Mol Gen Genet (1991) 225:38–48
002689259100006R

MGG

The regulatory status of the *fixL*- and *fixJ*-like genes in *Bradyrhizobium japonicum* may be different from that in *Rhizobium meliloti*

Denise Anthamatten and Hauke Hennecke

Mikrobiologisches Institut, Eidgenössische Technische Hochschule, ETH-Zentrum, Schmelzbergstrasse 7, CH-8092 Zürich, Switzerland

Received June 26, 1990 / August 1, 1990

Summary. The cloning, sequencing and mutational analysis of the *Bradyrhizobium japonicum* symbiotic nitrogen fixation genes *fixL* and *fixJ* are reported here. The two genes were adjacent and probably formed an operon, *fixLJ*. The predicted FixL and FixJ proteins, members of the two-component sensor/regulator family, were homologous over almost their entire lengths to the corresponding *Rhizobium meliloti* proteins (approx. 50% identity). Downstream of the *B. japonicum fixJ* gene was found an open reading frame with 138 codons (ORF138) whose product shared 36% homology with the N-terminal part of FixJ. Deletion and insertion mutations within *fixL* and *fixJ* led to a loss of approximately 90% wild-type symbiotic nitrogen fixation (Fix) activity, whereas an ORF138 mutant was Fix^+. In *fixL*, *fixJ* and ORF138 mutant backgrounds, the aerobic expression of the *fixRnifA* operon was not affected. NifA itself did not regulate the expression of the *fixJ* gene. Thus, the *B. japonicum* FixL and FixJ proteins were neither involved in the regulation of aerobic *nifA* gene expression nor in the anaerobic NifA-dependent autoregulation of the *fixRnifA* operon; rather they appeared to control symbiotically important genes other than those whose expression was dependent on the NifA protein. The *fixL* and *fixJ* mutant strains were unable to grow anaerobically with nitrate as the terminal electron acceptor. Therefore, some of the FixJ-dependent genes in *B. japonicum* may be concerned with anaerobic respiration.

Key words: Gene regulation – Nitrogen fixation – Oxygen control – Symbiosis – Two-component systems

Introduction

In root and stem nodule symbiosis with legumes, *Bradyrhizobium*, *Rhizobium* and *Azorhizobium* species fix molecular nitrogen under conditions of very low oxygen tension. Expression of the *nif* and *fix* genes in these bacteria is subject to a complex regulatory network involving both positive and negative control. In all systems studied, oxygen limitation has been recognized as being the major, if not the only, trigger that leads to *nif* and *fix* gene expression (Fischer and Hennecke 1987; Thöny et al. 1989; Ditta et al. 1987; Virts et al. 1988; David et al. 1988; Ratet et al. 1989).

In *Bradyrhizobium japonicum*, the root nodule bacterium of soybean, the NifA protein is the oxygen-responsive gene regulator (Fischer and Hennecke 1987). Under conditions of microaerobiosis and anaerobiosis, NifA is active and positively regulates the expression of the nitrogenase genes and other *nif* and *fix* genes or operons. During aerobiosis, the NifA protein is inactive and, hence, no NifA-dependent genes are expressed. A second mode of control exists at the level of the expression of the *nifA* gene. The *nifA* gene, which is part of the *fixRnifA* operon (Thöny et al. 1987), is expressed at a certain basal level under aerobic conditions. Under microaerobiosis, or when cells are growing anaerobically with nitrate, the expression of the *fixRnifA* operon is increased at least fivefold (Thöny et al. 1989). This increase is not observed in a *nifA* mutant background, which suggests that the NifA protein is responsible, directly or indirectly, for the anaerobic (auto)regulation of the *fixRnifA* operon. The aerobic expression of the *fixRnifA* operon may also be subject to regulation. This inference is based on the observation that a DNA sequence located around nucleotide position −66 from the transcriptional start site is required for maximal aerobic expression of the *fixRnifA* operon, a finding consistent with positive control of the *fixR* promoter by an unknown activator binding to that DNA region (Thöny et al. 1987, 1989).

In *Rhizobium meliloti*, the alfalfa symbiont, two nitrogen fixation regulatory genes, *fixL* and *fixJ*, have been identified (David et al. 1988; Virts et al. 1988; Banfalvi et al. 1989). They belong to the widespread family of two-component regulatory systems (for reviews see Albright et al. 1989; Gross et al. 1989; Stock et al. 1989, 1990). These systems are composed of a sensor compo-

Offprint requests to: H. Hennecke

nent and a response regulator. The sensor, a histidine-protein kinase, recognizes a particular environmental signal and transduces this signal to the response regulator by phosphorylation. FixL of *R. meliloti*, the sensor component, has been suggested to recognize the absence of oxygen as an environmental stimulus. The response regulator FixJ then activates the expression of two other regulatory genes. One of them is *nifA*, which encodes the transcriptional activator of *nif* and *fix* genes such as *nifHDKE* and *fixABCX* (David et al. 1988). The other is *fixK*, which codes for a regulatory protein with positive and negative functions: the FixK protein activates the expression of the *fixN* region and, at the same time, represses its own synthesis and that of NifA (Batut et al. 1989). Thus, oxygen control of *nif* and *fix* genes in *R. meliloti* is primarily mediated via the FixL and FixJ proteins. However, the *R. meliloti* NifA protein, like that of *B. japonicum*, has also been shown to be oxygen sensitive (Beynon et al. 1988; Huala and Ausubel 1989).

Comparison of the nitrogen fixation regulatory circuits in *B. japonicum* and *R. meliloti* shows that one major difference lies in the way the *nifA* gene is controlled in these two bacteria. The discovery of the *fixLJ* genes in *R. meliloti* prompted us to see whether or not analogous genes are also present in *B. japonicum*. Despite the fact that the *fixRnifA* operon is expressed aerobically, but in view of the postulated positive control and additional anaerobic induction of that operon, we felt it was necessary to test any possible involvement of *fixL* and *fixJ* in its regulation. We report here that *fixL*- and *fixJ*-like genes do exist in *B. japonicum*, but in contrast to *R. meliloti*, the *nifA*-dependent regulatory circuit may be uncoupled from that governed by *fixLJ*.

Materials and methods

Bacterial strains and plasmids. Strains, vectors and recombinant plasmids relevant for this work are listed in Table 1. Further description of newly constructed strains and plasmids is given in the text. *Escherichia coli* strains RR28 and HB101 were used as recipients in plasmid transformations. *E. coli* S17-1 and *E. coli* HB101 (carrying pRK2013 as a helper plasmid) were used for mobilization of pSUP202 and pRK290 derivatives, respectively. *E. coli* JM101 was used as recipient for transfection with M13 derivatives.

Media and growth of cells. E. coli strains were grown in LB medium (Miller 1972) at 37° C with the following concentrations of antibiotics: 200 μg/ml ampicillin; 30 μg/ml kanamycin; 10 μg/ml tetracycline. *B. japonicum* strains were usually grown aerobically in PSY medium (Regensburger and Hennecke 1983) at 28° C. Selection after matings was achieved by adding antibiotics at the following concentrations: 200 μg/ml kanamycin; 100 μg/ml tetracycline and, additionally, 10 μg/ml chloramphenicol for counterselection against *E. coli* donors. To monitor β-galactosidase activity, aerobic and anaerobic growth of *B. japonicum* strains was done in YEM medium (Daniel and Appleby 1972) with 10 mM KNO_3. Maintenance of pRK290 derivatives was achieved by adding 50 μg/ml tetracycline.

