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Analysis of DNA Sequences Transcribed at High Levels in *Bradyrhizobium japonicum* Bacteroids but Not Necessary for Symbiotic Effectiveness

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Five regions of the *Bradyrhizobium japonicum* genome, which are transcribed at high levels in nitrogen-fixing soybean (*Glycine max*) nodules, were identified. None of these regions contained previously identified genes (e.g., *nif*, *nod*, and *fix* genes) that are known to be essential for development of functional nitrogen-fixing nodules. To assess the role of these regions in the development of the *B. japonicum*-soybean symbiosis, we cloned and used them to construct *B. japonicum* strains, in which large DNA segments (2.0–6.8 kilobases) containing the highly transcribed regions were deleted. The deletion strains were examined for symbiotic effectiveness and were found to be indistinguishable from the wild-type strain. Transcription of the cloned regions under a variety of physiological conditions and in several defined mutant *B. japonicum* strains was also examined. The transcriptional start sites for one pair of divergent transcripts were determined; the promoters do not contain any of the conserved sequences found in *B. japonicum* genes involved in symbiosis or nitrogen metabolism.

Additional keywords: bacteroids, differential gene expression.

The development of a successful symbiotic relationship between the nitrogen-fixing bacterium *Bradyrhizobium japonicum* (Kirchner) Jordan, and its host, *Glycine max* (L.) Merr. (soybean), involves a complex series of events in both organisms. A set of plant gene products (nodulins) are produced in developing soybean root nodules and not in uninfected roots (as reviewed in Nap and Bisseling 1990). Similarly, a number of gene products have been shown to be present in bacteroids, the fully differentiated form of the bacterium, and not in free-living *B. japonicum* cells (Verma *et al.* 1986). The extent to which the developmental program in the bacterium is controlled at the level of transcription is not completely understood. Although the transcription of bacterial genes that encode the subunits of the nitrogenase enzyme is highly induced during nodule development (Corbin *et al.* 1982; Gubler and Hennecke 1987), other bacterial genes essential for proper development of nitrogen-fixing nodules (e.g., *nod* genes) are expressed at low levels (Mulligan and Long 1985; Kosslak *et al.* 1987). In a study of gene expression in *B. japonicum* bacteroids, we used differential hybridization techniques to isolate DNA sequences that are expressed at higher levels in bacteroids than in cells grown in culture (Scott-Craig *et al.* 1991). The objective of this study was to ascertain if these *B. japonicum* genes, identified by their transcriptional regulation during nodule development, were necessary for the development of an effective symbiosis. Five regions of the *B. japonicum* genome, which contained such genes, were subcloned; *B. japonicum* strains, from which these regions had been deleted, were constructed. The fine structure of the promoters of two divergent transcripts from one region was determined.

*Deceased 2 September 1987.

Nucleotide sequence data have been submitted to GenBank as accession numbers M87001 (promoter 1) and M87281 (promoter 2).

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacterial growth conditions. Bacterial strains and plasmids are listed in Table 1. *B. japonicum* was grown at 30° C in YEM (0.04% yeast extract [w/v], 1% mannitol [w/v], 3 mM K_2PO_4, 0.8 mM $MgSO_4$, 1.1 mM NaCl) or in YEGG (YEM with 0.5% sodium gluconate and 0.1% sodium glutamate replacing the mannitol). The minimal medium was described by Manian and O'Gara (1982) and contained xylose at 0.3% (w/v). For the low oxygen experiments, *B. japonicum* strain BJ110d was grown in YEM or YEM plus 10 mM KNO_3 in 10-L fermenters sparged with a mixture of 0.2% oxygen (v/v) and 99.8% nitrogen (v/v) as described (Adams and Chelm 1988). For the nitrogen limitation experiments, BJ110d was grown in minimal medium with 1 mM NH_4Cl as the sole nitrogen source as described (Carlson *et al.* 1987).

Manipulation of nucleic acids. Southern hybridizations, colony hybridizations, and isolation of plasmid DNA were carried out as previously described (Adams *et al.* 1984). Total genomic DNA was isolated by phenol extraction (Marmur and Doty 1962). The cosmid library of BJ110d DNA in vector pLAFR1 was described by Adams *et al.* (1984). The isolation of total bacterial RNA, cDNA synthesis, and the removal of ribosomal cDNA sequences from the cDNA were performed as described (Scott-Craig *et al.* 1991). We prepared and hybridized slot blots of RNA

by using the method of Schloss *et al.* (1984). We transferred RNA samples to cellulose nitrate by using a Minifold II Slot-Blotter (Schleicher & Schuell, Keene, NH). The hybridization signals were quantitated by using a Model 300 Series Computing Densitometer (Molecular Dynamics, Sunnyvale, CA).

Gene-directed mutagenesis. Gene-directed mutagenesis was performed as described by Guerinot and Chelm (1986). Seven different plasmids containing *B. japonicum* DNA inserts, in which the central portion had been deleted and replaced by the neomycin phosphotransferase (*nptII*) gene, were constructed in *Escherichia coli* (Migula) Castellani and Chalmers; standard recombinant DNA methods (Maniatis *et al.* 1982) were used. These plasmids were mated into *B. japonicum* strain BJ110d, and kanamycin-resistant exconjugants were screened by colony hybridization for the absence of vector (pBR322) sequences.

Plant tests, acetylene reduction assays, and poly-β-hydroxubutyrate (PHB) determinations. The ability of the mutated *B. japonicum* strains to form an effective symbiosis with soybeans (*G. max* 'Amsoy') was assessed as described (Guerinot and Chelm 1986). Six to eight nodules from each plant were then surface-sterilized, and the resident bacteria were extracted and checked for phenotype as described by Guerinot and Chelm (1986). PHB was extracted from frozen *B. japonicum* nodules by the method of Wong and Evans (1971), and the dry weight was determined by the method of Law and Slepecky (1961).

DNA sequence and nuclease protection analysis. A 1,000-bp *Pst*I-*Hin*dIII *B. japonicum* DNA fragment from plasmid pBJ216 was cloned into vector pUC8 to make plasmid pBJ296. A set of nested deletions was generated by using exonuclease BAL-31 as described by Poncz *et al.* (1982). The set was ligated into the M13 vector mp19 and transformed into *E. coli* strain JM101. Single-stranded phage DNA was purified as described by Messing (1983). The nucleotide sequence of the 1,000-bp *B. japonicum* DNA fragment was determined by the dideoxy chain termination method of Sanger *et al.* (1977) as described in the manual published by Amersham (1983). For nuclease protection analysis, single-stranded DNA probes were synthesized by primer extension as described by Holben *et al.* (1988). The 215-bp *Acc*I-*Sty*I and the 272-bp *Sty*I-*Sal*I fragments isolated from plasmid pBJ296 were also used to prepare end-labeled single-stranded DNA probes as described by Adams and Chelm (1984). We used both types of probes in S1

