Organization and Characterization of Genes Essential for Symbiotic Nitrogen Fixation from *Bradyrhizobium japonicum* I110

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A total of 96 independent Tn5 insertions within a 39-kilobase-pair (kbp) segment of chromosomal DNA containing the three structural genes for nitrogenase (*nifH*, *nifD*, and *nifK*) from *Bradyrhizobium japonicum* I110 were obtained in *Escherichia coli* and transferred to the wild-type strain by marker exchange. Individual transconjugants containing a Tn5 insertion were inoculated onto *Glycine max* cv. Wilkin (soybeans) and analyzed for their effect on symbiotic nitrogen fixation. In addition to the three structural genes, genes essential for nitrogen fixation (*fix* genes) were located in three separate regions: (i) 9 kbp upstream of the *nifDK* operon; (ii) 1.5 kbp downstream of the *nifDK* operon; (iii) 4.5 kbp upstream of *nifH*. All of the *fix*::Tn5 insertion strains formed nodules which contained low or undetectable levels of nitrogenase activity. Bacteroids isolated from these nodules had approximately the same levels of the *nifDK* and *nifH* transcripts as those detectable from nodules formed by the wild-type strain. Western blot analysis of bacteroid proteins from nodules formed by the *fix*::Tn5 mutants or the wild-type strain showed the presence of similar levels of the nitrogenase protein subunits. The region upstream of *nifH* was characterized further by DNA sequence analysis and was shown to contain the *nifB* gene. The coding sequence of the *nifB* gene consisted of 1,494 nucleotides and was preceded by putative promoter (5' GTGG-10 base pairs [bp] TTGCA 3') and upstream activator (5' TGT-4 bp-T-5 bp-ACA 3') sequences.

The symbiosis between the bacteria of the genus *Rhizobium* and members of the plant family *Leguminosae* results in the reduction of atmospheric nitrogen to ammonia by the bacteria and its subsequent assimilation into the plant for protein synthesis (44). The reduction of nitrogen is carried out by the enzyme nitrogenase which is composed of three polypeptides encoded by the *nifH*, *nifD*, and *nifK* genes (32). The *nif* genes are located on large indigenous plasmids (21, 34) and are transcribed as a single transcriptional unit in the order *nifHDK* in several fast-growing species including *Rhizobium meliloti* (36) and *Rhizobium leguminosarum* (22). In contrast, these genes are found on the chromosome separated into the two operons *nifH* and *nifDK* in the slow-growing species *Bradyrhizobium japonicum* (unpublished data; 12) and *Bradyrhizobium* sp. (isolated from *Vigna unguiculata* [cowpea plant]) (20, 47).

In addition to *nifH*, *nifD*, and *nifK*, other genes essential for nitrogen fixation (*fix* genes) have been best characterized from *R. meliloti*. For example, adjacent to the *nifHDK* operon in *R. meliloti* are genes clustered in three transcriptional units that are required for nitrogen fixation (11). One operon is comprised of three genes, *fixA*, *fixB*, and *fixC* (29), but the function of their products is unknown. A gene homologous to *Klebsiella pneumoniae nifB* which is involved in the synthesis of the iron-molybdenum cofactor of nitrogenase lies in a second operon (5). Between the *fixABC* and *nifB* operons is the *nifA* gene (5) which codes for a transcriptional activator of the *nifHDK* operon (41). Linkage of essential nitrogen fixation genes to the nitrogenase structural genes has also been found in the slow growers. In

Bradyrhizobium sp. (*Vigna*), site-directed Tn5 mutagenesis was used to identify *fix* genes within 10 kilobase pairs (kbp) of DNA immediately downstream of the *nifDK* operon (19). On the basis of interspecies hybridizations and partial DNA sequence analysis, putative *nifB* and *fixBC* genes were found linked to *nifH* in *B. japonicum* (14). Adams et al. (2) showed hybridization of the *nifA* gene of *K. pneumoniae* to several regions in the *B. japonicum* genome. Fisher et al. (13) also identified a region in *B. japonicum* that showed homology to *K. pneumoniae nifA*. This region was linked to the common nodulation genes. When this region was deleted, the dinitrogenase reductase protein (the product of *nifH*) was shown to be missing from bacteroid protein preparations.

In this report we describe the isolation of overlapping cosmid clones carrying the structural genes for nitrogenase from *B. japonicum* I110. A correlated physical-genetic map of 39 kbp of DNA which contains all three structural genes was established with site-directed Tn5 mutagenesis. *fix* genes were found to be separated into three regions that we refer to as *fix* regions I, II, and III. These regions were located 9 kbp upstream (*fix* region I) and 1.5 kbp downstream (*fix* region II) of the *nifDK* operon and 4.5 kbp upstream (*fix* region III) of the *nifH* operon. The effect of Tn5 mutagenesis of these three regions on both the transcription and translation of the *nifDK* and *nifH* operons was examined. In addition, the complete DNA sequence of *fix* region III was determined. Within this region were found three consecutive open reading frames (ORFs). One ORF corresponded to the *nifB* gene, as determined by comparative DNA sequence analysis with the *nifB* genes of *R. meliloti* and *K. pneumoniae*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Source or reference
E. coli		
HB101	Str^r *hsdM hsdR recA proA leuB6 thi*	8
JA221	*hsdM$^+$ hsdR recA leuB6*	10
NS433	λ *Eam4 b2 red3 cI ts857 Sam7*	40
NS428	λ *Aam11 b2 red3 cI ts857 Sam7*	40
S605	*thi thr leu supF lac met*::Tn*5* Km^r	38
SM10	*hsmM$^+$ hsdR$^+$ recA thi thr leu supF lac* RP-4-2-Tc::Mu	38
JM109	*hsdM$^+$ hsdR17 recA1* Δ(*pro-lac*) *endA1 gyrA96 thi-1 supE44 relA1/F traD36 proAB$^+$ lacI^{q}ZΔM15*	J. Messing
B. japonicum		
I110	Fix$^+$ isolate of strain 3I1b110	A. R. J. Eaglesham
Plasmids		
pSUP106	Tc^r Cm^r	38
pSUP201	Ap^r Cm^r	38
pSUP202	Ap^r Tc^r Cm^r	38
pRmB3.8H	Ap^r 3.8-kbp *R. meliloti, nifA* in *Hind*III	41
pRH231	Ap^r Tc^r, 21.0-kbp *Bradyrhizobium* sp. (*Vigna*) *nifK* in *Hind*III	R. G. Hadley
pRM2	Ap^r Tc^r, 9.6-kbp *Bradyrhizobium* sp. (*Vigna*) *nifD* in *Hind*III	R. G. Hadley
pRH232D	Ap^r Tc^r, 5.1-kbp *Bradyrhizobium* sp. (*Vigna*) *nifH* in *Hind*III	R. G. Hadley
M13 mp18	Derivative of M13 wild type	27
M13 mp19	Derivative of M13 wild type	27
pMJ14-6	Tc^r	This study

