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Bradyrhizobium japonicum Has Two Differentially Regulated, Functional Homologs of the σ^{54} Gene (*rpoN*)

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Recognition of −24/−12-type promoters by RNA polymerase requires a special sigma factor, σ^{54} (RpoN NtrA GlnF). In the nitrogen-fixing soybean symbiont *Bradyrhizobium japonicum*, two functional, highly conserved *rpoN* genes (*rpoN*$_1$ and *rpoN*$_2$) were identified and sequenced. The two predicted *B. japonicum* RpoN protein sequences were 87% identical, and both showed different levels of homology to the RpoN proteins of other bacteria. Downstream of *rpoN*$_2$ (but not of *rpoN*$_1$), two additional open reading frames were identified that corresponded to open reading frames located at similar positions in *Klebsiella pneumoniae* and *Pseudomonas putida*. Both *B. japonicum rpoN* genes complemented the succinate- and nitrate-negative phenotypes of a *Rhizobium meliloti rpoN* mutant. *B. japonicum* strains carrying single or double *rpoN* mutations were still able to utilize C_4-dicarboxylates as a carbon source and histidine, proline, or arginine as a nitrogen source, whereas the ability to assimilate nitrate required expression of at least one of the two *rpoN* genes. In symbiosis both *rpoN* genes could replace each other functionally. The *rpoN*$_{1/2}$ double mutant induced about twice as many nodules on soybeans as did the wild type, and these nodules lacked nitrogen fixation activity completely. Transcription of a *nifH'-'lacZ* fusion was not activated in the *rpoN*$_{1/2}$ mutant background, whereas expression of a *fixR'-'lacZ* fusion in this mutant was affected only marginally. By using *rpoN'-'lacZ* fusions, *rpoN*$_1$ expression was shown to be activated at least sevenfold in microaerobiosis as compared with that in aerobiosis, and this type of regulation involved *fixLJ*. Expression of *rpoN*$_2$ was observed under all conditions tested and was increased fivefold in an *rpoN*$_2$ mutant. The data suggested that the *rpoN*$_1$ gene was regulated in response to oxygen, whereas the *rpoN*$_2$ gene was negatively autoregulated.

In eubacteria promoter recognition specificity is provided to the RNA polymerase core enzyme $\alpha_2\beta\beta'$ by an additional protein factor, the σ factor. Binding of the RNA polymerase holoenzyme $\alpha_2\beta\beta'\sigma$ to a promoter sequence results in the formation of a closed complex, which is then converted to an open complex characterized by local melting of the double-stranded DNA. Once transcription is initiated, the σ factor is released from the complex and the core enzyme continues with RNA synthesis (for a review, see reference 34).

In *Escherichia coli* the most abundant σ factor required for the expression of many housekeeping genes is σ^{70}, the product of the *rpoD* gene. It allows recognition of and transcription from canonical −35/−10 promoters. Several alternative σ factors have been identified in enteric bacteria which enable the cells to transcribe specific sets of genes in response to environmental stimuli. Examples are the heat shock σ factor σ^{32} (RpoH [29]); an alternate heat shock factor, σ^{E} (23); σ^{F}, which is used for expression of flagellar, chemotaxis, and motility genes (6); and σ^{54} (RpoN, NtrA), originally described as a σ factor involved in the expression of nitrogen-regulated (*ntr*) genes (50, 56).

Promoters recognized by RNA polymerase containing σ^{54} show characteristic sequence motifs around positions −24 and −12 relative to the start of transcription (5'-CTGGCAC-N_5-TTGCA-3' [8]). Data accumulated during the recent years clearly demonstrate that −24/−12-type promoters not only are confined to nitrogen-controlled genes but also are present in front of a great variety of other genes within gram-negative bacteria (for reviews, see references 48 and 85). In all cases investigated, transcription from these promoters was shown to depend on a specific activator protein whose activity was modulated by physiological signals.

rpoN-like genes or the corresponding gene products have been identified genetically or functionally in the following bacteria: *E. coli* (41, 50, 78), *Salmonella typhimurium* (38, 50), *Klebsiella pneumoniae* (19, 56, 58), *Pseudomonas aeruginosa* (43, 86), *Pseudomonas facilis* (72), *Pseudomonas putida* (42, 45, 46), *Alcaligenes eutrophus* (71, 72), *Azotobacter vinelandii* (57), *Rhizobium meliloti* (73), *Rhizobium* sp. strain NGR234 (81, 88), *Rhodobacter capsulatus* (1, 44, 47), and *Thiobacillus ferrooxidans* (7). The DNA sequences of the *rpoN* genes of several species have been determined, and the deduced protein sequences were clearly homologous (see Fig. 4). Interestingly, no substantial homology to other known bacterial σ factors was observed. Consistent with the finding that, within a given species, −24/−12 promoters were associated with functionally unrelated genes, many *rpoN* mutants showed a pleiotropic phenotype. For example, an *R. meliloti rpoN* mutant was not only unable to fix nitrogen symbiotically but also was affected in nitrate assimilation, transport of C_4-dicarboxylates, and nodulation efficiency (22, 73).

In the soybean root nodule endosymbiont *Bradyrhizobium japonicum*, numerous genes involved in nitrogen fixation (*nif* and *fix* genes) have been identified in at least two chromosomal gene clusters (35). Most of them were shown by sequence analysis and transcript mapping to be preceded by −24/−12 promoters (36). In addition, −24/−12 promoters were identified upstream of the *B. japonicum glnII* and *glnB*

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genes, which are involved in nitrogen metabolism (11, 52). The transcriptional activator protein of the latter two genes is NtrC, whereas *nif* and *fix* genes are regulated by NifA (3, 26, 30, 52). As in *K. pneumoniae*, upstream activator sequences having the consensus sequence 5′-TGT-N_{10}-ACA-3′ were identified around 100 to 150 bp upstream of the transcriptional start sites of *B. japonicum nif* and *fix* genes (2, 10, 36). For the *K. pneumoniae nifH* promoter there is evidence that NifA binds to the upstream activator sequence, thereby activating transcription by causing the isomerization of a closed σ^{54}-RNA polymerase-DNA complex to an open complex (10, 62, 63). A similar mechanism may occur for *B. japonicum nif* gene activation. Indirect evidence that predicted the existence of a σ^{54}-like protein in *B. japonicum* came from the observation that activation of *B. japonicum nifH′-* and *nifD′-′lacZ* translational fusions in *E. coli* was dependent on the presence of a functional *E. coli rpoN* gene (3).

In *B. japonicum* the NifA protein, whose synthesis and activity are controlled by the cellular oxygen status, is encoded in the *fixRnifA* operon, which is preceded by a putative −24/−12-type promoter (27, 83, 84). However, this promoter is not activated in *E. coli*, and mutations in the −12 region but not those in the −24 region reduced its activity in *B. japonicum* (84). Under aerobic conditions the *fixRnifA* operon is expressed at a basal level. This expression depends on an upstream DNA sequence element that is located around position −66 relative to the transcriptional start site (83, 84). An unknown protein present in *B. japonicum* crude extracts binds to this DNA element and is postulated to function as a transcriptional activator of the *fixR* promoter (83). Under microaerobic or anaerobic conditions, *fixRnifA* expression is increased at least fivefold by a mechanism involving NifA (83). In contrast to the *B. japonicum* gene, the *R. meliloti nifA* gene is not expressed aerobically but is induced under low oxygen conditions by FixJ (17, 89). This protein is an activator protein which, together with the FixL protein, forms an oxygen-responsive two-component regulatory system. Recently *fixLJ*-like genes were also identified in *B. japonicum*, but they were probably not involved in regulation of *fixRnifA* expression (4). Therefore, to further analyze the structure and regulation of the complex *fixR* promoter, we were interested in obtaining *B. japonicum rpoN* mutants.

Here we report the identification and analysis of two highly homologous *rpoN* genes in *B. japonicum*. Single and double *rpoN* mutants were created, and both *rpoN* genes were thus shown to be functional in free-living and symbiotic conditions. The effect of *rpoN* mutations on *nifH* and *fixR* promoter activities was studied under different growth conditions. Finally, we provide evidence that expression of one *rpoN* gene is oxygen controlled by a mechanism that involves *fixLJ* and that the other *rpoN* gene is negatively autoregulated.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids used in this work are listed in Table 1.

Media and growth of cells. For growth of *E. coli* cells, LB medium (61) was used. PSY (69) or YEM (16) medium was used for routine growth of *B. japonicum* and *R. meliloti* cells. For characterization of the *B. japonicum rpoN* mutants, RDM minimal medium (73, 74) with glucose, succinate, fumarate, or malate (20 mM) as the carbon source and NH_4Cl (5 mM) or KNO_3 (10 mM) as the nitrogen source was used. Complementation tests with the *R. meliloti rpoN* mutant strain were performed as described previously (73). Oxygen-limited cultures were grown under a gas atmosphere that initially contained 2% oxygen. Anaerobic cultures were grown under argon in YEM medium containing 10 mM KNO_3 and spectinomycin as the only antibiotic. The concentrations of antibiotics in *E. coli* or *B. japonicum* cultures were as follows (micrograms per milliliter): ampicillin, 200; chloramphenicol, 20; kanamycin, 30 or 100; spectinomycin, 20 or 100; streptomycin, 50 or 100; tetracycline, 10 or 120. In *B. japonicum* liquid cultures, tetracycline at 50 μg ml^{-1} was used. For *R. meliloti*, streptomycin and tetracycline were used at 100 and 15 μg ml^{-1}, respectively.

Recombinant DNA work. For routine work with recombinant DNA, established protocols were used (51). Plasmid pNtr3.5EB (73) was used as the source for a specific *R. meliloti rpoN* DNA probe. A 2,033-bp *Apa*I-*Hin*dIII fragment was isolated and radioactively labeled by nick translation. This fragment contained 49 bp of noncoding DNA from the *rpoN* 5′ region, the complete *R. meliloti rpoN* gene, and 413 bp of 3′ DNA including 53 codons of the open reading frame (ORF) located downstream of *rpoN*. Homologous hybridizations were performed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 68°C, whereas 5× SSC at 58°C was used for heterologous (interspecies) hybridizations.

