A *Bradyrhizobium japonicum* Gene Essential for Nodulation Competitiveness Is Differentially Regulated from Two Promoters

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We report the identification and nucleotide sequence of a new symbiotic gene (*nfeC*) from the soybean root nodule bacterium, *Bradyrhizobium japonicum*. A Tn*5* insertion (NAD14) in this gene did not affect nitrogen fixation but caused a significant delay in soybean nodulation. In addition, this mutant exhibited a reduction in its competitive ability to nodulate soybean when coinoculated with the wild type. Sequence analysis of the mutated region revealed that the NAD14 Tn*5* insertion mapped within an open reading frame of 825 bp. Primer extension using *B. japonicum* mRNA from three different growth conditions, aerobic, anaerobic, and bacteroids (i.e., symbiotic form) indicated that the upstream region of the gene contained two promoters, which were differentially regulated in response to the growth conditions. One promoter was expressed in bacteroids, but not under aerobic or anaerobic free-living conditions. The other promoter was expressed only under aerobic conditions.

Additional keywords: competition, gene expression.

Legume nodulation by *Rhizobium*, *Azorhizobium*, and *Bradyrhizobium* species is a complex process requiring the coordinated expression of both bacterial and plant genes. Molecular genetic studies have identified a large number of bacterial genes that contribute, directly or indirectly, to the formation of an effective symbiosis. For example, *nif* and *fix* genes are required for the biosynthesis of the enzymatic machinery for nitrogen fixation, as well as maintenance of the symbiotic state (David *et al.* 1988; Hennecke 1990). The *nod/nol* gene products are required for infection of the plant and formation of the nodule structure (Barbour *et al.* 1992; Long 1989).

Recently, a few bacterial genes involved in nodule competition have been reported (Murphy *et al.* 1987; Murphy *et al.* 1988; Sanjuan and Olivares 1989; Sanjuan and Olivares 1991; Soto *et al.* 1993; Triplett 1988). The ability of certain strains of *Rhizobium* and *Bradyrhizobium* to dominate nodulation in a multistrain environment has been termed competitiveness (for review, see Triplett and Sadowsky 1992). When effective nitrogen-fixing strains of root nodule bacteria are used as inoculants on legume seeds, the plants are often nodulated by inferior nitrogen-fixing strains. Therefore, interstrain competition can be a significant agronomic problem in that field inoculation with efficient rhizobial strains can fail to improve yield due to the inability of inoculant strains to compete against indigenous strains for nodule occupancy. Despite the importance of interstrain competition, the molecular mechanism of competition is poorly understood. In *Rhizobium meliloti* strain GR4, Sanjuan *et al.* (1989) identified a DNA region, named *nfe* (nodule formation efficiency), involved in nodulation efficiency and competitiveness on alfalfa roots. Mutation in this region caused a delay in nodule formation as well as a reduction in nodulation competitiveness. In addition, expression of the *nfe* genes was found to be dependent on the NifA-RpoN regulatory system. Bhagwat *et al.* (1991) also recently identified a DNA region of *Bradyrhizobium japonicum* strain USDA110 that, when mutated by Tn*5*, resulted in a reduction in nodulation efficiency and competition on soybean plants. However, the sequence and possible function of this region has not been reported.

Previously, a Tn*5*-induced delayed nodulation mutant NAD14 of *B. japonicum* was isolated in our laboratory and was found to be linked to the known nodulation gene cluster (i.e., *nodYABC*) (Deshmane 1988). In this report, we provide a molecular and phenotypic description of the mutated region. The gene identified by the NAD14 mutation appears to be important for the ability of *B. japonicum* to efficiently nodulate soybean. This gene has many features in common with *nfe* genes identified in *R. meliloti* (Sanjuan and Olivares 1989; Soto *et al.* 1993). It has been therefore designated *nfeC*, according to the convention of Sanjuan *et al.* (1989) and Soto *et al.* (1993). Most interesting is the fact that the gene has two promoters, one specifically expressed *in planta* (i.e., bacteroids), while the other provides aerobic expression.

RESULTS

Nodulation efficiency and competition phenotypes of *B. japonicum* mutant NAD14.