Construction of pMJ14-6. The 1.4-kbp *Bam*HI fragment (*mob* gene) from pSUP201 was blunt-ended with the Klenow fragment of DNA polymerase, and *Eco*RI linkers were added. This fragment was then ligated to *Eco*RI-digested pBR325 (Ap^r Tc^r Cm^r) (7) to create plasmid pMJ14 (Ap^r Tc^r). To add a unique *Kpn*I site, plasmid pMJ14 was then digested with *Pst*I, blunt-ended with T4 DNA polymerase, and ligated to *Kpn*I linkers. The resulting plasmid was pMJ14-6 (Tc^r).

DNA isolations. Plasmid DNA from *Escherichia coli* was prepared either on a large scale as described by Morris et al. (26) or on a small scale as described by Birnboim and Doly (6). The small-scale preparation of total DNA from *Bradyrhizobium* cells was done by the method of Jagadish and Szalay (19). Total DNA for construction of the gene library was isolated as described by Rosenberg et al. (35).

Construction of *B. japonicum* I110 gene library. The gene bank was constructed as described by Morris et al. (26) with the following modifications. Total DNA, partially digested with *Eco*RI, was fractionated on 10 to 40% sucrose gradients to enrich for fractions in the size range of 20 to 40 kbp. Plasmid pSUP106 was ligated to fractionated strain I110 DNA and packaged in vitro. The packaged cosmid clones were transduced into *E. coli* HB101, and the tetracycline-resistant colonies were screened by colony hybridization, as described by Hanahan and Meselson (17).

Site-directed Tn*5* mutagenesis. The plasmids pSUP202 and pMJ14-6 can replicate in *E. coli* but not in *B. japonicum*. Isolated *Eco*RI or *Kpn*I fragments from the 39 kbp of strain I110 DNA were ligated into either pSUP202 or pMJ14-6, respectively. Tn*5* mutagenesis was performed in *E. coli* S605 by the method of Jagadish and Szalay (19). The location of Tn*5* within each individually isolated plasmid was first determined. The fragments carrying each Tn*5* insertion were then transferred by conjugation from *E. coli* SM10 to *B. japonicum* I110, and Km^r transconjugants were selected. Total DNA was isolated from Km^r transconjugants that were Tc^s, digested with *Eco*RI, and hybridized in separate experiments with ^{32}P-labeled pSUP202, pMJ14-6, and Tn*5*. A double reciprocal crossover resulting in the exchange of the Tn*5*-interrupted fragment on the plasmid for the corresponding wild-type genomic DNA was indicated by a single hybridization band with Tn*5* as the probe. This band was equal in length to Tn*5* (Tn*5* has no *Eco*RI sites) plus the *Eco*RI fragment in which it was inserted. A double reciprocal crossover was further indicated by the absence of vector sequences in the total DNA of the tested transconjugants. The presence of Tn*5* in *Bradyrhizobium* cells isolated from nodules was confirmed by hybridizing total DNA prepared from these cells with Tn*5* as the probe. Typically, three to four nodules from one plant replicate were pooled and crushed aseptically as described previously (19). The nodule suspension was spread onto minimal agar medium (19) without antibiotics, and total DNA was prepared from a loopful of the bacterial lawn.

DNA-DNA hybridization conditions. DNA fragments were transferred to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) by the method of Smith and Summers (39) and hybridized as described by Morris et al. (26).

Plant growth experiments and acetylene reduction determination. The procedures described by Jagadish and Szalay (19) were followed for all plant growth experiments and acetylene reduction activity determinations. Seeds of *Glycine max* cv. Wilkin (soybean) were inoculated at planting with broth cultures of *Bradyrhizobium*, and plants were harvested 40 days later. *Bradyrhizobium* cells were isolated from nodules as described previously (19). Typically, three to four nodules per plant and 20 isolates per plant were assayed for kanamycin resistance.

Nodule RNA isolation and Northern analysis. Nodules (2 g) obtained from *G. max* at 40 days were pulverized in liquid nitrogen with a mortar and pestle and suspended in a buffer adapted from Corbin et al. (11). Bacteroids were isolated from the crude homogenate by centrifugation through a self-generating Percoll gradient as described by Reibach et al. (30). Centrifugation was done in an ultracentrifuge rotor (Ti60; Beckman Instruments, Inc., Fullerton, Calif.) for 45 min at 48,400 × *g*. The bacteroids were disrupted with 12,000 lb/in² of pressure in a French press (SLM Instruments, Inc.), and RNA was isolated from the broken bacteroids by centrifugation through a cushion of CsCl as described by Glisin et al. (16).