DNA sequence analysis. The dideoxynucleotide chain termination method was used (77). Defined restriction fragments covering the *B. japonicum* $rpoN_1$ and $rpoN_2$ regions were cloned into M13 vectors mp18 and mp19, and single-stranded DNA for sequencing was obtained by conventional methods. In addition, starting with plasmids pRJ7693 and pRJ7694 (Fig. 1A), a set of ordered $rpoN_1$ deletion clones was constructed by using the exonuclease III-mung bean nuclease system (Stratagene, La Jolla, Calif.). Computer-assisted DNA and protein sequence analyses were performed by using the UWGCG (Genetics Computer Group of the University of Wisconsin, Madison) and the PC GENE (Genofit, Geneva, Switzerland) software packages. Multiple sequences were aligned with the program MULTALIN (14).

Construction of *B. japonicum rpoN* mutants. Site-directed mutagenesis of the *B. japonicum* $rpoN_1$ and $rpoN_2$ genes was performed by marker exchange via reciprocal homologous recombination. Suitable constructs were cloned into the vector pSUP202 and mobilized from *E. coli* S17-1 to *B. japonicum* as described previously (32). In mutant N50 a 258-bp $rpoN_1$-internal *Sal*I fragment was replaced by a 2,347-bp *Xho*I fragment from Tn*5* carrying the kanamycin resistance marker (*aphII* gene) (Fig. 1A). Similarly, in mutant N63 this kanamycin resistance cassette was used to replace a 327-bp $rpoN_2$-internal *Xho*I fragment (Fig. 1B). Mutant N97 was constructed by replacing a 258-bp $rpoN_2$-internal *Sal*I fragment with a 2-kb *Sma*I fragment that originated from the interposon Ω and conferred resistance to streptomycin (Fig. 1B). Thus, mutant strains N63 and N97 differed only by the location and type of the resistance gene inserted into $rpoN_2$. Strains N50 and N63 were used in a comparative analysis of the growth characteristics of individual *B. japonicum rpoN* mutants, since they allowed application of identical antibiotic selection conditions. To obtain the double mutant strain N50-97, the $rpoN_2$ gene was mutagenized in the $rpoN_1$ mutant strain N50; the second mutation was created in the same way that the wild type was mutated to give strain N97. In all four mutants the resistance genes were in opposite orientation to the *rpoN* genes. The Tn*5* insertion mutations downstream of $rpoN_1$ and $rpoN_2$

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
HB101	Sm^r *hsdR hsdM recA13*	18
MC1061	Sm^r Δ(*lacIPOZYA*)*X74 hsdR*	12
RR28	Sm^r *hsdR hsdM recA lac*	37
JM101	*supE thi*Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ* ΔM15]	59
JM103	*supE thi strA* Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ* ΔM15]	60
S17-1	Sm^r Sp^r *hsdR* (RP4-2 *kan*::Tn*7 tet*::Mu, integrated in the chromosome	79
R. meliloti		
2011	Wild type	C. W. Ronson
1681	Sm^r Km^r *rpoN*::Tn*5*	73
B. japonicum		
110*spc*4	Sp^r (wild type)	69
110*spc*4-48	Sp^r Tc^r *nifH′-′lacZ* integrated in the chromosome	30
110*spc*4-7290R	Sp^r Tc^r *fixR′-′lacZ* integrated in the chromosome	82a
8003	Sp^r Tc^r *rpoN*$_1$*′-′lacZ* integrated in the chromosome	This work
8015	Sp^r Tc^r *rpoN*$_2$*′-′lacZ* integrated in the chromosome	This work
N50	Sp^r Km^r *rpoN*$_1$::*aphII*	This work
N63	Sp^r Km^r *rpoN*$_2$::*aphII*	This work
N97	Sp^r Km^r *rpoN*$_2$::Ω	This work
N50-97	Sp^r Km^r Sm^r *rpoN*$_1$::*aphII rpoN*$_2$::Ω	This work
N50-97H	Sp^r Km^r Sm^r Tc^r *rpoN*$_1$::*aphII rpoN*$_2$::Ω *nifH′-′lacZ* integrated in the chromosome	This work
N50-97R	Sp^r Km^r Sm^r Tc^r *rpoN*$_1$::*aphII rpoN*$_2$::Ω *fixR′-′lacZ* integrated in the chromosome	This work
7361	Sp^r Km^r *fixJ*::*aphII*	4
7404	Sp^r Km^r *fixL*::*aphII*	4
Plasmids		
pUC18	Ap^r	66
pUR2	Ap^r	75
M13 mp18		66
M13 mp19		66
pSUP202	Ap^r Cm^r Tc^r, *oriT* from RP4	79
pBluescript II KS$^+$	Ap^r f1 (+) *ori*	Stratagene, La Jolla, Calif.
pBluescript II KS$^-$	Ap^r f1 (−) *ori*	Stratagene
pMC1403	Ap^r *′lacZYA*	12
pSUP202::Tn*5*-17	Ap^r Cm^r Tc^r Km^r, *oriT* from RP4	31a
pHP45::Ω	Ap^r Sm^r Sp^r	67
pUC4-KIXX	Ap^r Km^r	Pharmacia LKB, Uppsala, Sweden
pRK2013	Km^r *tra*$^+$	25
pRK290X	Tc^r	2
pRK290lac	Tc^r (pRK290X) *′lacZYA*	25a
pPP375	Tc^r (pRK290) polylinker in *Eco*RI	67a
pNtr3.5BE	Ap^r (pUC8), *R. meliloti rpoN*	73
pL3-6H	Tc^r (pLAFR1), *B. japonicum rpoN*$_2$	25a
pRJ6048	Ap^r Tc^r (pSUP202) *nifH′- ′lacZ*	30
pRJ7290	Ap^r Tc^r (pSUP202) *fixR′-′lacZ*	82a
pRJ7688	Ap^r (pUC18), *B. japonicum rpoN*$_1$, 4.1-kb *Eco*RI-*Bam*HI fragment	This work
pRJ7693	Ap^r (pBluescript II KS$^-$), *B. japonicum rpoN*$_1$, 1,738-bp *Eco*RI-*Hin*dIII fragment	This work
pRJ7694	Ap^r (pBluescript II KS$^+$), *B. japonicum rpoN*$_1$, same insert as pRJ7693	This work
pRJ7697	Tc^r (pPP375), *B. japonicum rpoN*$_1$, 1.8-kb *Bam*HI-*Hin*dIII fragment from pRJ7693	This work
pRJ7722	Ap^r (pUC18), *B. japonicum rpoN*$_2$, 9-kb *Eco*RI-*Bam*HI fragment	This work
pRJ7734	Ap^r (pBluescript II KS$^+$), *B. japonicum rpoN*$_2$, 2,982-bp *Eco*RI-*Cla*I fragment	This work
pRJ8000	Ap^r (pMC1403) *rpoN*$_1$*′-′lacZ*	This work
pRJ8002	Tc^r (pRK290X) *rpoN*$_1$*′-′lacZ*	This work
pRJ8003	Ap^r Tc^r (pSUP202) *rpoN*$_1$*′-′lacZ*	This work
pRM8005	Tc^r (pRK290X), *R. meliloti rpoN*, 3.5-kb *Eco*RI-*Bam*HI fragment from pNtr3.5BE, *Bam*HI converted to *Eco*RI with *Eco*RI linker	This work
pRJ8009	Ap^r (pMC1403) *rpoN*$_2$*′-′lacZ*	This work
pRJ8013	Tc^r (pRK290X) *rpoN*$_2$*′-′lacZ*	This work
pRJ8015	Ap^r Tc^r (pSUP202) *rpoN*$_2$*′-′lacZ*	This work
pRJ8019	Tc^r (pRK290X), *B. japonicum rpoN*$_2$, 3-kb *Bam*HI-*Bgl*II fragment from pRJ7734	This work

