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Sequence and Analysis of the *rpoN* Sigma Factor Gene of *Rhizobium* sp. Strain NGR234, a Primary Coregulator of Symbiosis

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We report the nucleotide sequence of the *rpoN* gene from broad-host-range *Rhizobium* sp. strain NGR234 and analyze the encoded RPON protein, a sigma factor. Comparative analysis of the deduced amino acid sequence of RPON from NGR234 with sequences from other gram-negative bacteria identified a perfectly conserved RPON box unique to RPON sigma factors. Symbiotic regulatory phenotypes were defined for a site-directed internal deletion within the coding sequence of the *rpoN* gene of *Rhizobium* strain NGR234: they included quantitative nodulation kinetics on *Vigna unguiculata* and microscopic analysis of the Fix$^-$ determinate nodules of *V. unguiculata* and *Macroptilium atropurpureum*. RPON was a primary coregulator of nodulation and was implicated in establishment or maintenance of the plant-synthesized peribacteroid membrane. Phenotypes of *rpoN* in *Rhizobium* strain NGR234 could be grouped as symbiosis related, rather than simply pleiotropically physiological as in free-living bacteria such as *Klebsiella pneumoniae* and *Pseudomonas putida*.

The gene *rpoN* (*ntrA*, *glnF*) encodes an alternative sigma factor, σ^{54}. In enteric bacteria, σ^{54}, also termed RPON, is a positive regulator of expression of *glnA* (encoding glutamine synthetase) and of the *aut*, *hut*, and *put* operons (encoding amino acid transport and degradative enzymes). The nitrogen fixation (*nif*) promoters of *Klebsiella pneumoniae* also require σ^{54}-RNA polymerase (RNAP). All promoters recognized by σ^{54}-RNAP are characterized by an invariant GG doublet at −24 and a GC doublet at −12 upstream of the transcriptional start site. Recognition complexes between σ^{54}-RNAP and the promoter are closed and nonproductive transcriptionally. Isomerization to the productive open complex requires binding of diverse activator proteins >100 nucleotides upstream of the transcription site. In the case of *glnA* and *nifLA* of *K. pneumoniae*, the activator is NTRC (34); in the case of other *nif* operons, it is NIFA (28, 30).

In *Rhizobium meliloti*, the nitrogen-fixing endosymbiotic bacterium of *Medicago sativa*, the *rpoN* locus has been mutated and sequenced (36) and gene expression has been analyzed (2). A mutant formed Fix$^-$ alfalfa nodules and could not grow on C_4-dicarboxylates, which are likely to be the plant-supplied substrate for nitrogen fixation in indeterminate nodules of alfalfa. The dicarboxylate permease gene (*dctA*) of *R. meliloti* has an RPON-RNAP consensus sequence 60 base pairs (bp) upstream of the first ATG and binding sites for the activator proteins DCTD (19) and NIFA (10).

NGR (New Guinea *Rhizobium*) 234 is a broad-host-range strain (45). The *rpoN* (*ntrA*) gene of NGR234 has been cloned and mutated; its phenotype was pleiotropic and included a measurable effect on nodulation gene expression. Preliminary analysis of a site-directed interposon mutant, NGR234*rnl*, and its minimally complementing subclone pSD23, was reported elsewhere (40). The *rnl* locus was mapped on the NGR234 chromosome between *ade-1* and *his-2* (32). In the present report we provide the nucleotide sequence analysis of *rpoN* of NGR234. We compare the locus and encoded RPON protein with those of *R. meliloti* and *Pseudomonas putida*, which have *rpoN* genes respectively very homologous and dissimilar to that of NGR234. We also analyze the phenotype of NGR234*rn3*, a site-directed mutant carrying an internal deletion in the *rpoN* coding sequence. We present evidence that the *rpoN*-encoded alternative sigma factor is required for symbiotic functions: normal nodulation kinetics and determinate nodule organogenesis as well as "late" nodule functions like dicarboxylate tranport and nitrogen fixation. We hypothesize that RPON is a primary coregulator of the endosymbiotic life-style of members of the genus *Rhizobium*.

MATERIALS AND METHODS

Bacteriology and genetic techniques. Bacterial strains, plasmids, and bacteriophages are described in Table 1. Complex (LB, TY, or YM) and defined (RM) media, growth conditions for *Rhizobium* strain NGR234R and *Escherichia coli*, and antibiotic concentrations were as described previously (40). *Azotobacter vinelandii* was cultured in YM (yeast mannitol) or YS (yeast succinate; 15 mM) medium with antibiotic concentrations as described elsewhere (44). Bacterial conjugations were performed as described previously, and pRK7813 derivatives were mobilized with the helper plasmids pRK2013 or pRK600. Transconjugants were selected on complex medium prior to screening for *rpoN*-related phenotypes. Bacteriophage M13 was propagated in *E. coli* TG1 (27).