The RNA pellet was dissolved in 50% formamide (deionized with mixed bed resin AG501-X8; Bio-Rad Laboratories, Richmond, Calif.)–6% formaldehyde–12 mM Tris–6 mM sodium acetate–0.3 mM EDTA (pH 7.5), heated at 60°C for 15 min, and electrophoresed in a 1% agarose gel. Electrophoresis was conducted in the presence of the same buffer without formamide for 18 h at 2 V cm^{-1}. The separated RNA was transferred to Gene Screen Plus (New England Nuclear Corp., Boston, Mass.) by procedures described by the manufacturer. The RNA blots were prehybridized in 0.45 M
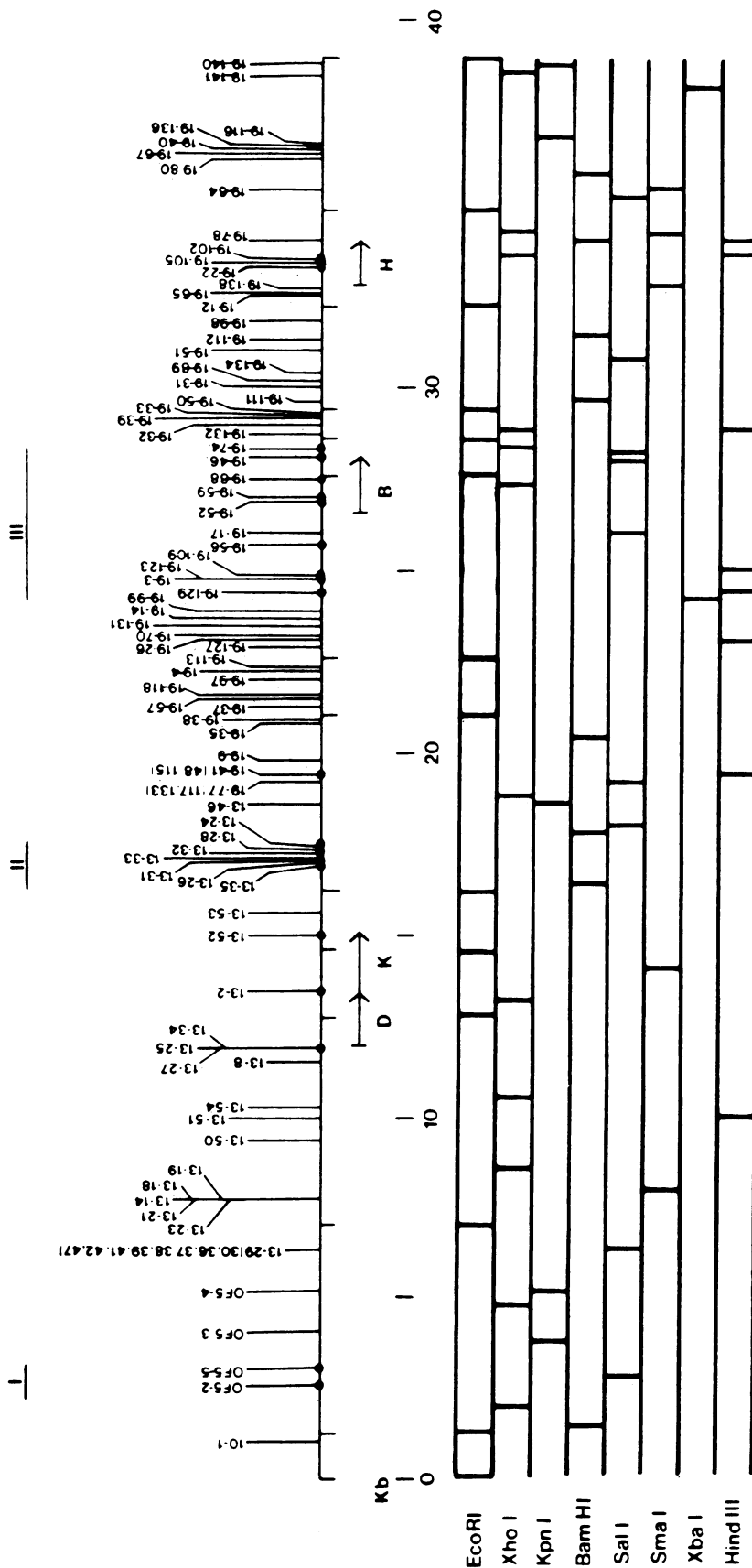
FIG. 1. Restriction endonuclease map and location of Tn5 insertions within B. japonicum I110 DNA in pJN110-22. Tn5 insertions within three regions (fix regions I, II, III) resulted in a Fix⁻ or low Fix phenotype and are indicated with closed circles. All other Tn5 insertions resulted in a Fix⁺ phenotype. Several independently generated Tn5 insertions were in the same location (branching vertical lines). EcoRI sites are shown below the location of the Tn5 insertions.

TABLE 2. Analysis of *G. max* inoculated with the wild-type and mutant strains of I110, at 40 days[a]

Tn5 insertion	Nodule			Total nodule dry wt (g)	Shoot dry wt (g)	ARA[b]
	No.	Color	Size			
fix I						
OF-2	61	P	S	0.065	0.81	0.5
OF-5	75	P	S	0.071	0.79	0.4
nifDK						
13-27	47	P	S	0.079	0.58	0.2
13-25	95	80 %P	S	0.070	0.62	0.2
13-34	78	90 %P	S	0.071	0.74	0.3
13-2	60	85 %P	S	0.071	0.78	0
13-52	71	P	S	0.069	0.08	0.3
fix II						
13-35	66	P	S	0.065	0.55	0.1
13-26	72	P	S	0.068	0.88	0.4
13-31	55	P	S	0.091	0.62	0.1
13-33	68	P	S	0.097	0.91	0.3
13-32	71	P	S	0.094	0.87	0.2
13-28	56	P	S	0.084	0.83	0.1
13-24	78	90 %P	S	0.071	0.74	0.3
19-41	115	P	L	0.383	4.09	19.2
fix III						
19-129	82	P	S	0.128	1.40	5.3
19-3	85	P	S	0.120	1.55	3.2
19-123	70	P	S	0.115	1.07	2.5
19-109	65	P	S	0.092	0.77	2.8
19-56	100	P	S	0.176	1.28	4.5
19-52	62	P	S	0.088	0.79	0
19-59	90	P	M	0.239	1.84	9.8
19-88	70	P	S	0.071	1.41	2.7
19-46	70	P	S	0.077	1.88	0.4
19-74	105	50 %P	S	0.104	0.68	1.9
nifH						
19-22	90	65 %P	S	0.057	0.73	0.1
19-105	65	P	S	0.053	0.89	0.3
19-102	65	70 %P	S	0.047	0.73	0.5
Uninoculated	0				0.92	0
No Tn5 insertion (wild-type I110)	67	P	L	0.303	7.41	58.79

[a] Abbreviations; P, pink; S, 0.10 to 0.15 mm in diameter; M, 0.15 to 0.25 mm in diameter; L, 0.25 to 0.40 mm in diameter. An average of five plant replicates per strain was determined.

[b] ARA, Acetylene reduction activity; values represent μmol of C_2H_4 produced per hour per plant.