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source
Escherichia coli		
ED8654	*met gal hsdR supE supF*	Borck *et al.* 1976
JM83	Host for pUC8 clones	Vieira and Messing 1982
JM101	Host for M13 mp18 and M13 mp19 clones	Yanisch-Perron *et al.* 1985
Bradyrhizobium japonicum		
BJ110d	Wild type	Guerinot and Chelm 1986
BJ27147	*ntrC*::*nptII*, Km^r	Martin *et al.* 1988
B. japonicum[a]		
BJ241	3.3-kbp *Hin*dIII-*Sma*I fragment of pBJ214	This study
BJ242	3.3-kbp *Hin*dIII fragment of pBJ216	This study
BJ243	3.4-kbp *Sal*I fragment of pBJ227	This study
BJ261	2.0-kbp *Bam*HI-*Cla*I fragment of pBJ261	This study
BJ281	2.5-kbp *Pst*I fragment of pBJ270	This study
BJ282	2.7-kbp *Cla*I fragment of pBJ282	This study
BJ283	6.8-kbp *Eco*RI-*Bam*HI fragment of pBJ283	This study
Plasmids		
pBJ33	*nifH* from *B. japonicum*	Adams *et al.* 1984
pBJ53A	*glnA* from *B. japonicum*	Carlson *et al.* 1985
pBJ110	*hemA* from *B. japonicum*	Guerinot and Chelm 1986
pBJ142	*rDNA* operon in *B. japonicum*	Scott-Craig *et al.* 1991
pBJ196A	*glnII* from *B. japonicum*	Carlson and Chelm 1986
pBJ214	5.3-kb *Eco*RI fragment from cosmid 3-61 cloned in pRL425	
pBJ216	5.1-kb *Bam*HI fragment from cosmid 3-61 cloned in pRL425	
pBJ227	6.0-kb *Bam*HI-*Eco*RI fragment from cosmid 14-8 cloned in pBR322	
pBJ241	pBJ214 with 3.3-kb *Hin*dIII-*Sma*I fragment replaced with *nptII* cassette	
pBJ242	pBJ216 with 3.3-kb *Hin*dIII fragment replaced with *nptII* cassette	
pBJ243	pBJ227 with 3.4-kb *Sal*I fragment replaced with *nptII* cassette	
pBJ261	2.9-kb *Cla*I and 1.4-kb *Bam*HI fragments from cosmid 4-51 flanking *nptII* cassette	
pBJ270	4.0-kb *Sst*I fragment from λB15 cloned into pRL425	
pBJ273	6.3-kb *Bam*HI fragment from cosmid 4-51 cloned in pRL425	
pBJ282	2.0-kb *Bam*HI-*Cla*I and 1.7-kb *Cla*I-*Bam*HI fragments from pBJ273 flanking *nptII* cassette	
pBJ283	1.5-kb *Eco*RI and 3.1-kb *Bam*HI fragments from cosmid 4-51 flanking *nptII* cassette	
pBJ285	2.5-kb *Pst*I fragment from pBJ270 cloned in pBR322	
pBJ296	1,000-bp *Pst*I-*Hin*dIII fragment from pBJ216 cloned in pUC8	
pBR322	Amp^r, Tet^r, cloning vector	Bolivar *et al.* 1977
pKC7	Amp^r, Kan^r, cloning vector	Rao and Rogers 1979
pRL425	Amp^r, cloning vector	Elhai and Wolk 1988
pUC8	Amp^r, cloning vector	Vieira and Messing 1982

[a] Deletion strains in which the indicated restriction fragment was replaced by the *nptII* cassette.

nuclease protection experiments to determine the direction of transcription and the approximate location of the transcription initiation site. S1 protection analysis was carried out by the method of Berk and Sharp (1977) as adapted by Adams and Chelm (1984). We synthesized single-stranded DNA probes, for precise determination of the transcription initiation site, by using gene-specific oligonucleotide primers as described by Adams and Chelm (1988) and Carlson *et al.* (1987). The sequences of the two primers used were 5′-TCATCGCGGTCTACGAG (oligonucleotide 1) and 5′-GCAAGGACGTAATATGC (oligonucleotide 2). DNA sequence comparisons were performed by using the MicroGenie software system (Beckman, Palo Alto, CA).

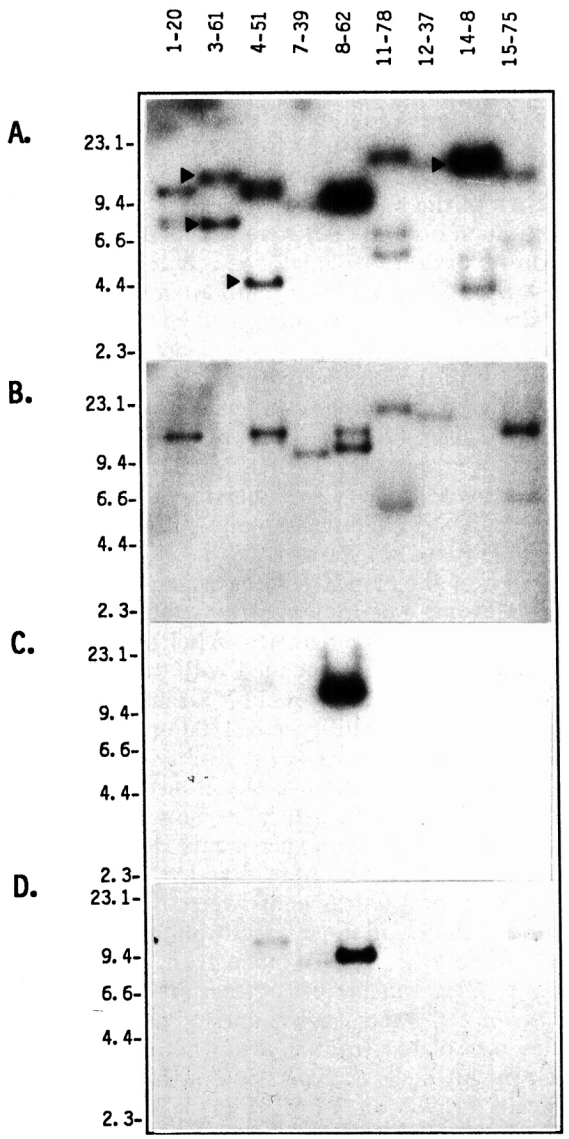

Fig. 1. Differential expression of *Bradyrhizobium japonicum* DNA sequences that are transcribed at high levels in bacteroids. DNA from nine cosmids was digested with restriction endonucleases and transferred onto cellulose nitrate filters. The filters were probed with random-primed cDNA prepared with RNA from four different *B. japonicum* cell types. **A,** Bacteroids; **B–D,** cells grown in culture (0.04% yeast extract [w/v], 1% mannitol [w/v], 3 mM K_2PO_4, 0.8 mM $MgSO_4$, 1.1 mM NaCl) and harvested at **B,** exponential; **C,** late logarithmic; and **D,** stationary phases. The *B. japonicum* bacteroids were isolated from 5-wk-old soybean root nodules. Arrows (▶) in A indicate the bands from particular cosmids that showed differential hybridization signals. Molecular weights are indicated in the left margin in kilobases

RESULTS

Identification, mapping, and subcloning of DNA sequences transcribed at high levels in bacteroids. DNA from nine independent cosmids containing sequences transcribed at high levels in bacteroids (Scott-Craig *et al.* 1991) was digested with *Eco*RI, run out on an agarose gel, and transferred to cellulose nitrate. The filters were hybridized with cDNA synthesized with total RNA from bacteroids or from free-living cells. Four DNA fragments from three different cosmids (3-61, 4-51, and 14-8) that produced the best differential signals (indicated by arrows in Fig. 1) were subcloned into plasmid vectors, restriction-mapped, and probed again with the same cDNAs to further localize the regions that were actively transcribed in bacteroids (Fig. 2). Recombinant bacteriophage λB15, identified in the original library screen, showed no homology to any member of the cosmid library but represented a fifth differentially expressed region of the *B. japonicum* genome. A 4-kb *Sst*I fragment of DNA was subcloned directly from λB15 into plasmid pRL425, and plasmid pBJ270 was created. The sequences from cosmids 3-61, 4-51, and 14-8, which were selected for analysis, are not expressed appreciably at any phase in the growth of cultured cells and are highly expressed in bacteroids (Fig. 1). Other cosmids (e.g., 11-78 and 15-75 in Fig. 1) clearly contained sequences that were expressed both in bacteroids and during exponential growth of cultured cells but not in stationary phase.