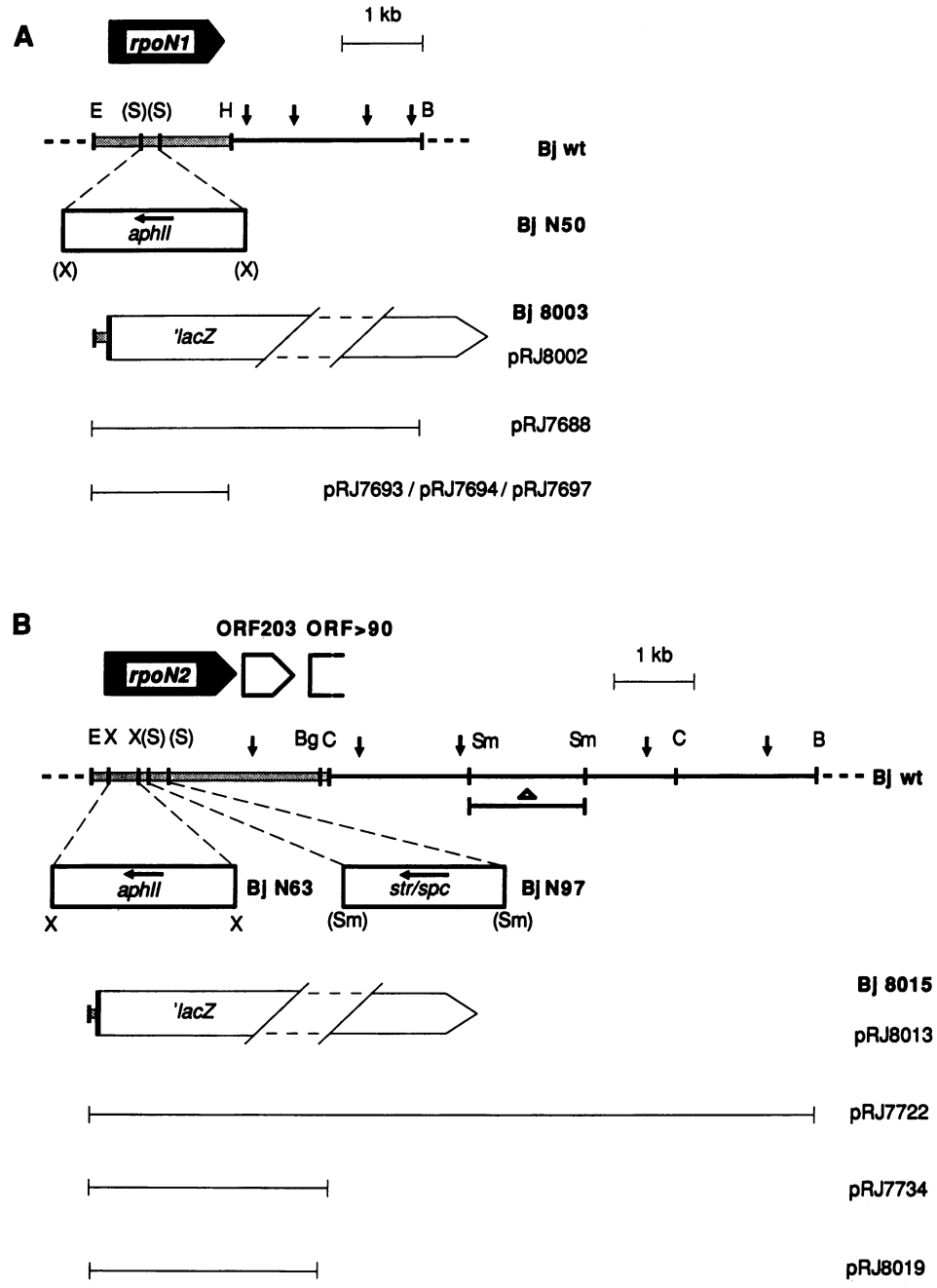

FIG. 1. Physical maps of the *B. japonicum* $rpoN_1$ (A) and $rpoN_2$ (B) regions. The regions that were sequenced (Fig. 2 and 3) are shown as dotted bars. The structures of the $rpoN_1$ and $rpoN_2$ deletion-replacement mutants N50, N63, and N97 are shown below the wild-type maps. The vertical arrows indicate positions of Tn*5* insertions. In panel B the 1.4-kb *Sma*I fragment that was deleted in one of the mutant strains is marked (△; for details, see the text). The structures of the extrachromosomal and chromosomally integrated *rpoN'-'lacZ* fusions are presented together with the corresponding plasmid and strain numbers, respectively. The relevant DNA fragments cloned during this work and the corresponding plasmid numbers are shown in the lower parts of both panels. Restriction sites within parenthesis were lost during cloning procedures. Only the relevant *Sal*I restriction sites are shown. Restriction sites are abbreviated as follows: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Sm, *Sma*I; X, *Xho*I.

were obtained by fragment-specific mutagenesis with the inserts of plasmids pRJ7688 ($rpoN_1$; Fig. 1A) and pRJ7722 ($rpoN_2$; Fig. 1B) as targets (32, 33). To confirm the genomic structures of the mutants, total genomic DNA from selected clones was analyzed by appropriate Southern blot hybridization.

Construction of *rpoN'-'lacZ* fusions. To construct the translational $rpoN_1$'-'*lacZ* fusion a 247-bp *Eco*RI-*Pvu*II fragment (nucleotides 1 to 247 in Fig. 2) containing 187 bp of noncoding DNA upstream of $rpoN_1$ and 20 codons of $rpoN_1$ was cloned into pMC1403 linearized with *Eco*RI and *Sma*I. From the resulting plasmid pRJ8000 the $rpoN_1$'-'*lacZ* fusion was

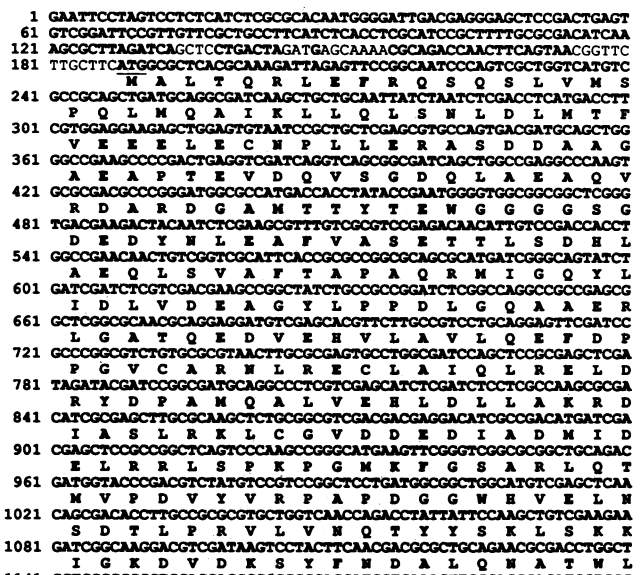

FIG. 2. Nucleotide sequence of a 1,738-bp *Eco*RI-*Hin*dIII fragment carrying the *rpoN*$_1$ gene. The amino acid sequence of the predicted RpoN$_1$ protein is shown below the nucleotide sequence. The putative start codon is underlined. Nucleotide sequence motifs present upstream of both *rpoN* genes are indicated in normal print (see Fig. 3).

isolated as a 6.45-kb *Eco*RI-*Sal*I fragment and subcloned into pRK290X digested with *Eco*RI and *Xho*I. This yielded plasmid pRJ8002 (Fig. 1A), which was mobilized into *B. japonicum*. The analogous plasmids, pRJ8009 (Table 1) and pRJ8013 (Fig. 1B), carrying the *rpoN*$_2$'-'*lacZ* fusion were constructed similarly starting from a 166-bp *Eco*RI-*Pvu*II fragment (nucleotides 1 to 166 in Fig. 3) containing 116 bp of noncoding DNA upstream of *rpoN*$_2$ and 20 codons of *rpoN*$_2$. The *Eco*RI-*Dra*I fragments (approximately 3.4 kb) of the *rpoN*$_1$'- and *rpoN*$_2$'-'*lacZ* fusion plasmids pRJ8000 and pRJ8009 were subcloned into the *Eco*RI site of pSUP202. For this purpose the *Dra*I sites were converted to *Eco*RI sites by ligation of *Eco*RI linkers. The resulting plasmids, pRJ8003 and pRJ8015, were cointegrated into the chromosome of wild-type *B. japonicum* yielding *B. japonicum* 8003 and 8015 (Fig. 1; see below).

Integration of *lacZ* fusions into the *B. japonicum* chromosome. For gene expression studies in bacteroids it was necessary to integrate *lacZ* fusions into the *B. japonicum* chromosome because plasmid-borne fusions were rapidly lost in the absence of antibiotic selection in symbiosis. Translational *lacZ* fusions to *rpoN*$_1$ (pRJ8003) and *rpoN*$_2$ (pRJ8015) were integrated in separate experiments into the chromosome of wild-type *B. japonicum*. The chromosome of the *rpoN* double mutant strain N50-97 was the target for the

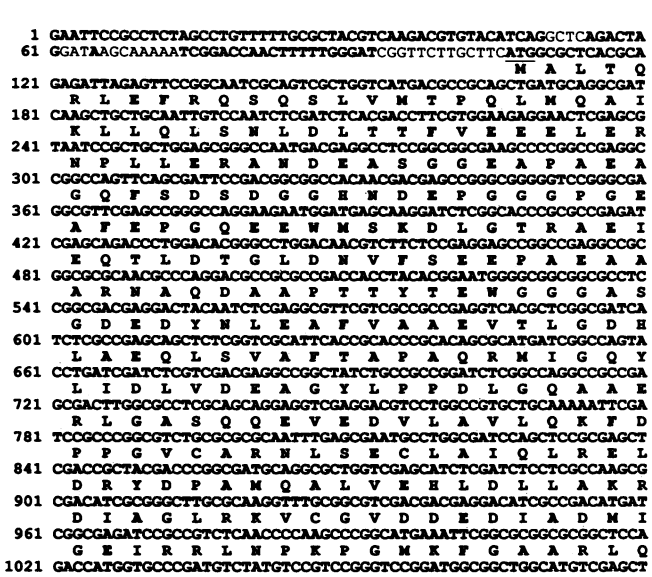

FIG. 3. Nucleotide sequence of a 2,982-bp *Eco*RI-*Cla*I fragment carrying the *rpoN*$_2$ gene, ORF203, and the 5' end of ORF>90. The amino acid sequences of the predicted proteins are given below the nucleotide sequence. The putative translational start codons and potential, purine-rich Shine-Dalgarno sequences in front of ORF203 and ORF>90 are underlined. The nucleotides shown in normal print upstream of *rpoN*$_2$ refer to sequence motifs also found in front of *rpoN*$_1$ (Fig. 2).

individual integration of a *nifH'-'lacZ* fusion (pRJ6048) and a *fixR'-'lacZ* fusion (pRJ7290). Integration was achieved by conjugational transfer of the respective plasmids into the recipient strains and selection for the desired exconjugants with tetracycline. In the resulting strains (*B. japonicum* 8003, 8015, N50-97H, N50-97R), the *lacZ* fusions were integrated by recombination upstream of the homologous gene and expressed from the corresponding promoter. In *B. japonicum* 8003 and 8015, intact copies of both *rpoN* genes were present besides the *lacZ* fusion. The genomic structure of all strains was confirmed by appropriate Southern blot hybridization.

Mating procedures. Plasmids were introduced into strains of *B. japonicum* or *R. meliloti* either by matings with *E. coli* S17-1 as a donor (79) or by triparental matings with pRK2013 as the mobilizing helper plasmid.

β-Galactosidase assay. Determination of β-galactosidase activity in *B. japonicum* cells carrying *lacZ* fusions was done as described by Miller (61) and modified by Thöny et al. (84). Microaerobic cultures were grown in 7-ml Bijou bottles containing 5 ml of PSY medium. β-Galactosidase activity in root nodule bacteroids was assayed as reported by Gubler and Hennecke (30).

Plant infection test. Infection of soybean seedlings and determination of nitrogenase activity in bacteroids have been described previously (28, 32).