DNA sequencing and computer analysis. The 2.5-kilobase (kb) insert fragment of pSD23 was subcloned into M13 derivatives. DNA was sequenced (37) with Sequenase (42) and [^{35}S]ATP (Amersham Corp.). Both strands were sequenced after hybridization and S1 nuclease analysis, which determined the orientations and sizes of subcloned fragments (1). Overlapping clones were generated by directed cloning and preparation of a set of nested deletions by the kilo-sequencing method (3). Sequence compressions were

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TABLE 1. Bacterial strains, plasmids, and phages

Organism, plasmid, or phage	Relevant characteristics	Origin (reference)
Rhizobium spp.		
NGR234R	Nod$^+$ Fix$^+$ Rif^r	M. Trinick (45)
NGR234R*rn3*	NGR234, *rpoN* *Bgl*IIf::GmI-3	This report
NGR234R*rn1*	NGR234, *rpoN*::GmI-1	Stanley et al. (40)
Rm5419	*R. meliloti*, *ntrA74*::Tn*5*	Finan et al. (12)
E. coli		
TG1	Δ(*lac pro*) *supE thi hsdD5* (F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15), host for M13 derivatives	T. J. Gibson
FM15R	F$^-$ Δ *lac pro thi lacZ recA* Rif^r	Stanley et al. (40)
A. vinelandii		
MV700	*ntrA1*::Tn*5*	A. Toukdarian and C. Kennedy (44)
Plasmids		
pRK7813	IncP1, *cos*, RK2 derivative, pUC9 polylinker, Tc^r	Jones and Gutterson (20)
pRK2013	*oriV* ColE1, Tra RK2, Km^r	Ditta et al. (7)
pRK600	pRK2013 *npt*::Tn*9*, Cm^r	Finan et al. (11)
pSD21	pRK7813, 6.5 kb f, RpoN$^+$, *Hin*dIIIf	Stanley et al. (40)
pSD21Ω S1	pRK7813, 8.6-kb f ORF1::Ω Spc	Stanley et al. (40)
pSD23	pRK7813, 2.5-kb *Hin*dIII-*Eco*RIf of NGR234 *rpoN*$^+$	Stanley et al. (40)
Bacteriophages		
M13mp10	Derivative of M13	J. Messing
M13mp18	Derivative of M13	Yanisch-Perron et al. (50)
M13mp19	Derivative of M13	Yanisch-Perron et al. (50)

resolved with 7-deaza-dGTP (29). Synthetic oligonucleotides were used to prime synthesis next to three regions of interest (see Fig. 1). Sequencing gels (50 cm) were fixed in 10% methanol–10% acetic acid and vacuum dried for autoradiography. DNA sequences were assembled and analyzed with the PC/Gene system (IntelliGenetics/Genofit) and its subprograms.

Tropical legume plant assays and root nodule microscopy. Seeds of legume host plants of NGR234 were obtained from Wright Stephenson Seeds (Seven Hills, Australia). Nodulation tests were done as previously described (39) on *Macroptilium atropurpureum*, *Vigna unguiculata* cv. Red Caloona, and nine other host plants (Table 2). Acetylene reduction was measured 30 days after innoculation with a Dani 8521 gas chromatograph equipped with a Porapak T column. Plants were grown in washed vermiculite held in modified "Magenta" jars (39) in a culture room with a day temperature of 28°C, a night temperature of 20°C, and a light phase of 16 h (150 μmol of photosynthetically active radiation per m^2 per s). The kinetics of nodulation in growth pouches was determined (4) with 100 μl of inoculum (10^7 cells) applied per root tip. NGR234R was compared with NGR234*rn3* three times on *V. unguiculata* cv. Red Caloona. Light and electron microscopy of determinate nodule sections was carried out as previously described (49) using nodules harvested from *V. unguiculata* cv. Red Caloona and *M. atropurpureum* 21 or 30 days after inoculation by NGR234R or NGR234*rn3*, respectively.

TABLE 2. Symbiotic acetylene reduction[a]

Plant	C_2H_4 production (μmol/h per plant)	
	NGR234R	NGR234*rn3*
Calopogonium caeruleum	1.41	0
Desmodium intortum cv. Greenleaf	2.8	0
Desmodium uncinatum cv. Silverleaf	1.5	0
Glycine max (Australian) cv. Davis	10	0
Glycine max cv. Peking	1.6	0
Glycine soja	3.0	0
Flemingia congesta	0.67	0
Macroptilium atropurpureum	0.52	0
Psophocarpus tetragonolobus	24	0
Tephrosia vogelii	14	0
Vigna unguiculata	14	0

[a] Plants were grown under conditions optimized for tropical legumes (see Materials and Methods).