We had previously isolated and described a delayed nodulation mutant NAD14 of *B. japonicum* that was generated by site-directed Tn*5* insertion mutagenesis (Deshmane 1988). To identify other mutants mapping close to NAD14, we screened a battery of Tn*5*-insertion mutants that had previously been obtained by random transposon mutagenesis of the cosmid clone pRjUT14 (Russell *et al.* 1985). Tn*5* insertions were

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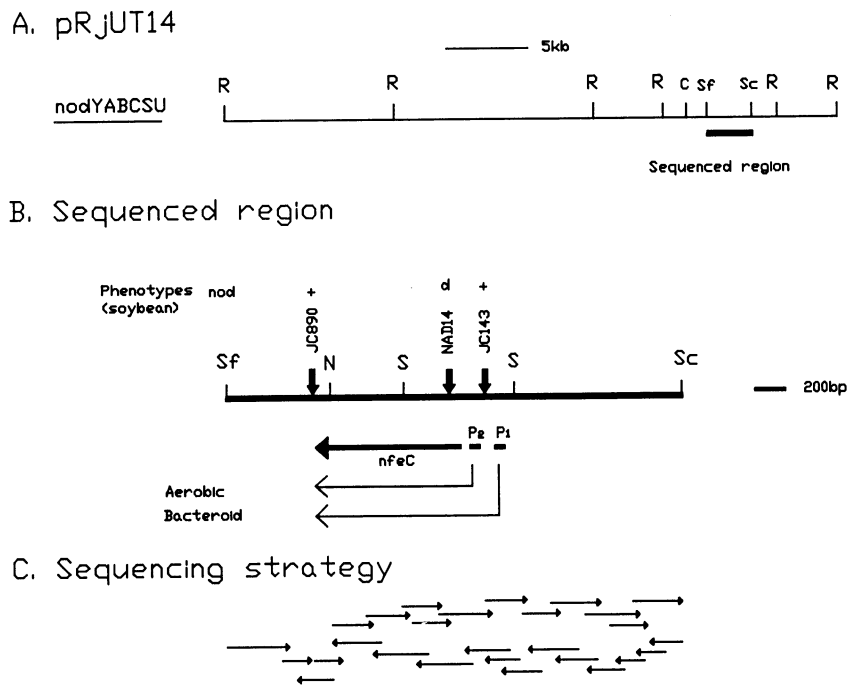

Fig. 1. Physical map of the *Bradyrhizobium japonicum nfeC* gene. **A,** The *nfeC* gene is located on the right about 43 kb from the *nodYABCSU* operon. **B,** The vertical arrows indicate the position of Tn*5* insertions based on sequencing data. The phenotypes of the Tn*5* inserts: +, Wild-type; d, delayed nodulation on soybean. The transcription of the *nfeC* gene is from right to left. Promoter regions: P1, bacteroid-specific RpoN-type; P2, aerobic-specific *Escherichia coli* consensus type. Restriction endonuclease sites: R, *Eco*RI; C, *Cla*I; Sf, *Sfu*I; Sc, *Sac*I; S, *Sal*I. **C,** The arrows indicate the sequencing strategy. The tail end of the arrow represents the end of a deletion, and the length of the arrow represents the sequence determined from that deletion.

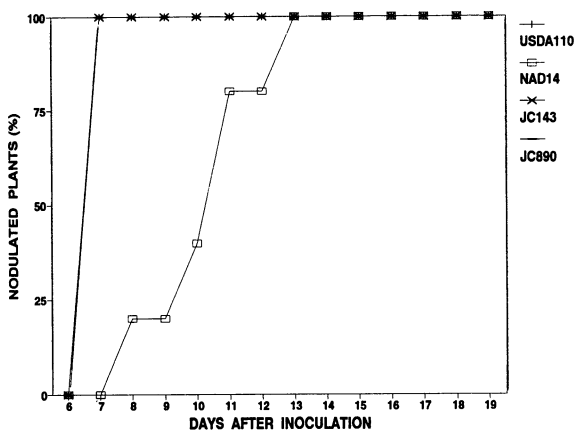

Fig. 2. Nodulation kinetics of wild-type *Bradyrhizobium japonicum* USDA110 and mutants NAD14, JC143, and JC890 on soybean *Glycine max* 'Essex.' The results are typical of two independent tests.

Table 1. Results of mixed-infection experiments with *B. japonicum* strains USDA110 and mutants

Ratio of strains within coinoculated mixture			Nodule occupied by the following strain[a] (%)			
			USDA110	NAD14	USDA110	JC143
A		B	A	B	A	B
USDA110			100	0	ND[b]	ND
		NAD14	0	100	ND	ND
1	:	1	88 ± 5	12 ± 5	49	51
1	:	10	53 ± 3	47 ± 3	4	96
1	:	100	11 ± 1	89 ± 1	0	100
10	:	1	100	0	ND	ND
50	:	1	100	0	ND	ND

[a] Thirty-two to fifty nodules were used for nodule occupancy determination. The results are typical of three replicate experiments.
[b] Not determined.

mapped by restriction endonuclease analysis and two were found to flank the site of Tn*5* insertion in NAD14 (Fig. 1). These Tn*5* insertions ND143 and ZB890 were marker exchanged into the *B. japonicum* genome, generating JC143 and JC890, respectively. Strains JC143 and JC890 mutants were tested for the ability to nodulate soybean (i.e., cv. Essex). Figure 2 shows the nodulation kinetics of mutants NAD14, JC143, and JC890. NAD14 exhibited a 6-day delay in nodule formation as compared with the wild-type strain. The average number and weight of nodules per plant induced by this mutant was similar to that of the wild type when the nodules were harvested at 19–20 days after inoculation. Acetylene reduction assays indicated that the mutants did not affect the level of nitrogen fixation (data not shown). Flanking insertions JC143 and JC890 were indistinguishable from wild type. Therefore, these two mutants delimit the symbiotic locus identified by the NAD14 mutation.