Construction of deletion strains. As depicted in Figure 2, seven regions of *B. japonicum* genomic DNA, ranging

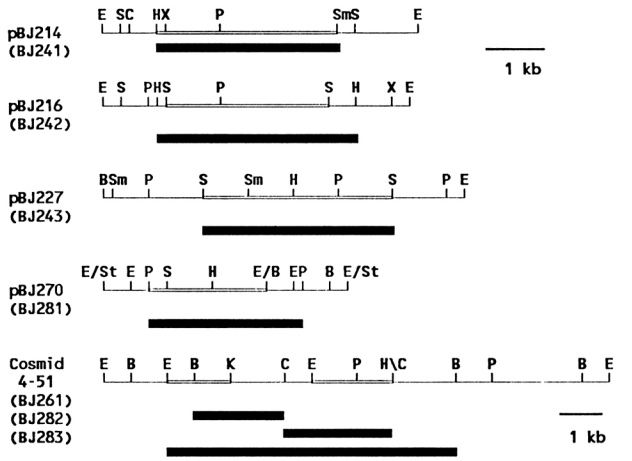

Fig. 2. Restriction endonuclease maps of five regions of the *Bradyrhizobium japonicum* genome. The regions contain sequences that are transcribed at high levels in bacteroids. Double-lined regions indicate DNA segments to which the cDNA, synthesized from bacteroid RNA, hybridized differentially. Heavy black lines indicate the portions of the highly transcribed regions that were deleted and replaced by the *nptII* gene. Below the name of each of the five plasmids (pBJ214, pBJ216, pBJ227, pBJ270, and cosmid 4-51) are listed, in parentheses, the names of the *B. japonicum* deletion strains derived from that plasmid. B = *Bam*HI, C = *Cla*I, E = *Eco*RI, H = *Hin*dIII, K = *Kpn*I, P = *Pst*I, S = *Sal*I, Sm = *Sma*I, St = *Sst*I, X = *Xho*I.

in size from 2.0 to 6.8 kb, were replaced *in vitro* by DNA fragments containing the *nptII* gene that encodes neomycin phosphotransferase II and confers resistance to the antibiotic kanamycin (Kan^r). Plasmids carrying these constructions were mated into *B. japonicum* wild-type strain BJ110d, and genomic DNA from kanamycin-resistant exconjugants was examined by Southern blot analysis to confirm that the gene replacements had occurred properly (data not shown).

To confirm the apparent induction of the cloned sequences in bacteroids, we transferred RNA from bacteroids and cells grown in culture to cellulose nitrate filters and probed them with radio-labeled clones of the regions that had been deleted in each of five of the constructed strains. As shown in Figure 3C–E, the RNA corresponding to each deleted region is present in greater amounts in bacteroids (Fig. 3, lane 1) than in cells grown to stationary phase (Fig. 3, lane 2) or in cells growing exponentially in culture (Fig. 3, lane 3). The expression of a gene (*nifH*; Fig. 3A) known to be induced in bacteroids (Corbin *et al.* 1982) and a gene (*glnA*; Fig. 3B) known to be only modestly regulated in all three cell types (Carlson *et al.* 1985) were used as controls. RNA homologous to the *nifH* gene is present at approximately 15-fold higher levels in *B. japonicum* bacteroids than in cells grown to stationary phase in culture (Fig. 3A, lanes 1,2). Under similar conditions, the *glnA* gene shows a threefold induction (Fig. 3B). The sequences isolated in this study all showed between six- and 15-fold induction (Fig. 3C–E). The filter probed with the radiolabeled rDNA plasmid (Fig. 3F) demonstrates that the samples from each cell type contained equal amounts of RNA.

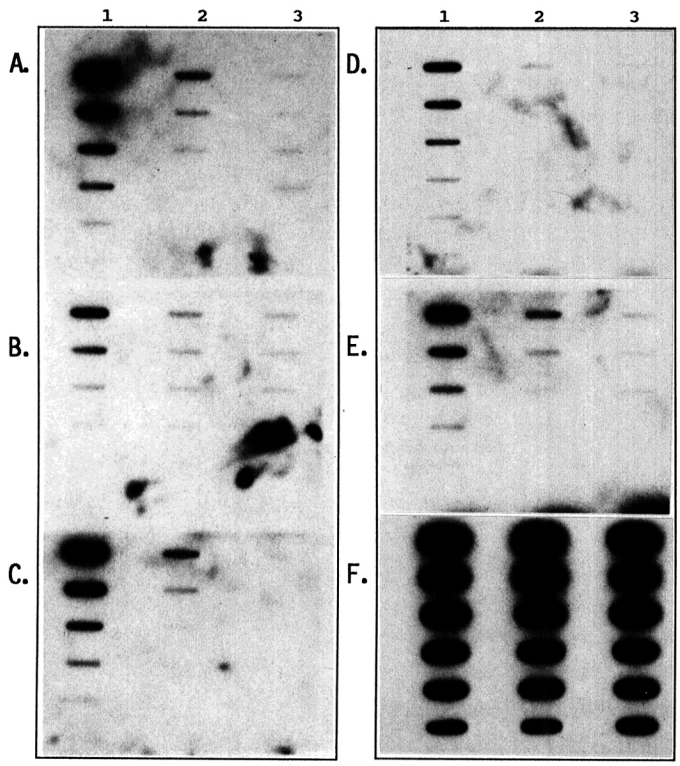

Fig. 3. Slot blots of *Bradyrhizobium japonicum* RNA probed with radiolabeled plasmids containing control genes or one of the sequences replaced in the deletion constructions. Lane 1 = bacteroid RNA; 2 = stationary culture (0.04% yeast extract [w/v], 1% mannitol [w/v], 3 mM K_2PO_4, 0.8 mM $MgSO_4$, 1.1 mM NaCl [YEM]) RNA; and 3 = late exponential culture (YEM) RNA. **A–E,** RNA from each source was loaded in a vertical array of six slots with (from top to bottom) 3.0, 1.0, 0.3, 0.1, 0.03, and 0.01 μg of RNA. **F,** RNA concentrations were lowered to cover the range 0.3–0.001 μg and, thus, obtain more readily measurable signals. The probes used were **A,** pBJ33 (*nifH*); **B,** pBJ49 (*glnA*); **C,** pBJ214; **D,** pBJ227; **E,** pBJ285; **F,** pBJ142 (rDNA).