RESULTS

Genetic organization of the locus. We determined the nucleotide sequence of the *Hin*dIII-*Eco*RI insert of pSD23 (40), a DNA region of 2,501 bp (see Fig. 1). We scanned (PC/Gene NMAPUS) for homologies with the sequenced *rpoN* loci of *K. pneumoniae*, *A. vinelandii*, and *R. meliloti*. Three open reading frames (ORFs) were identified by scanning all predicted ORFs generated with the universal code, initiation codons AUG/ATG and GUG/GTG, for a minimum size of 10 amino acids (PC/Gene COD-FICK, PC/Gene TRANSL). The positions and orientations of the ORFs could be determined relative to the organization of the *rpoN* locus of *R. meliloti*. The coding sequence of the largest ORF was bisected by two *Bgl*II sites separated by 210 bp. The internal *Bgl*II fragment of the wild-type gene cloned in pSD21 was deleted and replaced by the Gm^r casette, yielding pSD21::GmI-3. This construct was homogenotized into NGR234R as previously described (39, 40), generating an internal deletion mutant. A genomic Southern blot analysis was made of this mutant, NGR234*rn3*, NGR234R, and various wild-type gram-negative bacteria under medium stringency conditions (2× SSC [1× is 0.15 M NaCl plus 0.015 M sodium citrate], 0.01% sodium dodecyl citrate; 65°C) with the 2.5-kb *Hin*dIII-*Eco*RI fragment from pSD23 as a probe. NGR234*rn3* was confirmed as a precise replacement of pSD21::GmI-3 in the *rpoN* locus. Homologous fragments were observed in the genomes of *R. meliloti* (*Bam*HI-*Hin*dIII, 3.5 kb) and *Agrobacterium tumefaciens* (*Bam*HI-*Hin*dIII, 4.2 kb). Weak homology existed with *A. vinelandii* (*Bam*HI-*Hin*dIII, 5.7 kb), and there was negligible homology with *K. pneumoniae* or *E. coli* (data not shown). The capacity of pSD23 to complement *rpoN* mutations in other gram-negative bacteria was tested. *A. vinelandii* MV700 carries the *rpoN* mutation *ntrA1*::Tn*5* (44) and cannot grow on 10 mM succinate as carbon source in YS medium. Conjugative transfer of pSD23 to MV700 restored wild-type growth on YS medium.

Sequence analysis of *rpoN*. The strategy for sequencing *rpoN* is outlined in Fig. 1. Both strands were sequenced with sets of overlapping clones generated by (i) subcloning *Sal*I, *Pst*I, and *Xho*I-*Sal*I fragments or (ii) kilo-sequencing. Six

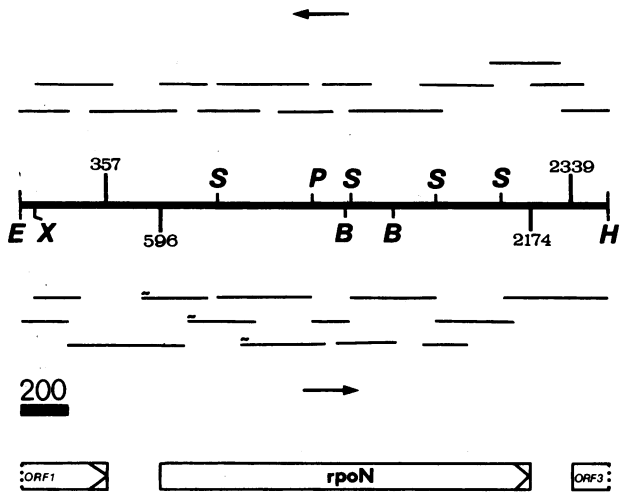

FIG. 1. Physical map of *rpoN* locus, nucleotide sequencing strategy, and distribution of ORFs. The 2.5-kb *Eco*RI-*Hin*dIII fragment insert of pSD23 is shown; the nucleotide sequence was numbered from the *Eco*RI site (nucleotide 1) to the *Hin*dIII site (nucleotide 2501). Subfragments below the line represent the coding strand, sequenced in the direction shown by the arrow. Subfragments above the line represent the noncoding strand, sequenced in the direction of the arrow. Synthesis was primed with the M13 17-mer, except in the case of three subclones (~), where priming was with synthetic oligonucleotides. Restriction sites were *Bgl*II (B), *Eco*RI (E), *Hin*dIII (H), *Pst*I (P), *Sal*I (S), and *Xho*I (X). Nucleotides 357, the stop codon for ORF1, and 2339, the initiation codon for ORF3, are indicated. Interposon GmI replaced the internal *Bgl*II subfragment within *rpoN* (nucleotides 596 to 2174) in the mutation NGR234*rn3*. Below the size bar (in kilobases) are shown the limits of the three ORFs.

phases of translation of this DNA revealed start and termination codons consistent with one major and complete ORF. This ORF exhibited strong homology to the *R. meliloti rpoN* sequence, permitting comparative analysis of the topology (PC/Gene NALIGN) and potential regulatory sites (PC/Gene SIGNAL). The ATG start codon at nucleotides 596 to 599 was preceded by a potential ribosome-binding site, GGAGT, at nucleotides 580 to 584. A putative promoter region at −35 to −10 was located at nucleotides 397 to 402 and 420 to 425.