NAD14 was further characterized for its ability to compete for nodule formation against the wild-type strain. Coinoculation experiments showed that NAD14 was less competitive relative to wild-type strain USDA110 (Table 1). When the rate of the coinoculation was increased in the wild-type strain at a 10:1 or 50:1 ratio (USDA110/NAD14), 100% of nodules were occupied by the wild-type strain. However, mutant NAD14 occupied less than 12% of nodules when coinoculated with the wild type at a 1:1 ratio, whereas at a 1:10 or 1:100 ratio (USDA110/NAD14), the mutant formed 47 and 89% of the

nodules, respectively. These results indicate that the mutant NAD14 has approximately a 10-fold decrease in competitive ability when compared to USDA110. To test if this was a nonspecific effect caused by the insertion of Tn*5* in NAD14, a Tn*5*-induced mutant JC143 that has a wild-type nodulation phenotype was used as a control strain in competition experiments. JC143 retained its competitive ability under the same assay conditions, indicating that the reduction of competition observed with mutant NAD14 was not due to a nonspecific effect caused by the presence of the transposon. Bhagwat *et al.* (1991) reported that strains of *B. japonicum* with a delayed nodulation phenotype retained their competitive ability. In addition, the growth rate of NAD14 was similar to the wild-type strain USDA110 in rich (i.e., RDY) or minimal media (data not shown). Therefore, these data suggest that the DNA region mutated in strain NAD14 contains a gene involved in interstrain competition for nodulation.

Nucleotide sequencing and identification of *nfeC*.

To identify the gene mutated by the NAD14 insertion, a 2.2-kb DNA region from the wild-type *B. japonicum* strain USDA110 was sequenced. This region encompasses the location of the Tn*5* insertions NAD14, JC143, and JC890. The sequencing strategy is shown in Figure 1.

The resulting nucleotide sequence of 2,190 bp of *B. japonicum* DNA is given in Figure 3. A major open reading frame (ORF) was identified extending from nucleotide position 1254–2078. A putative ribosome binding sequence is present eight base pairs upstream of the most likely ATG start codon. This ORF was designated *nfeC* and encodes 275 amino acids with a deduced molecular weight of 31,352. Comparison of the DNA sequence of *nfeC* with sequences in the GenBank and EMBL databases showed no significant similarities.

The exact position of the Tn*5* insertions in mutants NAD14, JC143, and JC890 was determined by sequencing *Bam*HI fragments of pND63, pND143, and pZB890, respectively, which contain the kanamycin resistance gene of Tn*5* plus the flanking region of *B. japonicum* DNA. The insertion point for transposon NAD14 lies within the *nfeC* coding sequence. By contrast, strains JC143 and JC890, which display no altered symbiotic phenotype, have the Tn*5* inserted 152 bp upstream from the start codon of *nfeC* and 4 bp downstream from the end of *nfeC*, respectively. Thus, as indicated by restriction mapping, these mutations bracket the NAD14 insertion and identify *nfeC* as important to soybean nodulation.

Transcriptional regulation of *B. japonicum nfeC* gene.

To determine the transcription start sites of the *nfeC* gene, we isolated RNA from a wild-type *B. japonicum* strain USDA110 grown under free-living aerobic (Fig. 4, lane 1) and anaerobic (lane 2) conditions or from bacteroids isolated from soybean root nodules (lane 3). Two synthetic oligonucleotide primers complementary to the coding region of *nfeC* (see Materials and Methods) were used to carry out both the primer extension reaction with RNA, and a DNA sequencing reaction using plasmid pJY105 as template (for *nfeC*). These experiments revealed two major extension products beginning at positions 184 (t_1) and 40 (t_2) bp upstream of the putative translational start codon. The mapped transcription start site (t1) coincided with the presence of a putative –24/–12 promoter consensus sequence (i.e., RpoN/NtrA binding site) (Morett and Buck 1989; Thöny and Hennecke 1989). The conserved GC doublet of this sequence lies 13 bp upstream of the transcriptional start site. The transcript, t_1, was observed only when using bacteroid RNA as template, suggesting that the t_1 promoter requires a bacteroid-specific factor(s) for its expression. By contrast, t_2 was obtained only when the RNA was from aerobically grown *B. japonicum* cells. Several reasons support the assertion that the transcript, t_2, is not an artifact caused by nonspecific extension, hairpin secondary structure, or the degradation of the primary transcript. First, there are no obvious regions of secondary structure between t_1 and the putative start codon. Second, if the extension product was an artifact, the product should be observed with RNA from all three growth conditions. Third, two different primers produced the same extension products in the mRNA from the aerobic growth conditions and bacteroids. In addition, the DNA region upstream of the t2 start has significant similarity to the consensus for constitutively expressed *E. coli* promoters (Harley and Reynolds 1987; Hawley and McClure 1983). The transcription start sites are indicated in the DNA sequence shown in Figure 3.

