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Regulation of the *fixA* Gene and *fixBC* Operon in *Bradyrhizobium japonicum*

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The transcriptional start site of the *Bradyrhizobium japonicum fixBC* operon was identified by nuclease S1 mapping. It was located approximately 700 base pairs upstream of *fixB* and was preceded by a promoter sequence that showed strong homology to the *B. japonicum fixA* promoter and thus to the general *nif* consensus promoter sequence. Further transcript mapping experiments revealed that *fixA* and *fixBC* transcription in *B. japonicum* strictly depended on the presence of the regulatory gene *nifA* and on low oxygen partial pressure. Consistent with these data, chromosomally integrated *fixA*- and *fixB-lacZ* fusions expressed β-galactosidase activity only in the wild type but not in a *nifA* mutant and only under microaerobic but not aerobic growth conditions. The presence of *nifA* accounted for a 19-fold and 44-fold activation of the *fixA* and *fixB* promoters, respectively. These results show that the *fixA* and *fixBC* genes are regulated in a way similar to that of the nitrogenase genes *nifH* and *nifDK*. A very peculiar finding was that the *fixA* and *fixB* promoters, when they were located on plasmids, could hardly be activated by the NifA protein, irrespective of whether this was tested in *Escherichia coli* or *B. japonicum* backgrounds. This is in clear contrast to the situation with *nifH* and *nifD* promoters.

Symbiotic nitrogen fixation genes which are not homologous to any of the known *nif* genes in the free-living diazotroph *Klebsiella pneumoniae* are called *fix*. A group of *fix* genes, *fixA*, *fixB*, and *fixC*, was first detected in the alfalfa root nodule bacterium *Rhizobium meliloti* (11, 35, 43). Homologous genes have since then been detected in other root and stem nodule bacteria, namely, *Bradyrhizobium japonicum* (23, 25), *Azorhizobium sesbaniae* (15), and *Rhizobium leguminosarum* (24). Circumstantial evidence suggests that, in aerobic nitrogen-fixing bacteria, these genes may code for functions involved in electron transport to nitrogenase: (i) in *B. japonicum* and *A. sesbaniae* they were found to be indispensible not only for symbiotic but also for microaerobic N_2 fixation under free-living conditions (15, 25); (ii) they were found to hybridize to DNA of other nonrhizobial, (micro)aerobic diazotrophs such as *Azospirillum brasilense* and *Azotobacter vinelandii* (18, 22, 25); (iii) as predicted from the nucleotide sequence of the *R. meliloti fixC* gene, the FixC protein may contain a signal sequence for membrane insertion (18); (iv) immediately adjacent to the 3′ end of the *fixC* gene, and probably cotranscribed with it, a so-called *fixX* gene has been found in several *Rhizobium* species to encode a ferredoxinlike protein (17, 18, 24, 29).

In fast-growing rhizobia the *fixA*, *fixB*, *fixC*, and *fixX* genes are on one operon, *fixABCX* (5, 18, 35). The *R. meliloti fixA* promoter has been identified and found to contain the characteristic *nif* consensus promoter sequence (5), which implies that it is probably recognized by the product of *ntrA* (a specific sigma factor of RNA polymerase) and is positively controlled (see reference 26 for a review). In fact, it has been shown that no *fixABC(X)* transcript was synthesized in a *R. meliloti nifA* mutant (47) which suggests that the *fixA* promoter is activated by the product of *nifA*. In conflict with this result, however, it was reported that the *fixA* promoter could be activated neither by the *K. pneumoniae* nor by the *R. meliloti* NifA protein in *Escherichia coli* (4, 26). Under nitrogen-limiting growth conditions ex planta (free living) the *R. meliloti fixA* promoter appears to be activated by the product of the nitrogen-regulatory gene, *ntrC* (46). Since *R. meliloti* is unable to fix N_2 under these growth conditions, the physiological meaning of this seemingly futile expression is not understood (46).

In the soybean root nodule symbiont *B. japonicum*, the *fixA* gene has been found to be separated from the *fixB* and *C* genes (23, 25). The *fixB* and *fixC* genes are adjacent and form an operon, *fixBC*; these genes are located in the chromosomal cluster I of *B. japonicum* symbiotic genes (28). The *fixA* gene is located in the cluster II region (28); its promoter has been mapped, and its sequence was found to be of the *nif* consensus promoter type (23). In the present work we wished to address the following questions. (i) Is the *fixB* promoter sequence similar to the *fixA* promoter, and is the expression from both promoters coordinately controlled? (ii) Are *fixA* and *fixBC* regulated by the *nifA* gene product and by oxygen like the *B. japonicum* nitrogenase genes *nifH* and *nifDK* (2, 21)? (iii) Are the *fix* genes expressed at a high level similar to that of the nitrogenase genes? To answer these questions, we carried out transcript mapping studies and determined the expression of *fix-lacZ* fusions in different mutant backgrounds and under various physiological conditions.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains, vectors, and recombinant plasmids are listed in Table 1. *E. coli* strains were used for construction and maintenance of plasmids (HB101), for β-galactosidase assays (MC1061), and for mobilization of pSUP202 derivatives (S17-1) or pRK290 derivatives (HB101 carrying pRK2013 as a helper plasmid).

Media and growth of cells. *E. coli* strains were routinely grown in LB medium (33). *E. coli* strains to be tested for β-galactosidase activity were grown in NFDM medium (14)

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TABLE 1. Bacterial strains and plasmids