DNA manipulations. Recombinant DNA work such as cloning, restriction analysis and plasmid preparations was done as described by Maniatis et al. (1982). Isolation of chromosomal DNA from *B. japonicum* strains was performed as described previously (Hahn and Hennecke 1984). Nick-translated, ^{32}P-labelled plasmid DNAs were used as probes for heterologous Southern blot hybridizations. Probes for homologous hybridizations were labelled using the non-radioactive labelling kit from Boehringer, Mannheim, FRG.

DNA sequence analysis. For DNA sequence analysis overlapping M13 subclones were used for both strands. The sequencing reactions were done by the chain termination method (Sanger et al. 1977) using M13-specific fluorescent dye primers and analysed on a DNA sequencer (Sequencer model 370 and dye primers from Applied Biosystems, Foster City, Calif.). Protein alignments were done using programs of the University of Wisconsin Genetics Computer Group (Madison, Wis.).

Construction of mutations for marker exchange mutagenesis. To construct *fixL* deletion mutants, plasmid pRJ7354 (harbouring a 3 kb *Eco*RI fragment spanning the whole *fixLJ*ORF138 gene region; Fig. 1) was digested with *Bam*HI. After a fill-in reaction and *Eco*RI linker ligation, a shortened 2.4 kb *Eco*RI fragment was ligated with *Eco*RI-linearized pGem-1 vector. The resulting plasmid was cut with *Sac*I and ligated with a 1.7 kb *Sac*I fragment from pUC4-KIXX (containing the kanamycin resistance gene) in both orientations. A 3.4 kb *Eco*RI fragment from these plasmids was cloned into the *Eco*RI site of pSUP202, leading to plasmids pRJ7403 (with the *aph* gene having the same orientation as *fixL*) and pRJ7404 (with the *aph* gene reading in the opposite direction). To generate insertional mutations in *fixJ*, vector pUC18 was first digested with *Hin*dIII and *Bam*HI and religated after filling in the protruding 5′ ends. The corresponding vector pUC18HB was then digested with *Eco*RI and ligated with a 3 kb *Eco*RI fragment from pRJ7354. Plasmid pUC4-KIXX was cut with *Sma*I, and after ligation of a *Sal*I linker, a 1.3 kb *Sal*I fragment containing the *aph* gene was ligated in both orientations with the *Sal*I-linearized pUC18HB derivative. A 4.3 kb *Eco*RI fragment from the resulting plasmids was then cloned into the *Eco*RI site of pSUP202. The pSUP202 derivatives were named pRJ7360 (in which the *aph* gene has the same orientation as *fixJ*) and pRJ7361 (with the kanamycin resistance gene having the opposite orientation). For insertion mutagenesis of ORF138, plasmid pRJ7354 was partially digested with *Bam*HI and ligated again in both orientations with a 1.7 kb *Bam*HI fragment from pUC4-KIXX (harbouring the *aph* gene region of Tn*5*). A 4.7 kb *Eco*RI fragment was then ligated with *Eco*RI-linearized pSUP202. The corresponding pSUP202 derivatives were called pRJ7413 (in which the *aph* gene has the same orientation as ORF138) and pRJ7414 (with the kanamycin resistance gene in the op-

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference
Escherichia coli		
RR28	*hsdR hsdM recA pheS12 thi leu pro lac gal ara mtl xyl supE44 endA*	Hennecke et al. (1982)
S17-1	*hsdR*/RP4-2 *kan::*Tn*5 tet::*Mu, integrated in the chromosome	Simon et al. (1983)
HB101	*hsdR hsdM recA13* Str^r	Davis et al. (1980)
JM101	*Δlac−proAB thi supE* F′(*traD36 proAB*$^+$ *lacI*^{q}*Z ΔM15*)	Messing (1983)
Bradyrhizobium japonicum		
110*spc*4	Spc^r (wild type)	Regensburger and Hennecke (1983)
7403[a]	Spc^r Km^r, *fixL::aph*	This work
7403R	Spc^r Km^r Tc^r, *fixL::aph fixR′−′lacZ*	This work
7403A	Spc^r Km^r Tc^r, *fixL::aph nifA′−′lacZ*	This work
7404[a]	Spc^r Km^r, *fixL::aph*	This work
7404R	Spc^r Km^r Tc^r, *fixL::aph fixR′−′lacZ*	This work
7404A	Spc^r Km^r Tc^r, *fixL::aph nifA′−′lacZ*	This work
7360[a]	Spc^r Km^r, *fixJ::aph*	This work
7360R	Spc^r Km^r Tc^r, *fixJ::aph fixR′−′lacZ*	This work
7360A	Spc^r Km^r Tc^r, *fixJ::aph nifA′−′lacZ*	This work
7361[a]	Spc^r Km^r, *fixJ::aph*	This work
7361R	Spc^r Km^r Tc^r, *fixJ::aph fixR′−′lacZ*	This work
7361A	Spc^r Km^r Tc^r, *fixJ::aph nifA′−′lacZ*	This work
7413[a]	Spc^r Km^r, ORF138*::aph*	This work
7413A	Spc^r Km^r Tc^r, ORF138::*aph nifA′−′lacZ*	This work
7414[a]	Spc^r Km^r, ORF138*::aph*	This work
7414R	Spc^r Km^r Tc^r, ORF138*::aph fixR′−′lacZ*	This work
7414A	Spc^r Km^r Tc^r, ORF138*::aph nifA′−′lacZ*	This work
7417	Spc^r Tc^r, *nifA′−′lacZ*	This work
7290	Spc^r Tc^r, *fixR′−′lacZ*	This work
A9	Spc^r Km^r, *nifA::aph*	Fischer et al. (1986)
Plasmids		
pSUP202	Ap^r Tc^r Cm^r, *oriT* from RP4	Simon et al. (1983)
pRK290X	Tc^r	Alvarez-Morales et al. (1986); Ditta et al. (1980)
pRK2013	Km^r, *tra*$^+$	Figurski and Helinski (1979)
pNM480	Ap^r *′lacZ*	Minton (1984)
pNM482	Ap^r *′lacZ*	Minton (1984)
pNM480B	Ap^r (pNM480) *′lacZ* *Bam*HI linker insertion in *Stu*I site	M. Göttfert, ETH
pNM482B	Ap^r (pNM482) *′lacZ* *Bam*HI linker insertion in *Stu*I site	M. Göttfert, ETH
pUC4-KIXX	Ap^r, *lacZ::aph*	Pharmacia LKB Biotechnology Uppsala, Sweden
pGem-1	Ap^r, SP6 and T7 promoters	Promega Biotech, Madison, Wis
pMC1403	Ap^r, *′lacZYA*	Casadaban et al. (1983)
pUC18	Ap^r	Norrander et al. (1983)
pUC18HB	Ap^r (pUC18) *ΔHin*dIII−*Bam*HI fragment	This work
pDD5	Ap^r Tc^r (pBR322) *fixLJ* (*Rhizobium meliloti*)	David et al. (1987)
pRJ7349[b]	Ap^r (pGem-1) 3 kb *Sal*I fragment	This work
pRJ7354[b]	Ap^r (pGem-1) 3 kb *Eco*RI fragment	This work
pRJ7403	Ap^r Tc^r (pSUP202) *fixL::aph*	This work
pRJ7404	Ap^r Tc^r (pSUP202) *fixL::aph*	This work
pRJ7360	Ap^r Tc^r (pSUP202) *fixJ::aph*	This work
pRJ7361	Ap^r Tc^r (pSUP202) *fixJ::aph*	This work
pRJ7413	Ap^r Tc^r (pSUP202) ORF138 ::*aph*	This work
pRJ7414	Ap^r Tc^r (pSUP202) ORF138*::aph*	This work
pRJ7407	Tc^r (pRK290X) out-of-frame *fixJ′−′lacZ*	This work
pRJ7408[b]	Tc^r (pRK290X) in-frame *fixJ′−′lacZ*	This work
pRJ7548	Ap^r (pMC1403) *nifA′−′lacZ*	H.M. Fischer, ETH