Examination of the nucleotide sequence upstream of nucleotide 357 showed an incomplete ORF (Fig. 1) with very strong homology to ORF1 of *R. meliloti*. Analysis of the intergenic region between ORF1 and *rpoN* was facilitated by complementation studies of the insertion mutation in pSD21::ΩS1 which introduces a strong transcriptional terminator into the *Eco*RI site located at nucleotide 1 of Fig. 1. Since this mutant plasmid complemented *rpoN* mutations of both NGR234*rn3* and Rm5419, transcriptional readthrough from ORF1 cannot be required for *rpoN* expression in NGR234. The stop codon of *rpoN* was located at nucleotides 2174 to 2177. No [*rho*]-independent transcriptional terminator was found in the intergenic space (165 bp) preceding nucleotides 2339 to 2341, the start codon of the downstream ORF (Fig. 1). The sequenced 160 bp of this ORF exhibited strong homology to ORF3 of *R. meliloti*. A putative ribosome-binding site, GAGGA, for this ORF3 was found at nucleotides 2328 to 2332.

Analysis of RPON protein. The deduced composition of RPON of NGR234 (NGRRPON; 77 acidic and 69 basic amino acids) confirms that it is a large acidic protein of 57.8 kilodaltons with a probable isoelectric point of 4.9 (if the N-terminal methionine residue is included in the calculation). It was very similar to the *R. meliloti* polypeptide (RmRPON) but markedly less so to that of *P. putida* (PpRPON), with which we compare it in Fig. 3. A pairwise comparison was made between this protein, NGRRPON, and the RPON proteins of five other species by using alignment of polypeptides with a structure-genetic matrix (PC/Gene PALIGN). Data for these polypeptides was as follows (numbers in parentheses are the number of identical equivalently positioned amino acids divided by the total number of amino acids in that protein). RmRPON was 86% homologous (identical plus similar amino acid residues) (450/523); PpRPON was 52.7% homologous (262/497); *Rhodobacter capsulatus* RPON (RcRPON) was 48.2% homologous (205/426); *A. vinelandii* RPON (AvRPON) was 54.8% homologous (275/502); and *K. pneumoniae* (KpRPON) was 50.1% homologous (239/477).

Applying the method of Merrick et al. (25) to the alignment of the predicted amino acid sequences of NGRRPON and five other RPON proteins confirmed that NGRRPON contains three major regions (Fig. 2). Region I of NGRRPON (Met-1 to Glu-50 inclusive) was a conserved N-terminal domain, 98% homologous with that of RmRPON but with lower homology (~70%) to such domains in KpRPON, AvRPON, PpRPON, and RcRPON. Region II of NGRRPON (Glu-50 to Lys-152 inclusive) showed variability between polypeptides (RmRPON 76% homology; all others, less than 10% homology). NGRRPON contained two more amino acids in this region than RmRPON. Region III of NGRRPON ran from Lys-152 to Arg-512 inclusive. Here, both rhizobial proteins (90.4% homology) contained 18 more amino acids at their carboxy termini than did PpRPON, KpRPON, or AvRPON. Four subregions within region III can be identified. They were (inclusive numbers) regions IIIA, (Leu-198 to Pro-217), IIIB (Trp-357 to His-386), IIIC (Leu-396 to Ser-415), and IIID (Ala-484 to Arg-492). NGRRPON subregion IIIA (Leu-198 to Pro-217) is the 20-amino-acid sequence with homology (25) to the conserved region of bacterial sigma factors such as RPOD of *E. coli* or SPOIIAC of *Bacillus subtilis*. Similarly (25), NGRRPON subregions IIIB (Trp-357 to His-386) can be aligned with the N-terminal 30 amino acids of the β′ subunit of *E. coli* RNAP (>50% homology). Subregion IIIC was identified by our program PC/Gene REGULAT as a helix-turn-helix motif typical of DNA-binding proteins that lay between Leu-396 and Ser-415. Two potential alpha helices of eight and nine residues (Leu-396 to Ile-403 and Glu-407 to Ser-415) are separated by a beta turn (Lys-405 to His-406). We considered subregion IIID particularly interesting, since we found it conserved between most similar (NGRRPON and RmRPON) and dissimilar (NGRRPON versus PpRPON and RcRPON) proteins (Fig. 3). A 9-amino-acid sequence (Ala-484 to Arg-492) is perfectly conserved between NGRRPON, RmRPON, PpRPON, KpRPON, AvRPON, and RcRPON. We term this the core element of an RPON box, which can be expanded to 13 amino acids if 1 preceding and 3 following homologous amino acids are taken into account. Interspecies alignment of this RPON box is shown in Fig. 3. The RPON box spans the following amino acid residues in the respective polypeptides: NGRRPON, 484 to 492; RmRPON, 482 to 490; PpRPON, 474 to 482; KpRPON, 454 to 462; AvRPON, 479 to 487; and RcRPON, 400 to 409.