Symbiotic effectiveness of deletion strains. Six individual soybean plants were inoculated with each of the deletion mutant strains. After 4 wk, the plants were examined for appearance, nodule formation, and the ability of excised nodules from each plant to reduce acetylene to ethylene. In all cases, the mutant *B. japonicum* strains were similar to the wild-type strain BJ110d (data not shown). To ensure that the phenotype seen on plants inoculated with deletion strains was not due to contamination by wild-type *B. japonicum*, we crushed six nodules from each plant and determined the antibiotic resistance phenotype of the resident bacteria. All of the bacteria isolated from mutant nodules from each plant (100 of 100) were resistant to kanamycin, and all of the bacteria isolated from control plants inoculated with BJ110d were sensitive to kanamycin.

The ultrastructure of nodules incited by each mutant strain was examined by transmission electron microscopy. No significant differences were observed when each of the mutant strains was compared to wild-type BJ110d (data not shown). Bacteria purified from nodules incited by four of the mutant strains (BJ214, BJ216, BJ227, and BJ261) were also assayed for levels of the storage polymer PHB and were found to contain amounts similar to wild-type strain BJ110d (data not shown).

Expression of the cloned regions under various physiological conditions. The mutant strains were examined by comparison with BJ110d for growth on rich medium (YEGG) and on minimal medium amended with 0.3% xylose. The growth rate of each of the mutant strains was indistinguishable from that of wild-type BJ110d under both conditions (data not shown). Because the strains grew identically, we examined the expression of the cloned regions under a variety of conditions to determine how these genes are regulated. In each of the experiments described below, cosmid or plasmid DNAs were digested with restriction endonucleases, and the fragments were transferred to cellulose nitrate filters after gel electrophoresis. The filters were hybridized with cDNA made from total RNA extracted from *B. japonicum* cells of various genotypes. The *B. japonicum* cells were grown under particular cultural conditions or isolated from soybean nodules. The possibility that the low level of expression of the cloned regions seen in cells grown on YEM (Fig. 3) was due to the particular stage in the growth curve when the cells were harvested was examined. Samples of cells were collected at exponential, late logarithmic, and stationary phase. As shown in Figure 1, with the exception of sequences on cosmid 8-62 (which are highly expressed in each cell type), each cosmid contains one or more bands that produce a greater signal when probed with bacteroid cDNA than with cDNA prepared from cultured cells (see arrows).

Possible carbon source effects were also examined, and cDNA was prepared from cells grown on minimal medium containing either xylose or formate as the sole source of

carbon. Neither growth on the "good" carbon source (xylose) nor on the "poor" carbon source (formate) induced transcription of the cloned regions (data not shown). To determine if the level of free oxygen might be involved in the transcriptional regulation of the sequences isolated in this study, we prepared cDNA by using total RNA isolated from *B. japonicum* cells grown under microaerobic conditions. Cells were grown in 10-L fermenters containing YEM either with or without 10 mM KNO_3 as a terminal electron acceptor and were sparged with nitrogen containing 0.1% O_2. Transcripts homologous to pBJ227 were induced by low oxygen levels (Fig. 4C, lane 4), and transcripts homologous to pBJ227 and pBJ270 were induced by low oxygen levels in the presence of nitrate (Fig. 4D, lanes 4,5). Although the signals obtained were below levels seen in bacteroids (compare Fig. 4A, lanes 4,5 to 4C, lane 4 and 4D, lanes 4,5), they were similar to those for pBJ33 (*nifH*) (Fig. 4D, lane 1).

We conducted two sets of experiments to examine if the global nitrogen regulatory (*ntr*) genes (Magasanik 1982) affect the expression of the highly expressed sequences. RNA extracted from cultures of BJ110d grown with a limited supply of fixed nitrogen was used to prepare a cDNA probe that was hybridized to cellulose nitrate filters similar to those used in the preceding experiments. The low signal levels obtained (Fig. 5B, lanes 1–6) indicate that induction of the *ntr* system alone is insufficient to promote induction of the *nifH* gene or the sequences isolated in this study. The signal resulting from the induction of *glnII*, a gene known to be under *ntr* control (Carlson *et al.* 1987), can be seen in Figure 5B, lane 7. In addition, when cDNA prepared from total RNA extracted from nodules incited by an *ntrC* strain of BJ110d (Martin *et al.* 1988) was used as probe, all of the sequences were expressed at levels approximating those seen in wild-type nodules (Fig. 6B, lanes 2–6). In this case, the lack of a hybridization signal from an *ntrC*-regulated gene (*glnII*) confirms that the *ntrC* gene product was indeed inactivated (Fig. 6B, lane 7).

Identification of promoter regions. Initial S1 nuclease protection experiments with uniformly labeled single-stranded DNA probes indicated that total RNA isolated from soybean nodules incited by *B. japonicum* BJ110d protected portions of both strands of the DNA present on plasmid pBJ296. The DNA sequence of the 1,000-bp *B. japonicum* *Pst*I-*Hin*dIII fragment contained in pBJ296 was determined (data not shown). The transcription initia-

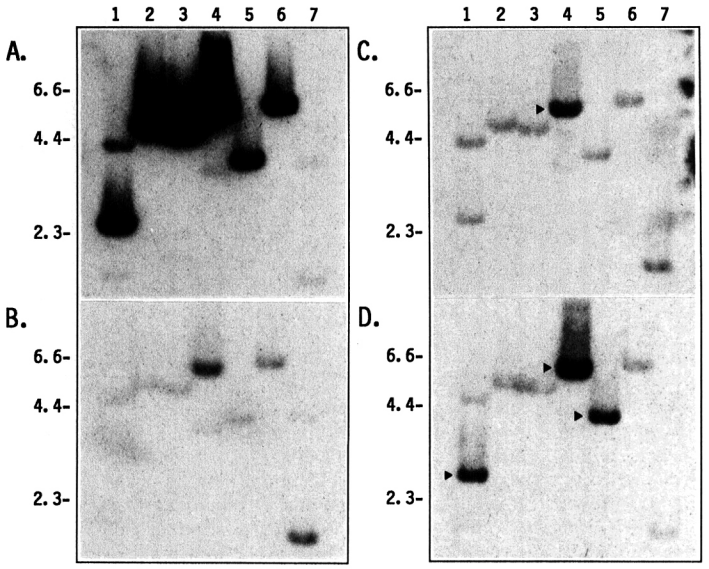

Fig. 4. Effect of lowered oxygen tension on the expression of *Bradyrhizobium japonicum* sequences that are transcribed at high levels in bacteroids. **A–D,** Four identical sets of plasmid restriction endonuclease digests were size-fractionated on agarose gels and transferred onto cellulose nitrate filters. The filters were hybridized with cDNA synthesized with **A,** RNA isolated from bacteroids isolated from 4-wk-old soybean nodules; **B,** cells grown aerobically in YEM (0.04% yeast extract [w/v], 1% mannitol [w/v], 3 mM K_2PO_4, 0.8 mM $MgSO_4$, 1.1 mM NaCl); **C,** cells grown microaerobically (0.2% O_2) in YEM; and **D,** cells grown microaerobically (0.2% O_2) in YEM with 10 mM potassium nitrate. The plasmids contain the *nifH* gene (lane 1); five regions of the *B. japonicum* genome that are highly transcribed in bacteroids (lanes 2–6 = pBJ214, pBJ216, pBJ227, pBJ270, and pBJ273); and the *glnII* gene (lane 7). Arrows in C and D indicate the sequences in which expression is induced. Molecular weights are indicated in the left margin in kilobases.