[a] The genomic structures of these mutants are shown in Fig. 1
[b] The inserts of these plasmids are shown in Fig. 1

posite orientation). All pSUP202 derivatives were mobilized from *E. coli* S17-1 to the *B. japonicum* strain 110*spc*4 (Hahn and Hennecke 1984). After marker replacement mutagenesis, the corresponding *B. japonicum* mutants were named 7403, 7404, 7360, 7361, 7413 and 7414 (see Fig. 1). The genomic structures of these mutations were confirmed by appropriate Southern blot analysis of total DNA.

Symbiotic nitrogen fixation assays. Plant infection tests with soybean seedlings (*Glycine max* L. Merr. cv. Williams) were performed as described previously (Hahn and Hennecke 1984). Twenty days after inoculation, nitrogen fixation activity of whole root nodules was determined by the acetylene reduction assay (Turner and Gibson 1980).

Gel retardation assays. B. japonicum strains were grown aerobically in PSY medium. Preparation of soluble proteins from crude extracts and gel retardation assays were done as described (Thöny et al. 1989).

Genomic integration of fixR'- *and* nifA'—'lacZ *fusions.* Plasmid pRJ7548 (Table 1) was digested with *Dra*I. After *Eco*RI linker ligation, a 5.8 kb *Eco*RI fragment containing the 5' region of *fixR*, the *fixR* gene and a translational *nifA'—'lacZ* fusion was introduced into the *Eco*RI site of pSUP202. A pSUP202 derivative containing the 5' region of *fixR* and a translational *fixR'—'lacZ* fusion was kindly provided by B. Thöny. Matings were performed as described previously (Hahn and Hennecke 1984). Cointegration of the pSUP202 derivatives was achieved by selection on tetracycline-containing plates after the matings. *B. japonicum* wild-type, *fixL*, *fixJ* and ORF138 mutant strains served as recipients for the matings. Cointegrations generated by homologous recombination within the *fixRnifA* operon were confirmed by Southern blot analysis of total DNA.

Construction of pRK290 derivatives to test extrachromosomal lacZ *fusions.* To generate translational in-frame and out-of-frame *fixJ'—'lacZ* fusions, a 3 kb *Sal*I fragment from pRJ7349, covering the 5' region of *fixL*, the *fixL* gene itself and part of the *fixJ* gene (see Fig. 1), was isolated and cloned into the *Sal*I sites of pNM482B and pNM480B. For the cloning of these *fixJ'—'lacZ* fusions into pRK290X, a 6 kb *Bam*HI fragment from the pNM482B and pNM480B derivatives was ligated with *Bgl*II-linearized pRK290X. The corresponding plasmids were called pRJ7408 (Fig. 1), harbouring the in-frame *fixJ'—'lacZ* fusion, and pRJ7407, containing the out-of-frame fusion. For both *fixJ'—'lacZ* fusions, the reading frame was confirmed by DNA sequence analysis.

β-Galactosidase assays. B. japonicum strains to be tested for their expression of translational *lacZ* fusions were grown either aerobically for 2 days or anaerobically for 3 days in supplemented YEM medium at 28° C. Maintenance of pRK290 derivatives was achieved by antibiotic selection. At least ten independent cultures were assayed twice. The assay was performed with 50 µl samples of the cultures as described by Miller (1972).

Results

Identification and cloning of a fixLJ*-like DNA region*

To identify *fixLJ*-like genes in *B. japonicum*, total genomic DNA of *B. japonicum* was probed in heterologous Southern blot hybridizations with fragments containing the *fixLJ* genes of *R. meliloti*. To obtain a specific *fixLJ* internal probe, the 6 kb *Hin*dIII—*Bal*I fragment from plasmid pDD5, containing *R. meliloti fixL* and most of *fixJ*, was subcloned into vector pGem-1 cut with *Hin*dIII and *Sma*I. A 1 kb *Eco*RI fragment (encoding the C-terminal part of FixL and the N-terminal part of FixJ) from the resulting plasmid was purified and used as a probe in hybridization experiments. Hybridization of this heterologous probe to *B. japonicum* total DNA was found even under conditions of high stringency (data not shown). A 3 kb hybridizing *Sal*I fragment from *B. japonicum* total DNA was cloned into *Sal*I-linearized pGem-1 vector, leading to plasmid pRJ7349 (Fig. 1). To confirm the hybridization data, the nucleotide sequence of a hybridizing 124 bp *Sph*I—*Sal*I fragment at one end of pRJ7349 was established and found to show 70% homology to *fixJ* of *R. meliloti* (David et al. 1988). To obtain additional, adjacent DNA, pRJ7349 DNA was hybridized to a *B. japonicum* λ gene bank (kindly provided by G. Acuña). An overlapping 3 kb *Eco*RI fragment from a hybridizing λ clone was ligated with *Eco*RI-linearized pGem-1 vector, leading to plasmid pRJ7354 (Fig. 1).