Phenotypes of NGR234*rn3*. Inability to utilize nonoverlapping groups of amino acids is a phenotype of *K. pneumoniae* when compared with *P. putida*. In this context, we investigated whether *Rhizobium* strain NGR234 would exhibit a

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362   GCGCAGGCAAATTGCGCCCTACAACGCCTCGGTGCTTGACCAAATCCCGGTAATAAGCAA

422   TTTTTGGCCAACTACAGCGCCCGAGTCTTTTCAGACGCACAAAGGACGCTGTAGCACTTT

482   AAATTGCTGCATGTACTTATCCTTAAATCGGTTACGATTTCAGGAACATGCAGTAAGAAT

542   TGGTCCCGTCCACGCAAGACTGGTCCGGAGATTTGACGGGAGTTTCGCGTCAGCATGGCT
                                                *****          METAla

602   TTGTCCGCCAGCCTACATCTGCGACAATCACAGTCGCTGGTCATGACGCCGCAACTGATG
      LeuSerAlaSerLeuHisLeuArgGlnSerGlnSerLeuValMetThrProGlnLeuMet

662   CAGTCGATCCAGCTGCTTCAGATGACGCATCTCGAACTCGGTCAGTTCATCGCCCAGGAA
      GlnSerIleGlnLeuLeuGlnMetThrHisLeuGluLeuGlyGlnPheIleAlaGlnGlu

722   GTCGAGAAGAACCCTTTGCTTGAGGTTCCAATCGCCGATGAGGCGGTTTCGGTTCGGATC
      ValGluLysAsnProLeuLeuGluValProIleAlaAspGluAlaValSerValArgIle
                              50

782   GGCCCGAGCGTGATGAGACACGTGACGGCCGAGGCGGGCGGCGCGGAGGAATCCGTCGAC
      GlyProSerValMetArgHisValThrAlaGluAlaGlyGlyAlaGluGluSerValAsp

842   CACGGCGATCTCTACGACAGCGCGACGACAAGTCCCGGCGAACGGCTCAGATCCGAACTC
      HisGlyAspLeuTyrAspSerAlaThrThrSerProGlyGluArgLeuArgSerGluLeu

902   GACGCCGACTTCGCCAACGTGTTCCAGGACGACACGGCGCCGCAGCGCGACGCGCCGGAA
      AspAlaAspPheAlaAsnValPheGlnAspAspThrAlaProGlnArgAspAlaProGlu

962   CTGTTGGGCCAATGGAAATCGATGCCGGGCGCCGGCGGCAGCAACGACGGCGAGGGATAC
      LeuLeuGlyGlnTrpLysSerMetProGlyAlaGlyGlySerAsnAspGlyGluGlyTyr

1022  GATCTCGACGATTCGTCGCCGGTCGGAAAACTCCGCGAGACGCTGATCGAGCAGGTCGCC
      AspLeuAspAspSerSerProValGlyLysLeuArgGluThrLeuIleGluGlnValAla
                                  152

1082  TTCGCGTTTGCCGCCGCCGCCGACCGGCTGATTGCCCAGCATCTCATCGACCAGCTCGAC
      PheAlaPheAlaAlaAlaAlaAspArgLeuIleAlaGlnHisLeuIleAspGlnLeuAsp

1142  GAAGCGGGCTATCTGCATGCGGAGATCGCTGAAACGGCGGCCAGGCTTGCGGCGAGCGCG
      GluAlaGlyTyrLeuHisAlaGluIleAlaGluThrAlaAlaArgLeuAlaAlaSerAla
                                                    198------------

1202  GCAGATGTGACGCGCGTCCTTGTCGTGCTGCAGCAGTTCGATCCGCCCGGCGTCTTCGCG
      AlaAspValThrArgValLeuValValLeuGlnGlnPheAspProProGlyValPheAla
      ----------------------------------------217

1262  CCGCACGCTCAGTGAATGCCTCGCCATCCAGCTCCGGTTGCGCAATGGCCTCGATCCGCG
      ArgThrLeuSerGluCysLeuAlaIleGlnLeuArgLeuArgAsnGlyLeuAspProAla

1322  ATGGAGGCGCTTGTCGCCAATCTCGAACTTCTGGGCCGCCGCGACTTCGCAAGCCTCAAG
      MetGluAlaLeuValAlaAsnLeuGluLeuLeuGlyArgArgAspPheAlaSerLeuLys

1382  AAGATCTGCGGGGTCGACGAGGAGGATCTCATCGAGATGCTCGCTGAAATCCGCAAACTC
      LysIleCysGlyValAspGluGluAspLeuIleGluMetLeuAlaGluIleArgLysLeu