Nucleotide sequence accession number. The nucleotide sequences of *B. japonicum krpoN1* (Fig. 2) and *rpoN2* ORF203 ORF>90 (Fig. 3) have been deposited in GenBank under accession numbers M59242 and M59243, respectively.

RESULTS

Identification and cloning of the *B. japonicum rpoN*$_1$ and *rpoN*$_2$ genes. Total genomic *B. japonicum* DNA digested with different restriction endonucleases was hybridized with a radioactively labeled probe specific for the *R. meliloti rpoN* gene (see Materials and Methods). Two *Eco*RI-*Bam*HI fragments of approximately 4.1 and 9 kb in size hybridized strongly, suggesting the presence of two *rpoN*-like genes (data not shown). To clone the 4.1-kb fragment, *B. japonicum* genomic DNA cut with *Eco*RI and *Bam*HI was size fractionated on an agarose gel, and the fragments in the 3.5- to 5-kb range were isolated. This fraction was ligated to vector pUC18 linearized with *Eco*RI and *Bam*HI, and the ligation products were transformed into *E. coli* MC1061. Colonies hybridizing with the *R. meliloti rpoN* probe were shown to contain plasmids (pRJ7688) with the expected 4.1-kb *Eco*RI-*Bam*HI fragment (Fig. 1A). Restriction analysis and hybridizations delimited the presumptive *B. japonicum rpoN*$_1$ gene on a 1,738-bp *Eco*RI-*Hin*dIII fragment that was subcloned in plasmids pRJ7693 and pRJ7694 (Fig. 1A).

When pRJ7688 was used to probe a *B. japonicum* cosmid library, the cosmid pL3-6H was found to hybridize strongly, but its restriction pattern was different from that of the *rpoN*$_1$ region cloned in pRJ7688 (data not shown). Further subcloning showed that the hybridizing region on pL3-6H was located on a 9-kb *Eco*RI-*Bam*HI fragment (pRJ7722; Fig. 1B) and, more precisely, on a 2,982-bp *Eco*RI-*Cla*I fragment (pRJ7734; Fig. 1B). It was concluded that *B. japonicum* contains two homologous chromosomal regions encoding presumptive *rpoN* genes (*rpoN*$_1$ and *rpoN*$_2$). As observed initially (see above), both regions hybridized with the *R. meliloti rpoN* probe.

DNA sequence analysis of the *rpoN*$_1$ and *rpoN*$_2$ regions. To analyze the two *rpoN* homologous regions of *B. japonicum* in more detail, their nucleotide sequences were established (Fig. 2 and 3). On the sequenced 1,738-bp *Eco*RI-*Hin*dIII fragment of the *rpoN*$_1$ region (Fig. 1A) an ORF of 1,452 bp was identified which encoded a predicted 484-amino-acid (aa) protein with a molecular weight of 53,772. The ATG at position 188 (Fig. 2) was assigned as translational start codon because the homology of the deduced RpoN$_1$ protein sequence to other known RpoN proteins started thereafter (Fig. 4). However, no obvious Shine-Dalgarno-like sequence was detected at the appropriate distance in front of this presumptive ATG.

Sequence analysis of the 2,982-bp *Eco*RI-*Cla*I fragment spanning the *rpoN*$_2$ region (pRJ7734; Fig. 1B) revealed the presence of three ORFs with the same orientation (Fig. 3). Based on sequence homology, the first 1,611-bp ORF was named *rpoN*$_2$. This gene could be translated into a predicted protein of 537 aa with a molecular weight of 58,831. For the assignment of the ATG start codon at position 107 (Fig. 3), the same criteria were applied as for the start of *rpoN*$_1$ (see above). As with *rpoN*$_1$, no *E. coli*-like Shine-Dalgarno sequence was found in front of *rpoN*$_2$. In fact, the 13 nucleotides immediately 5′ to the ATGs were identical in both *rpoN* genes. Further upstream, separated by 32 bp (*rpoN*$_1$) or 33 bp (*rpoN*$_2$) from the translational starts, the following sequence element was present in both genes: 5′-GCTC-7 bp-GATRAGCAAAA-3′ (Fig. 2 and 3). Whether this element plays a functional role has not been investigated. Beyond this element no further similarities were detected in the sequenced 5′ regions. In the 3′ regions the homology between *rpoN*$_1$ and *rpoN*$_2$ ended within the coding sequence at about 50 bp (*rpoN*$_1$) or 70 bp (*rpoN*$_2$) before their respective stop codons. Results from hybridization experiments suggested that the regions downstream of *rpoN*$_1$ and *rpoN*$_2$ were not homologous (data not shown).

Amino acid sequence comparison between RpoN proteins. Comparison of the amino acid sequences (Fig. 4) revealed an 87% identity and a 91% similarity between the two *B. japonicum* RpoN proteins. A similarity coefficient (S_{AB} value) of 0.82 was calculated. Forty-eight of the 53 aa by which RpoN$_2$ was longer than RpoN$_1$ were located between a conserved short NH_2-terminal domain (72 aa) and the long remainder of the protein (approx. 350 aa) that was also highly conserved. Interestingly, the spacer separating the short NH_2-terminal and the long COOH-terminal conserved domains is the least conserved region in all seven RpoN protein sequences shown in Fig. 4. Apart from this extra stretch of amino acids present in RpoN$_2$, the *B. japonicum* RpoN proteins differed in 70 aa positions; only 50 of these 70 changes (20 at the very COOH-terminal ends) were nonconservative (applying the amino acid similarity groups as defined by Helmann and Chamberlin [34]).

The comparison of seven RpoN protein sequences showed a fairly good overall conservation (Fig. 4). The similarity between the RpoN proteins of *B. japonicum* and five other bacterial species decreased in the order *R. meliloti* > *P. putida*, *K. pneumoniae*, *A. vinelandii* > *R. capsulatus*. Stretches of particularly high homologies were located in the NH_2-terminal parts (about 50 aa) and in the COOH-terminal regions (about 100 to 125 aa). A sequence of 9 contiguous, absolutely conserved aa (ARRTVAKYR) was detected in all seven proteins (between positions 344 and 352 in the *B. japonicum* RpoN$_1$ protein). Recently, this element, which is also present in the RpoN proteins of *E. coli* (78) and *Rhizobium* sp. strain NGR234 (88), was termed a core RpoN box (88).