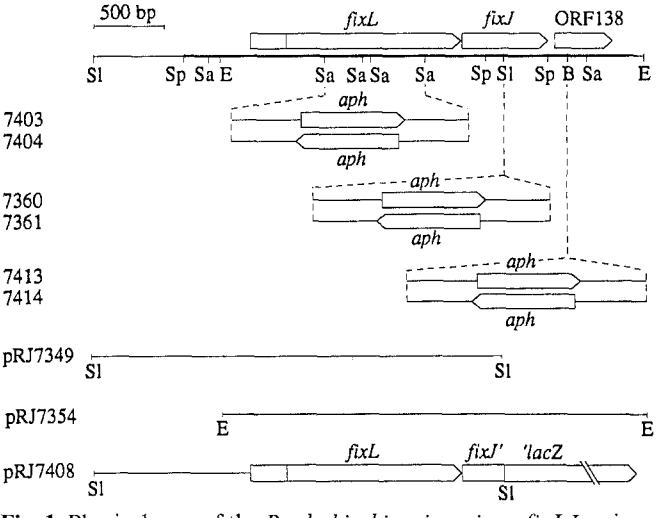

Fig. 1. Physical map of the *Bradyrhizobium japonicum fixLJ* region. The *bold line* within the map indicates the region that was sequenced. The numbers of the *fixL*, *fixJ* and ORF138 mutant strains carrying an *aph* insertion are given on the left side. The two clones covering the whole DNA region are shown in the lower part. At the bottom, the clone carrying the *fixJ'—'lacZ* fusion is shown. Restriction sites: B, *Bam*HI; E, *Eco*RI; Sa, *Sac*I; Sl, *Sal*I; Sp, *Sph*I

Fig. 2. Nucleotide sequence of the *fixLJ* DNA region. Translation of the three open reading frames *fixL*, *fixJ* and ORF138 is shown. Shine-Dalgarno-like sequences are *underlined*. Potential start codons are *boxed* and their corresponding amino acids are printed in *bold face*. TTG- and GTG-encoded amino acids (Land V) would have to be substituted for methionine (M) if they are N-terminal

DNA sequence of fixLJ

Nucleotide sequence analysis of a 3.24 kb region revealed the presence of three open reading frames (Fig. 2) with high coding probabilities, all of them having the same orientation. The first ORF was 1515 bp long, predicting a protein of 505 amino acids. The corresponding TTG start codon at position 468 was preceded by a Shine-Dalgarno-like sequence (Fig. 2). Because of its homology to the *fixL* sequence of *R. meliloti* (David et al. 1988) this ORF was named *fixL*. There was a second start codon (GTG) at position 735 which was preceded by a less well-conserved Shine-Dalgarno-like sequence. The shorter ORF of 1248 bp would lead to a protein of only 416 amino acids. The next ORF located downstream of *fixL* was called *fixJ* because it showed homology to *fixJ* of *R. meliloti* (David et al. 1988). It started with an ATG 3 bp downstream of the stop codon of *fixL* and was 615 bp long, predicting a protein of 205 amino acids. The closely adjacent *fixL*–*fixJ* arrangement suggested that both genes formed an operon. At 43 bp downstream of the *fixJ* stop codon there was a third ORF of 414 bp, which would lead to a protein of 138 amino acids. No Shine-Dalgarno-like sequence could be detected in front of this third ORF. ORF138 showed homology over its entire length to the first half of *fixJ* (see next paragraph).

Amino acid sequence homologies to R. meliloti *FixL and FixJ and other two-component systems*

FixL and FixJ of *R. meliloti* belong to the conserved family of two-component regulatory systems (David et al. 1988). The homology among the sensors of two-component systems consists of a conserved domain of about 250 amino acids, generally in the C-terminal part of the protein. (Exceptions where the conserved domain is situated in the central part of the sensor have been reviewed: Albright et al. 1989; Gross et al. 1989; Stock et al. 1989, 1990.) The amino acid sequence of FixL of *B. japonicum* was found to share quite high homology

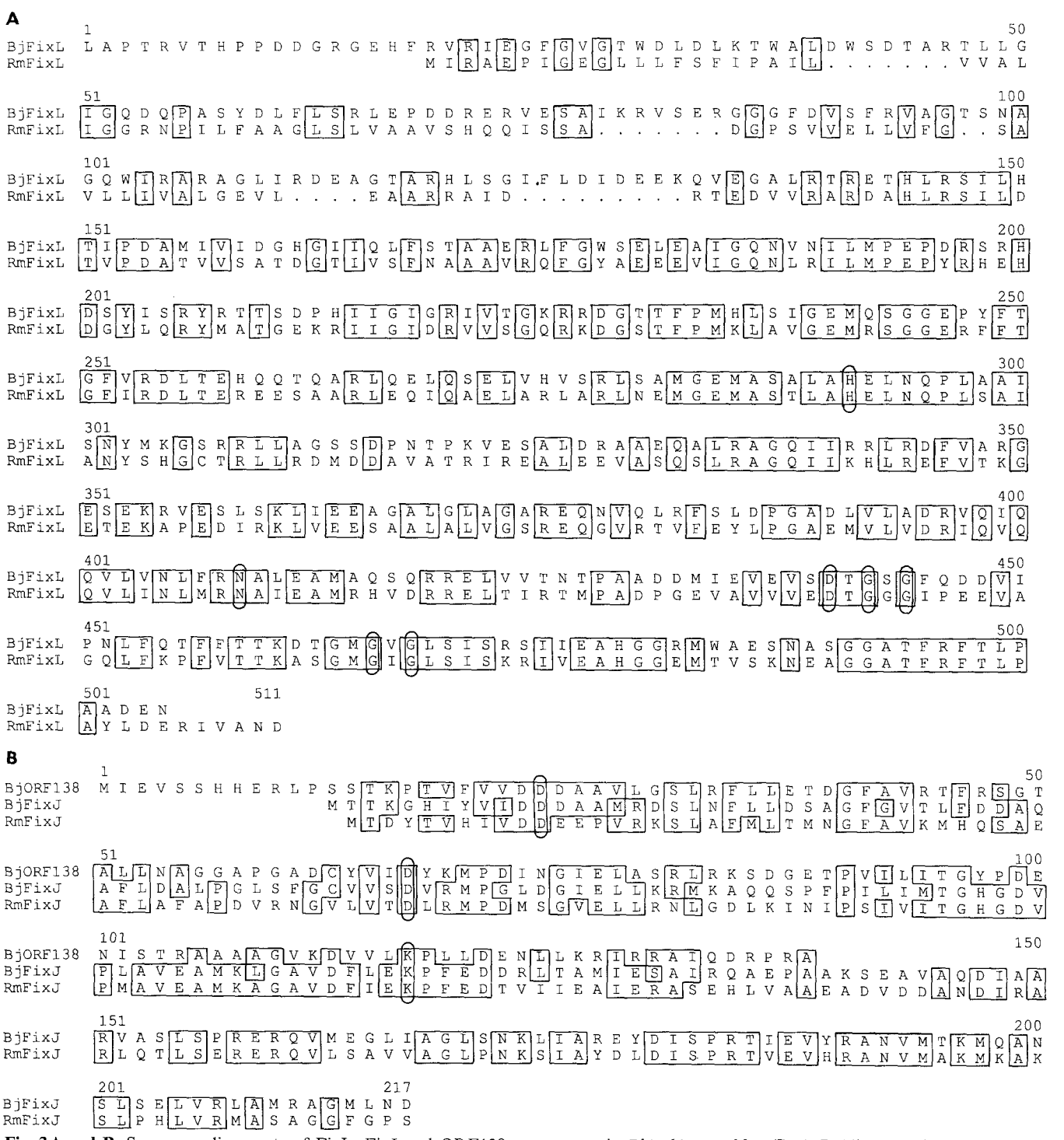

Fig. 3A and B. Sequence alignments of FixL, FixJ and ORF138. Identical amino acids are *boxed.* Amino acids found to be conserved in all known two-component systems are *circled.* **A** Alignment between FixL of *Bradyrhizobium japonicum* (Bj) and its counterpart in *Rhizobium meliloti* (Rm). **B** Alignment between ORF138 and FixJ of *B. japonicum* and FixJ of *R. meliloti.* The *R. meliloti* sequences were taken from David et al. (1988)

(50%) with FixL of *R. meliloti.* This homology was not only restricted to the conserved region of the sensor family, but extended over at least 370 amino acids from the C-terminus of the two proteins (Fig. 3A). In the very N-terminal part of the proteins the homology dropped considerably. In contrast to FixL of *R. meliloti* (David et al. 1988), no significant hydrophobic domains were present in the N-terminus of *B. japonicum* FixL. There are other examples known of sensors without potential membrane-bound domains: NtrB, DegS, SpoIIJ, CheA and FrzE (for reviews see Albright et al. 1989; Gross et al. 1989; Stock et al. 1989, 1990).