Strains and plasmids	Relevant characteristics	Source or reference
E. coli		
HB101	*hsdR hsdM recA13* Str^r	12
S17-1	*hsdR*/RP4-2 *kan*::Tn*7* *tet*::Mu, integrated in the chromosome	45
MC1061	Δ(*lacIPOZYA*)*X74 hsdR*	10
B. japonicum		
110*spc*4	Spc^r (wild type)	36
UB518[a]	Spc^r Km^r, *aph* insertion upstream of *fixB*	This work
UB1108[a]	Spc^r Km^r, *aph* insertion upstream of *fixB*	This work
UC417[a]	Spc^r Km^r, *aph* insertion upstream of *fixB*	This work
UC913[a]	Spc^r Km^r, *aph* insertion upstream of *fixB*	This work
A9	Spc^r Km^r, *nifA*::*aph*	20
A10[b]	Spc^r Km^r, *nifA*::*aph*	20
110*spc*4-46[b]	Spc^r Tc^r, Φ(*fixA'-'lacZ*)Hyb integrated in the chromosome	This work
110*spc*4-47	Spc^r Tc^r, Φ(*fixB'-'lacZ*)Hyb integrated in the chromosome	This work
110*spc*4-48	Spc^r Tc^r, Φ(*nifH'-'lacZ*)Hyb integrated in the chromosome	This work
A10-46[b]	Spc^r Km^r Tc^r, *nifA*::*aph* Φ(*fixA'-'lacZ*)Hyb integrated in the chromosome	This work
A10-47	Spc^r Km^r Tc^r, *nifA*::*aph* Φ(*fixB'-'lacZ*)Hyb integrated in the chromosome	This work
A10-48	Spc^r Km^r Tc^r, *nifA*::*aph* Φ(*nifH'-'lacZ*)Hyb integrated in the chromosome	This work
Plasmids		
pGEM-1	Ap^r, SP6 and T7 promoters	Promega Biotech, Madison, Wis.
pSUP202	Ap^r Tc^r Cm^r, *oriT* from RP4	45
pMC1403	Ap^r, *'lacZYA*	10
pRK290X	Tc^r	1, 13
pRK2013	Km^r, tra^+	19
pMC71A	Cm^r (pACYC184), *Kp nifA*(Con)[c]	6
pRJ6012[d]	Ap^r (pMC1403), Φ(*fixA'-'lacZ*)Hyb	This work
pRJ6017[d]	Ap^r (pMC1403), Φ(*fixB'-'lacZ*)Hyb	This work
pRJ6018[d]	Ap^r (pMC1403), Φ(*fixB'-'lacZ*)Hyb	This work
pRJ6019[d]	Ap^r (pMC1403), Φ(*fixB'-'lacZ*)Hyb $nifH^+$	This work
pRJ6020[d]	Ap^r (pMC1403), Φ(*fixB'-'lacZ*)Hyb *nifH*	This work
pRJ1009	Ap^r (pMC1403), Φ(*nifH'-'lacZ*)Hyb	2
pRJ6027	Tc^r (pRK290X), Φ(*fixA'-'lacZ*)Hyb of pRJ6012	This work
pRJ6029	Tc^r (pRK290X), Φ(*fixB'-'lacZ*)Hyb of pRJ6018	This work
pRJ1025	Tc^r (pRK290X), Φ(*nifD'-'lacZ*)Hyb	1
pRJ6046	Ap^r Tc^r (pSUP202), Φ(*fixA'-'lacZ*)Hyb	This work
pRJ6047	Ap^r Tc^r (pSUP202), Φ(*fixB'-'lacZ*)Hyb	This work
pRJ6048	Ap^r Tc^r (pSUP202), Φ(*nifH'-'lacZ*)Hyb	This work

[a,b] The relevant genomic structure of these mutants is depicted in Fig. 1 and 4, respectively.
[c] Constitutive *K. pneumoniae (Kp) nifA* expression.
[d] The structure of these *lacZ* fusions is shown in Table 3.

supplemented with filter-sterilized glutamine, Casamino Acids, and yeast extract (each at 200 μg/ml). Antibiotic concentrations were as follows: ampicillin, 100 μg/ml; chloramphenicol, 40 μg/ml; kanamycin, 50 μg/ml; tetracycline, 10 μg/ml.

B. japonicum strains were usually grown in PSY medium (36). For selection after matings, antibiotics were used at the following concentrations: spectinomycin, 200 μg/ml; kanamycin, 200 μg/ml; tetracycline, 120 μg/ml; chloramphenicol, 10 μg/ml (for counterselection against *E. coli* donors). *B. japonicum* strains to be tested in β-galactosidase assays were grown in minimal medium (36). Microaerobic cultures were grown as described previously (36).

Soybean nodulation and nitrogen fixation assays. Inoculation of soybean (*Glycine max* L. Merr. cv. Clark L1) seedlings and growth of the plants was done as described previously (27). Whole root nodule nitrogen fixation activity was determined by the acetylene reduction assay (48) 21 days after inoculation.

β-Galactosidase assays. *E. coli* strains harboring *fix-* or *nif-lacZ* fusion derivatives of pMC1403 were grown overnight at 28°C in 7-ml, air-tight, screw-capped plastic bottles containing 5 ml of supplemented NFDM medium. Antibiotic selection was applied for maintenance of plasmids. *B. japonicum* strains harboring *fix-* or *nif-lacZ* fusions on pRK290X or integrated in the chromosome were grown at 28°C in minimal medium for 2 days (aerobic) or for 7 days (microaerobic) before assaying β-galactosidase activity. At least two independent cultures of every *E. coli* and *B. japonicum* strain were assayed twice. Samples (100 μl) of cells were treated as described by Miller (33), and calculation of enzyme activities was done identically for *E. coli* and *B. japonicum*. Root nodule bacteroids were suspended in bacteroid isolation buffer (see below), and serial dilutions were then assayed for β-galactosidase activity. The units of enzyme activity were based on measurements of the optical density (A_{600}) of the bacteroids. The relative units obtained nicely compare to values obtained on a per-protein basis (1).

Isolation of root nodule bacteroids. Root nodules from 21-day-old plants were harvested into liquid nitrogen and kept at −80°C for long-term storage. The nodules from 3 to 10 plants were ground to a fine powder in a mortar cooled

with liquid nitrogen. The powder was suspended in bacteroid isolation buffer (0.5 M mannitol, 20 mM sodium succinate, 5 mM sodium dithionite, 50 mM Tris hydrochloride, pH 7.5), and the bacteroids were separated from particulate nodule debris by differential centrifugation as follows. After a 3-min spin at 3,000 rpm (Sorvall SS34 rotor; 4°C) the supernatant solution was poured into a new tube, and the centrifugation step was repeated once. The supernatant solution was again transferred into a new tube, and the bacteroids were sedimented by centrifugation at 12,000 rpm for 6 min at 4°C. The pellet containing mainly bacteroids was suspended in a small volume of buffer appropriate for the following experimental step.

RNA isolation. Free-living *B. japonicum* (aerobic or microaerobic cultures) and root nodule bacteroids were harvested by centrifugation and suspended in 20 mM sodium acetate (pH 5.5)–1 mM EDTA–0.5% sodium dodecyl sulfate. An equal volume of hot (65°C) phenol equilibrated with 20 mM sodium acetate (pH 5.5), was added, mixed, and incubated at 65°C for 5 min. The extraction was repeated with phenol-chloroform (1:1) at room temperature followed by two extractions with 2 volumes of diethyl ether. The nucleic acids were precipitated by addition of 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol and left at −20°C for storage.