1442  GATCCGAAGCCAGGAACCAGCTTCGAGACCAGCGTTACCGAGGCGATCATTCCCGATGTC
      AspProLysProGlyThrSerPheGluThrSerValThrGluAlaIleIleProAspVal

1502  GTCGTACGTTCGCGGCCCGATGGCGGCTGGCTGGTCGAGCTCAATCCCGACGCATTGCCA
      ValValArgSerArgProAspGlyGlyTrpLeuValGluLeuAsnProAspAlaLeuPro
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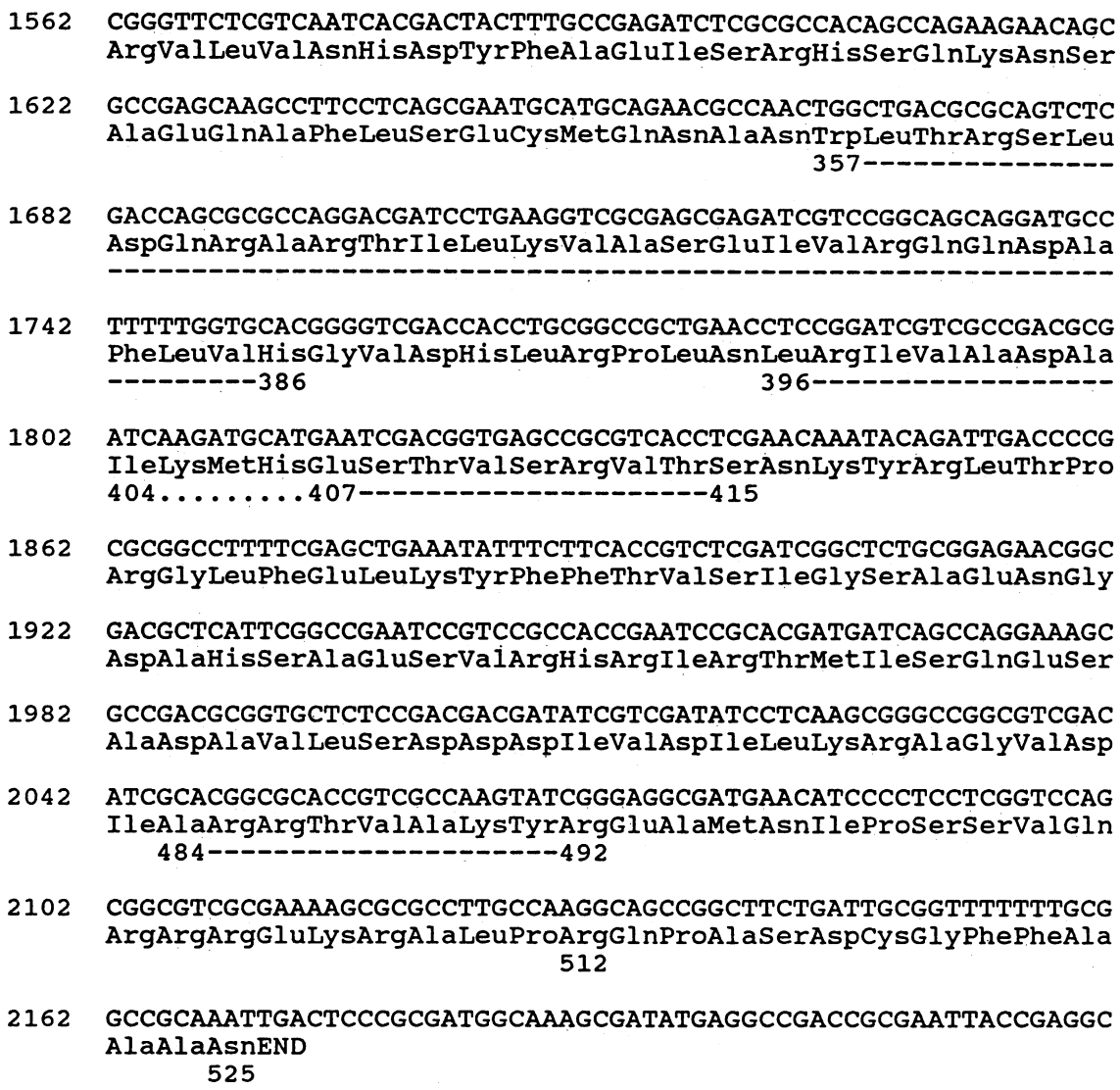

FIG. 2. Nucleotide and deduced amino acid sequences of *Rhizobium* strain NGR234 *rpoN* gene. Numbering of the sequence was made from the first nucleotide of the *Eco*RI site of the sequenced fragment. Potential −35 and −10 regions are underlined. The possible ribosome-binding site is indicated by asterisks. The initiation codon ATG is at nucleotide 596, and the termination codon is at nucleotide 2171. The deduced sequence of 525 amino acids is also shown. Comparative analysis with other RPON proteins (see Results) identified three major regions: Met-1 to Glu-50 (region I), Glu-50 to Lys-152 (region II), and Lys-152 to Asn-525 (region III). Within region III, four conserved subregions are shown by dashed underlining between the first and last amino acids.