The bacteroid-specific expression from t_1 is intriguing in light of the presence of a putative RpoN-binding sequence. All other such *B. japonicum* promoters are expressed both symbiotically and under anaerobic conditions (Hennecke *et al.* 1988; Thöny *et al.* 1989). Therefore, to confirm that the growth conditions used were indeed anaerobic, we hybridized a 1.4-kb *Eco*RI fragment from pJY400, carrying parts of *nifD* and *nifK*, to the mRNA used in the primer extension experiments. Positive hybridization was found to mRNA isolated from anaerobically grown cells and bacteroids, but not from cells grown aerobically (data not shown).

As shown in Figure 3, the Tn*5* position of JC143 lies between the t_1 and t_2 start sites, but JC143 has a wild-type nodulation phenotype. This is likely due to the documented nonpolarity of Tn*5* caused by the presence of an outward reading promoter within Tn*5* (Corbin *et al.* 1983; Fisher *et al.* 1987; Horvath *et al.* 1986; Mulligan and Long 1985). As evidence, slot blot hybridization using the internal fragment (0.4-kb *SalI*/*Nru*I) of *nfeC* as a probe showed that this fragment hybridized to the *B. japonicum* mRNA from JC143 but not from NAD14 (data not shown).

DISCUSSION

This paper reports the identification and nucleotide sequence of a new symbiotic gene from *B. japonicum*. The gene appears to be involved in nodule formation efficiency. A mutation in this gene does not affect nitrogen fixation, but causes a delay in nodule formation. More importantly, the mutant is significantly reduced in its competitive ability for nodulation when compared to the wild type.

Other mutants in nodulation efficiency and competition ability have been reported from *R. meliloti* (i.e., *nfe1*, *nfe2*) (Sanjuan and Olivares 1989), *R. fredii* (McLoughlin *et al.* 1987), and *B. japonicum* (Bhagwat *et al.* 1991). Nucleotide sequence information is available for the *nfe1* and *nfe2* genes from *R. meliloti* (Sanjuan and Olivares 1989; Soto *et al.* 1993). The coding sequence of both of these genes is preceded by a RpoN-type promoter. Similar to *nfeC*, the *nfe1* gene is expressed from a different promoter as well as the