NaCl–0.45 M sodium citrate (pH 7.2)–50% (vol/vol) formamide–0.2% Ficoll–0.2% polyvinylpyrrolidone–0.2% bovine serum albumin–0.5% sodium dodecyl sulfate–salmon sperm DNA (100 μg/ml) for 6 h at 37°C. DNA fragments were purified twice from agarose gels by electroelution onto dialysis membranes (45), labeled with [α-^{32}P]dTTP by nick translation (31), and used as probes. After 24 h at 37°C, the filters were washed by the methods described by the manufacturer (New England Nuclear) and subjected to autoradiography.

Immunological detection of nitrogenase. Total protein was obtained from isolated bacteroids disrupted by sonic oscillation in a sonicator (Lab-line Instruments, Inc., Melrose Park, Ill.) and separated in a sodium dodecyl sulfate-polyacrylamide gel by the method of Ames (4). *B. japonicum* nitrogenase components were detected by the method of Towbin et al. (43) with antiserum made against components

I and II of *R. leguminosarum* nitrogenase (supplied by O. M. Aguilar) and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

M13 cloning and sequencing. DNA fragments to be sequenced were cloned into the M13 vectors M13 mp18 or M13 mp19 and transfected into strain JM109. Dideoxy sequencing was performed as described previously (25). The sequences were analyzed with sequencing programs (DNASTAR, Inc., Madison, Wis.).

RESULTS

Isolation of cosmid clones containing *nif* genes. A cosmid gene bank of *B. japonicum* I110 was screened by colony hybridization with the *nifK*, *nifD*, and *nifH* genes (in plasmids pRH231, pRM2, and pRH232D, respectively) that were previously isolated in our laboratory from *Bradyrhizobium* sp. (*Vigna*) IRc78. One cosmid, pJN110-22, was isolated which contained a 39-kbp insert that carried all three genes for nitrogenase. A physical map of pJN110-22 was determined with eight restriction endonucleases (Fig. 1). Restriction endonuclease analysis showed that the *nifD* and *nifK* genes were linked and separated from the *nifH* gene by 17.2 kbp. The integrity of this region was determined by comparing the hybridization patterns obtained when pJN110-22 and total DNA from strain I110 were digested with several enzymes and hybridized with ^{32}P-labeled pJN110-22. The hybridization patterns were identical (excluding the vector-insert junction fragments) between the total genomic and cloned DNA. This confirmed that the 39 kbp of strain I110 DNA in pJN110-22 was from a contiguous region of the chromosome. The direction of transcription of the *nifH* and *nifDK* operons was previously determined by Fischer and Hennecke (12).

Identification of *fix* genes by site-directed Tn5 mutagenesis. Tn5 mutagenesis in *E. coli* was used to generate 96 independently derived Tn5 insertions distributed throughout the 39 kbp of strain I110 DNA containing the *nif* structural genes (Fig. 1). Following the exchange by homologous recombination in *B. japonicum* I110 of a single Tn5-interrupted sequence for the corresponding wild-type sequence, each strain was inoculated onto *G. max*. The location of Tn5 in *Bradyrhizobium* cells isolated from nodules 40 days after inoculation was the same as that determined prior to inoculation (data not shown).

All 96 strains formed 47 to 145 nodules per plant on *G. max*; therefore, none of the insertions were in genes required for nodulation. Eight insertions resulting in essentially the complete loss of nitrogenase activity in nodules were within the nitrogenase genes (Fig. 1 and Table 2). The *nifK* and *nifD* genes were within a 4.1-kbp region bounded by insertions 13-8 and 13-53, which had no effect on nitrogen fixation. The *nifH* gene was within a 1.2-kbp region bounded by insertions 19-138 and 19-78, which also did not affect fixation. Twenty insertions distributed in three distinct regions located 9 kbp upstream of *nifDK* (*fix* region I), 1.5 kbp downstream of *nifDK* (*fix* region II), and 4.5 kbp upstream of *nifH* (*fix* region III) resulted in a complete or partial loss of nitrogenase activity. *fix* region II most likely is not part of the *nifDK* operon because Tn5 insertion 13-53, which has no effect on nitrogen fixation, is between the two regions. *Fix* region III spans 4 kbp of DNA. Because insertion 19-17 also had no effect on nitrogen fixation, this suggests that the *fix* genes within this region are not all part of the same operon. Except for insertions 19-52 and 19-59, strains with Tn5 insertions in
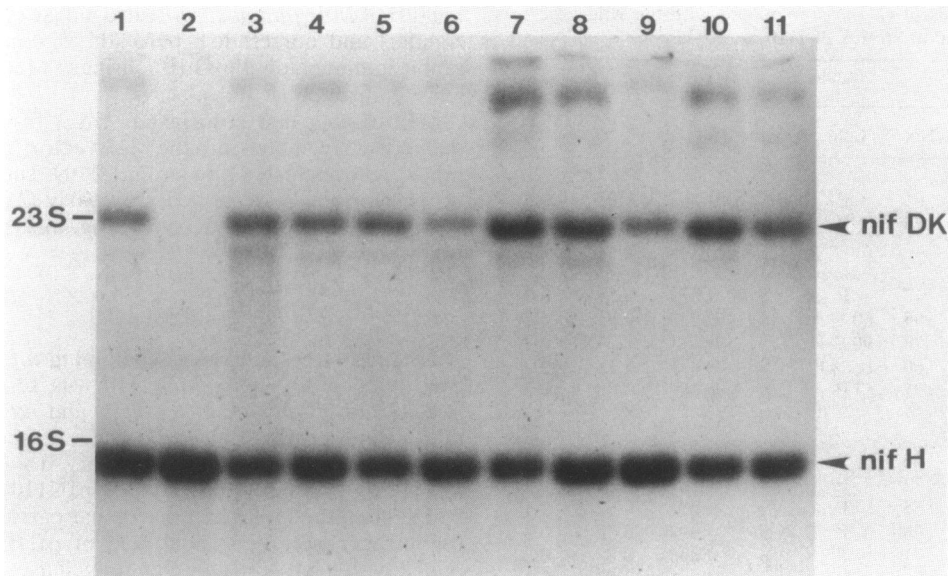
FIG. 2. Northern analysis of nodule RNA from *G. max* inoculated with wild-type and mutant strains of I110. Bacteroid RNA was isolated from nodules formed by wild-type strain I110 (lane 8) or by strains with Tn5 insertion: OF-2, lane 1; 13-25, lane 2; 13-28, lane 3; 13-35, lane 4; 19-129, lane 5; 19-109, lane 6; 19-56, lane 7; 19-52, lane 9; 19-46, lane 10; 19-74, lane 11. The filter was hybridized with ^{32}P-labeled 1.35-kbp *Eco*RI-*Sma*I (*nifDK*) and 1.40-kbp *Sma*I (*nifH*) fragments from pJN110-22. The high-molecular-weight bands above the position of 23S RNA resulted from hybridization of the probes to residual DNA in the RNA preparations.

fix region III formed nodules that expressed 3 to 9% of the nitrogenase activity found in nodules formed by the wild-type strain. This low level of nitrogenase activity was insufficient to support normal plant growth, as *G. max* inoculated with these *fix*::Tn5 insertion strains were similar in weight to the uninoculated plants (Table 2).