The regulators of the two-component systems are defined by a conserved region of about 100 amino acids

Table 2. Symbiotic nitrogen fixation (acetylene reduction) activity of *Bradyrhizobium japonicum* wild-type and mutant strains

Strains	Relevant genotype	Specific activity[a]	% of wild type
110*spc*4	Wild type	139	100
7403	*fixL::aph* (→)[b]	14	11
7404	*fixL::aph* (←)[b]	7	5
7360	*fixJ::aph* (→)[b]	19	14
7361	*fixJ::aph* (←)[b]	6	4
7413	ORF138*::aph* (→)[b]	135	97
7414	ORF138*::aph* (←)[b]	155	112

[a] All nodules from at least nine independent plants were measured 20 days after inoculation. Specific activity is expressed as micromoles of C_2H_4 formed per hour per gram nodule dry weight

[b] The arrows indicate the orientation of the *aph* gene with respect to the *fixLJ*ORF138 reading frames as shown in Fig. 1

in the N-terminus of these proteins. The amino acid sequence of FixJ of *B. japonicum*, however, was found to be highly homologous (48%) over its entire length to FixJ of *R. meliloti* (Fig. 3B). Interestingly, ORF138 was also found to be homologous (36%) to the N-terminal part of FixJ of *B. japonicum* (Fig. 3B). The regulators can be grouped into subfamilies according to the homologies in their C-terminal domains. ORF138 is like CheY (Macnab 1987; Stewart and Dahlquist 1987) and SpoOF (Losick et al. 1986; Smith 1989); these are about the same length and consist only of the conserved N-terminal domain. FixJ belongs to another subfamily with extended homology into the C-terminal part. Other members of this subfamily, which are thought to function as transcriptional activators, are: NarL, the activator of the nitrate reductase genes (Stewart 1988); UhpA, the regulator of the hexose phosphate uptake system (Weston and Kadner 1988); UvrC–ORF2, whose function is unknown (Moolenaar et al. 1987); ComA, which leads to competence behaviour in *B. subtilis* (Albano et al. 1987; Hahn et al. 1987); and DegU, involved in secretion of enzymes (Ayusaw et al. 1975; Henner et al. 1988).

The fixL *and* fixJ *genes are involved in symbiosis*

To test a possible involvement of *fixL*, *fixJ* and ORF138 in establishing an effective symbiosis between *B. japonicum* and soybean, different mutants were constructed by marker exchange mutagenesis (see Materials and methods and Fig. 1). Gene disruption of *fixJ* and ORF138 was achieved by insertion of the Tn*5*-derived aminoglycoside phosphotransferase gene cartridge (*aph*) into the *Sal*I and the *Bam*HI site, respectively. To obtain *fixL* mutants, three *fixL* internal *Sac*I fragments were deleted and replaced by the *aph* gene. Because of an expected out-reading promoter activity of the inserted fragment, the *aph* gene cartridge was inserted in each case in both orientations. The mutants were tested for nodulation and symbiotic nitrogen fixation activity on soybean (*G. max*). The nodulation behaviour of the mutants and the frequency of nodulation were not altered when compared with roots infected by the *B. japonicum* wild-type strain. Nevertheless, the average dry weight per nodule infected by *fixL* and *fixJ* mutants was only about one-half of the wild-type level (data not shown). Electron microscopic analysis showed the presence of vacuolar structures within nodule cells infected with *fixL* and *fixJ* mutants, a possible sign of beginning bacteroid degradation within these cells (D. Studer, personal communication). The ORF138 mutants 7413 and 7414 were clearly Fix^+ (Table 2), which meant that ORF138 was not essential for symbiosis. The *fixL* and *fixJ* mutant strains showed more than 85% reduced Fix activity (Table 2). This justified *fixL* and *fixJ* being called "*fix* genes".

FixL *and* fixJ *mutant strains fail to grow anaerobically with nitrate*

The *B. japonicum* wild type is able to grow anaerobically with nitrate as terminal electron acceptor. Since the *R. meliloti fixLJ* regulatory system has been suggested to respond to oxygen as a signal (David et al. 1988), we were prompted to test the different *B. japonicum* mutant strains for their ability to grow anaerobically on nitrate. Mutants 7413 and 7414 (ORF138) could grow like the wild-type strain under these growth conditions (not shown). Strain 7403, in which the *aph* gene insertion within the *fixL* gene has the same orientation as *fixL* and *fixJ*, showed a reduced growth rate when compared with the wild type (Fig. 4). By contrast, mutants 7404, 7360 and 7361 showed hardly any growth. This suggested that FixL and especially FixJ were important for switching from aerobic to anaerobic respiration, whereas ORF138 had no function in this process. Possible reasons why mutant 7403 maintained slow anaerobic growth will be discussed later.

Aerobic expression of the fixRnifA *operon is independent of FixL and FixJ*

As shown above, the *B. japonicum* FixL and FixJ proteins are highly homologous to their counterparts in

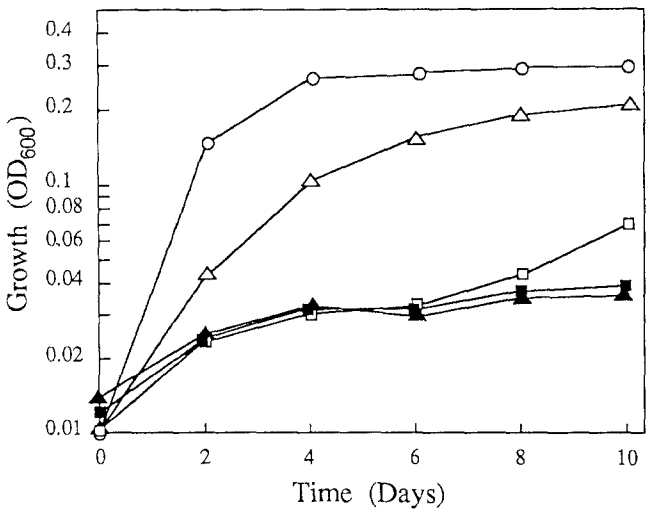

Fig. 4. Growth curves of *Bradyrhizobium japonicum* wild-type and different mutant strains. The strains were grown anaerobically in YEM medium with KNO_3. Growth was determined by measuring the density of the cultures at 600 nm (OD_{600}). ○, wild type; △, strain 7403; ▲, strain 7404; □, strain 7360; ■, strain 7361