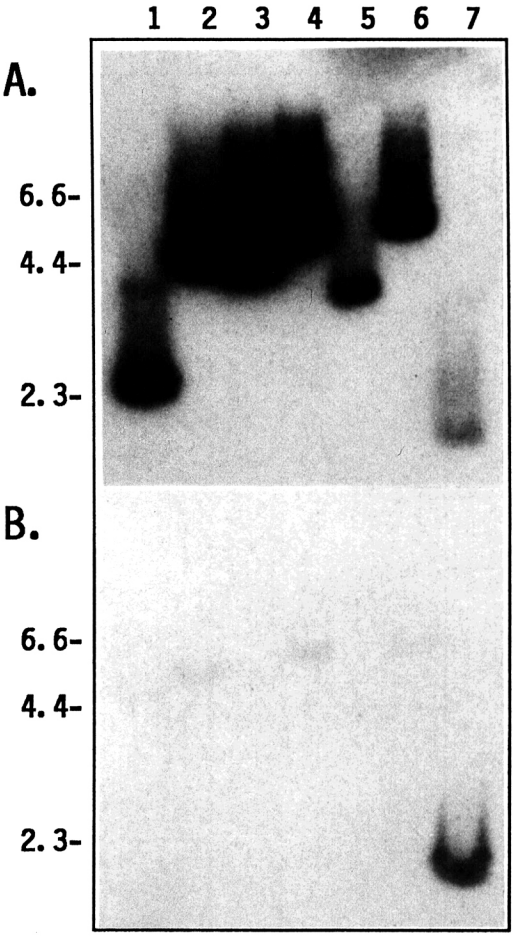

Fig. 5. Expression of the highly transcribed sequences in *Bradyrhizobium japonicum* cultures grown under nitrogen-limiting conditions. **A, B,** Restriction endonuclease digests of plasmid DNAs were size-fractionated on agarose gels and transferred onto cellulose nitrate filters. The filters were hybridized with cDNA synthesized with RNA isolated from **A,** *B. japonicum* bacteroids or **B,** aerobic cultures in which the supply of fixed nitrogen became limiting as the cells grew. The plasmids contained *nifH* (lane 1), the sequences isolated in this study (lanes 2–6 = pBJ214, pBJ216, pBJ227, pBJ270, and pBJ273), or *glnII* (lane 7). Molecular weights are indicated in the left margin in kilobases.

tion sites for both transcripts were localized by nuclease S1 protection analysis with end-labeled DNA fragments as probes (Fig. 7A,C). The transcription initiation sites were then precisely identified by comparison of the protected portion of end-labeled oligonucleotide-generated probes to a DNA sequence ladder generated by using the same oligonucleotides as primers (Fig. 7B,D). The promoter regions were then compared with conserved regions of the *E. coli* consensus promoter (Hawley and McClure 1983), and some degree of similarity was found (Fig. 8). DNA sequence comparisons of the two promoters with those of the promoters of the *B. japonicum nifDK*, *hemA*, *glnA*, and *glnII* genes as well as the entire GenBank data base did not identify any regions of DNA with significant similarities.

DISCUSSION

An underlying assumption in this effort to isolate genes that are transcribed at high levels in *B. japonicum* bacteroids was that such genes would have functions necessary for the proper development of the *B. japonicum*-soybean symbiosis. In *Rhizobium* and *Bradyrhizobium*, the best characterized example of developmentally regulated genes are the *nifH,D,K* genes, which encode the subunits of nitrogenase (Krol *et al.* 1980; Prakash *et al.* 1982; Corbin *et al.* 1982). The *nif* genes are induced at high levels specifically in bacteroids (Corbin *et al.* 1982), and a mutation in any one of the three structural genes prevents the reduction of dinitrogen to ammonia (Ruvken and Ausubel 1981). Although none of the highly transcribed sequences isolated and analyzed in this study share sequence similarity with known *nif* or *fix* genes (Scott-Craig *et al.* 1991), all

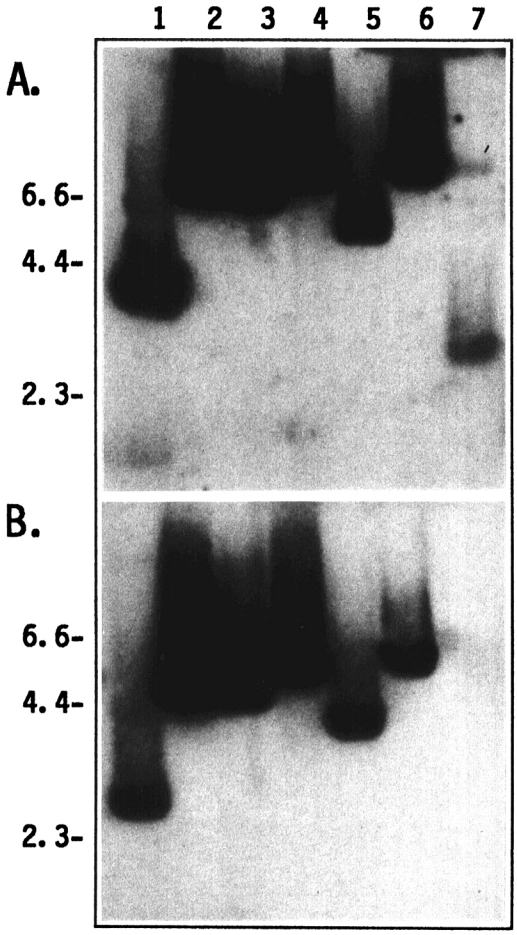

Fig. 6. Expression of the highly transcribed sequences in a *Bradyrhizobium japonicum ntrC* insertion strain. **A, B,** Restriction endonuclease digests of plasmid DNAs were size-fractionated on agarose gels and transferred onto cellulose nitrate filters. The filters were hybridized with cDNA made with RNA isolated from **A,** nodules incited by wild-type strain BJ110d or **B,** *ntrC* insertion strain BJ262. The plasmids contained *nifH* (lane 1), the bacteroid-specific sequences isolated in this study (lanes 2–6 = pBJ214, pBJ216, pBJ227, pBJ270, and pBJ273), or *glnII* (lane 7). Molecular weights are indicated in the left margin in kilobases.