Cloning and analysis of recombinant DNA. The methods used for the preparation of plasmid DNA and the application of enzymes for restriction and modification of DNA were as described by Maniatis et al. (30). DNA restriction fragments were purified from low-melting-point agarose (Sea plaque agarose; FMC Corp.) after electrophoretic separation by the method of Weislander (49). *B. japonicum* total DNA was prepared essentially as described previously (27), and Southern blot analysis of *B. japonicum* mutants generated by marker exchange mutagenesis or plasmid cointegration was performed (25). The probes for Southern blot hybridizations were either nick-translated, ^{32}P-labeled plasmid DNA (30) or in vitro-synthesized, ^{32}P-labeled RNA transcripts from DNA fragments linked to the SP6 promoter of pGEM-1 (32).

Plasmid constructions. The *fixA*- and *fixB-lacZ* translational fusions depicted in Table 3 are derivatives of the fusion vector pMC1403 (10). The *fixA-lacZ* fusion in pRJ6012 was constructed by first isolating a 600-base-pair (bp) *Cla*I-*Xho*I fragment covering the 5′ region of the *fixA* gene and ligating it (after filling in the protruding 5′ ends) into the *Sma*I site of pMC1403. This generated an out-of-frame *fixA-lacZ* fusion, which was cut at the regenerated *Xho*I fusion site. The 5′ ends were again filled in with Klenow DNA polymerase I, and religation resulted in the addition of 4 bp and a continuous *fixA-lacZ* reading frame.

The *fixB-lacZ* fusion in pRJ6017 was constructed by first isolating a DNA fragment covering the *fixB* 5′ region which has been progressively shortened by *Bal* 31 digestion to remove most of the *fixB* 3′-coding region. The fragment mixture was ligated to the *Sma*I site of pMC1403, which generated in-frame and out-of-frame fusions with the *lacZ* gene. In-frame fusions were recognized on the basis of the more intensive blue color of MC1061 transformants on indicator plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (2). The *fixA*- and *fixB-lacZ* fusion sites were sequenced, which confirmed the correct reading frame in both fusion constructs. The fusions were thus created at the 42nd codon of *fixA* and the 45th codon of *fixB*.

Additional plasmid derivatives of the *fixB-lacZ* fusion were constructed, each differing with respect to the length of DNA upstream of the *fixB* promoter. Plasmid pRJ6018 was generated by ligation of the adjacent 1.3-kilobase-pair (kb) *Eco*RI-*Cla*I DNA fragment into pRJ6017 cut with *Eco*RI and partially with *Cla*I, thus replacing the cloning vector portion (see Table 3) and providing the *lacZ* fusion with 1.2 kb of original sequence upstream of the *fixB* promoter. In a similar way pRJ6019 was constructed by insertion of the 3.0-kb *Eco*RI fragment covering the *nifH* region upstream of the *fixBC* operon into the *Eco*RI site of pRJ6018. Deletion of the 750-bp *Hin*dIII fragment from pRJ6019 removed the *nifH* promoter and most of the *nifH*-coding region and resulted in plasmid pRJ6020.

For the integration of *lacZ* fusions into the *B. japonicum* chromosome, pSUP202 derivatives were constructed. Plasmids pRJ6012, pRJ6018, and pRJ1009 were restricted with *Dra*I, which cuts 65 bp downstream of the *lacZ* 3′ end and at several additional sites but leaves intact the *lacZ* gene and the *fix* and *nif* DNAs upstream of the fusion sites. In a subsequent reaction the pRJ6018-derived fragment was cut with *Stu*I to reduce the stretch of DNA upstream of the *fixB-lacZ* fusion site. *Eco*RI linkers were ligated to all blunt ends created by *Dra*I and *Stu*I. Restriction digestion with *Eco*RI removed all linker concatemers and cleaved the single *Eco*RI site in all three fusion constructs. Three DNA fragments of 3.7, 3.8, and 4.0 kb containing the *fixA*-, *fixB*-, and *nifH-lacZ* fusions, respectively, were isolated and ligated to pSUP202 linearized with *Eco*RI, which resulted in plasmids pRJ6046, pRJ6047, and pRJ6048.

Marker exchange mutagenesis. The mutagenesis method was described in detail previously (25, 27, 42). For insertion mutagenesis, the *Xho*I fragment from Tn*5* coding for kanamycin resistance was used. Mobilization of the mutagenized *B. japonicum* DNA cloned in vector pSUP202 into the wild-type strain 110*spc*4 was done with the *E. coli* donor strain S17-1. Marker exchange events in *B. japonicum* generated by homologous recombination were confirmed by Southern blot analysis of total DNA.

Nuclease S1 mapping. All probes used for S1 mappings were double-stranded DNA fragments covering the promoter regions of *fixA* or *fixB* or *aph*. The probes were labeled at the 5′ ends with T4 polynucleotide kinase and [γ-^{32}P]ATP (31). After the DNA probes were cut once asymmetrically with an appropriate restriction enzyme, the fragments were separated on a low-melting-point agarose gel and reisolated. The DNA probes and the RNA samples were denatured, hybridized, and digested with nuclease S1 as described by Berk and Sharp (3), except that hybridization was done at 45°C for 2 h and nuclease S1 digestion was done at 37°C for 30 min with 300 U of S1 (Boehringer GmbH, Mannheim, Federal Republic of Germany). The digestion products were analyzed on 6% polyacrylamide sequencing gels alongside sequencing ladders.

DNA sequencing. *fixA*- and *fixB-lacZ* fusion frames were confirmed by sequencing the fusion sites by the chain-termination method of Sanger et al. (44). Sequencing of the *fixB* promoter region and for S1 mapping experiments was done by the method of Maxam and Gilbert (31).