Six *fix*::Tn5 insertion strains formed white and pink nodules on the same plant. Bacteria isolated from both types of nodules still retained Tn5 in the same location. Additionally, *Bradyrhizobium* cells isolated from either white or pink nodules were able to induce both nodule types on the same plant. Five of the six Tn5 insertions were within the *nifDK* and *nifH* operons. The significance of these observations, however, remains unclear.

One Tn5 insertion (insertion 19-41) 4 kbp downstream of *nifDK* resulted in a plant shoot dry weight of 50% and nitrogenase activity of 33% of those obtained with the wild-type strain.

Effect of Tn5 insertions on the transcription of the *nif* genes. The Fix$^-$ phenotype of *B. japonicum* strains carrying Tn5 insertions outside of the structural genes for nitrogenase was also correlated with either low or undetectable nitrogenase activity in the nodules formed by these strains (Table 2). The effect of these insertions on the levels of the *nifDK* and *nifH* transcripts was determined by Northern blot analysis of bacteroid RNA isolated from these nodules. Only nodules clustered around the primary root (early induced nodules) were used. Approximately equal amounts of RNA (10 μg) from nodules formed by either the wild-type strain or by the *fix*::Tn5 insertion strains were electrophoresed through an agarose gel and transferred to Gene Screen Plus. A 1.35-kbp *Eco*RI-*Sma*I fragment containing *nifD* and *nifK* gene sequences and a 1.40-kbp *Sma*I fragment containing *nifH* were isolated from pJN110-22 (Fig. 1) and used as probes. As expected with a Tn5 insertion in the promoter-proximal portion of an operon, insertion 13-25 (Fig. 2, lane 2) resulted in the absence of any detectable transcript from the *nifDK*

operon. However, none of the Tn5 insertions outside of the nitrogenase structural genes prevented the transcription of the *nifDK* and *nifH* operons, although there was variability in the amounts of these transcripts relative to those found in nodules formed by the wild-type strain. This variability was most evident in the levels of the *nifDK* transcripts in nodules formed by strains with insertions 19-109, 19-52, and 19-74 (Fig. 2, lanes 6, 9, and 11).

Effect of Tn5 insertions on the translation of the *nif* genes. To determine if the Fix$^-$ phenotypes of the strains shown in Table 2 were caused by a failure of the *nifDK* and *nifH* transcripts to be translated, bacteroid protein preparations were assayed for the presence of the nitrogenase subunits. The individual subunits of nitrogenase were detected in preparations of bacteroid proteins after incubation with antisera made against component I (the products of *nifD* and *nifK*) and component II (the product of *nifH*) from *R. leguminosarum*. The bands corresponding to each component in *B. japonicum* were identified on the basis of their molecular weights. As a control, *R. leguminosarum* bacteroid proteins were also incubated with antiserum (Fig. 3, lane 25). The strain with a Tn5 insertion in the *nifDK* operon (insertion 13-34) produced only component II (Fig. 3, lane 12). Neither component I nor component II was detected in total protein isolated from wild-type strain I110 grown aerobically (Fig. 3, lane 11). The results of this experiment (Fig. 3) show not only that the three polypeptides of nitrogenase are synthesized but also that the levels of components I and II in the Fix$^-$ strains were approximately the same as those found in the wild-type strain. There were also few differences in the individual component levels detected in nodules either 32 or 40 days after inoculation. Although the band corresponding to the *nifK* gene product of *B. japonicum* (Fig. 3, the upper band of the two bands identified as component I) appeared to be more intense on the RNA blots, no conclusions could be made concerning the amount of the *nifK* and *nifD* gene products relative to each other. This is,
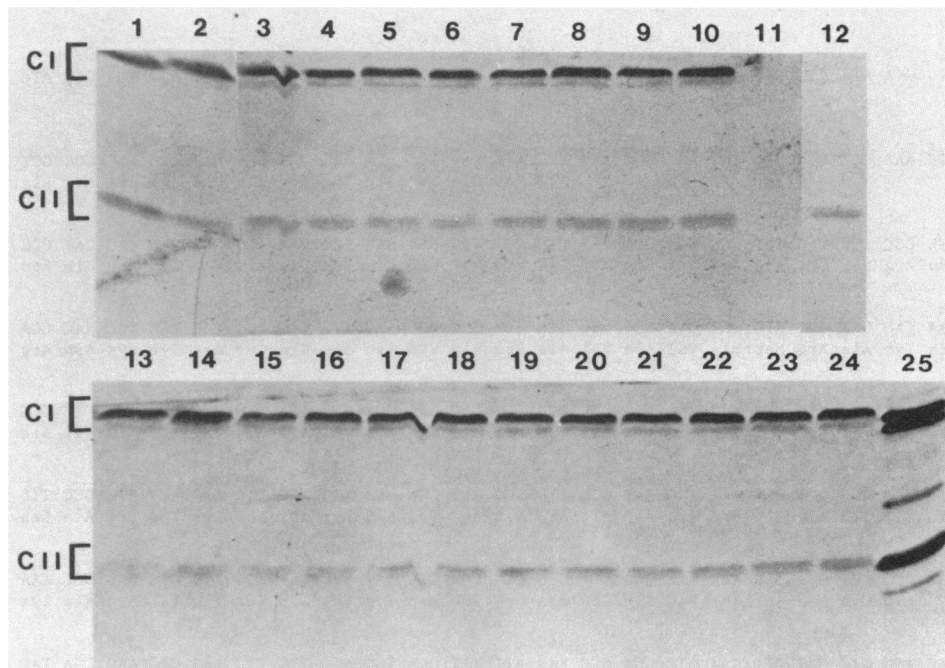
FIG. 3. Immunological detection of the nitrogenase subunits in bacteroid proteins. The lanes contain bacteroid protein isolated from nodules 32 and 40 days after inoculation from nodules formed by wild-type strain I110, lanes 23 and 24, respectively; or by strains with Tn5 insertion OF-2, lanes 1 and 2; 13-28, lanes 3 and 4; 13-35, lanes 5 and 6; 19-123, lanes 7 and 8; 19-129, lanes 9 and 10; 19-109, lanes 13 and 14, 19–56, lanes 15 and 16; 19-52; lanes 17 and 18; 19-46, lanes 19 and 20; 19-74, lanes 21 and 22. Lane 25, R. leguminosarum bacteroid protein; lane 11, protein from strain I110 grown aerobically; lane 12, bacteroid protein from nodules at 32 days formed by strain with Tn5 insertion 13-34. Each lane contains 60 µg of protein that was reacted with antisera against components I and II of R. leguminosarum.