Because of the high similarity between the *B. japonicum*

```
BJ1   1 MALTQRLEFRQSQSLVMSPQLMQAIKLLQLSNLDLMTFVEEELECNPLLERASDDAAGAEAPTEVDQVSGDQ........
BJ2   1 MALTQRLEFRQSQSLVMTPQLMQAIKLLQLSNLDLTTFVEEELERNPLLERANDEASGGEAPAEAGQFSDSDGGHNDEPG
RM    1 MALSASLHLRQSQSLVMTPQLMQSIQLLQMNHLELSHFIAQEVEKNPLLEVQPADEPTISDREDAGPHPAETGGETDEAA
PP    1   MKPSLVLKMGQQLTMTPQLQQAIRLLQLSTLDLQQEIQEALESNPMLERQ.EDGEDFDNSDPMADNAENK..PAAEVQ
AV    1   MKPSLVLKMGQQLTMTPQLQQAIRLLQLSTLDLQQEIQEALDSNPMLERQ.EDAEDYDSPDMLGEHGDQS..TLDTTP
KP    1   MKQGLQLRLSQQLAMTPQLQQAIRLLQLSTLELQQELQQALDSNPLLEQT.D.........LHDEVETK..EAED..
RC    1   MELAQTLSQRQTMQMAGQMLHSLAILGMSSQDLSEHLTEQATSNPFLTYR..........................

con       M   L LRQSQ L MTPQL QAI LLQLS LDL     E LESNPLLER   D


BJ1  73 ..........................L............AEAQVRDARDGAMTTYTEWGGGGSGDEDYNLEAF.VASE
BJ2  81 GGPGEAFEPGQEEWMSKDLGTRAEIEQTLDTGLDNVFSEEPAEAAARNAQDAAPTTYTEWGGGASGDEDYNLEAF.VAAE
RM   81 G.QSDLYDSA....MSRS.GER..LSEGLDADFANVF...PDDTAPQRADAPELLGQWKSMPGAGDAEGYDLDDF.VGGR
PP   76 ...DNSFQE..........STVSADN..LE...DGEWSERIPNELPVDTAWEDIYQTSASSLPSNDDDEWDFTT.RTSAG
AV   76 GSYQEGYES..........GAASEDGGTLE...EGDWHERIPSELPVDTAWEDIYQTSASNLPSTDEDEWDFTT.RTSTG
KP   64 ...RESLDT..........VDA......LE...QKE....MPEELPLDASWDEIYTAGTPSGNGVDYQDDELPVYQGETT
RC   51 ..................................................APPAFIARGGEDFDAVGAVAAHK

con                                  L                P DA                 D ED DL


BJ1 112 TTLSDHLAEQLSVAFTAPAQRMIGQYLIDLVDEAGYLPPDLGQAAERLGAT...QEDVEHV.LAVLQEFDPPGVCARNLR
BJ2 160 VTLGDHLAEQLSVAFTAPAQRMIGQYLIDLVDEAGYLPPDLGQAAERLGAS...QQEVEDV.LAVLQKFDPPGVCARNLS
RM  149 KTLRETLAEQLPFALSAVSDRLIARYFIDQLDDAGYLHADLAETAETLGAA...GEDVARV.LHVLQQFDPPGVFARTLG
PP  137 ESLQSHLLWQLNLAPMSDTDRLIAVTLIDSINGQGYLEDTLEEICAGFDPELDIELDEVEAVLHRIQQFEPAGVGARNLG
AV  142 ESLQSHLLWQLNLTPMSDTDRLIAVTLIDSINSDGYLEAALEEILASLDPELGVELDEVEMVLRRIQQFEPAGIAARDLS
KP  118 QSLQDYLMWQVELTPFTDTDRAIATSIVDAVDDTGYLTISVEDIVESIGDD.EIGLEEVEAVLKRIQRFDPVGVAAKDLR
RC   74 PSLMAHVVDQIEMAFTETPDRLLALRFAEALEPSGWLGQSLDSIALAAGVS....LSRAESMLAVLQGFEPTGLFARDLS

con      SL  HL  QL  A     DRLIA  LID  D  GYL   L  IAE LG      LD  E  L VLQ FDP GV AR L


BJ1 188 ECLAIQLRELD....RYDPAMQALVEHLDLLAKRDIASLRKLCGVDDEDIADMIDELRRLSPKPGMKFGSARLQTMVPDV
BJ2 236 ECLAIQLRELD....RYDPAMQALVEHLDLLAKRDIAGLRKVCGVDDEDIADMIGEIRRLNPKPGMKFGAARLQTMVPDV
RM  225 ECLAIQLRARN....RLDPAMEALVANLELLARRDFASLKKICGVDEEDLIDMLAEIRKLDPKPGTSFETGVFEAIIPDV
PP  217 ECLLLQLRQLPATTPWMTEAKRLVTDFIDLLGSRDYSQLMRRMKIKEDELRQVIELVQSLNPRPGSQIESSEPEYVVPD.
AV  222 ESLLLQLRQLPPDTPWLEEAKRLAKDYLDLLGNRDFTQLMRRMKLKEEELRPVIELIQSLNPRPGAQIESSEPEYVVPD.
KP  197 DCLLVQLSQFAKETPWIEEARLIISDHLDLLANHDFRSLMRVTRLKEEVLKEAVNLIQSLDPRPGQSIQTGEPEYVIPD.
RC  150 DCLILQAREAD....ILTWEVETLIRNIRLIAENRLSDLADLCDCDIGDIPEIIKQIRHLNPKPGLAFDHQPTPVFPPD.

con      ECL  QLR L         A   L   LDLLA RD   L   C  DEEDL   I  IR LNPKPG  F     E  VPD


BJ1 264 YVRPAPDGGWHVELNSDTLPRVLVNQTYYSKLSKKIGKD.VDKSYFNDALQNATWLVRALDQRARTILKVATEIVRQQDG
BJ2 312 YVRPGPDGGWHVELNSDTLPRVLVNQTYYSELSKKIGKD.GDKSYFTDALQNATWLVRALDQRARTILKVATEIVRQQDG
RM  301 VVRAAPDGGWLVELNPDALPRVLVNHDYFTEISRSSRKNSGEQAFLNECLQNANWLTRSLDQRARTIMKVASEIVRQQDA
PP  296 VIVRKDSDRWLVELNQEAIPRLRVNPQ.YAGFVRRADTS.ADNTFMRNQLQEARWFIKSLQSRNETLMKVATQIVEHQRG
AV  301 VYVRKHNDRWLVELNQEAVPRLRINPH.YAGFIRRADAS.ADNTFMRNQLQEARWFIKSLQSRNETLMKVSTQIVEHQRG
KP  276 VLVRKVNDRWVVELNSDSLPRLKINQQ.YAAMGNSTRND.ADQGFIRSNLQEARWLIKSLESRNDTLLRVSRCIVEQQQA
RC  225 LIAVRGAEGWTVELNRATSPTITVREDRFADGTADAKAR.AERR.RRGRGPGAG...EALERRRDTLLRTAAVLVARQSA

con      V       GW VELN D LPR  VN   YA          AD  F R  LQ A WL  SL  R  TLLKVAT IV QQ G


BJ1 343 FFTLGVAHLRPLNLKAVAEAIQMHESTVSRVTANKYMATNRGTFELKYFFTASIPSADGGEAHSAEAVRHRIKQLIESEE
BJ2 391 FFTHGVAHLRPLNLKAVADAIQMHESTVSRVTANKYMATNRGTFELKYFFTASIASADGGEAHSAEAVRHHIKQLIDSEA
RM  381 FLIHGVGHLRPLNLRIVADAIKMHESTVSRVTSNKYMLTPRGLFELKYFFTVSIGSAENGDAHSAESVRHRIRTMINQES
PP  374 FLDHGDEAMKPLVLHDIAEAVGMHESTISRVTTQKYMHTPRGIYELKYFFSSHVSTSEGGEC.SSTAIRAIIKKLVAAEN
AV  379 FLDYGEEAMKPLVLHDIAEAVGMHESTISRVTTQKYMHTPRGIYELQYFFSSHVSTAEGGEC.SSTAIRAIIKKLIAAEN
KP  354 FFEQGEEFMKPMVLADIAQAVEMHESTISRVTTQKYLHSPRGIFELKYFFSSHVNTEGGGEA.SSTAIRALVKKLIAAEN
RC  300 FLDKGPAHLVPLTLEDVASELGLHASTISRAVSGRMIQTQTRALPLRAFFSRAVSTQGGGEAVSRDSL.DFVQRTWAAKI

con      FL  G  HL PL L DVA A  MHESTISRVT  KYM TPRG FELKYFFS  V TA GGEA S  A R  IK LIAAE


BJ1 423 PSAVLSDDAIVERLRVSGIDIARRTVAKYREAMRIRSSVQRRRDN.......MWSTMNSRASGGTGLDK*  484 aa
BJ2 471 PAAILSDDTIVERLRASGIDIARRTVAKYREAMRIPSSVQRRRDKQSALGNVLSTAMSDRSRNPEPA*    537 aa
RM  461 ADAVLSDDDIVDVLQQAGVDIARRTVAKYREAMSIPSSVQRRREKRA....LPRPRDSERCRQAASA*    523 aa
PP  453 QKKPLSDSKIAGLLEAQGIQVARRTVAKYRESLGIAPSSERKRLM*                          497 aa
AV  458 PKKPLSDSKIAGLLEEQGIQVARRTVAKYRESLSIAPSSFRKRLM*                          502 aa
KP  433 PAKPLSDSKLTTMLSDQGIMVARRTVAKYRESLSIPPSNQRKQLV*                          477 aa
RC  379 RQNPLSDDAIVTLAERAGLRIARRTVAKYRSTLGLASSYERRRAA.............AAR*          426 aa

con      P  PLSDD IV  L   GI IARRTVAKYRE L I SS QRRR                R
```

FIG. 4. Amino acid sequence alignment of the RpoN proteins from *B. japonicum* (BJ1, BJ2), *R. meliloti* (RM) (73), *P. putida* (PP) (42), *A. vinelandii* (AV) (57), *K. pneumoniae* (KP) (56), and *R. capsulatus* (RC) (44). The consensus line (con) shows the positions where four or more proteins had identical amino acid residues.

$RpoN_1$ and $RpoN_2$ proteins, each of the two showed almost identical levels of conservation with respect to the RpoN proteins from the other bacterial species listed in Fig. 4. These homologies were as follows (expressed in S_{AB} values): to *R. meliloti*, 0.55; to *P. putida*, *A. vinelandii*, and *K. pneumoniae*, 0.36; to *R. capsulatus*, 0.30.

Sequence analysis of the region downstream of *rpoN*$_2$. At 159 bp downstream of *rpoN*$_2$ we detected the start of an ORF (ORF203) extending over 609 bp that predicted a protein of 203 aa with a molecular weight of 21,626. The putative translational start codon ATG at position 1877 was preceded by a potential purine-rich Shine-Dalgarno-like sequence (Fig. 3). In addition, 225 bp downstream of ORF203, at position 2711, we identified the start of another ORF

```
BJORF203     1 MTLRISGKSVSVGEALRGRVSDRTEEVLRKYFDGNYSGHITLSKDGFGFRTDCALHLDSGITLEADSNAP
                *  * ***    *   * *     *    ***** **   *  * *  *  ** **** *  * *   *
RMORF>104    1 MSVRVSGKHMEIGDSFRVRIGEQIEQAVTKYFDGGYSSQVTVEKSGSRFSADCKLHLDTGVVLQANGQAN
                * *  **      *  *    * *       **   * **              **        **
AVORF107     1 MQVNISGHQLDVTDALRDYVEEKISR.LERHFDRITSVQVIMTVEKLKQKIEATLHVSGA.EVVANAEHE
                *******    **  ***** **  *  * *** ** ***** **** ** ****   *  *********
PPORF102     1 MQVNISGQHVEVTQPLRDYVLEKLAR.VESHFDKITNVQVIMKVEKLQQKVEATLQIPGG.EVVANAEHE
                ** ** *  ** *   ** *  *     *  ** *  *    ****  *   * *   ** *  * **
KPORF95      1 MQLNITGHNVEITPAMRDFVTAKFSK.LEQFFDRINQVYIVLKVEKVTQIADANLHVNGG.EIHASAEGQ

consensus      MQVNISG  VEVT ALRD V EK    LE  FD I SVQV   VEK  Q  DA LH  GG E  ANAE


BJORF203    71 DAYASADQALVMIEKRLKRYKSRLKDRSARKAHVASAAL...   plus 94 aa*        203 aa
                  * *   ***** ***  ***
RMORF>104   71 EPQSAFDAASERIEKRLRRYKRKLKDHHNGNGQN...                          >104 aa
                  * *         *   * *      *
AVORF107    70 DMYAAIDLLADKLDRQLIKHKEKQIERQQGQRPADSPVP*                        107 aa
               ******* ********* ******    **
PPORF102    70 DMYAAIDALADKLDRQLKKHKEKQQSLLQGAAAR*                            102 aa
               ******* * *** *** *** *
KPORF95     70 DMYAAIDGLIDKLARQLTKHKDKLKQH*                                    95 aa

consensus      DMYAAID L DKL RQL KHK KLK
```

FIG. 5. Amino acid sequence comparison of the proteins predicted by *B. japonicum* (BJ) ORF203 and corresponding ORFs located downstream of the *rpoN* genes in *R. meliloti* (RM) (73), *A. vinelandii* (AV) (55), *P. putida* (PP) (42), and *K. pneumoniae* (KP) (55). The COOH-terminal extension of 94 aa of the ORF203 protein (Fig. 3) is not shown here. The sequence of *R. meliloti* ORF>104 is incomplete. Asterisks mark positions of identical amino acids in adjacent sequences. The consensus sequence line shows positions where three or more sequences had identical amino acid residues.