RESULTS

Mutagenesis of the *fixB* 5′-flanking region. In the DNA sequence (not shown) immediately upstream of the *fixB* 5′ end we did not detect a *nif* consensus promoter sequence. To test the possibility that the *B. japonicum fixBC* operon extends further into the direction where the *nifH* gene was located (Fig. 1), we constructed four new insertion mutants. The Tn*5*-derived *Xho*I fragment encoding the aminoglyco-

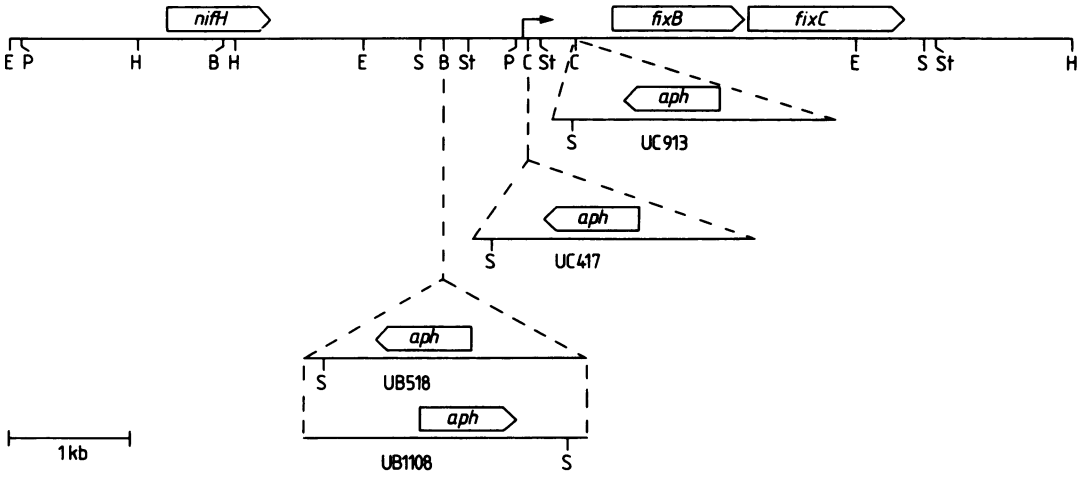


FIG. 1. Physical map of the *B. japonicum fixBC* region. The numbers of the newly generated mutants carrying *aph* insertions upstream of the *fixB* gene are indicated below the inserted fragments. The *fixB* transcription start site is marked by an arrow. Restriction sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H; *Hin*dIII; P, *Pvu*II; S, *Sal*I; St, *Stu*I.

side phosphotransferase gene (*aph*) was inserted into either of the two *Cla*I sites and a *Bam*HI site upstream of *fixB*. The orientations of the *aph* gene are indicated in Fig. 1. The mutations were transferred to the *B. japonicum* genome by marker exchange, which resulted in mutants UC913, UC417, UB518, and UB1108 (Fig. 1). The symbiotic nitrogen fixation (Fix) activities of these mutants are given in Table 2. Mutants UB518 and UB1108 were Fix^+, whereas UC913 and UC417 were Fix^-, which delimited the upstream extension of the *fixBC* operon maximally to the *Bam*HI site. The mutations in strains UC913 and UC417 must be considered to be polar on *fixBC* expression. It was thus likely that the *fixBC* promoter was to be found several hundred base pairs upstream of *fixB*.

Determination of the transcription initiation site and *fixB* promoter sequence. In a first attempt to localize the *fixB* promoter a low-resolution S1 mapping experiment was carried out with a DNA probe covering the *fixB* 5′ end and the region 800 bp upstream of it. Total RNA from soybean root nodule bacteroids protected the probe against S1 nuclease digestion and gave rise to a signal indicating a transcription start site approximately 700 bp upstream of the *fixB* 5′ end (data not shown). No additional signals closer to *fixB* were found. Once the approximate transcription start site was known, a high-resolution S1 mapping experiment was performed. The 200-bp *Pvu*II-*Stu*I DNA fragment of the wild-type DNA (Fig. 1) that was expected to contain the *fixB* promoter was used as a probe for S1 mapping and sequencing reactions. Figure 2 shows the autoradiograph of the sequencing gel on which the *fixB* transcription start point was clearly identifiable by three major bands in lanes 1 and 2. The signals were generated by using RNA from wild-type root nodule bacteroids (lane 1) and from microaerobically grown *B. japonicum* wild type (lane 2). The central band (usually the strongest) of the three prominent S1 signals was taken to assign the first nucleotide (+1) of the *fixBC* transcript. The sequence between nucleotide positions −7 and −27 (Fig. 2) shows extensive homology to the *B. japonicum fixA* promoter (23) and, hence, to the overall *nif* consensus promoter sequence (26). This supports the idea that the *fixA* and *fixBC* genes in *B. japonicum* are regulated coordinately, and that they may be subject to *nifA*-mediated activation as has been shown for *K. pneumoniae nif* (9), *B. japonicum nif* (2), and *R. meliloti nif* and *fix* promoters (4, 47).

Oxygen- and *nifA*-controlled transcription of *fixA* and *fixBC*. The following transcript mapping experiments were designed to test the influence of aerobic growth conditions and of a *nifA* mutant *B. japonicum* genetic background on *fixA* and *fixBC* expression. Since both conditions were known to lead to drastic, pleiotropic changes in the overall cellular physiology (20, 37), it was necessary to include an internal standard, the constitutively expressed *aph* gene, as a control to check the RNA recovery from the cells. The RNA was isolated (i) from the microaerobically grown *nifA* mutant (strain A9), which contained a *nifA*-internal, 837-bp deletion that had been replaced by an *aph* fragment (20), and (ii) from the aerobically grown Fix^+ (wild-type-like) strain UB518 (Fig. 1), which contained the same *aph* fragment within a nonessential site. These RNA preparations were used to protect a mixture of three DNA probes against nuclease S1 digestion; the mixture contained (i) a 355-bp *Pst*I-*Xho*I fragment carrying the *fixA* promoter region (23), (ii) the 200-bp *Pvu*II-*Stu*I fragment with the *fixB* promoter (Fig. 1 and 2), and (iii) a Tn*5*-derived 94-bp *Pvu*II-*Bgl*II fragment containing the presumptive *aph* promoter region (41). It was thus possible to potentially perform three S1 mappings at once with the same RNA preparation. Neither the RNA from aerobically grown UB518 nor the RNA from microaerobically grown A9 produced S1 signals for *fixA* and *fixBC* as they are normally found with RNAs from bacteroids

TABLE 2. Symbiotic nitrogen fixation (acetylene reduction) activity of *B. japonicum* strains carrying mutations upstream of *fixB*

Strain	Sp act[a]	% of wild type
110*spc*4	125.1	100
UB518	118.0	94
UB1108	114.5	92
UC417	0.03	0
UC913	0.04	0

[a] All nodules from two independent plants were measured 21 days after inoculation. Specific activity is expressed as micromoles of C_2H_4 formed per hour per gram of nodule dry weight.