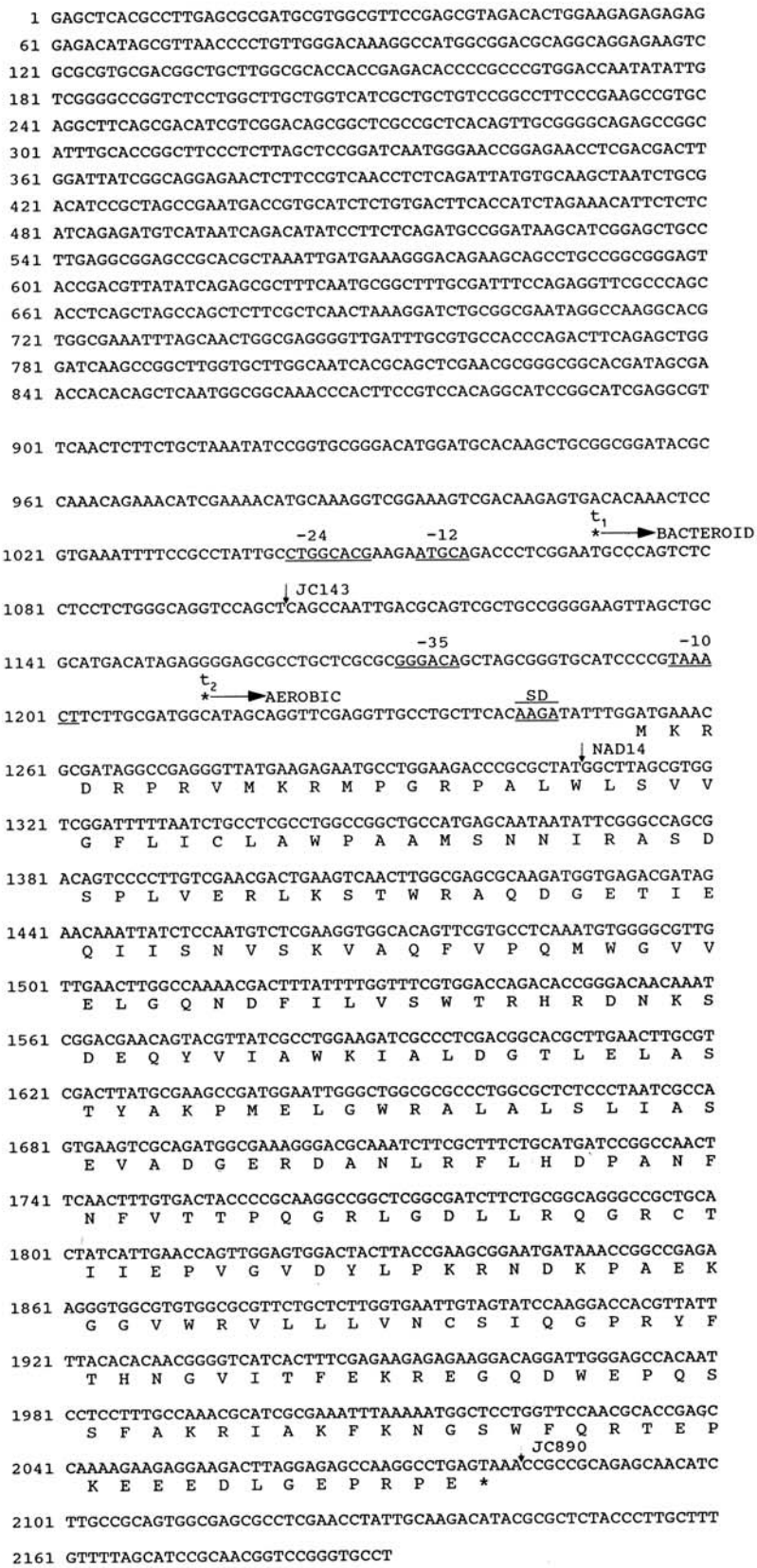

Fig. 3. Nuceotide sequence of the *nfeC* gene with predicted amino acid sequence. The presumptive ribosome-binding site (SD) is indicated and the sites of the Tn*5* insertion in the mutants, NAD14, JC143, and JC890 are marked by arrows. The two transcriptional start sites are designated by asterisks. The RpoN consensus promoter upstream of t_1 (–12/–24) and similarity to the *Escherichia coli* consensus promoter upstream of t_2 (–10/–35) are underlined.

RpoN-type promoter. Expression of the *nfe* genes (*nfe1* and *nfe2*) was found to be activated in microaerobically grown free-living cells and in alfalfa nodules, but not in aerobically grown cells.

Transcription of *nfeC* is initiated from two closely spaced but independently regulated promoters. One promoter, P1 (corresponding to the t_1 start), is expressed only in bacteroids. The other promoter, P2 (corresponding to the t_2 start), is expressed only under aerobic conditions. The P1 promoter is preceded by a RpoN-regulated promoter consensus sequence (Thöny and Hennecke 1989). In contrast, the sequence upstream of the P2 promoter has similarity to an *E. coli* consensus promoter (Harley and Reynolds 1987; Hawley and McClure 1983). Therefore, the regulation of the *nfeC* gene is likely different from that of the *nfe1* and *nfe2* genes identified in *R. meliloti*. Indeed, the *nfeC* gene of *B. japonicum* and the *nfe* genes of *R. meliloti* may have unrelated functions. However, *nfeC* has many features in common with the *nfe1* and *nfe2* genes with respect to mutant phenotypes and promoters of the genes; hence, our designation of this gene as *nfeC*. *B. japonicum* has two functionally interchangeable *rpoN* genes (Kullik *et al.* 1991). The $rpoN_{1/2}$ double mutant (N50-97) induced nodules, but these contained fewer bacteroids and lacked nitrogen fixation activity. Since the *nfeC* promoter (P1) is active only in bacteroids and the $rpoN_{1/2}$ mutant is defective in bacteroid formation, it is difficult to prove directly whether the bacteroid-specific promoter (P1) of *nfeC* is regulated by RpoN.