correlated phenotype by testing the effect of deletion *rn3* on its capacity to utilize all amino acids as sole nitrogen or carbon source. All amino acids except tyrosine served as an N or C source for NGR234, as did homoserine and ornithine. NGR234*rn3* exhibited the same Dct$^-$ Nas$^-$ (nitrate assimilation) phenotype as mutant NGR234*rn1* (40). The kinetics of legume nodulation by mutant and wild type was examined for *V. unguiculata* (cowpea) by using the growth pouch method to score nodule formation as a function of time. Nodulation kinetics of NGR234*rn3* and NGR234R are presented in Fig. 4. The mutant formed only Fix$^-$ nodules on *V. unguiculata*, and there was a delay of 9 days. By 30 days after inoculation, the mutant had formed approximately half the number of nodules formed by the parent. All plants were nodulated by the mutant. Both wild type and mutant gave sigmoidal curves, indicating that the infection process was parallel although its kinetics was delayed (Nod^d).

In order to evaluate possible host plant-specific effects on symbiotic nitrogen fixation by the mutant, 11 legume host plants of NGR234 were inoculated with wild-type or mutant bacteria. Growth conditions were optimized for tropical legumes (see Materials and Methods). Acetylene reduction (nitrogenase) activities for NGR234R and NGR234*rn3* are shown in Table 2.

We also analyzed Fix$^-$ (*rn3*) and Fix$^+$ (wild-type) nodules of *V. unguiculata* and *M. atropurpureum* by light and electron microscopy. These experiments indicated that fewer cells were infected by the *rpoN* mutant in nodules of either plant (Fig. 5A and D and 6A and D). There was no other observable difference in these low-power tissue maps, which revealed true determinate root nodules in all four cases. In the case of *rn3* nodules (Fig. 5E and 6E), plant cell cytoplasm was electron lucent and showed little evidence of organelles. This contrasted with wild-type infected-cell cy-

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NGRRPON     MALSASLHLRQSQSLVMTPQLMQSIQLLQMTHLELGQFIAQEVEKNPLLEVPIAD  55
RMRPON      MALSASLHLRQSQSLVMTPQLMQSIQLLQMNHLELSHFIAQEVEKNPLLEVQPAD  55
PPRPON      MK--PSLVLKMGQQLTMTPQLQQAIRLLQLSTLDLQQEIQEALESNPMLERQEDG  53

NGRRPON     EA-VSVRIGPSVMRHVTAEAGGAEESVDHGDLYDSATTSPGERLRSELDADFANV  109
RMRPON      EPTISDREDAG--PHPAETGGETDEAAGQSDLYDSAMSRSGERLSEGLDADFANV  108
PPRPON      E-----------------------------DFDNSDP-------------------  61

NGRRPON     FQDDTAPQR-DAPE-------------------------------------------  122
RMRPON      FPDDTAPQRADAPE-------------------------------------------  122
PPRPON      -MADNAENKPAAEVQDNSFQESTVSADNLEDGEWSERIPNELPVATAWEDIYQTS  115

NGRRPON     ---------------------------LLGQWKSMPGAGGSNDGEGYDLDDSSPV  150
RMRPON      ---------------------------LLGQWKSMPGAG---DAEGYDLDDF--V  145
PPRPON      ASSLPSNDDDEWDFTTRTSAGETLQSHLLWQLKLAPMSD----------------  154

NGRRPON     G---KLRETLIEQVAFAFAAAADRLIAQHLIDQLDEAGYLHAEIAETAARLAAS-  201
RMRPON      GGRKTLRETLAEQLPFALSAVSDRLIARYFIDQLDDAGYLHADLAETAETLGAA-  199
PPRPON      ---------------------TDRLIAVTLIDSMNGQGYL----EDTLEEISAGF  184

NGRRPON     -------AADVTRVLVVLQQFDPPGVFARTLSECLAIQLR---LRNGLDPAMEAL  246
RMRPON      -------GEDVARVLHVLQQFDPPGVFARTLGECLAIQLR---ARNRLDPAMEAL  244
PPRPON      DPELDIELDEVEAVLHRIQQFEPAGVGARNLGECLLLQLRQLPATTPWMTEAKRL  239

NGRRPON     VAN-LELLGRRDFASLKKICGVDEEDLIEMLAEIRKLDPKPGTSFETSVTEAIIP  300
RMRPON      VAN-LELLARRDFASLKKICGVDEEDLIDMLAEIRKLDPKPGTSFETGVFEAIIP  298
PPRPON      VTDFIDLLGRRDYSQLMRRMKIKEDELRQVIELVQTLNPRPGSQIESSEPEYVVP  294