(Fig. 3, lane 2) or a microaerobic culture of the wild type (lane 3). That the two RNA preparations from UB518 and A9 were intact was demonstrated by the fact that both gave rise to the same *aph*-specific S1 signal (lanes 1 and 4, respectively). These experiments clearly showed that the transcription of *fixA* and *fixBC* was dependent on the presence of the *nifA* gene and was repressed by oxygen.

A byproduct of these experiments was the mapping of the transcription start site of the *aph* gene as it is used in *B. japonicum*. The strongest S1 signal (Fig. 3) pointed to an A (position +1 on the noncoding strand); it was located only five nucleotides downstream of the sequence 5′-TAAGGT-3′, which has previously been proposed to be the −10 region of the *aph* promoter solely on the basis of its homology with the *E. coli* model promoter sequence (40, 41).

Expression of plasmid-borne *fixA*- and *fixB-lacZ* fusions in *E. coli*. Translational fusions of the *fixA* and *fixB* genes to *lacZ* were created, and their expression was measured as β-galactosidase activity to obtain an estimate (i) of the strength of *fixA* and *fixB* expression in comparison to previously studied *nif* genes and (ii) of the factor of activation in response to the NifA protein. Previous data have shown that the *B. japonicum nifH* and *nifD* promoters can be activated more than 100-fold in *E. coli* by providing the *K. pneumoniae* NifA protein in *trans*, and that an upstream activator

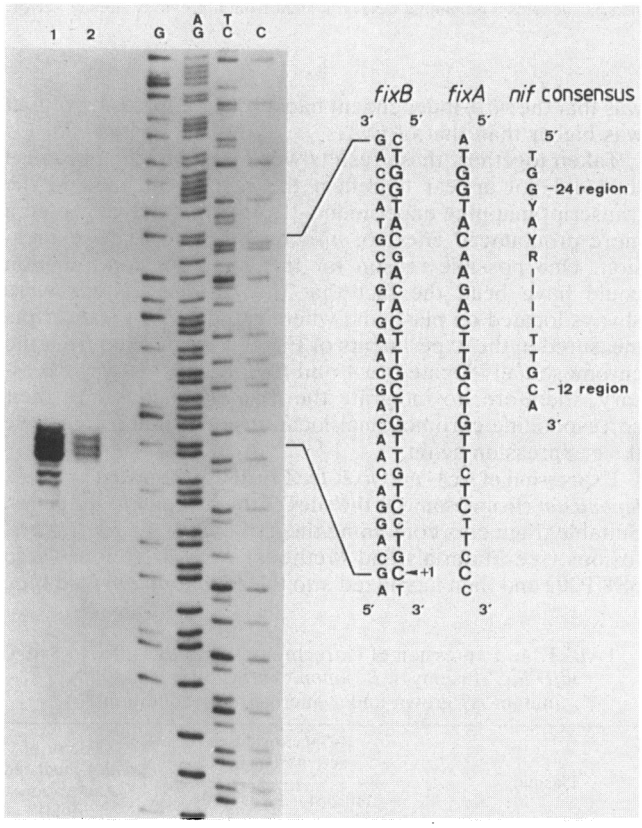


FIG. 2. High-resolution S1 mapping of the *fixB* transcription start site. The probe was the end-labeled 200-bp *Pvu*II-*Stu*I fragment containing the *fixB* promoter. The RNA used was from wild-type bacteroids (lane 1) and from microaerobically grown *B. japonicum* wild-type cells (lane 2). The transcription start site is indicated by an arrowhead (+1). The right margin shows the comparison of the *fixB* promoter with the *fixA* promoter (23) and of both with the overall *nif* consensus promoter sequence. Identical nucleotides in the *fixB* and *fixA* promoters are emphasized by enlarged letters.

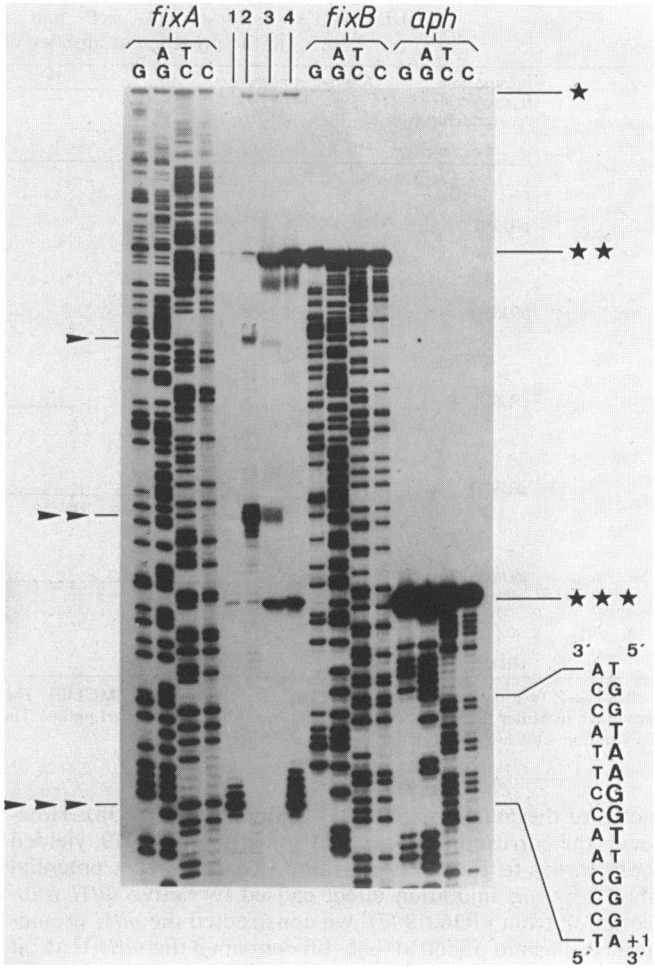
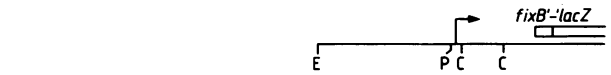
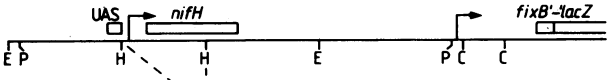
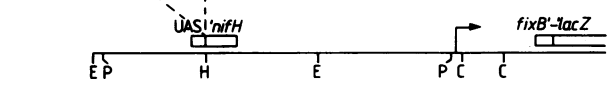