in part, because the antiserum against component I is a mixture of antibodies against both the nifD and nifK gene products, and the titers for each have not been determined.

Sequence analysis. To more completely characterize fix region III, DNA sequence analysis was performed. The 4-kbp region between insertions 19-99 and 19-132 was sequenced and shown to contain the nifB gene (Fig. 1) by comparative sequence analysis with the R. meliloti and K. peumoniae nifB sequences (5; F. M. Ausubel and W. J. Buikema, personal communication). The GAP program of the DNASTAR system was used to determine the best alignments to these sequences. A single unambiguous ORF of 1,494 nucleotides preceded by a putative promoter (5' GTGG-10 base pairs [bp]-TTGCA 3') at position −65 that correlates well with the nif-promoter consensus sequence (5' ^C_GTGG-8 bp-TTGC^A_T 3') was found (Fig. 4). Additionally, the upstream activator sequence (5' TGT-4 bp-T-5 bp-ACA 3') (9) was found at position −213 (data not shown). The first ATG of the ORF was chosen as the translational initiation codon because it was immediately preceded by a putative Shine-Dalgarno sequence. On the basis of the positions of the putative promoter sequence and the ORF, the direction of transcription of nifB is the same as that of the nifDK and nifH operons.

The DNA sequence of nifB of R. japonicum was 54 and 47% homologous, respectively, to the nifB genes of R. meliloti and K. pneumoniae. Similarly, 51 and 45% of the amino acids of the R. meliloti and K. pneumoniae genes, respectively, were conserved. Furthermore, those cysteine residues that were conserved among the nifB genes of R. meliloti, K. pneumoniae, and R. leguminosarum were also conserved in B. japonicum. The transcribed but untranslated

leader regions among the nifD genes and among the nifH genes of several slow-growing species are highly conserved (1, 47). However, the sequence between the putative promoter and the first ATG of B. japonicum showed no significant homology to either of these leader sequences.

In addition to the ORF corresponding to the nifB gene, two other ORFs were found within fix region III (J. D. Noti, O. Folkerts, A. N. Turken, A. A. Szalay, manuscript in preparation). The nifB coding sequence is immediately followed by an ORF of 831 nucleotides. The nifB gene and this ORF were separated by an AG-rich region of seven nucleotides (Fig. 4) which could serve as a possible ribosome-binding site. This finding suggests that nifB and the small ORF are part of the same transcriptional unit. In K. pneumoniae the nifQ gene lies downstream of nifB and is part of the same operon (5, 24; F. M. Ausubel and W. J. Buikema, personal communication). A comparison of this ORF with nifQ, however, did not reveal any significant sequence homology.

The second ORF was upstream of the nifB gene and began between Tn5 insertions 19-99 (Fix⁺) and 19-129 (Fix⁻) and ended between insertions 19-56 (Fix⁻) and 19-17 (Fix⁺) (data not shown). The ORF was 1,356 nucleotides long and was preceded at position −53 (upstream from the putative initiation codon) by a putative promoter sequence (5' CTGG-9 bp-TTGCT 3'). A comparison of this ORF with the nifA genes of K. pneumoniae and R. meliloti did not reveal any significant sequence homology.

DISCUSSION

We cloned a 39-kbp region of DNA from B. japonicum I110 that contains all three structural genes for nitrogenase.

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        -120                              -90                              -60
ACG GCA TGC AAG TTG CTA ATC TTC CTG AAG CGC GCT CTA GGA TGA TCT GTG GGC GTC GAT GCT TGC AGG GGA GTG ATC TCG GTC GTG GAG


        -30                              1                               30
CGC GGA AAA TAT ATC AAA GCA GCG GTC AAT AGC GGG AAG ATC ATG CAG TCC ATA ACC GAG CAT AAG GGC TGC CGC GCT TCG GCG AAG ACC
                                                     Met Gln Ser Ilu Thr Glu His Lys Gly Cys Arg Ala Ser Ala Lys Thr


        60                               90                              120
GGG CGG GCG AGG CTG CGC TCG CAG GCC GGC CGA GGC GAT CTG CCG GTC GAA ATC TGG GAA AGG GTG AAA AAC CAT CCC TGT TAC AGC GAG
Gly Arg Ala Arg Leu Arg Ser Glu Ala Gly Arg Gly Asp Leu Pro Val Glu Ilu Trp Glu Arg Val Lys Asn His Pro Cys Tyr Ser Glu


        150                              180                             210
GAT GCG CAC CAT CAT TAC GCT CGC ATG CAT GTC GCG GTC GCA CCT GCC TGC AAT ATC CAG TGC AAC TAC TGC AAC CGA AAA TAC GAC TGC
Asp Ala His His His Tyr Ala Arg Met His Val Ala Val Ala Pro Ala Cys Asn Ilu Gln Cys Asn Tyr Cys Asn Arg Lys Tyr Asp Cys


        240                              270                             300
GCC AAT GAA TCG CGT CCG GGT GTG GTG AGC GAG AAG CTC ACC CCT GAG CAG GCA GTG AGA AAA GTG ATC GCG GTC GCG ACG ACC ATT CCG
Ala Asn Glu Ser Arg Pro Gln Val Val Ser Glu Lys Leu Thr Pro Glu Gln Ala Val Arg Lys Val Ilu Ala Val Ala Thr Thr Ilu Pro