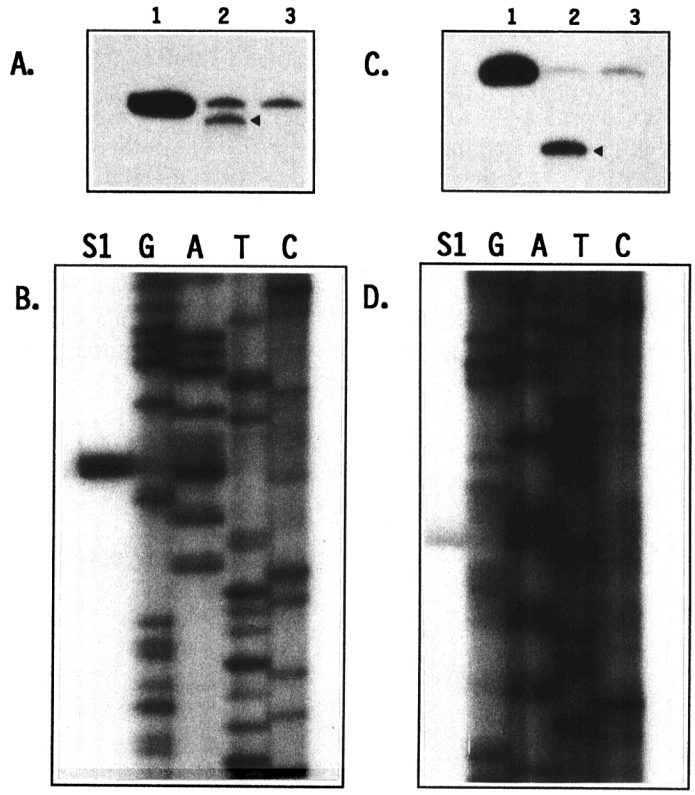

Fig. 7. Nuclease S1 protection analysis of two divergently transcribed *Bradyrhizobium japonicum* sequences that are expressed at high levels in bacteroids. **A, C,** Total bacteroid RNA (10 μg), purified from bacteroids isolated from root nodules incited by *B. japonicum* strain BJ110d, was hybridized with end-labeled, strand-separated DNA probes prepared from **A,** the 272-bp *Sty*I-*Sal*I and **C,** the 215-bp *Acc*I-*Sty*I fragments from plasmid pBJ296. Lane 1 contains a DNA probe that was not digested with S1 nuclease. Lane 2 contains a DNA probe that was hybridized with RNA and then subjected to digestion with S1 nuclease. Lane 3 contains a DNA probe that was placed under hybridization conditions without added RNA and then subjected to digestion with S1 nuclease. The band in lane 2 indicated by the arrow is the probe DNA partially protected by RNA. The band that migrates at the same size as the untreated probe DNA in both lanes 2 and 3 represents full length protection of the probe by DNA hybridization. **B, D,** The transcription initiation sites for the two divergently transcribed sequences were precisely localized. Single-stranded DNA probes were generated by primer extension of two specific end-labeled 17-bp oligonucleotides (see text). These probes were hybridized with total bacteroid RNA (10 μg) and then subjected to digestion by S1 nuclease. The protected DNA fragments were accurately sized by subjecting them to electrophoresis next to a DNA sequencing ladder that had been produced by primer extension of the same oligonucleotide.

are differentially expressed in bacteroids at levels approaching those seen for the *nif* genes (Fig. 3). The results of the deletion analysis, however, indicate that none of these highly transcribed regions is necessary for nodulation or nitrogen fixation. The simplest explanation for these results is that the *B. japonicum* genome contains an additional copy or copies of these sequences. Examples of gene duplication have been reported in various rhizobia (Kullik *et al.* 1991; Applebaum *et al.* 1988; Renalier *et al.* 1987; Honma *et al.* 1985; Quinto *et al.* 1982). With the exception of the sequence contained on plasmid pBJ227, however, hybridization of radio-labeled plasmid DNA from the deleted regions to total *B. japonicum* genomic DNA did not indicate the existence of other copies of the cloned regions (data not shown). The hybridization conditions employed in these experiments were of sufficiently moderate stringency so that closely related sequences would have been revealed. It is possible that the functions carried out by the products of these regions are duplicated by other nonhomologous regions of the genome. The glutamine synthetase genes in *B. japonicum* provide an example of such a duplication. Although the two gene products perform the same enzymatic function, the two proteins differ in physical properties and mode of regulation (Carlson *et al.* 1987). Mutational analysis revealed that deletion of either gene alone did not result in glutamine auxotrophy and did not affect symbiotic competence, but a strain carrying deletions in both genes required glutamine for growth in culture and formed ineffective nodules (Carlson *et al.* 1987). If the highly transcribed sequences isolated in this study were functionally duplicated by nonhomologous sequences located elsewhere in the *B. japonicum* genome, the lack of a symbiotic phenotype in nodules incited by the deletion strains would be expected.

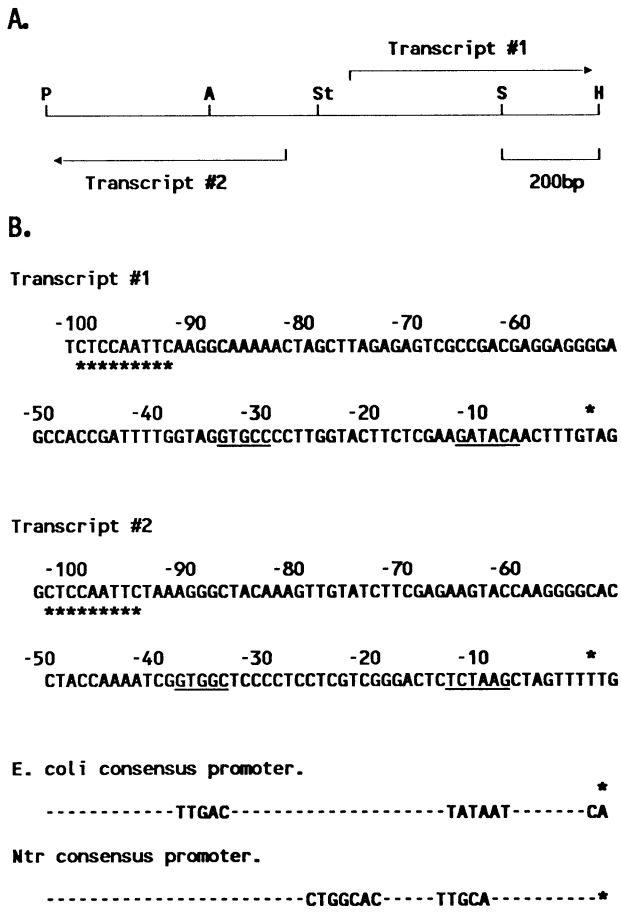

Fig. 8. A, Restriction endonuclease map of plasmid pBJ296. The initiation sites and direction of transcription of the two divergent transcripts are indicated. A = *Acc*I, H = *Hin*dIII, P = *Pst*I, S = *Sal*I, and St = *Sty*I. **B,** DNA sequence of the promoter regions of two *Bradyrhizobium japonicum* symbiotically regulated sequences. The transcription initiation sites are labeled with an asterisk above the nucleotides. The regions showing similarity to the *Escherichia coli* promoter consensus sequences at −10 nt and −35 nt upstream of the transcription initiation sites are underlined. A 9-bp sequence present in both promoters is centered at position −97 nt in transcript 1 and at position −99 nt in transcript 2; this sequence is underlined with asterisks.