FIG. 3. Transcription from *fixA* and *fixB* promoters: dependence on *nifA* and repression by oxygen. ^{32}P-labeled *fixA*-, *fixB*-, and *aph*-specific DNA probes (see the text) were mixed and hybridized with total RNA from strain UB518 grown aerobically (lane 1), wild-type bacteroids (lane 2), wild type grown microaerobically (lane 3), and *nifA* mutant A9 grown microaerobically (lane 4). The transcription start signals are marked on the left margin with one arrowhead for *fixA*, two arrowheads for *fixB*, and three arrowheads for *aph*. Signals representing the renatured probes, which are thus protected against nuclease S1 digestion, are marked on the right margin with one (*fixA*), two (*fixB*), and three asterisks (*aph*). The sequencing ladders of the *fixA*, *fixB*, and *aph* promoter regions are indicated on top. The right margin also shows the sequence of the previously proposed −10 region of the *aph* promoter (41: enlarged letters) and the first transcribed nucleotide (+1) in *B. japonicum*.

sequence (UAS) located approximately 100 bp upstream of each promoter was required for maximal activation (1, 2). In similar in vivo assays (Table 3) the newly constructed *fixA-lacZ* fusion on plasmid pRJ6012 which carries about 400 bp of *fixA*-upstream DNA was already expressed at a comparatively high level in *E. coli* without the *K. pneumoniae* NifA protein and was activated only two- to threefold by NifA. The original *fixB-lacZ* fusion (on pRJ6017) had only 70 bp of *B. japonicum* upstream DNA and was also activated threefold by *K. pneumoniae* NifA (Table 3). The presence of more *fixB*-upstream DNA (1.2 kb) in pRJ6018 did not improve the weak activation (Table 3). We then tested the possibility that the strong UAS coupled to *nifH*, which is located 3.2 kb upstream of the *fixB* gene (Fig. 1), might

TABLE 3. Expression of *fixA*-, *fixB*-, and *nifH-lacZ* translational fusions in *E. coli* MC1061 in the presence or absence of the *K. pneumoniae nifA* gene[a]

Plasmid with *B. japonicum fix-lacZ* fusion	Relevant structure	β-Galactosidase activity: − *nifA*	β-Galactosidase activity: + *nifA* (pMC71A)	Factor of *nifA*-mediated activation
pRJ6012		344	834	2.4
pRJ6017		15	49	3.2
pRJ6018		32	70	2.2
pRJ6019		22	82	3.7
pRJ6020		24	129	5.4
pRJ1009	*nifH'-'lacZ*	32	5300	166

[a] All *lacZ* fusion plasmids (left column) are based on vector pMC1403. The *nifA* gene was constitutively expressed from pMC71A. β-Galactosidase activities are given in Miller units (33). Arrows represent transcription start points. The hatched bar denotes a remaining DNA fragment from a previous cloning vector. Restriction sites are abbreviated as in Fig. 1

activate the *fixB* promoter at a long distance (8, 38). However, the corresponding plasmid construct, pRJ6019, yielded only three- to fourfold activation. To rule out a potential multicopy *nif* inhibition effect caused by active *nifH* transcription from pRJ6019 (7), we constructed the *nifH* promoterless plasmid pRJ6020 that still contained the *nifH* UAS; it resulted in about fivefold activation of *fixB* by the NifA protein (Table 3). The experiments of Table 3 were also performed by providing the *B. japonicum* NifA protein (20) in *trans* and using all plasmids except pRJ6019 and pRJ6020. Essentially the same results were obtained as with the *K. pneumoniae* NifA protein; no activation was seen with the *K. pneumoniae* NtrC protein (data not shown).

Thus, although we did observe activation of *fixA* and *fixB* by NifA, the activation factors were rather poor in comparison to, for example, the 166-fold activation of a *nifH-lacZ* fusion (Table 3). To exclude the possibility that this was due to the heterologous nature of the in vivo assay, we performed similar experiments in the *B. japonicum* genetic background.

Expression of plasmid-borne *fixA*- and *fixB-lacZ* fusions in microaerobic *B. japonicum* cultures. The *fixA*- and *fixB-lacZ* fusion plasmids pRJ6012 and pRJ6018 (Table 3) were digested with *Eco*RI and *Sal*I, and the fragments carrying the entire *fix-lacZYA* region were cloned into pRK290X, resulting in plasmids pRJ6027 and pRJ6029, respectively. These plasmids were mobilized into the *B. japonicum* wild type and into the *nifA* mutant strain A9 by triparental mating. β-Galactosidase activities were determined in cultures grown microaerobically. For comparison, a strain harboring a *nifD-lacZ* fusion (pRJ1025) was also tested. Whereas the presence of *nifA* in the wild type accounted for a 36-fold activation of the *nifD* promoter, there was only a marginal activation of the *fixA* and *fixB* promoters (Table 4). Thus, these results were comparable to the data obtained with the *E. coli* experiment. The only difference from the *E. coli* experiment was that the *nifA*-independent background expression of *fixB* was higher than that of *fixA*.

Taken together, these results were very puzzling because they did not appear to reflect the results obtained in the transcript mapping experiments, from which we expected a more pronounced effect of *nifA* on *fixA* and *fixBC* expression. One possible reason for this apparent contradiction could have been the fact that the *fix-lacZ* fusions were always located on plasmids, whereas the mRNA transcripts measured in the experiments of Fig. 3 were derived from the chromosomally located *fixA* and *fixBC* genes. It was necessary, therefore, to integrate the *fix-lacZ* fusions into their corresponding chromosomal locations and then to measure their expression again.

Expression of *fixA*- and *fixB-lacZ* fusions integrated in the *B. japonicum* chromosome at the sites of the *fixA* and *fixBC* genes. Suitable fragments containing the *fixA*-, *fixB*-, and *nifH-lacZ* fusions (see Materials and Methods) were first cloned into pSUP202 and then mobilized into the *B. japonicum* wild type

TABLE 4. Expression of extrachromosomal *fixA*-, *fixB*-, and *nifD-lacZ* fusions in *B. japonicum* wild type and *nifA* mutant A9 grown under microaerobic conditions[a]

Plasmid	β-Galactosidase activity (U) in: 110*spc*4 (wild type)	β-Galactosidase activity (U) in: A9 (*nifA*)	Factor of *nifA*-mediated activation (fold)
pRJ6027 (*fixA-lacZ*)	157	103	1.5
pRJ6029 (*fixB-lacZ*)	861	437	2
pRJ1025 (*nifD-lacZ*)	5,951	167	36

[a] Tetracycline selection for plasmid maintenance under these growth conditions was not possible. Plasmid loss was determined after each experiment by plating on nonselective and selective media. For the calculation of β-galactosidase activities, the loss of plasmids (between 40 to 60%) was taken into account.