        330                              360                             390
CAG ATG ACG GTA CTT GGC ATC GCT GGT CCC GCG GAT GCC CTG GCC AAT CCA GCA AAG ACG TTC AAA ACG CTC GCG TTG GTC ACC GAG GCT
Gln Met Thr Val Leu Gly Ilu Ala Gly Pro Ala Asp Ala Leu Ala Asn Pro Ala Lys Thr Phe Lys Thr Leu Ala Leu Val Thr Glu Ala


        420                              450                             480
GCT CCT GAC ATC AAG CTG TGT CTG TCA ACC AAC GGA CTA GCG CTG CCA GAC TAT GTC GAT ACC ATC GTG AGG GCC AAA GTT GAC CAC GTC
Ala Pro Asp Ilu Lys Leu Cys Leu Ser Thr Asn Gly Leu Ala Leu Pro Asp Tyr Val Asp Thr Ilu Val Arg Ala Lys Val Asp His Val


        510                              540                             570
ACC ATC ACC ATC AAC ATG GTC GAT CCT GAA ATC GGA GCC AAG ATT TAT CCA TGG ATC TTC TTC AAC CAC AAG CGA TAC ACC GGC ATC GAG
Thr Ilu Thr Ilu Asn Met Val Asp Pro Glu Ilu Gly Ala Lys Ilu Tyr Pro Trp Ilu Phe Phe Asn His Lys Arg Tyr Thr Gly Ilu Glu


        600                              630                             660
GCG GCA AGG ATA CTC ACC AAT CGC CAG CTT CAA GGG CTC GAG ATG CTT AGC GAA CGG GGC ATT TTG TGC AAG ATC AAC TCG GTG ATG ATC
Ala Ala Arg Ilu Leu Thr Asn Arg Gln Leu Gln Gly Leu Glu Met Leu Ser Glu Arg Gly Ilu Leu Cys Lys Ilu Asn Ser Val Met Ilu


        690                              720                             750
CCC AAT ATC AAT GAT GAC CAC CTG GTC GAG GTC AAC AAG GCG GTC ACG TCG CGC GGT GCC TTC CTT CAC AAT ATC ATG CCG CTG ATC TCC
Pro Asn Ilu Asn Asp Asp His Leu Val Glu Val Asn Lys Ala Val Thr Ser Arg Gly Ala Phe Leu His Asn Ilu Met Pro Leu Ilu Ser


        780                              810                             840
GTA CCC GAG CAC GGA ACA GCA TTT GGC CTC AAC GGT CAG CGC GGT CCG ACG GCT CAA GAA TTG AAG ACG CTG CAA GAT GCT TGC GAA GGG
Val Pro Glu His Gly Thr Ala Phe Gly Leu Asn Gly Gln Arg Gly Pro Thr Ala Gln Glu Leu Lys Thr Leu Gln Asp Ala Lys Glu Gly


        870                              900                             930
AAG ATA AAC ATG ATG CGG CAT TGC GGC AGT GCC GCT GAT GCG GTC GGT CTA CTC GGC GAG GAT CGC AGC GCG GAG TTC ACC AAT GAT CAG
Lys Ilu Asn Met Met Arg His Cys Gly Ser Ala Ala Asp Ala Val Gly Leu Leu Gly Glu Asp Arg Ser Ala Glu Phe Thr Asn Asp Glu


        960                              990                             1020
GTG ATG AAA ATG GAC GTC CAT TAT GAC CTA GAG ATG CGC AAG GCT TAT CAA AAA AGG GTA GAG AAT GAG CGC GTC TCC AAA GTC GCG GCC
Val Met Lys Met Asp Val His Tyr Asp Leu Glu Met Arg Lys Ala Tyr Gln Lys Arg Val Glu Asn Glu Arg Val Ser Lys Val Ala Ala


        1050                             1080                            1110
GGT CAA AAG GAG TTG GCG GGA GTC TCC GGA GAG ATG AGC GCG ATC ACT GTT CTA GTA GCC GTC GCA ACT AAG GGC TCG GGA TTG ATC AAC
Gly Gln Lys Glu Leu Ala Gly Val Ser Gly Glu Met Ser Ala Ilu Thr Val Leu Val Ala Val Ala Thr Lys Gly Ser Gly Leu Ilu Asn


        1140                             1170                            1200
GAG CAC TTT GGA CAT GCA AAG GAA TTC CAA CTG TAC GAG CTC TCC ACA TCC GGC GCC AAG TTC GTC GGG CTA CGT CGT GTG GAG GGT TAC
Gln His Phe Gly His Ala Lys Glu Phe Gln Leu Tyr Gln Leu Ser Thr Ser Gly Ala Lys Phe Val Gly Leu Arg Arg Val Glu Gly Tyr


        1230                             1260                            1290
TGC CAG GCC GGC TAT GGC GAG GAA GAT AGG CTA TCC GTG ATC ATG CGC GAC ATC CGT GAC TGC CAC GCG GTC TTC GTG GCT AAG ATC GGC
Cys Gln Ala Gly Tyr Gly Glu Gln Asp Arg Leu Ser Val Ilu Met Arg Asp Ilu Arg Asp Cys His Ala Val Phe Val Ala Lys Ilu Gly


        1320                             1350                            1380
GGC TGC CCC AAG AGC GGC CTG ATC AAG GCT GGG ATC GAG CCG GTC GAT CAA TTC GCC TAT GAG TAC ATT GAG AAG TCG ACG ATC GCT TGG
Gly Cys Pro Lys Ser Gly Leu Ilu Lys Ala Gly Ilu Glu Pro Val Asp Gln Phe Ala Tyr Glu Tyr Ilu Glu Lys Ser Thr Ilu Ala Trp


        1410                             1440                            1470
TTC AGG GCC TAT GTC GGC AAA GTA AAG CGC GGG GAG ATC CAG CAT GTG CAG CGC GGC GTG CCC CCG CGT TGG CCT GGA GAT CGG ATC TCT
Phe Arg Ala Tyr Val Gly Lys Val Lys Arg Gly Gln Ilu Gln His Val Gln Arg Gly Val Pro Pro Arg Trp Pro Gly Asp Arg Ilu Ser