The role of transcriptionally regulated genes in development has been examined in a variety of systems. Using a transposon Tn*5*-derived promoter probe (Tn*5-lac*), Kroos *et al.* (1986) examined 2,374 Tn*5-lac* insertion-containing *Myxococcus xanthus* Beebe strains for both development-specific expression and for developmental phenotype. Thirty-six strains, which had significantly increased levels of β-galactosidase activity when placed under conditions known to induce fruiting body formation, were identified. Only three of these *M. xanthus* strains (and eight of the original 2,374) also caused abnormal fruiting body development, indicating that far fewer genes are essential for development than are regulated during development (Kroos *et al.* 1990).

Employing a method similar to the one used to identify the clones characterized in this study, Mathiopoulos and Sonenshein (1989) identified *Bacillus subtilis* Mendelson genes expressed early during sporulation. Insertion mutations in two of these genes had no effect on sporulation, leading the researchers to speculate that multiple overlapping pathways control the onset of this developmental process.

In *Aspergillus nidulans* (Eidam) G. Wint., genes transcribed specifically during conidiation have been isolated by differential screening (Zimmerman *et al.* 1980). A 38-kb region of DNA containing at least 14 developmentally regulated transcripts was identified (Gwynne *et al.* 1984), but when the entire segment of DNA was removed from the genome, conidiation was unaffected (Aramayo *et al.* 1989). These authors also speculate that the deleted genes may be part of redundant metabolic networks.

A second possible explanation for the failure of the *B. japonicum* deletion strains to produce a symbiotic phenotype is that the gene products encoded by the deleted regions are not involved in the processes of nodulation or nitrogen fixation but are active at a later stage of the interaction (e.g., senescence). To examine this possibility, we determined the PHB content of nodules incited by the deletion strains, because PHB is accumulated during nodule development and hydrolyzed during nodule senescence (Wong and Evans 1971). No evidence was found, however, for altered levels of PHB accumulation in the deletion strains. It would seem unlikely, moreover, that all of the isolated sequences would fall into the "late gene" category. The fact that the sequences were actively transcribed in nodules harvested at 4 (Fig. 4A) and 5 (Fig. 2A) wk after infection

also argues against this explanation.

Examination of the expression of the highly expressed sequences in *B. japonicum* wild-type cells grown under microaerobic conditions (0.2% O_2 +/− 10 mM KNO_3) provides insight into the mechanism by which two of the sequences are regulated. As was previously demonstrated for the *nif* genes (Adams and Chelm 1988), lowered oxygen tension is sufficient to induce transcription of the sequences contained on plasmids pBJ227 and pBJ270 (Fig. 4, lanes 4,5). The diffusion of oxygen in soybean root nodules is thought to be facilitated by leghemoglobin, the apo-protein portion of which is not synthesized until the nodule structure begins forming at approximately 10 days after infection (Gloudemans *et al.* 1987). If functional leghemoglobin is necessary for the expression of oxygen-regulated genes in *B. japonicum*, then the gene products encoded by these genes are most likely used, like nitrogenase, after nodule development is well underway.

Transcripts homologous to the highly expressed sequences examined in this study are not induced under conditions that induce the nitrogen regulatory (*ntr*) genes (Fig. 5). In addition, the levels of expression of the transcripts in *B. japonicum* nodules incited by the *ntrC* insertion strain do not differ from those seen in nodules incited by wild-type strain BJ110d (Fig. 6). These results confirm directly that the global nitrogen regulatory (*ntr*) system is not involved in the transcriptional regulation of these sequences. The two divergent promoters cloned on plasmid pBJ216 did not contain any of the conserved sequences found upstream of *nif*, *nod*, or nitrogen-regulated genes (Gussin *et al.* 1986; Rostas *et al.* 1986). Some similarity to the *E. coli* consensus promoter was found (Hawley and McClure 1983), and one 9-bp motif of unknown significance was present at the same position in both promoters.

The role in symbiotic development of the highly transcribed sequences isolated in this study is not readily apparent. The expression of these transcripts at high levels in bacteroids would lead to the assumption that they are involved in some aspect of metabolism related to nitrogen fixation or assimilation. The most probable explanation for the unaltered symbiotic competence of the strains specifically deleted for these regions is that functional but non-homologous duplications exist elsewhere in the *B. japonicum* genome.

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LITERATURE CITED

Adams, T. H., and Chelm, B. K. 1988. Effects of oxygen levels on the transcription of *nif* and *gln* genes in *Bradyrhizobium japonicum*. J. Gen. Microbiol. 134:611-618.

Adams, T. H., and Chelm, B. K. 1984. The *nifH* and *nifDK* promoter regions from *Rhizobium japonicum* share structural homologies with each other and with nitrogen-regulated promoters from other organisms. J. Mol. Appl. Genet. 2:392-405.

Adams, T. H., McClung, C. R., and Chelm, B. K. 1984. Physical organization of the *Bradyrhizobium japonicum* nitrogenase gene region. J. Bacteriol. 159:857-862.

Amersham Corporation. 1983. M13 Cloning and Sequencing Handbook. Amersham Corp., Arlington Heights, IL.

Applebaum, E. R., Thompson, D. V., Idler, K., and Chartrain, N. 1988. *Rhizobium japonicum* USDA 191 has two *nodD* genes that differ in primary structure and function. J. Bacteriol. 170:12-20.

Aramayo, R., Adams, T. H., and Timberlake, W. E. 1989. A large cluster of highly expressed genes is dispensable for growth and development in *Aspergillus nidulans*. Genetics 122:65-71.

Berk, A. J., and Sharp, P. A. 1977. Sizing and mapping early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell 12:721-732.

Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heynecker, H. L., Boyer, H. W., Crosa, J. H., and Falkow, S. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95-113.

Borck, K., Beggs, J. D., Brammar, W. J., Hopkins, A. S., and Murray, N. E. 1976. The construction *in vitro* of transducing derivatives of phage lambda. Mol. Gen. Genet. 146:199-207.

Carlson, T. A., and Chelm, B. K. 1986. Apparent eukaryotic origin of glutamine synthetase II from the bacterium *Bradyrhizobium japonicum*. Nature (London) 322:568-570.

Carlson, T. A., Guerinot, M. L., and Chelm, B. K. 1985. Characterization of the gene encoding glutamine synthetase I (*glnA*) from *Bradyrhizobium japonicum*. J. Bacteriol. 162:698-703.

Carlson, T. A., Martin, G. B., and Chelm, B. K. 1987. Differential transcription of the two glutamine synthetase genes of *Bradyrhizobium japonicum*. J. Bacteriol. 169:5861-5866.

Corbin, D., Ditta, G., and Helinski, D. R. 1982. Clustering of nitrogen fixation (*nif*) genes in *Rhizobium meliloti*. J. Bacteriol. 149:221-228.

Elhai, J., and Wolk, C. P. 1988. A versatile class of positive-selection vectors based on the nonviability of palindrome-containing plasmids that allows cloning into long polylinkers. Gene 68:119-138.

Gloudemans, T., de Vries, S., Bussink, H.-J., Malik, N. S. A., Franssen, H. J., Louwerse, J., and Bisseling, T. 1987. Nodulin gene expression during soybean (*Glycine max*) nodule development. Plant Mol. Biol. 8:395-403.