Table 4. Expression of an extrachromosomal *fixJ'—'lacZ* fusion (on plasmid pRJ7408) in *Bradyrhizobium japonicum* wild-type and different mutant strains under aerobic and anaerobic growth conditions[a]

Strains	Relevant chromosomal background	β-Galactosidase activity (U)[b]	
		Aerobic growth	Anaerobic growth
110*spc*4	Wild type	239 (±64)	94 (± 5)
A9	*nifA::aph*	239 (±54)	87 (±10)
7360	*fixJ::aph*	231 (±56)	NA[c]
7361	*fixJ::aph*	231 (±58)	NA[c]

[a] For both conditions, cells were grown in YEM medium containing 10 mM KNO_3
[b] The wild-type strain without any *lacZ* fusion and the out-of-frame *fixJ'—'lacZ* fusion showed less than 2 Miller units (U)
[c] NA, not applicable because these strains do not grow anaerobically (see Fig. 4)

Table 3. Expression of chromosomally integrated *fixR'*- and *nifA'—'lacZ* fusions in *Bradyrhizobium japonicum* wild-type and different mutant strains under aerobic growth conditions[a]

Relevant genetic background	β-Galactosidase activity (U) from strains with a chromosomally integrated *lacZ* fusion[b]			
	Strain	*fixR'—lacZ'* fusion	Strain	*nifA'—'lacZ* fusion
Wild type	7290	490 (± 86)	7417	36 (± 7)
fixL::aph (→)[c]	7403R	346 (±142)	7360A	31 (± 8)
fixL::aph (←)[c]	7404R	316 (± 84)	7404A	31 (± 7)
fixJ::aph (→)[c]	7360R	260 (± 32)	7360A	29 (± 5)
fixJ::aph (←)[c]	7361R	257 (± 26)	7361A	29 (± 7)
ORF138::*aph* (→)[c]	ND[d]	ND[d]	7413A	30 (±10)
ORF138::*aph* (←)[c]	7414R	267 (± 35)	7414A	28 (± 8)

[a] Cells were grown in YEM medium containing 10 mM KNO_3
[b] The wild-type strain without any *lacZ* fusion showed less than 2 Miller units (U)
[c] The arrows indicate the orientation of the *aph* gene with respect to the *fixLJ*ORF138 reading frames as shown in Fig. 1
[d] ND, not done

R. meliloti, and are involved in symbiosis. We therefore tested whether they controlled *nifA* gene expression as they did in *R. meliloti*. In a previous study (Thöny et al. 1989) the *fixRnifA* operon of *B. japonicum* was found to be expressed under aerobic conditions. Therefore the expression of chromosomally integrated *fixR'—'lacZ* and *nifA'—'lacZ* fusions in the different *fixL*, *fixJ* and ORF138 mutant backgrounds (see Materials and methods) was determined under aerobic growth conditions. None of the mutants affected the expression of the *fixR'*- or *nifA'—'lacZ* fusions (Table 3). Also, the tenfold higher expression of the *fixR'—'lacZ* fusion as compared with the *nifA'—'lacZ* fusion (Thöny et al. 1987) remained undisturbed (Table 3). This showed that *fixLJ* of *B. japonicum* was not involved in any possible aerobic activation of the *fixRnifA* operon. Previously, gel retardation experiments had been carried out with a 32 bp synthetic double-stranded DNA fragment covering the *cis*-acting UAS (upstream activator sequence) element upstream of the *fixRnifA* operon. Protein extracts from the *B. japonicum* wild type were found to contain a protein binding to this sequence (Thöny et al. 1989). Similarly, protein extracts of mutants 7360 and 7361 still contained that protein (data not shown), suggesting that it was not encoded by *fixJ*.

Expression of fixJ *is not controlled by NifA*

Translational in-frame and out-of-frame *fixJ'—'lacZ* fusions were constructed (see Materials and methods). As *fixJ* probably formed an operon with *fixL*, the fusion

constructs carried *fixL* and 1 kb of upstream DNA (Fig. 1). After cloning the fusions into the broad host range vector pRK290X they were mobilized into the *B. japonicum* wild type, into a *nifA* mutant, and into the *fixJ* mutant strains. Expression of the *fixJ'—'lacZ* fusion was determined under aerobic and, if possible, also under anaerobic growth conditions (Table 4). The out-of-frame *fixJ'—'lacZ* fusion showed no β-galactosidase activity. When introduced into the wild-type strain, the in-frame *fixJ'—'lacZ* fusion was expressed at a relatively high level under aerobic conditions (Table 4). Under anaerobic growth conditions this expression was reduced to about 40%. The expression of the *fixJ'—'lacZ* fusion in the *nifA* mutant was the same as in the wild type, which we interpret to mean that *fixJ* is not controlled by NifA. It was not possible to test the influence of *fixJ* on its own expression under anaerobiosis, because the *fixJ* mutants were unable to grow anaerobically with nitrate (see Fig. 4).

Discussion

The occurrence of *fixL*- and *fixJ*-like genes in *B. japonicum* implies that they function similarly in this bacterium and in *R. meliloti*, in which these genes were first found (David et al. 1988; Virts et al. 1988; Banfalvi et al. 1989). In fact this is probably true to some extent; however, as shown in this report, there are also considerable differences. It is because of these differences that one may question whether the newly detected *B. japonicum* genes are indeed homologues of the *R. meliloti fixL* and *fixJ* genes. To find a solution to this problem one has to weigh the following observations and arguments.

1. Among all the two-component regulatory systems known to date, the *B. japonicum fixL*- and *fixJ*-like genes and products share the greatest degree of similarity with the *fixL* and *fixJ* genes and their products from *R. meliloti*. This is particularly evident in the case of the FixL protein. Usually, the sensor proteins of the family of two-component systems are homologous only within a C-terminal stretch of about 250 amino acids in length (Albright et al. 1989; Gross et al. 1989; Stock et al. 1989, 1990). By contrast, the *R. meliloti* and *B. japonicum* FixL proteins are highly homologous over a stretch of at least 370 amino acids (approx. 55% identity therein). Only the first 135 amino acids at the N-terminus are quite divergent. In this poorly conserved region the *R. meliloti* FixL protein is considerably more hydrophobic than the *B. japonicum* protein. By analogy with many other sensors, the *R. meliloti* FixL protein has been suggested to be bound to the cytoplasmic membrane (David et al. 1988). A similar prediction cannot be made as readily for FixL of *B. japonicum*.

2. Strains of *R. meliloti* and *B. japonicum* that have insertion mutations in the *fixLJ* genes are defective in symbiotic nitrogen fixation. The *R. meliloti fixLJ* mutants have been reported to be completely Fix^- whereas the *B. japonicum fixLJ* mutants always had some 5% to 10% residual acetylene reduction activity. While this suggests an involvement of the *B. japonicum* FixL and FixJ proteins in the regulation of one or several symbiotic N_2 fixation genes, this result also shows that the *fixLJ* genes are not absolutely essential for symbiosis. Either the functions of the FixLJ proteins or the functions of the products of genes controlled by them can be substituted to some extent by other cellular systems.