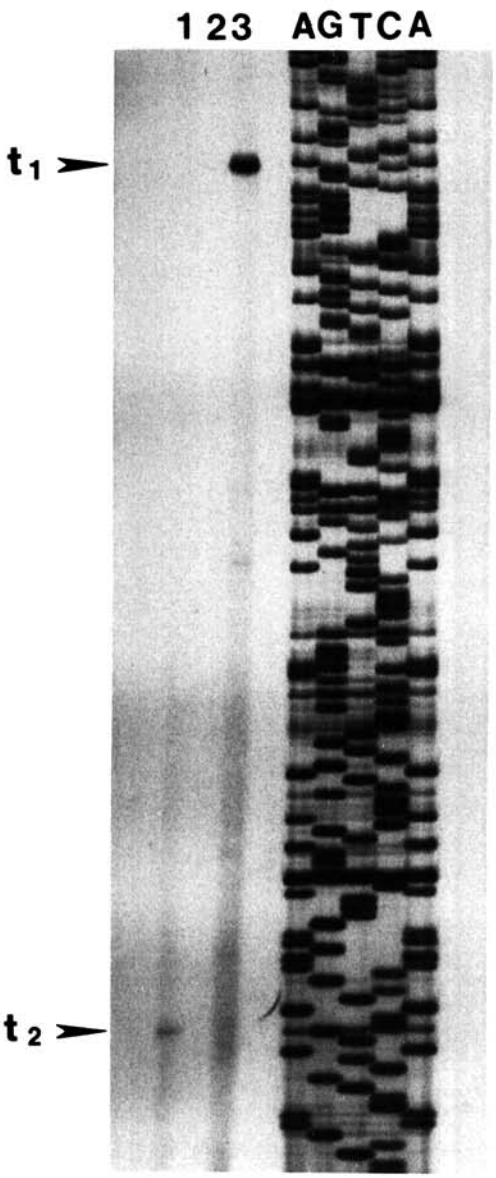

Fig. 4. Determination of the transcriptional initiation sites of the *nfe* transcripts. Primer extension with 20 μg of total *Bradyrhizobium japonicum* RNA extracted from three different growth conditions; lane 1, free-living aerobic; lane 2, free-living anaerobic; lane 3, bacteroids (bacteria isolated from soybean nodules) compared to a DNA ladder. Transcripts t_1 and t_2 are indicated.

It should be noted that the existence of two promoters in highly regulated prokaryotic operons is not uncommon. For example, the tandem promoters for the *glnB* gene from *B. japonicum* (Martin *et al.* 1989) and the *glnA* gene from *K. pneumoniae* (Dixon 1984) are differentially regulated. In both cases one promoter resembles a consensus nitrogen fixation gene promoter while the other promoter is *E. coli*-like. The *nfeC* tandem promoters resembled those of the *glnB* and *glnA* genes in that they all contain a putative RpoN-type promoter and an *E. coli* consensus promoter.

A common feature of RpoN-dependent promoters is that they are usually activated by binding of an additional regulatory protein upstream from the promoter (Buck *et al.* 1986; Ames and Nikaido 1985; Gussin *et al.* 1986; Ames and Nikaido 1985; Inouye *et al.* 1987; Johnson *et al.* 1986). The RpoN-type promoter of *nfeC* gene is expressed only in bacteroids but not under free-living conditions. These results suggest the presence of an upstream activator responsible for bacteroid-specific expression. When the free-living bacteria undergo conversion to bacteroids, massive changes in the cellular protein composition take place (Werner 1992), indicating specific regulation of gene expression in bacteroids. Therefore, it is not surprising to find a bacteroid-specific promoter. However, the *nfeC* promoter identified in this study is different from other known active genes in bacteroids (Gussin *et al.* 1986; Morett and Buck 1989; Murphy *et al.* 1988). For example, *nif* gene expression is high in bacteroids and requires RpoN, but these genes are also expressed under anaerobic growth conditions (Hennecke *et al.* 1988; Thöny *et al.* 1989). Thus, to our knowledge, the P1 promoter of *nfeC* is the first report of bacteroid-specific promoter in *B. japonicum* that does not appear to be under oxygen control. Further study of this gene should add to our understanding of the regulation of bacteroid-specific genes. Scott-Craig and Chelm (1992) have identified two promoters activated at high levels in *B. japonicum* bacteroids. However, expression of both promoters was also detected in microaerobically grown cells. Sequence analysis of the region upstream of the transcription start sites revealed no similarity between these two promoters and *nfeC*.

Since little is known about genes repressed under anaerobic conditions in *B. japonicum*, no canonical promoter sequence for such genes is known. The oxygen-dependent promoter (P2) of the *nfeC* gene has an *E. coli* consensus-like sequence, but it is unknown how this promoter is regulated during the transition from aerobic growth conditions to anaerobic conditions. Recently, Gabel and Maier (1993) reported that cyto-

chrome aa_3 in *B. japonicum* has a promoter repressed under low levels of oxygen. However, this gene was also expressed at reduced levels in bacteroids. Thus, this promoter is likely different from the P2 promoter of *nfeC*. Again, there is no apparent similarity between the promoter regions of the cytochrome aa_3 gene and *nfeC*. Further investigation of *nfeC* expression should provide interesting insights into bacteroid-specific gene expression in *B. japonicum*.