        1500                             1530                            1560
GCG GCG TAA GGA GGA ATA TGT GCC ATT CAA AAT CAT CGC CTC GCA GTG CAC GAG CTG CTC AGC TTG CGA GCC TTT ATG CCC GAA CGT TGC
Ala Ala
```

FIG. 4. Nucleotide sequence of the *nifB* gene of *B. japonicum* I110. The first nucleotide of the putative translation initiation codon for *nifB* is numbered 1, and the coding sequence ends at position 1494 with the termination codon (TAA). A putative initiation codon for the downstream ORF begins at position 1506.

These genes were previously shown to be separated into two operons, nifDK and nifH (2, 12, 28). By Northern blot analysis of bacteroid RNA we found that the nifDK and nifH transcripts are approximately 3,000 and 900 nucleotides, respectively (Fig. 2). These experimentally determined lengths correlated well with those that could be predicted from the lengths of the coding sequences of the nifD, nifK, and nifH genes (1,545, 1,554, and 882 nucleotides, respectively [15, 23, 42]) and their established transcriptional start sites. Thus it appears that only these three genes comprise the two operons.

A total of 96 Tn5 insertions distributed throughout this 39-kbp region defined the boundaries of these two operons and revealed three additional regions, referred to as fix regions I, II, and III, that are essential for nitrogen fixation. From results of preliminary studies in our laboratory, the nifE gene of K. pneumoniae (33) was found to hybridize to fix region II. Because the nifE gene is required for the synthesis of an active iron-molybdenum cofactor for the nitrogenase enzyme, it may be that this B. japonicum gene is involved in the cofactor synthesis. DNA sequence analysis, currently in progress, should help elucidate the function of this region.

The findings that the nif genes in K. pneumoniae and R. meliloti are positively regulated by the nifA gene and that the nif-promoter sequences of the nitrogenase genes in slow growers (1, 47) are similar to those of the fast growers suggest that these organisms may have a similar regulatory mechanism. Of particular interest was fix region III, which corresponds to the region between the two operons nifDK and nifH that was previously reported to show homology to K. pneumoniae nifA (2). Interestingly, we detected weak homology of the internal portion of the R. meliloti nifA gene to this region. We were, therefore, encouraged to determine whether the expression of either the nifDK or nifH operon was influenced by this region or by the other essential regions identified in this study. In preliminary experiments we found that Tn5 insertions within fix region III resulted in reduced levels of the nifH transcripts in bacteroid RNA preparations from nodules formed by strains carrying these insertions (28). Because of the difficulties encountered in isolating undegraded bacteroid RNA, however, we reexamined our original method of isolation. An alternate protocol was adopted that allowed bacteroids first to be isolated essentially free of contaminating plant nucleases on a Percoll gradient. By this procedure we found that the levels of the nifDK and nifH transcripts in nodules formed by the strains carrying Tn5 insertions were similar to those found in nodules formed by the wild-type strain I110 (Fig. 2). Furthermore, immunological detection of components I and II of nitrogenase in the protein fraction of these bacteroids showed that translation of these transcripts is unaffected. These findings suggest that the putative regulatory gene must be located in a region not included in this study.

DNA sequence analysis of these three regions was performed to further characterize these essential fix genes. One of these regions, fix region III, was completely sequenced. Within this region were located three consecutive ORFs, one of which was found to be homologous to the nifB genes of R. meliloti and K. pneumoniae. The availability of long stretches of flanking sequences allowed for a best-fit alignment with the nifB sequences of R. meliloti and K. pneumoniae. The location of the ORF corresponding to nifB was in agreement with the positions of the Tn5 insertions in this region that resulted in a Fix⁻ phenotype (Fig. 1 and Table 2). It is also consistent with the findings of Fuhrmann et al. (14).

They concluded that a putative nifB gene is present within this same region after they compared the sequence of the nifB gene of R. leguminosarum with a 150-bp DNA sequence of a region of DNA from B. japonicum. The complete length of the B. japonicum nifB ORF (1,494 nucleotides) is comparable to the length of the nifB ORF of R. meliloti (1,470 nucleotides) (5; F. M. Ausubel and W. J. Buikema, personal communication).

In K. pneumoniae a small ORF of 500 bp was found within the region proposed to contain nifQ (5; F. M. Ausubel and W. J. Buikema, personal communication). The putative ribosome-binding site and the translational initiation codon of nifQ overlap the 3' end and stop codon, respectively, of nifB. With the finding of an ORF immediately downstream of nifB, the possibility was raised that nifQ may also follow nifB in B. japonicum. However, as was also shown by Ausubel and Buikema (personal communication) in the region downstream of nifB in R. meliloti, no significant homology to K. pneumoniae nifQ was found. The insertion of Tn5 within this small ORF would help to determine whether another fix gene is indeed present. The location of Tn5 insertion 19-74 has not been unambiguously established; the best approximation is that it is either within the putative 5' portion of this small ORF or within the last 100 nucleotides of the nifB gene. The exact location of this insertion is currently being determined by DNA sequence analysis.

The presence of a large ORF preceded by a nif-promoter consensus sequence shows that another fix gene lies upstream of nifB. The orientation of the nif-promoter sequence and the ORF with respect to nifB shows that this gene is transcribed in the same direction as nifB. This gene is not part of the nifB operon, as evidenced from the presence of a nif-promoter consensus sequence that precedes the nifB ORF and also by the finding that a Tn5 insertion (19-17) between these genes does not affect nitrogen fixation. DNA sequence analysis showed that no other nif-promoter sequence lies within the 620 nucleotides that separate these two ORFs (data not shown). The function of this gene is unknown, but apparently it does not affect the synthesis of components I and II of nitrogenase (Fig. 3). It is interesting that none of the Tn5 insertions within this gene resulted in the complete absence of nitrogen fixation or acetylene reduction activity (Table 2), a phenotype that is also found with nifF, nifM, and nifS mutants of K. pneumoniae (18, 33). Interspecies hybridization experiments and in vitro assays with purified cofactor and nitrogenase components are now in progress to elucidate the role of this gene.

Although we have not associated a Fix⁻ phenotype with the region downstream of nifH, this region was not saturated with Tn5 insertions (Fig. 1). It is very probable, however, that this region contains the fixB and fixC genes, as suggested by Fuhrmann et al. (14).

Because of the difficulties experienced in isolating intact transcripts of the nifDK and nifH operons, a more precise method of monitoring the regulation of transcription of these promoters was desired. To this end, nif-promoter-lacZ fusions were constructed and transferred to the wild-type strains and to the strain carrying the Tn5 insertions used in this study, as described in the accompanying paper (46).

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