3. Perhaps some, but certainly not all of the target genes regulated by the *fixLJ* system are similar in *B. japonicum* and *R. meliloti*. One apparent difference is the *nifA* gene, which is activated by FixJ in *R. meliloti* but probably not in *B. japonicum*. It is shown in this paper that the aerobic expression of the *B. japonicum fixRnifA* operon is not affected in *fixL* and *fixJ* mutant backgrounds. Furthermore, it is unlikely that the *fixLJ* genes are involved in the fivefold enhancement of *fixRnifA* operon expression under anaerobiosis. As shown previously (Thöny et al. 1989), this increase is NifA dependent and, therefore, an involvement of the *fixLJ* genes could only be rationalized if these genes were regulated by NifA. We report here that this is not the case. However, an unanswered question remains: what is the nature of the gene-activating system that accounts for the residual anaerobic *fixRnifA* expression that is not *nifA* dependent? In a *nifA* mutant background this anaerobic expression was approximately 20% of the maximal, wild-type level (Thöny et al. 1989) and thus in the same range as the aerobic expression level. We have argued previously (Thöny et al. 1989) that the same, as yet unidentified, positive control system takes care of activating the *fixRnifA* operon to a basal level under both aerobic and anaerobic conditions. In the light of our discovery of a *fixLJ*-like system in *B. japonicum*, however, we cannot rule out the possibility that the basal anaerobic, non-*nifA*-dependent level of *fixRnifA* expression is mediated by FixJ whereas a different activator is responsible for expression under aerobiosis. Unfortunately, there are technical obstacles to testing this contention experimentally. First, one cannot test anaerobic *fixRnifA* expression in *fixJ* mutant backgrounds because these strains do not grow under such conditions. Second, using microaerobic growth conditions we found it difficult to see a reproducible difference to the aerobic *fixRnifA* expression level even in the wild type. Third, in symbiosis the physiological state of *fixJ* mutant bacteroids differs considerably from that of wild-type bacteroids which not only affects the recovery of bacteroid isolates but also makes any result from a comparative analysis of *fixRnifA* expression quite dubious. In conclusion, the question of whether or not the *fixLJ* system contributes to some extent to anaerobic *fixRnifA* expression must be left open.

In addition to the *nifA* gene, another *R. meliloti* gene known to be controlled by the *fixLJ* system is the *fixK* gene (Batut et al. 1989). Results obtained very recently in our laboratory seem to suggest that a *fixK*-like gene is also present in *B. japonicum* (B. Scherb and D. Anthamatten, unpublished observation). It will be of interest to see what the phenotype of a *B. japonicum fixK* mutant will be like and whether or not such a gene is controlled by *fixLJ*.

4. A characteristic feature of the *R. meliloti* FixL protein

is its role as a sensor of low oxygen concentration (Virts et al. 1988; David et al. 1988). This signal is transduced to the FixJ protein, which then activates *nifA* and *fixK*. It is not known whether the FixL and FixJ proteins of *B. japonicum* respond to a similar environmental stimulus. In this context it is of interest that *B. japonicum fixL* and *fixJ* mutants do not grow anaerobically with nitrate as terminal electron acceptor. It seems reasonable to suggest that there are *fixLJ*-dependent *B. japonicum* genes that code for components of the denitrification enzymes such as nitrate, nitrite or N_2O reductases. Moreover, it is conceivable that these "anaerobic" genes are regulated by oxygen, possibly via *fixLJ*. Having proposed this, however, it must be mentioned that Batut et al. (1989) have detected wild-type levels of respiratory nitrate reductase activity in microaerobically grown cells of *R. meliloti fixJ* and *fixK* mutants. It is not clear from their report whether or not these mutants could grow anaerobically with nitrate.

The above considerations do not allow an unambiguous conclusion to be made as to whether the *fixLJ* system of *B. japonicum* has a similar function to that in *R. meliloti*. The assumption that the *B. japonicum nifA* gene is not subject to regulation by *fixLJ*, as opposed to the situation in *R. meliloti*, does not exclude the possibility that there are other *fixLJ*-dependent genes common to both organisms (possibly *fixK*). Primarily because of the high sequence similarity we propose to adhere to "*fixL*" and "*fixJ*" as operational terms for the *B. japonicum* genes described in this work.

There is one additional aspect that requires comment: the phenotype of a *fixL* mutant with the inserted kanamycin resistance gene (*aph*) being transcribed towards *fixJ* and ORF138 was always more leaky than that of the *fixL* mutant with the *aph* gene reading in the opposite orientation. This was particularly evident when the rates of anaerobic growth with nitrate were compared. The constitutive expression of *fixJ* from the strong *aph* promoter may have led to increased FixJ synthesis resulting in a partial activation of FixJ-dependent genes even in the absence of a functional FixL protein. This observation is reminiscent of the work of Hertig et al. (1989) who have shown that overexpression of the *R. meliloti fixJ* gene (cloned on a multicopy plasmid) leads to *nifA* and *fixK* activation in the absence of *fixL*, at least in *E. coli*. Such a situation is not uncommon among members of two-component regulatory systems, and one explanation may be the existence of cross-talk with other cellular sensors (discussed by Stock et al. 1989, 1990). ORF138, located downstream of *fixJ*, also appears to encode a protein with homology to the regulator class of the two-component systems. Since no phenotype has as yet been associated with ORF138 insertion mutants, the role of this putative gene and its product remains obscure.

Acknowledgements. We wish to thank P. Boistard for plasmid pDD5. Our colleagues M. Göttfert, H.M. Fischer and B. Thöny kindly provided other plasmids and strains and G. Acuña provided the λ library. This work was supported by a grant from the Swiss National Foundation for Scientific Research.

References

Albano M, Hahn J, Dubnau D (1987) Expression of the competence genes in *Bacillus subtilis*. J Bacteriol 169:3110–3117

Albright LM, Huala E, Ausubel FM (1989) Procaryotic signal transduction mediated by sensor and regulator pairs. Annu Rev Genet 23:311–336

Alvarez-Morales A, Betancourt-Alvarez M, Kaluza K, Hennecke H (1986) Activation of the *Bradyrhizobium japonicum nifH* and *nifDK* operons is dependent on promoter-upstream DNA sequences. Nucleic Acids Res 14:4207–4227

Ayusaw D, Yoneda Y, Yamane K, Mauro B (1975) Pleiotropic phenomena in autolytic enzyme(s) content, flagellation and simultaneous production of extracellular α-amylase and protease in a *Bacillus subtilis* mutant. J Bacteriol 124:459–469

Banfalvi Z, Petkova V, Lados M, Slaska-Kiss K, Putnoky P, Ung CH, Kondorosi A (1989) Identification of two *fix* loci controlling the expression of *nif* genes in *Rhizobium meliloti* 41. Mol Gen Genet 215:345–348

Batut J, Daveran-Mingot ML, David M, Jacobs J, Garnerone AM, Kahn D (1989) *fixK*, a gene homologous with *fnr* and *crp* of *Escherichia coli*, regulates nitrogen fixation genes both positively and negatively in *Rhizobium meliloti*. EMBO J 8:1279–1286