MATERIALS AND METHODS

Bacterial strains and plasmids.

NAD14 is a Tn*5*-induced mutant of *B. japonicum* USDA110 and has been described previously (Deshmane 1988). JC143 and JC890 are Tn*5* and Tn*5*::*lacZ*-induced mutants, respectively, derived by marker exchanging pND143 and pZB890 into *B. japonicum* USDA110. The Tn*5* or Tn*5*::*lacZ* insertions of all strains were confirmed by appropriate Southern blot hybridization. Recombinant plasmids and phage used in this work are listed in Table 2.

Microbiological techniques.

B. japonicum strains were cultured at 30° C in minimal medium containing HM salts plus arabinose and gluconate (Cole and Elkan 1973) or RDY medium (Nieuwkoop *et al.* 1987). *E. coli* strains were grown in LB medium (Miller 1972) at 37° C. When appropriate, antibiotics were added to the meium at the following final concentrations (micrograms per milliliter): *B. japonicum*, kanamycin, streptomycin, or tetracycline (150 each); *E. coli*, ampicillin (50), kanamycin (50), tetracycline (20), and chloramphenicol (30). Conjugation conditions for *B. japonicum* strains were as described previously (So *et al.* 1987). Anaerobic growth of *B. japonicum* was achieved in KNO_3 medium containing 10 mM KNO_3 (Stacey *et al.* 1993). Isolation of bacteria from soybean nodules has been described previously (Weaver *et al.* 1991).

Recombinant DNA work.

Cloning, restriction mapping, transformation, plasmid isolation, nick-translation, Southern blotting, and hybridization were performed using standard protocols (Maniatis *et al.* 1982). DNA restriction endonuclease fragments used in subcloning were isolated by separation on agarose gels by electrophoresis and the Geneclean II kit (Bio 101 Inc., La Jolla, CA). Isolation of chromosomal DNA from *B. japonicum* was done as described (So *et al.* 1987).

Nodulation tests.

Plants were infected with USDA110, JC143, JC890, or NAD14 and grown as described previously (Nieuwkoop *et al.* 1987). Eighteen individual plants were used for the nodulation assay of each strain. After inoculation, the number of nodulated plants and the number of nodules per plant were recorded each day. Nitrogen fixation activity was determined by the acetylene reduction assay as previously described (Wacek and Brill 1976).

Table 2. Bacterial strains, plasmids, and phage used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Bacterial strains		
B. japonicum		
USDA110	Wild-type; colony type I110	Kuykendall and Elkan 1976
NAD14	USDA110::Tn*5* (Km^r, Sm^r)	Deshmane 1988
JC143	USDA110:Tn*5* (km^r, Sm^r)	This study
JC890	USDA110::Tn*5*::*lac* (Km^r)	This study
E. coli		
S17-1	*hsdR thi pro* recA; RP4-2 kan::Tn*7* tet::Mu integrated in the chromosome	Simon *et al.* 1983
XL1-blue	*recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1* (F′ *proAB lacI*^{q} *lacZ*Δ*M15* Tn*10*)	Stratagene
Plasmids		
pBluescript II		
SK + and −	Ap^r, sequencing vector	Stratagene
pUC18	Ap^r, cloning vector	Vieira and Messing 1982
pSUP202	Ap^r Cm^r Tc^r, Mob^+	Simon *et al.* 1983
pRjUT14	pHC79 clone of *B. japonicum*	Russell *et al.* 1985
pRK2013	Km^r, helper plasmid	Figurski and Helinski 1979
pZB890	Tn*5*::*lacZ* insertion in 7.0kb *Eco*RI fragment of pRjUT14	This laboratory
pND143	Tn*5* insertion in 7.0kb *Eco*RI fragment of pRjUT14	This laboratory
pND63	Tn*5* insertion in 7.0kb *Eco*RI fragment of pRjUT14	This laboratory
pRj676	amp^r, *nif* DK	Fisher and Hennecke 1984
pJY400	1.4kb *Eco*RI fragment containing parts of *nif*D and *nif*K from pRj676 subcloned in pBluescriptII SK+	This study
pJY14-7	7.0kb*Eco*RI fragment from pRjUT14 subcloned in pSUP202	This study
pJY100	3.8kb *Cla*I-*Sac*I fragment from pJY14-7 subcloned in pBluescriptII SK+	This study
pJY101	2.0kb *Nru*I-*Sac*I fragment from pJY100 subcloned in pBluescriptII SK+	This study
pJY102	2.0kb *Hind*III-*Sac*I fragment from pJY101 subcloned in pUC18	This study
pJY103	2.0kb *Hind*III-*Eco*RI fragment from pJY102 subcloned in pBluescriptII SK−	This study
pJY104	1.4kb *Nru*I fragment from pJY100 subcloned in pBluescriptII SK−	This study
pJY105	2.6kb *Sfu*I-*Sac*I fragment from pJY100 subcloned in pBluescriptII SK+	This study
pJY107	0.6kb *Sal*I fragment from JY101 subcloned in pBluescriptII SK−	This study
pJY108	0.4kb *Nru*I-*Sal*I fragment from pJY101 subcloned in pBluescriptII SK−	This study
Phage		
L408	Helper phage for single-stranded DNA isolation	Stratagene

Competition assays.