(ORF>90) of 272 bp that obviously extended beyond the *Cla*I site at the end of the sequenced region. A Shine-Dalgarno-like sequence was present at an appropriate distance before the putative ORF>90 start codon.

The amino acid sequence of the predicted ORF203 protein was compared with the amino acid sequences of proteins encoded by ORFs located downstream of the *rpoN* genes in *R. meliloti*, *A. vinelandii*, *P. putida*, and *K. pneumoniae* (Fig. 5). The NH_2-terminal part of the *B. japonicum* ORF203 protein showed significant homology with all compared proteins. It had maximal homology to the *R. meliloti* protein (43 identical aa within the 104 compared aa). The COOH-terminal portion with about 100 additional aa may be specific to the *B. japonicum* protein because the corresponding proteins of *A. vinelandii*, *P. putida*, and *K. pneumoniae* lacked this portion. It is not known whether this also holds true for the corresponding *R. meliloti* protein because the sequence of its ORF has not been completed (Fig. 5).

The incomplete *B. japonicum* ORF>90 encoded the NH_2-terminal portion of a protein that had homologous counterparts in *K. pneumoniae* (ORF162 [55]) and *P. putida* (incomplete ORF>88 [42, 55]). Of the 90 aa of the *B. japonicum* ORF>90 protein, 33 and 31 were found to be identical in the *K. pneumoniae* and *P. putida* proteins, respectively (data not shown). No sequence data are available for the corresponding *rpoN* downstream regions of *R. meliloti* and *A. vinelandii*.

Complementation of a *R. meliloti rpoN* mutant. Both *B. japonicum rpoN* genes were tested for their ability to complement the succinate- and nitrate-negative phenotypes of the *R. meliloti rpoN* mutant strain 1681. Plasmids pRJ7697 ($rpoN_1$) and pRJ8019 ($rpoN_2$) (Fig. 1) were introduced into *R. meliloti* 1681, and the resulting strains were analyzed for growth on succinate as the sole carbon source and nitrate as the sole nitrogen source. As a control we used *R. meliloti* 1681 containing the cloned *R. meliloti rpoN* gene on plasmid pRM8005 (Table 1). *B. japonicum* $rpoN_2$ present on pRJ8019 was able to restore both defective phenotypes when the strains were tested under normal aerobic conditions and under reduced (2%) oxygen conditions, whereas $rpoN_1$ on pRJ7697 could complement only under low oxygen conditions. This indicated that both *B. japonicum rpoN* genes were functional in *R. meliloti* and that they may be regulated differentially with respect to oxygen (see also below).

Phenotypes of *B. japonicum rpoN* mutants. To further analyze the functional role of the two *rpoN* genes in symbiosis, four *B. japonicum* mutant strains were constructed (for details see Materials and Methods). The single *rpoN* mutants N50 ($rpoN_1$) and N63 ($rpoN_2$) (Fig. 1) as well as the double mutant N50-97 were tested for (i) growth on dicarboxylic acids as the carbon source, (ii) utilization of selected amino acids as the nitrogen source, and (iii) assimilation of nitrate. When compared with the *B. japonicum* wild-type strain none of the mutants showed a significant difference in growth on minimal medium containing either succinate, malate (both tested over a range from 0.1 to 20 mM), or fumarate (20 mM) as the sole carbon source. Similarly, all mutants were able to utilize histidine, proline, or arginine as the nitrogen source. However, there was a marked difference between strain N63 and strain N50 or N50-97 in the ability to grow aerobically on nitrate as the sole nitrogen source (Fig. 6A). Cultures of wild-type *B. japonicum* and strain N50 grew very similarly to a final optical density at 600 nm of about 0.5, whereas strains N63 and N50-97 reached only about 20% of this level. When the growth curves were established from analogous cultures grown under reduced oxygen conditions (2% O_2) mutant N63 reached almost the same final density as the wild type or strain N50, although with a slower rate (Fig. 6B). The $rpoN_{1/2}$ double mutant N50-97 was still not able to grow under these conditions. This meant that *B. japonicum* required at least one intact copy of *rpoN* for the assimilation of nitrate and, in addition, that low oxygen conditions were essential for $rpoN_1$-dependent growth (see below).

To characterize the symbiotic properties of *B. japonicum rpoN* mutants, strains N50, N97, and N50-97 were inoculated on soybean seedlings, and their Nod and Fix phenotypes were determined (Table 2; mutant strain N63 exhibited the same phenotype as strain N97 [data not shown]). All mutants were able to elicit nodules. Apart from a slightly more dispersed location of the nodules on the root system, no other effects of the single *rpoN* mutations on the symbiotic properties were observed. Both the number of nodules

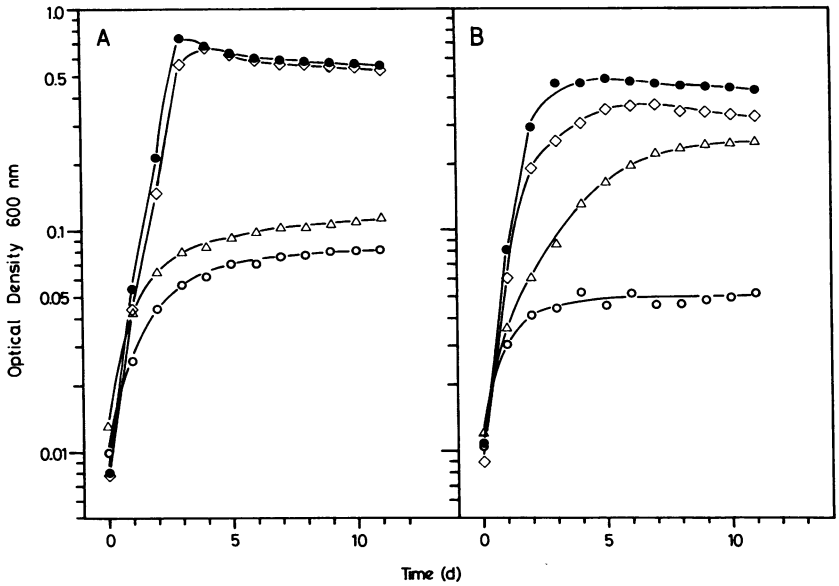

FIG. 6. Aerobic (A) and oxygen-limited growth (2% O_2) (B) of *B. japonicum rpoN* mutants in RDM medium containing 20 mM succinate and 10 mM KNO_3 as the sole carbon source and the sole nitrogen source, respectively. Symbols: ●, *B. japonicum* 110*spc*4 (wild type); ◇, $rpoN_1$ mutant N50; △, $rpoN_2$ mutant N63; ○, $rpoN_{1/2}$ mutant N50-97.

and the total nodule dry weight per plant were not affected by individual *rpoN* mutations. Moreover, the nitrogenase activity in the nodules was at least as high as that in the wild type. The double mutant strain N50-97, however, induced about twice the number of nodules as compared with the wild type, and these lacked Fix activity completely (Table 2). The N50-97-induced nodules were small in size and dispersed over the whole root system. In addition, their leghemoglobin content was about 25% of that of the wild-type nodules (data not shown). No differences were observed in the ultrastructure of nodules induced by the single *rpoN* mutants and by the wild-type strain. However, infected plant cells in nodules elicited by the double mutant strain N50-97 contained fewer bacteroids and showed enlarged peribacteroid spaces. Although empty peribacteroid vesicles were detected, the overall structure of these nodules remained intact, and necrosis was never observed (data not shown). Taken together, these results suggested that each individual *rpoN* gene was functional and could replace the other under symbiotic conditions.

Mutational analysis of the *B. japonicum* $rpoN_1$ and $rpoN_2$ downstream regions. The DNA regions downstream of $rpoN_1$ on the 4.1-kb *Eco*RI-*Bam*HI fragment (pRJ7688) and downstream of $rpoN_2$ on the 9-kb *Eco*RI-*Bam*HI fragment (pRJ7722) (Fig. 1) were analyzed by mutation for the presence of further symbiotic genes. Four Tn*5* insertions were introduced into the $rpoN_1$ region (Fig. 1A), and five were introduced into the $rpoN_2$ region (Fig. 1B). In addition, the $rpoN_2$ downstream region was mutagenized by replacing a 1.4-kb *Sma*I fragment with a 1.2-kb kanamycin resistance cassette from pUC4-KIXX (Fig. 1B). The Tn*5* insertion in ORF203 was shown by sequencing to be located between nucleotides A-2010 and T-2011 (Fig. 3), thereby interrupting this ORF at its 45th codon. All the resulting *B. japonicum* mutant strains were found to have a Nod^+ Fix^+ phenotype and were thus indistinguishable from the wild-type strain. In conclusion it seemed unlikely that the *B. japonicum* regions immediately downstream of $rpoN_1$ and $rpoN_2$ harbored symbiotically essential genes.

TABLE 2. Nod and Fix phenotypes of *B. japonicum rpoN* mutants[a]

Strain	Relevant genotype	No. of nodules per plant	mg (dry wt) per nodule	Nitrogenase activity (μmol of C_2H_4 g^{-1} h^{-1})
110*spc*4	Wild type	19 (4.7)	1.3 (0.2)	137 (23)
N50	$rpoN_1$	20 (7.1)	1.3 (0.4)	173 (47)
N97	$rpoN_2$	20 (6.0)	1.4 (0.2)	189 (52)
N50-97	$rpoN_{1/2}$	34 (6.9)	0.7 (0.1)	0

[a] Standard deviations are given within parentheses. Six plants were analyzed per strain.

Effect of *rpoN* mutations on expression of *nifH* and *fixR*. To determine more specifically the effect of *rpoN* mutations on *nifH* and *fixR* expression, we tested the activity of corresponding translational *lacZ* fusions in an *rpoN* mutant background. For this purpose, the fusions were integrated at the homologous site into the chromosome of the *rpoN* double mutant strain N50-97. *nifH* and *fixR* promoter activity was analyzed by measuring β-galactosidase activity in cells grown under different conditions. The double mutant strain N50-97 did not express the *nifH'-'lacZ* fusion under all conditions tested, whereas a several hundredfold induction occurred in the wild type under free-living, anaerobic conditions or in symbiotic root nodule bacteroids (Table 3). Surprisingly, the activity of the *fixR* promoter was affected only marginally by the *rpoN* mutations. Although the aerobic *fixR* expression in strain N50-97 was similar to that in the wild type, it was reduced to 70 and 58% under anaerobic conditions and in bacteroids, respectively. These results clearly showed that expression from the *B. japonicum* −24/−12-type promoter of *nifH* required σ^{54}, whereas expression from the *fixR* promoter was largely but not entirely independent of the $RpoN_1$ and $RpoN_2$ proteins.