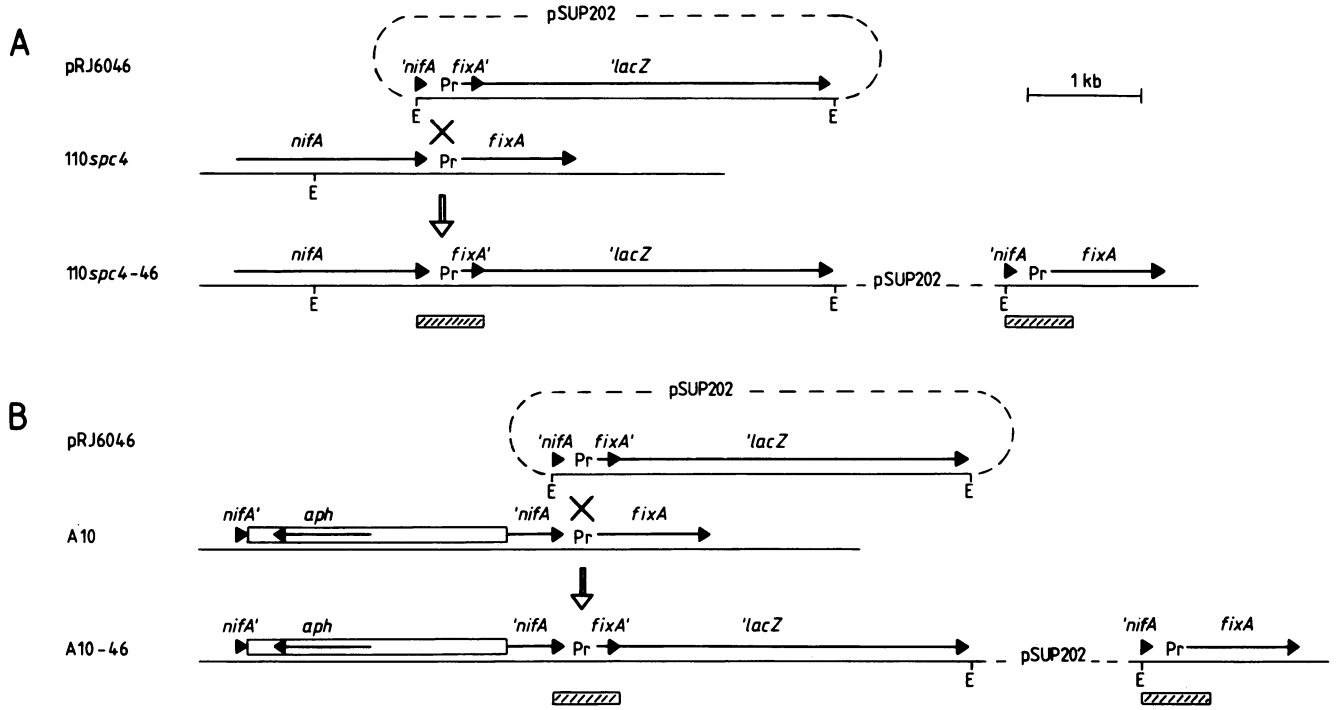


FIG. 4. Chromosomal integration of the *fixA-lacZ* fusion into *B. japonicum* wild type (A) and *nifA* mutant A10 (B) via homologous recombination. The procedure is explained in the text. The hatched bars indicate a 600-bp duplication. The arrows denote the extent and transcriptional direction of the genes. Pr, *fixA* promoter; E, *Eco*RI.

and into the *nifA* mutant strain A10. All pSUP202 derivatives (which are unable to replicate in *B. japonicum*) contained only a few hundred base pairs of *fix-nif* 5′-flanking DNA that was sufficient to generate cointegrates at the respective chromosomal *fixA*, *fixB*, and *nifH* locations by homologous recombination. These cointegrates were selectable as pSUP202-encoded tetracycline resistance. The correct DNA structure of the expected plasmid integrations was confirmed by analyzing the DNA of putative recombinants with appropriate Southern blot hybridizations (data not shown).

One example, representative for all cointegrate constructions, is given in Fig. 4 with *fixA-lacZ* and the wild type (A) and strain A10 (B) as recipients. Figure 4A shows that the pSUP202 derivative containing *fixA-lacZ* (pRJ6046) was cointegrated such that it generated a 600-bp duplication. The consequence of this was that the *fixA-lacZ* fusion was flanked by its natural, genomic DNA on the 5′ side and that the wild-type *fixA* gene on the right of the integrated plasmid was left intact (Fig. 4A); accordingly, the resulting *B. japonicum* strain 110*spc*4-46 was found to be symbiotically Fix$^+$. The *nifA* mutant A10 was chosen as a suitable recipient (Fig. 4B) because the *aph* gene inserted into *nifA* was transcribed divergently from *fixA* so that *aph* expression should not interfere with the expression of the adjacent *fixA-lacZ* fusion. Again, both the *fixA-lacZ* fusion and the *fixA* gene contained their natural promoter elements; nevertheless, the resulting strain A10-46 was Fix$^-$ because of the *nifA* mutation.