Twelve soybean plants were used for each strain in the competition assays. Sterilization of seeds and preparation of seedlings (*Glycine max* 'Essex') was described previously (Nieuwkoop *et al.* 1987). Competition experiments were designed to compare the mutants with wild-type strain USDA110 for nodule occupancy on soybean. Suspensions of two strains were adjusted to the same density (OD_{600} = 0.1) and mixed to the desired ratio 1:1, 1:10, 1:100, 10:1, and 50:1 (USDA110/Tn*5* mutant) before inoculation. This suspension was diluted to a density of 2×10^5 cells per milliliter, and 1 ml was applied immediately to each root of 3-day-old soybean seedlings in growth pouches (Vaughan's seed company, Downers Grove, IL). Actual cell concentrations were checked by viable plate counts. After 19–20 days, nodules were collected, rinsed in 70% ethanol, washed twice in sterile distilled water, and immersed in 0.1% $HgCl_2$ in 0.06 N HCl for 5 min. Nodules were then washed five times in sterile distilled water. Following this pretreatment, the nodules were crushed and streaked on RDY agar with or without the appropriate antibiotics (i.e., kanamycin for the presence of Tn*5*). Plates were incubated at 29° C for 5–7 days, and the identities of strains were determined based on the resistance to antibiotics.

RNA isolation.

B. japonicum strains were grown with shaking at 200 rpm in 500-ml flasks containing 100 ml of RDY medium with appropriate selection. Anaerobic growth in the presence of 10 mM KNO_3 was performed in 15-ml Falcon tubes that were filled to the top with nitrate medium inoculated with approximately 10^7 cells. When the cultures were in the late log phase, cells were harvested and frozen under liquid nitrogen, then stored at –70° C for later use. Total RNA from *B. japonicum* free-living culture cells (aerobic or anaerobic cultures) and the isolated bacteroid cells were isolated using the hot phenol extraction method as previously described (Wang *et al.* 1991). Any contaminating DNA in the RNA samples was removed by digestion with RNase-free DNase I (Promega, Madison, WI).

Primer extension.

To determine the transcriptional start site of *nfeC*, two oligonucleotide primers, 5′-GTCTTCCAGGCATTCTCTTCATA-3′ (23-mer) and 5′-AATCCGACCACGCTAAGCCATA-3′ (22-mer), were synthesized, which were complementary to bases +25 to +47 and +54 to +75 of the *nfeC* coding sequence, respectively. End labeling was carried out for 1 hr at 37° C by using a volume of 25 µl with 0.1 µg of DNA primer and 240 µCi of [γ-^{32}P]ATP and using 4 units of T4 polynucleotide kinase in the buffer suggested by the supplier (USB, Cleveland, Ohio). The kinase reaction was stopped by incubating at 65° C for 5 min. The unincorporated [γ-^{32}P]ATP was removed by precipitating twice with the addition of 25 µl of 4 M ammonium acetate and 250 µl of ethanol at –70° C for 30 min. The labeled primer resuspended in 100 µl of sterile distilled water was hybridized to 20 µg of RNA by precipitating with 4 M ammonium acetate, resuspending the pellet in 30 µl of hybridization buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 0.1mM EDTA), and denaturing at 100° C for 3 min, and then incubating at 63° C for 1.5 hr. Primer extensions were carried out by the method of Kassavetis and Geiduschek (1982). Size standards were obtained by using the same primer in a dideoxy sequencing reaction with plasmid pJY105, containing *nfeC*, as template.

DNA sequencing.

DNA sequences were determined by the dideoxy chain determination method (Sanger *et al.* 1977). A nested set of deletions of the cloned fragments in vectors pBluescript II SK +/– was generated by the exonuclease III digestion procedure of Henikoff (1984). Single-stranded DNA was isolated as recommended by the manufacturer (Stratagene, La Jolla, CA 1990). The sequencing strategy is shown in Figure 1. The sequence of transposon insertion positions was determined by sequencing the Tn*5*-flanking regions of mutants NAD14, JC143, and JC890, using a Tn*5*-specific oligonucleotide, 5′ CAGGACGCTACTTGT 3′, as primer.

Computer-assisted sequence analysis was done using programs of the University of Wisconsin Genetics Computer Group (Madison, WI).

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