Differential regulation of $rpoN_1$ and $rpoN_2$. The existence of two functional *rpoN* genes, the results from the complemen-

TABLE 3. Effect of a *B. japonicum* $rpoN_{1/2}$ double mutation on the expression of chromosomally integrated *nifH'-'lacZ* and *fixR'-lacZ* fusions under different growth conditions

B. japonicum strain	Relevant genotype	β-Galactosidase activity (U)[a]		
		Aerobic[b]	Anaerobic[b]	Bacteroids[c]
110*spc*4	Wild type	3 (2)	5 (2)	24 (3)
110*spc*4-48	*nifH'-'lacZ*	7 (3)	5,303 (498)	5,604 (827)
N50-97H	*nifH'-'lacZ* $rpoN_{1/2}$	6 (1)	7 (2)	50 (12)
110*spc*4-7290R	*fixR'-'lacZ*	413 (61)	1,914 (359)	1,780 (563)
N50-97R	*fixR'-'lacZ* $rpoN_{1/2}$	558 (206)	1,348 (163)	1,035 (50)

[a] Standard deviations are given within parentheses.
[b] Aerobic and anaerobic cultures were grown in YEM medium plus 10 mM KNO_3. Six cultures were assayed in duplicate.
[c] The nodules of five plants were pooled, and four serial dilutions of the bacteroid suspensions were assayed in duplicate.

tation experiments in *R. meliloti*, and the analysis of the nitrate phenotypes (see above) suggested a differential regulation of the *B. japonicum rpoN* genes in response to oxygen. To test this assumption directly, *lacZ* fusions to $rpoN_1$ and $rpoN_2$ were integrated into the *B. japonicum* chromosome (strains 8003 and 8015; for details, see Materials and Methods), and the expression of both *rpoN* genes was assayed under different oxygen conditions. The expression of $rpoN_1$ was hardly detectable under aerobic conditions and was induced about 25- and 12-fold under microaerobic conditions and in bacteroids, respectively (Table 4). In contrast, $rpoN_2$ was expressed at a constant intermediate level under all conditions tested. Thus, expression of $rpoN_1$, but not that of $rpoN_2$, was regulated by oxygen.

Similar experiments were performed with plasmid-borne *rpoN'-'lacZ* fusions introduced into various *B. japonicum* backgrounds (Table 5). As observed with the integrated fusions, the expression of $rpoN_1$ in the wild-type background was induced by microaerobiosis, whereas expression of $rpoN_2$ was not affected by the oxygen conditions. Interestingly, mutations in *fixL* or *fixJ* (*B. japonicum* 7404 and 7361) abolished the microaerobic induction of $rpoN_1$ but had no effect on $rpoN_2$ expression. The same results were obtained in *fixL* and *fixJ* mutants strains carrying the inserted Km^r cassettes in opposite orientations. A mutation in ORF138 located downstream of *B. japonicum fixJ* (4) did not affect the expression of $rpoN_1$ (data not shown). Thus, oxygen regulation of $rpoN_1$ specifically involved the *fixLJ* genes.

In the $rpoN_2$ mutant N63 and in the double mutant N50-97, $rpoN_2$ expression was increased at least fivefold over the wild-type level irrespective of the oxygen conditions used (Table 5). This effect was specific for expression of $rpoN_2$ and could not be overcome by $RpoN_1$ under microaerobic conditions. The Tn*5* insertion in ORF203 (Fig. 1B) had no effect on $rpoN_2$ expression (data not shown). Thus, in contrast to $rpoN_1$, the expression of $rpoN_2$ was found to be negatively autoregulated.

TABLE 4. Expression of chromosomally integrated *rpoN'-'lacZ* fusions in *B. japonicum* grown under the conditions indicated

Strain	Relevant genotype	β-Galactosidase activity (U)[a]		
		Aerobic[b]	Microaerobic[b]	Bacteroids[c]
110*spc*4	Wild type	2 (1)	4 (2)	26 (1)
8003	$rpoN_1$'-'*lacZ*	8 (3)	220 (24)	97 (2)
8015	$rpoN_2$'-'*lacZ*	139 (14)	148 (24)	74 (15)

[a] Standard deviations are given within parentheses.
[b] Aerobic and microaerobic cultures were grown in PSY medium (for details see Materials and Methods). Three to six cultures were assayed in duplicate.
[c] From each of two plants six nodules were selected and pooled, and two serial dilutions of the bacteroid suspensions were assayed in duplicate.

TABLE 5. Differential effects of oxygen conditions and host genotype on the expression of plasmid-borne $rpoN_1$'- and $rpoN_2$'-'*lacZ* fusions

Strain	Relevant genotype	β-Galactosidase activity (U)[a]			
		$rpoN_1$'-'*lacZ*		$rpoN_2$'-'*lacZ*	
		Aerobic	Microaerobic	Aerobic	Microaerobic
110*spc*4	Wild type	41 (15)	281 (71)	83 (11)	90 (13)
7404	*fixL*	13 (2)	13 (3)	90 (10)	90 (5)
7361	*fixJ*	15 (3)	13 (3)	103 (5)	94 (7)
N50	$rpoN_1$	31 (2)	235 (11)	93 (5)	91 (6)
N63	$rpoN_2$	29 (2)	250 (10)	613 (39)	498 (78)
N50-97	$rpoN_{1/2}$	20 (2)	232 (30)	566 (61)	540 (72)

[a] The cultures were grown in PSY medium as described in Materials and Methods. At least four cultures were assayed in duplicate. Standard deviations are given within parentheses. The $rpoN_1$'-'*lacZ* fusion was located on plasmid pRJ8002; the $rpoN_2$'-'*lacZ* fusion was on pRJ8015. The background level of β-galactosidase activity ranged from 3 to 9 U and was estimated from a wild-type *B. japonicum* strain carrying the promoterless *lacZ* fusion plasmid pRK290lac.

DISCUSSION

Using the *R. meliloti rpoN* gene as a probe, two homologous regions were cloned from the genome of *B. japonicum*. By sequence analysis both regions were shown to contain homologs of the σ^{54} gene, which we named $rpoN_1$ and $rpoN_2$. They do not map to either of the two clusters of *nif*, *fix*, and *nod* genes in *B. japonicum*, and their location on the chromosome is presently unknown. That both genes produced functional products was demonstrated by complementation experiments and by mutational analysis. Furthermore, it was shown that they were both involved in the expression of a *nifH'-'lacZ* fusion but only marginally involved in that of a *fixR'-'lacZ* fusion.

The major difference between the two *B. japonicum* RpoN proteins is represented by the presence of an extra stretch of 48 aa between positions 73 and 121 in $RpoN_2$. This region corresponds to the most diverged domain in all known RpoN proteins. An even larger gap than in the *B. japonicum* $RpoN_1$ protein is present in *R. capsulatus* RpoN, rendering this protein the smallest of all RpoN proteins. At least in *B. japonicum* the structure of this nonconserved region and the distance between the highly conserved NH_2-terminal and COOH-terminal protein domains do not seem to be critical for recognition of promoters essential for nitrate assimilation or symbiosis.

As was noticed for the σ^{54} proteins from *K. pneumoniae* and *A. vinelandii* (34, 57), the *B. japonicum* RpoN proteins showed only a very limited similarity to other bacterial σ factors. The only exceptions were observed by Merrick et al. (56, 57), who identified a 20-aa stretch in the RpoN proteins of *A. vinelandii*, *R. meliloti*, and *K. pneumoniae* that showed significant homology to other prokaryotic σ factors and found a helix-turn-helix motif that is characteristic for many DNA binding proteins such as σ factors. These two elements are also present in both *B. japonicum* RpoN proteins (positions 159 through 178 and 351 through 376 in $RpoN_1$; positions 207 through 226 and 399 through 424 in $RpoN_2$),

which may be taken as additional evidence for their proposed functional role. As in *K. pneumoniae* and *R. meliloti*, however, the putative helix-turn-helix motifs of both *B. japonicum* RpoN proteins lack the characteristic glycine residue that is normally present at position 9 in other helix-turn-helix motifs (9, 21) (in $RpoN_1$ we find Q-364; in $RpoN_2$ we find Q-412). Another conserved sequence motif, Lys-Tyr, is present three times in the COOH termini of both RpoN proteins (positions 377, 389, and 450 in $RpoN_1$; positions 425, 437, and 498 in $RpoN_2$). It is tempting to speculate that this corresponds to the motif "basic amino acid - aromatic amino acid" that has been proposed in bacterial σ factors to be involved in DNA melting during open complex formation (34).

Duplication of genes in nitrogen-fixing organisms is not without precedents. For example, multiple functional copies of the *nifH* gene have been found in *Rhizobium leguminosarum* biovar phaseoli (68) and in *Azorhizobium caulinodans* ORS571 (65). In *R. capsulatus* the region encompassing *nifA* and *nifB* is repeated, and both copies are functional (53). Two and three *nodD* genes are present in *B. japonicum* and *R. meliloti*, respectively, and in the latter case they have distinct regulatory properties (5, 31, 39, 64). The data presented here show that the two *B. japonicum rpoN* genes could replace each other in symbiosis and for nitrate assimilation, but it is possible that they also have specific individual functions. In fact, the autoregulation of $rpoN_2$ expression is an example for an $RpoN_2$-specific function (see also below).