Gubler, M., and Hennecke, H. 1988. Regulation of the *fixA* gene and *fixBC* operon in *Bradyrhizobium japonicum*. J. Bacteriol. 170:1205-1214.

Guerinot, M. L., and Chelm, B. K. 1986. Bacterial 5-aminolevulinic acid synthase activity is not essential for leghemoglobin formation in the soybean/*Bradyrhizobium japonicum* symbiosis. Proc. Natl. Acad. Sci. USA 83:1837-1841.

Gussin, G. N., Ronson, C. W., and Ausubel, F. M. 1986. Regulation of nitrogen fixation genes. Annu. Rev. Genet. 20:567-592.

Gwynne, D. I., Miller, B. L., Miller, K. Y., and Timberlake, W. E. 1984. Structure and regulated expression of the *SpoC1* gene cluster from *Aspergillus nidulans*. J. Mol. Biol. 180:91-109.

Hawley, D. K., and McClure, W. R. 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. 11:2237-2255.

Holben, W. E., Jansson, J. K., Chelm, B. K., and Tiedje, J. M. 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. 54:703-711.

Honma, M. A., Smith, C. A., Hirsch, A. H., Lang-Unnasch, N., and Ausubel, F. M. 1985. Evidence for additional *nodD*-like genes in *R. meliloti*. Page 120 in: Nitrogen Fixation Research Progress. N. J. Evans, P. J. Bottomley, and W. E. Newton, eds. Martinus Nijhoff Publishers, Dordecht, Netherlands.

Kosslak, R. M., Bookland, R., Barkei, J., Paaren, J. E., and Applebaum, E. R. 1987. Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*. Proc. Natl. Acad. Sci. USA 84:7428-7432.

Krol, A. J. H., Hontelez, J. G. J., Van den Bos, R. C., and Van Kammen, A. 1980. Expression of large plasmids in the endosymbiotic form the *Rhizobium leguminosarum*. Nucleic Acids Res. 8:4337-4347.

Kroos, L., Kuspa, A., and Kaiser, D. 1986. A global analysis of developmentally regulated genes in *Myxococcus xanthus*. Dev. Biol. 117:252-266.

Kroos, L., Kuspa, A., and Kaiser, D. 1990. Defects in fruiting body

development caused by Tn*5-lac* insertions in *Myxococcus xanthus*. J. Bacteriol. 172:484-487.
Kullik, I., Fritsche, S., Knobel, N., Sanjuan, J., Hennecke, H., and Fischer, H.-M. 1991. *Bradyrhizobium japonicum* has two differentially regulated, functional homologs of the σ^{54} gene (*rpoN*). J. Bacteriol. 173:1123-1138.
Law, J. H., and Slepecky, R. A. 1961. Assay of poly-β-hydroxybutyric acid. J. Bacteriol. 82:33-36.
Magasanik, B. 1982. Genetic control of nitrogen assimilation in bacteria. Annu. Rev. Genet. 16:135-168.
Manian, S. S., and O'Gara, F. 1982. Induction and regulation of ribulose-bisphosphate carboxylase activity in *Rhizobium japonicum* during formate dependent growth. Arch. Microbiol. 131:51-54.
Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Marmur, J., and Doty, P. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. 5:109-118.
Martin, G. B., Chapman, K. A., and Chelm, B. K. 1988. Role of the *Bradyrhizobium japonicum ntrC* gene product in differential regulation of the glutamine synthetase II gene (*glnII*). J. Bacteriol. 170:5452-5249.
Mathiopoulos, C., and Sonenshein, A. L. 1989. Identification of *Bacillus subtilis* genes expressed early during sporulation. Mol. Microbiol. 3:1071-1081.
Messing, J. 1983. New M13 vectors for cloning. Meth. Enzymol. 101:20-78.
Mulligan, J. T., and Long, S. R. 1985. Induction of *Rhizobium meliloti nodC* expression by plant exudate requires *nodD*. Proc. Natl. Acad. Sci. USA 82:6609-6613.
Nap, J.-P., and Bisseling, T. 1990. Developmental biology of a plant-prokaryote symbiosis: The legume root nodule. Science 250:948-954.
Poncz, M., Solowiejczyk, D., Ballantine, M., Schwartz, E., and Surrey, S. 1982. "Nonrandom" DNA sequence analysis in bacteriophage M13 by the dideoxy chain-termination method. Proc. Natl. Acad. Sci. USA 79:4298-4302.
Prakash, R. K., Van Brussel, A. A. N., Quint, A., Mennes, A. M., and Schilperoort, R. A. 1982. The map position of sym-plasmid regions expressed in the bacterial and endosymbiotic form of *Rhizobium leguminosarum*. Plasmid 7:281-286.
Quinto, C., de la Vega, H., Flores, M., Fernandez, L., Ballado, T., Soberon-Chavez, G., and Palacios, R. 1982. Reiteration of nitrogen fixation gene sequences in *Rhizobium phaesoli*. Nature (London) 299:724-726.
Rao, R. N., and Rogers, S. G. 1979. Plasmid pKC7: A vector containing ten restriction endonuclease sites suitable for cloning DNA segments. Gene 7:79-82.
Renalier, M. H., Batut, J., Ghai, J., Terzaghi, B., Gherardi, M., David, M., Garnerone, A.-M., Vasse, J., Truchet, G., Huguet, T., and Boistard, P. 1987. A new symbiotic cluster on the pSym megaplasmid of *Rhizobium meliloti* 2011 carries a functional *fix* gene repeat and a *nod* locus. J. Bacteriol. 169:2231-2238.
Rostas, K., Kondorosi, E., Horvath, B., Simoncsits, A., and Kondorosi, A. 1986. Conservation of extended promoter regions of nodulation genes in *Rhizobium*. Proc. Natl. Acad. Sci. USA 83:1757-1761.
Ruvkun, G. B., and Ausubel, F. M. 1981. A general method for site-directed mutagenesis in prokaryotes. Nature (London) 289:85-88.
Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain termination inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.
Schloss, J. A., Silflow, C. D., and Rosenbaum, J. L. 1984. mRNA abundance changes during flagellar regeneration in *Chlamydomonas reinhardtii*. Mol. Cell. Biol. 4:424-434.
Scott-Craig, J. S., Guerinot, M. L., and Chelm, B. K. 1991. Isolation of *Bradyrhizobium japonicum* DNA sequences that are transcribed at high levels in bacteroids. Mol. Gen. Genet. 228:356-360.
Verma, D. P. S., Fortin, M. G., Stanley, J., Mauro, V. P., Purohit, S., and Morrison, N. 1986. Nodulins and nodulin genes of *Glycine max*. Plant Mol. Biol. 7:51-61.
Vieira, J., and Messing, J. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259-268.
Wong, P. P., and Evans, H. J. 1971. Poly-β-hydroxybutyrate utilization by soybean (*Glycine max* Merr.) nodules and assessment of its role in maintenance of nitrogenase activity. Plant Physiol. 47:750-755.
Yanisch-Perron, C., Vieira, J., and Messing, J. 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103-119.
Zimmerman, C. R., Orr, W. C., Leclerc, R. F., Barnard, E. C., and Timberlake, W. E. 1980. Molecular cloning and selection of genes regulated in *Aspergillus* development. Cell 21:709-715.