Beynon JL, Williams MK, Cannon FC (1988) Expression and functional analysis of the *Rhizobium meliloti nifA* gene. EMBO J 7:7–14

Casadaban MJ, Martinez-Arias A, Shapira SK, Chou J (1983) β-Galactosidase gene fusions for analyzing gene expression in *E. coli* and yeast. Methods Enzymol 100:293–308

Daniel RM, Appleby CA (1972) Anaerobic nitrate, symbiotic and aerobic growth of *Rhizobium japonicum*: effects on cytochrome P_{450}, other haemoproteins, nitrate and nitrite reductases. Biochim Biophys Acta 275:347–354

David M, Domergue O, Pognonec P, Kahn D (1987) Transcription patterns of *Rhizobium meliloti* symbiotic plasmid pSym: identification of *nifA*-independent *fix* genes. J Bacteriol 169:2239–2244

David M, Daveran ML, Batut J, Dedieu A, Domergue O, Ghai J, Hertig C, Boistard P, Kahn D (1988) Cascade regulation of *nif* gene expression in *Rhizobium meliloti*. Cell 54:671–683

Davis RW, Botstein D, Roth JR (1980) Advanced bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Ditta GS, Stanfield S, Corbin D, Helinski DR (1980) Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank in *Rhizobium meliloti*. Proc Natl Acad Sci USA 77:7347–7351

Ditta GS, Virts E, Palomares A, Kim C (1987) The *nifA* gene of *Rhizobium meliloti* is oxygen regulated. J Bacteriol 169:3217–3223

Figurski DH, Helinski DR (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc Natl Acad Sci USA 76:1648–1652

Fischer HM, Hennecke H (1987) Direct response of *Bradyrhizobium japonicum nifA*-mediated *nif* gene regulation to cellular oxygen status. Mol Gen Genet 209:621–626

Fischer HM, Alvarez-Morales A, Hennecke H (1986) The pleiotropic nature of symbiotic regulatory mutants: *Bradyrhizobium japonicum nifA* gene is involved in control of *nif* gene expression and formation of determinate symbiosis. EMBO J 5:1165–1173

Gross R, Arico B, Rappuoli R (1989) Families of bacterial signal-transducing proteins. Mol Microbiol 3:1661–1667

Hahn J, Albano M, Dubnau D (1987) Isolation and characterisation of Tn*917lac*-generated competence mutants of *Bacillus subtilis*. J Bacteriol 169:3104–3109

Hahn M, Hennecke H (1984) Localized mutagenesis in *Rhizobium japonicum*. Mol Gen Genet 193:46–52

Hennecke H, Günther I, Binder F (1982) A novel cloning vector for the direct selection of recombinant DNA in *E. coli*. Gene 19:231–234

Henner DJ, Yang M, Ferrari E (1988) Localization of *Bacillus subtilis sacU(Hy)* mutations to two linked genes with similarity to the conserved procaryotic family of two-component signalling systems. J Bacteriol 170:5102–5109

Hertig C, Ya Li R, Louarn A-M, Garnerone A-M, David M, Batut J, Kahn D, Boistard P (1989) *Rhizobium meliloti* regulatory gene *fixJ* activates transcription of *R. meliloti nifA* and *fixK* genes in *Escherichia coli*. J Bacteriol 171:1736–1738

Huala E, Ausubel FM (1989) The central domain of *Rhizobium meliloti* NifA is sufficient to activate transcription from the *R. meliloti nifH* promoter. J Bacteriol 171:3354–3365

Losick R, Youngman P, Piggot PJ (1986) Genetics of endospore formation in *Bacillus subtilis*. Annu Rev Genet 20:625–669

Macnab RM (1987) Motility and chemotaxis. In: Neidhardt FC, Ingraham JL, Low KB, Magasanik B, Schaechter M, Umbarger HE (eds) *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington DC, pp 732–759

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Messing J (1983) New M13 vectors for cloning. Methods Enzymol 101:20–78

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Minton NP (1984) Improved plasmid vectors for the isolation of translational *lac* gene fusions. Gene 31:269–273

Moolenaar GF, van Sluis CA, Backendorf C, van der Putte P (1987) Regulation of the *Escherichia coli* excision repair gene *uvrC*. Overlap between the *uvrC* gene and the region coding for a 24 kD protein. Nucleic Acids Res 15:4273–4289

Norrander J, Kempe T, Messing J (1983) Construction of improved M13 vectors using oligonucleotide-directed mutagenesis. Gene 26:101–106

Ratet P, Pawlowski K, Schell J, de Bruijn FJ (1989) The *Azorhizobium caulinodans* nitrogen fixation regulatory gene, *nifA*, is controlled by the cellular nitrogen and oxygen status. Mol Microbiol 3:825–838

Regensburger B, Hennecke H (1983) RNA polymerase from *Rhizobium japonicum*. Arch Microbiol 135:103–109

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Simon R, Priefer U, Pühler A (1983) Vector plasmids for in vivo and in vitro manipulation of gram-negative bacteria. In: Pühler A (ed) Molecular genetics of the bacteria-plant interaction. Springer Verlag, Heidelberg, pp 98–106

Smith I (1989) The initiation of sporulation. In: Smith I, Slepecky R, Setlow P (eds) Regulation of procaryotic development. American Society for Microbiology, Washington DC, pp 185–210

Stewart RC, Dahlquist FW (1987) Molecular components of bacterial chemotaxis. Chem Rev 87:997–1025

Stewart V (1988) Nitrate respiration in relation to facultative metabolism in enterobacteria. Microbiol Rev 52:190–232

Stock JB, Ninfa AJ, Stock AM (1989) Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol Rev 53:450–490

Stock JB, Stock AM, Mottonen JM (1990) Signal transduction in bacteria. Nature 344:395–400

Thöny B, Fischer HM, Anthamatten D, Bruderer T, Hennecke H (1987) The symbiotic nitrogen fixation regulatory operon (*fixRnifA*) of *Bradyrhizobium japonicum* is expressed aerobically and is subject to a novel, *nifA*-independent type of activation. Nucleic Acids Res 15:8479–8499

Thöny B, Anthamatten D, Hennecke H (1989) Dual control of the *Bradyrhizobium japonicum* symbiotic nitrogen fixation regulatory operon *fixRnifA*: analysis of *cis*- and *trans*-acting elements. J Bacteriol 171:4162–4169

Turner GL, Gibson AH (1980) Measurement of nitrogen fixation by direct means. In: Bergersen FJ (ed) Methods for evaluating biological nitrogen fixation. John Wiley & Sons, Chichester, pp 111–138

Virts EL, Stanfield SW, Helinski DR, Ditta GS (1988) Common regulatory elements control symbiotic and microaerobic induction of *nifA* in *Rhizobium meliloti*. Proc Natl Acad Sci USA 85:3062–3065

Weston LA, Kadner RJ (1988) Role of *uhp* genes in expression of the *Escherichia coli* sugar-phosphate transport system. J Bacteriol 170:3375–3383

Communicated by A. Kondorosi