The newly generated *B. japonicum* strains were assayed for derepression of the integrated *lacZ* fusions in free-living, aerobic and microaerobic cultures by measuring β-galactosidase activities (Table 5). In the wild type, but not in the *nifA* mutant, there was a strong derepression not only of the *nifH* fusion but also of the *fixA* and *fixB* fusions. The *nifA*-mediated activation of the *fixA* and *fixB* promoters accounted for a 19- and 44-fold derepression, respectively, under conditions of microaerobiosis. No derepression was detectable when the same strains were grown aerobically (Table 5). Thus, these results were fully consistent with the transcript mapping data reported above. β-Galactosidase activities were also measured in root nodule bacteroids of the cointegrate-containing wild type. The ratio of *fixA*/*fixB*/*nifH* expression was 1:3:9.4 in nodules as compared with 1:2.3:20 in microaerobic culture (Table 5). With the exception of the twofold discrepancy in *nifH* expression, this result shows that the microaerobic cultures nicely reflect the

TABLE 5. Expression of chromosomally integrated *fixA*-, *fixB*-, and *nifH-lacZ* fusions in *B. japonicum* wild-type and *nifA*$^-$ strains under different growth conditions

Growth condition	Integrated *lacZ* fusion (*n*)	β-Galactosidase activity (U) in:	
		110 *spc*4 (wild type)	A10 (*nifA*)
Microaerobic	*fixA-lacZ* (46)	383	20
	fixB-lacZ (47)	885	20
	nifH-lacZ (48)	7,675	12
Aerobic	*fixA-lacZ* (46)	25	31
	fixB-lacZ (47)	24	13
	nifH-lacZ (48)	22	5
Root nodule bacteroides	*fixA-lacZ* (46)	1,600	NA[a]
	fixB-lacZ (47)	4,801	NA
	nifH-lacZ (48)	15,067	NA

[a] Not applicable because soybean nodules infected by *B. japonicum nifA* mutants are subject to severe disintegration (20).

bacteroid situation, i.e., the *nifH* gene is expressed much more strongly than the *fix* genes.

The stability of the integrated *lacZ* fusions in all strains was determined by plating serial dilutions of the aerobic and microaerobic cultures as well as of isolated ex-nodule bacteria first on nonselective medium, and then by replica plating about 2,500 colonies on tetracycline-containing plates: not a single tetracycline-susceptible clone was found, which documents a remarkable stability of the cointegrated plasmids.

DISCUSSION

These results clearly show that the *B. japonicum fixA* and *fixBC* genes are coordinately regulated. Their expression is strictly dependent on an intact *nifA* gene and on a microaerobic environment. This is consistent with previous findings that all *nifA*-dependent gene activations in *B. japonicum* are oxygen sensitive (20, 21). The *fixA* and *fixBC* genes are thus also coordinately regulated together with other *nif* genes such as the structural genes of the nitrogenase complex *nifH* and *nifDK* (2, 20). In view of the postulated function of the *fixA*, *fixB* and *fixC* products in electron transport to nitrogenase (18, 25), this coordinated control makes sense physiologically. Quantitative measurements with chromosomally integrated *fixA*-, *fixB*-, and *nifH-lacZ* fusions have shown that *nifH* was expressed at least 3- to 4-fold more strongly than *fixB* and 9 to 10-fold more strongly than *fixA*. A several-fold-higher nitrogenase gene expression as compared with accessory *nif* genes is quite common in diazotrophic bacteria (39). The validity of the β-galactosidase values, however, must be judged under the assumption that the different amino termini have a negligible influence on the activity of the hybrid β-galactosidases. The two- to threefold-higher *fixB* expression as compared with *fixA* expression is probably not due to differences in the *fixA* and *fixB* promoter sequences, because these were shown to be highly homologous. A significant difference between both promoters, however, is their distance from the structural gene. Whereas the transcriptional start of *fixA* is located 28 bp upstream of the *fixA* start codon (23), the distance between *fixB* and its promoter is reported here to be 700 bp. Recent work in our laboratory suggests that this 700-bp region does not contain an absolutely essential *nif* or *fix* gene (T. Zürcher, personal communication). Such a long mRNA leader region is quite unusual for procaryotes. It may be speculated that the spacing has been generated by a genomic rearrangement event which eventually led to the separation of the *fixA* and *fixBC* genes characteristically found in *B. japonicum* (23).

Previous work has shown that the *B. japonicum nifH* and *nifD* promoters can be strongly activated by the *K. pneumoniae* and *B. japonicum* NifA proteins both in heterologous *E. coli* and homologous *B. japonicum* backgrounds (2, 20). Similar activation of the *fixA* and *fixB* promoters, surprisingly, has not been possible. In contrast to chromosomally integrated *fix-lacZ* fusions, usually only a two- to threefold activation of plasmid-borne *fix*-lacZ fusions was observed, even though several hundred base pairs of DNA were present upstream of the target promoters. If missing UASs on the plasmids were to be made responsible for this striking difference, such UASs would have to act from a long distance on the chromosome. An artificially transplaced, *nif*-specific UAS has in fact been shown to be able to enhance transcription from a distance of 2 kb (8); however, the natural situation in all *nif* promoters investigated is the location of UASs at about 100 to 150 bp upstream of the transcription start site (1, 8). Lack of activation in *E. coli* has also been reported for the *R. meliloti fixA* promoter (4), despite the fact that it carries a perfect UAS (5′-TGT-N_{10}-ACA-3′) at a position of 130 bp upstream of the transcription start site (5, 18). It is also unlikely that an activating protein other than NifA is specifically involved in the control of the *fix* genes, because this would explain why activation has only been marginal in *E. coli* but would not explain why it has also been low in *B. japonicum*. Instead, we must conclude that *fix-lacZ* fusions allow far more sensitive measurements of gene expression when chromosomally integrated at their specific gene loci as compared with when they are borne on plasmids. In fact, this may also hold true for *nif-lacZ* fusions, because we observed that a *nifD-lacZ* fusion on pRK290 can be activated in *B. japonicum* by a factor of 36, whereas a chromosomally integrated *nifH-lacZ* fusion responds to NifA with 640-fold increase of expression. Provided that *nifD* and *nifH* normally are activatable in the same order of magnitude, there must ba a drastic difference in sensitivity depending on where (chromosome or plasmid) the *lacZ* fusions are located.

At present we can only offer two possible reasons for this phenomenon without being able to favor one of them. (i) Only a special DNA conformation present in the chromosome may allow the activation of *fix* and *nif* promoters through the action of the NifA protein. For example, the degree of supercoiling of plasmid DNA may prevent *nif* or *fix* promoters from being fully activated by NifA. In *E. coli* and *Salmonella typhimurium* it is well known that alterations in the superhelical nature of the chromosomal DNA can have a tremendous effect on the expression of a large number of genes, in either a positive or a negative way (16). A similar phenomenon has recently been described in the case of the uptake hydrogenase genes of *B. japonicum* (34). (ii) The *lac* fusions present on a multicopy plasmid might titrate out one of the factors required for *nif* and *fix* gene expression (such as NifA or NtrA) and thus cannot be activated to full extent.

As a result of our experience with the studies reported here, we suggest that the expression of chromosomal *nif*- or *fix-lacZ* fusions should be analyzed in cases where the activation of plasmid-borne *lacZ* fusions fails.

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