Downstream of $rpoN_2$ we identified one complete ORF (ORF203) and a second ORF (ORF>90) whose sequence was not completed. Their translation products showed significant homologies to the predicted products of similar ORFs located downstream of the *rpoN* genes in *R. meliloti*, *P. putida*, *A. vinelandii*, and *K. pneumoniae*. In a recent study, Merrick and Coppard (55) demonstrated that mutations in *K. pneumoniae* ORF95 or ORF162 increased the levels of expression from σ^{54}-dependent promoters via an unknown mechanism. This effect was dependent on the nitrogen conditions of the cultures and on the promoter studied. In the course of analyzing the downstream regions of the *B. japonicum* $rpoN_1$ and $rpoN_2$ genes, we isolated a mutant in which ORF203 was disrupted by Tn*5* insertion. However, this strain and all the other mutants examined in this context showed a wild-type phenotype in a plant infection test. Nevertheless, a functional role of *B. japonicum* ORF203 in the regulation of σ^{54} promoters cannot be ruled out completely, because the assay system used (acetylene reduction by infected soybean roots) may not be sensitive enough to detect subtle differences.

The involvement of the two *B. japonicum rpoN* genes in symbiotic nitrogen fixation and in diverse metabolic functions was analyzed by deletion-replacement mutagenesis. Since the promoters of the inserted resistance genes were in an orientation opposite that of the *rpoN* genes, any additional genes located downstream and belonging to the same operon could be affected by polarity. However, we also constructed a mutant strain similar to N50-97 in which the resistance genes read in the same direction as the two *rpoN* genes. This strain exhibited identical properties (data not shown). Furthermore, polarity seems to be unlikely because (i) the individual mutant strains N50, N63, and N97 showed identical (wild-type) symbiotic phenotypes even though the *rpoN* downstream regions were not homologous, and (ii) all insertion mutations generated downstream of both *rpoN* genes produced Nod^+ Fix^+ phenotypes. This suggested that the phenotype of the double mutant N50-97 could be attributed to the *rpoN* mutations rather than to polar effects on potential downstream genes. In the $rpoN_{1/2}$ mutant background the expression from the *nifH* promoter was abolished completely. Since we did not analyze the effect of individual *rpoN* mutations on *nifH* expression, we cannot rule out completely a differential dependence of the *nifH* promoter on the two *rpoN* genes. However, the symbiotic wild-type phenotype of the single mutant strains indicated that each of the two *rpoN* genes was sufficient to allow for a level of *nifH* gene expression that did not limit symbiotic N_2 fixation efficiency.

With regard to free-living phenotypic traits, we compared the wild type with the single and double *rpoN* mutant strains for their abilities to grow on C_4-dicarboxylates as carbon sources, to utilize histidine, proline, or arginine as a nitrogen source, and to assimilate nitrate. Unlike *rpoN* mutants of *K. pneumoniae* (19), all mutants could use any of the three amino acids tested as the sole nitrogen source, and all of them were glutamine prototrophs. The same result was described previously for *rpoN* mutants of *R. meliloti* and *Rhizobium* sp. strain NGR234 (73, 81). However, in contrast to the findings in the latter organisms, all *B. japonicum rpoN* mutants grew indistinguishably from the wild type on succinate, malate, or fumarate as the sole carbon source. This indicated that in *B. japonicum* an *rpoN*-independent C_4-dicarboxylate uptake system may be present. Succinate uptake systems have been analyzed in different strains of *B. japonicum* (40, 54, 76). Interestingly, in strain I-110, which is most related to the strain used here, a constitutively synthesized succinate uptake system was reported (76). Since the activity of many σ^{54}-dependent promoters is regulated via an activator protein in response to environmental signals (48), it is tempting to speculate that the constitutive uptake system for succinate in *B. japonicum* I-110 is not σ^{54} dependent. Nitrate assimilation by *B. japonicum* required expression of at least one functional *rpoN* gene. This indicates that one or several genes involved in the process of nitrate assimilation are under the control of a −24/−12-type promoter. The apparent nitrate-negative phenotype of the $rpoN_2$ mutant strain N63 under aerobic conditions could later be explained by the fact that $rpoN_1$ was not expressed under these conditions. Under reduced oxygen conditions, strain N63 was able to assimilate nitrate. In agreement with this finding the *B. japonicum* $rpoN_1$ gene was able to complement an *R. meliloti rpoN* mutant only under microaerobic conditions. The fact that expression of *B. japonicum* $rpoN_1$ is oxygen controlled also in *R. meliloti* implied that the necessary *trans*-acting elements were present in this background and that they could interact productively with the heterologous target DNA.

A model concerning *B. japonicum nif* and *fix* gene regulation proposed recently by Thöny et al. (83) suggested a σ^{54}-dependent expression of the regulatory operon *fixRnifA* because the *fixR* promoter was of the −24/−12 type and could also be autoregulated to some extent by NifA. However, the aerobic expression from the *fixR* promoter was not affected by mutations in both *rpoN* genes, and under anaerobic or symbiotic conditions at least a partial induction was observed in this background (Table 3). Thus, expression of the *fixRnifA* operon is almost, if not all, independent of the apparent −24/−12 promoter but must occur from an alternative yet unidentified promoter whose recognition does not require either of the two *B. japonicum* RpoN proteins. The *fixR* promoter-down phenotype of a mutation in the −12 region as well as transcript mapping data now suggest that

the apparent −24/−12 promoter and the postulated σ^{54}-independent promoter must overlap at least partially (84; see above). The observed expression of the *fixRnifA* operon in the $rpoN_{1/2}$ mutant N50-97 can explain why this strain elicited none of the necrotic nodules that are characteristic for *B. japonicum nifA* mutants (26, 82). This explanation includes the assumption that the NifA-dependent functions that must be involved in the suppression of the host plant defense response are σ^{54} independent. A hypothetical repressor activity of NifA would accommodate such an idea. Under anaerobic or symbiotic conditions NifA could bind independently of σ^{54} to specific target sites, thereby interfering with the expression of genes whose product would trigger a plant defense response. In addition, such a hypothetical gene product could act itself as a repressor on the expression of the *fixRnifA* operon under aerobic conditions, and its reduced synthesis would result in the enhanced expression of *fixRnifA* observed under anaerobic or symbiotic conditions (Table 3). In this context, it is of interest to note that *B. japonicum nifA* mutants grown under microaerobic conditions specifically derepressed the synthesis of a protein of M_r 35,000 that was absent in the wild type (26). The proposed dual function of NifA as an activator and repressor would be reminiscent of that of Fnr in *E. coli* and NtrC in *E. coli* and other enteric bacteria. These regulatory DNA binding proteins can act as activators at certain promoters and, at the same time, as repressors at others (15, 20, 70, 80). As an alternative to the repressor hypothesis, there is the remote possibility that a third, as yet undetected RpoN-like protein might exist. If this were the case, however, that σ factor must be highly specialized for the expression of the *nifA* gene and certain NifA-dependent genes but clearly is unable to recognize the *nifH* promoter.

Unlike the expression of $rpoN_2$, that of $rpoN_1$ was influenced by the oxygen conditions. This was revealed by three independent experimental findings. The complementation of the *R. meliloti rpoN* mutant by $rpoN_1$ as well as the $rpoN_1$-dependent nitrate assimilation of *B. japonicum* N63 required microaerobic conditions. In addition, the oxygen conditions specifically influenced the expression of the $rpoN_1$'-'*lacZ* fusion. The identical regulatory pattern of chromosomally integrated and plasmid-borne $rpoN_1$'-'*lacZ* fusion indicated that all of the *cis*-acting elements required for the regulated expression of $rpoN_1$ must be contained within the 247 bp of *B. japonicum* DNA present on plasmid pRJ8002. Similarly, the 176 bp of *B. japonicum* DNA cloned in plasmid pRJ8015 was sufficient to mediate expression and negative autoregulation of $rpoN_2$. The 5'-upstream regions of $rpoN_1$ and $rpoN_2$ showed only weak sequence similarity, and there was no obvious −24/−12- or −35/−10-type promoter present. Thus, in order to define more precisely the promoter elements of the *rpoN* genes, appropriate mutants are required and the transcriptional start sites need to be determined.

In *B. japonicum*, *nifA* has been shown to mediate oxygen control of *nif* and *fix* gene expression. In addition, *fixLJ*-like genes were identified recently in this organism (4). However, unlike *fixLJ* in *R. meliloti*, they are probably not involved in the regulation of *nifA* expression; instead, we demonstrate here that they are required for the induction of $rpoN_1$ under conditions of low oxygen tension. It will be interesting to examine whether $rpoN_1$ activation occurs directly by FixJ, or whether this involves a regulatory cascade via the *fixK*-like gene that was found most recently in *B. japonicum* (B. Scherb and D. Anthamatten, unpublished results). How $rpoN_2$ controls its own expression also remains to be elucidated. The absence of a −24/−12 promoter structure on either strand in the *B. japonicum* DNA sequence on plasmid pRJ8015 makes it seem unlikely that this regulation simply occurs via direct binding of an RNA polymerase-$RpoN_2$ complex. Rather, we tend to predict the existence of an unknown repressor protein whose synthesis is controlled by $RpoN_2$. In both cases, however, one cannot readily explain the specific $RpoN_2$ dependence of this regulatory circuit without attributing unique functions to the individual RpoN proteins.

Although the *rpoN* genes of *E. coli*, *K. pneumoniae*, and *R. meliloti* are expressed constitutively (13, 19, 58, 73), regulated expression of other bacterial σ factor genes has been reported. Examples include the *E. coli rpoH* (*htpR*) gene (24, 87), several σ factor genes involved in the process of endospore formation in *Bacillus subtilis* (49), and the *rpoN* (*nifR4*) gene of *R. capsulatus*, which is subject to transcriptional control by ammonia and oxygen (44). In the view of the simultaneous induction of numerous −24/−12 promoters during the onset of nitrogen fixation, it would make perfect sense for *B. japonicum* to increase the synthesis of the relevant σ factor, even though our results indicated that the contribution by the enhanced expression of $rpoN_1$ was not essential for a functional symbiosis. Nevertheless, this additional level of control in the complex regulatory system of *nif* and *fix* gene expression in *B. japonicum* may be advantageous for a rapid adaptation to changing environmental conditions.

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