on activation by FixK₂, 60 genes have orthologs in *S. meliloti*, and among them, 26 genes are also controlled by FixJ and FixK in microoxically cultivated *S. meliloti* cells (10). Prominent examples are the *fixGHIS* and *fixNOQP* operons, responsible for the biogenesis and function of the bacteroid-specific *cbb₃*-type respiratory cytochrome oxidase, and the *napEDABC* operon for the periplasmic nitrate reductase involved in denitrification. The *B. japonicum*-versus-*S. meliloti* transcriptome comparison in bacteroids was a little more complicated because only the FixJ regulon, but not the FixK regulon, was assessed in *S. meliloti* bacteroids (7, 10). Nevertheless, we recognized the existence of 55 *S. meliloti* orthologs of genes that belong to the combined FixJ-FixK₂ regulon in *B. japonicum* bacteroids. Of these 55 genes, only 11 and 20 depended on FixJ according to the results of the studies performed by Bobik et al. (10) and Barnett et al. (7), respectively. Again, the *cbb₃*-type cytochrome oxidase genes were among them. Yet, the differences in this respect between *B. japonicum* and *S. meliloti* were substantial, which among other reasons, might reflect differences in root nodule physiology and the smaller number of genes in the *S. meliloti* genomic repertoire (30, 38). In this context, it will now be of interest to compare more-closely related species, such as *Bradyrhizobium* sp. BTAi1, *Bradyrhizobium* ORS278, or even the nonsymbiotic, photosynthetic *Rhodospseudomonas palustris* (32, 43), once corresponding expression data have become available for these species.

A surprising result was that 166 genes were expressed in a FixK₂-dependent manner in soybean bacteroids which were not concomitantly included in the list of FixJ-regulated genes. This can be interpreted to mean that a basal level of FixK₂ protein may become activated in bacteroids by a new type of control (a novel regulatory protein?) which is uncoupled from FixJ control and is destined to activate a new group of genes. Also, strikingly, a large proportion of these genes does not overlap with the microoxic regulon (genes induced in microoxic conditions compared with oxic conditions) but instead belongs to the previously identified set of genes that are expressed in bacteroids only (62 genes out of 166) (56). Hence, a large number of genes activated by FixK₂ in the bacteroids appear to escape from oxygen control, suggesting that a signal other than oxygen limitation is integrated at the level of FixK₂.

On this occasion, we cannot help but dispute some recently published data of Chang et al. (14), who reported that not only *fixK₂* gene expression but also the expression of many well-known FixK₂ targets, such as the *fixNOQP* operon, is strongly downregulated in soybean bacteroids. These data conflict with our own data on *B. japonicum* bacteroids (56 and this work) and those of others on *S. meliloti* (7, 9, 10), and they are difficult to reconcile with the essential nature of FixK₂ and the *cbb₃*-type cytochrome oxidase in symbiotic nitrogen fixation in *B. japonicum*, *S. meliloti*, and other rhizobia.

The FixK₁ regulon. The FixK₂-activated *fixK₁* gene encodes an oxygen-sensitive FNR-like protein that is not essential for

symbiotic nitrogen fixation (4, 53). Information on target genes regulated by FixK₁ had been missing altogether. In the work reported here, using microoxic and nitrate-respiring, anoxic *B. japonicum* cells, we have identified 17 genes as being under positive control by FixK₁; however, only one gene, blr6062 (*cycS*), was strongly activated (see below). The most-stunning result was that, contrary to data for the FixJ and FixK₂ regulons, the FixK₁ regulon contained a substantial number of negatively controlled genes, most of which had been known from previous work to belong to the group of genes activated by the NifA protein (37). Hence, for the first time, our work has disclosed a peculiar regulatory interaction between the FixLJ-FixK₂-FixK₁ cascade and the RegSR-NifA cascade, in which FixK₁, directly or indirectly, exerts an antagonistic effect on genes activated by NifA. Judged by the changes in the gene regulation measured in microarrays, this effect is generally more pronounced in microoxic cultures than in anoxic cultures. In accordance with the disparate oxygen responsiveness of FixLJ and NifA in vivo (63), we propose here that a decrease in the oxygen concentration to intermediate levels (as in microoxic culture) induces the FixLJ-FixK₂ cascade but still keeps some NifA-dependent genes repressed via FixK₁. When the oxygen concentration drops further, cells may build up more and more of the active NifA protein, which gradually overrides the transient repression by FixK₁. How this attractive fine-tuning of NifA-dependent gene expression works mechanistically has yet to be elucidated. Curiously, a reverse type of cross-pathway control may exist in *S. meliloti* bacteroids, because the results of transcriptome profiling have shown that a set of FixK-dependent genes were upregulated in a $\Delta nifA$ mutant (10).

The *cycS* gene and its product. Particular attention was paid to the regulation of *cycS* (blr6062) because this gene is located adjacent but in divergent orientation to the *fixK₁* gene (bll6061), with only a single FixK₂ box in the middle of the intergenic region. Using semisynthetic CRP-dependent promoters, El-Robh and Busby (22) had shown that CRP bound to a single DNA site could activate transcription in divergent orientations in vitro and in vivo. We show here that this works similarly for a natural constellation. FixK₂ was able to activate in vitro transcription from the same DNA template not only into the *fixK₁* gene but also into the opposite *cycS* gene. While the *fixK₁* gene was classified by transcriptome analysis as a member of the FixK₂ regulon, the activation of *cycS* was puzzling insofar as this gene was originally found to be a specific FixK₁ regulon member. The answer to this problem could be that FixK₂ in vivo first activates both *fixK₁* and *cycS*, but it is the accumulating FixK₁ protein which may additionally boost *cycS* gene expression. In fact, we demonstrated experimentally that a constitutively expressed *fixK₁* gene could partially restore *cycS* gene expression in a *B. japonicum* mutant background in which the *fixJ* and *fixK₂* genes had been knocked out. Such a dual control of one target gene by two homologous transcription factors of the CRP/FNR family is not without precedent. We had previously shown that the maximal activation in vivo of the *B. japonicum* nitrite reductase gene *nirK* required both the FixK₂ and NnrR proteins in cells grown anoxically with nitrate (47). Therefore, a general caveat seems to be justified: even after the demonstration of direct transcription activation in vitro, such as by FixK₂ in the case of *cycS*, the situation in vivo

may be more complex in that additional transcription factors, especially homologous ones, potentially participate in the overall control of a given target gene.

The *cycS* gene was annotated as a cytochrome *c₆* gene (38). In fact, we show in this work that the CycS protein is a hitherto unrecognized soluble, low-molecular-mass *c*-type cytochrome. A total of four proteins of that class have now been identified in *B. japonicum*, the gene products of *cycA*, *cycB*, *cycC*, and *cycS* (11, 61, 66; this work). While none of them is essential for symbiotic nitrogen fixation, the CycA protein was shown to be involved in electron transfer to the copper-containing nitrite reductase (12), the periplasmic enzyme that catalyzes the reduction of nitrite to nitric oxide. Likewise, based on the delayed denitrification phenotype of a *cycS* knockout mutant, we suggest a role of the CycS protein in denitrification, although it is not essential for this process. The precise biochemical function in delivering electrons to one of the N-oxide intermediates is currently not known, and the question remains unanswered as to whether one of the three other soluble *c*-type cytochromes can at least partly replace CycS function.

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Socorro Mesa dedicates this article to the memory of her father, Francisco Mesa.

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