NGRRPON     DVVVRSRPDGGWLVELNPDALPRVLVNHDY-------------------FAEISR  336
RMRPON      DVVVRAAPDGGWLVELNPDALPRVLVNHDY-------------------FTEISR  334
PPRPON      DVIVRKDSDR-WLVELNQEAIPRLRVNPQYAGFVRRADTSADNTFMRNQ------  342

NGRRPON     HSQKNSAEQAFLSECMQNANWLTRSLDQRARTILKVASEIVRQQDAFLVHGVDHL  391
RMRPON      SSRKNSGEQAFLNECLQNANWLTRSLDQRARTIMKVASEIVRQQDAFLIHGVGHL  389
PPRPON      ---------------LQEARWFIKSLQSRNETLMKVATRIVEHQRGFLDHGDEAM  382
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NGRRPON     RPLNLRIVADAIKMHESTVSRVTSNKYRLTPRGLFELKYFFTVSIGSAENGDAHS  446
RMRPON      RPLNLRIVADAIKMHESTVSRVTSNKYMLTPRGLFELKYFFTVSIGSAENGDAHS  444
PPRPON      KPLVLHDIAEAVRMHESTISRVTTQKYMHTPRGIYELKYFFSSHVSTSE-GGECS  436

NGRRPON     AESVRHRIRTMISQESADAVLSDDDIVDILKRAGVDI ARRTVAKYR EAMNIPS  499
RMRPON      AESVRHRIRTMINQESADAVLSDDDIVDVLKQAGVDI ARRTVAKYR EAMSIPS  497
PPRPON      STAIRAIIKNLVAAENQKKPLSDSKIAGLLEGQGIQM ARRTVAKYR ESLGIAP  489
KPRPON                                           V ARRTVAKYR ESL
AVRPON                                           V ARRTVAKYR ESL
RCRPON                                           I ARRTVAKYR STL

NGRRPON     SVQRRREKRALPRQPASDCGFFAAAN                            525
RMRPON      SVQRRREKRALPRPRDSERCRQAASA                            523
PPRPON      SSERKRLM                                              497
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FIG. 3. Alignment of NGRRPON with most similar (RmRPON) and most dissimilar (PpRPON) proteins, showing the RPON box. Symbols: | , positions with identical residues in RPON proteins; :, positions with conservative substitutions. Conservative substitution were Ala-Gly, Ser-Thr, Asp-Glu, Asn-Gln, Arg-His-Lys, Ile-Leu-Met-Val, and Phe-Tyr-Trp. The box at the carboxy terminus aligns a 9-amino-acid sequence (region IIID of NGRRPON) with conserved residues of RmRPON and PpRPON and the relevant regions of AvRPON, KpRPON, and RcRPON. The 9-amino-acid sequence represents a core RPON box which expands to 13 amino acids if conservative substitutions are taken into account as shown (1 preceding, 3 following).

toplasm (Fig. 5B and 6B), where organelles were abundant. In the case of NGR234*rn3*, the infected plant cells contained a homogeneous population of "bacteroids" without normal peribacteroid membrane (pbm) vesicles. Although pbm might have existed in a very compromised form, it is clear that the normal massive synthesis (47) of this membrane accompanying bacteroid division was absent. Wild-type NGR234-infected plant cells contained abundant pbm (Fig. 5B and 6B). At a magnification of 10,000, wild-type NGR234 was seen to form one bacteroid per pbm envelope in either plant host. Synchronously dividing bacteroid-pbm envelope(s) was also observed. Wild-type nitrogen-fixing bacteroids contained much refractile storage material, presumably polyhydroxybutyric acid granules (Fig. 5C and 6C). These granules were not seen in *rn3* bacteroids (Fig. 5F and 6F). *rn3* bacteroids in *Macroptilium* nodules (Fig. 6F) appeared to be plasmolyzed.

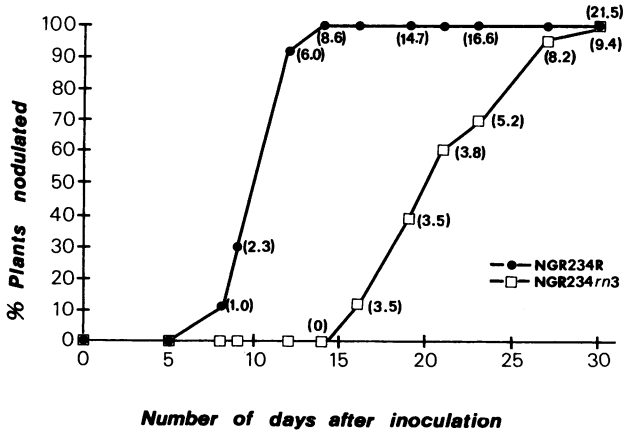
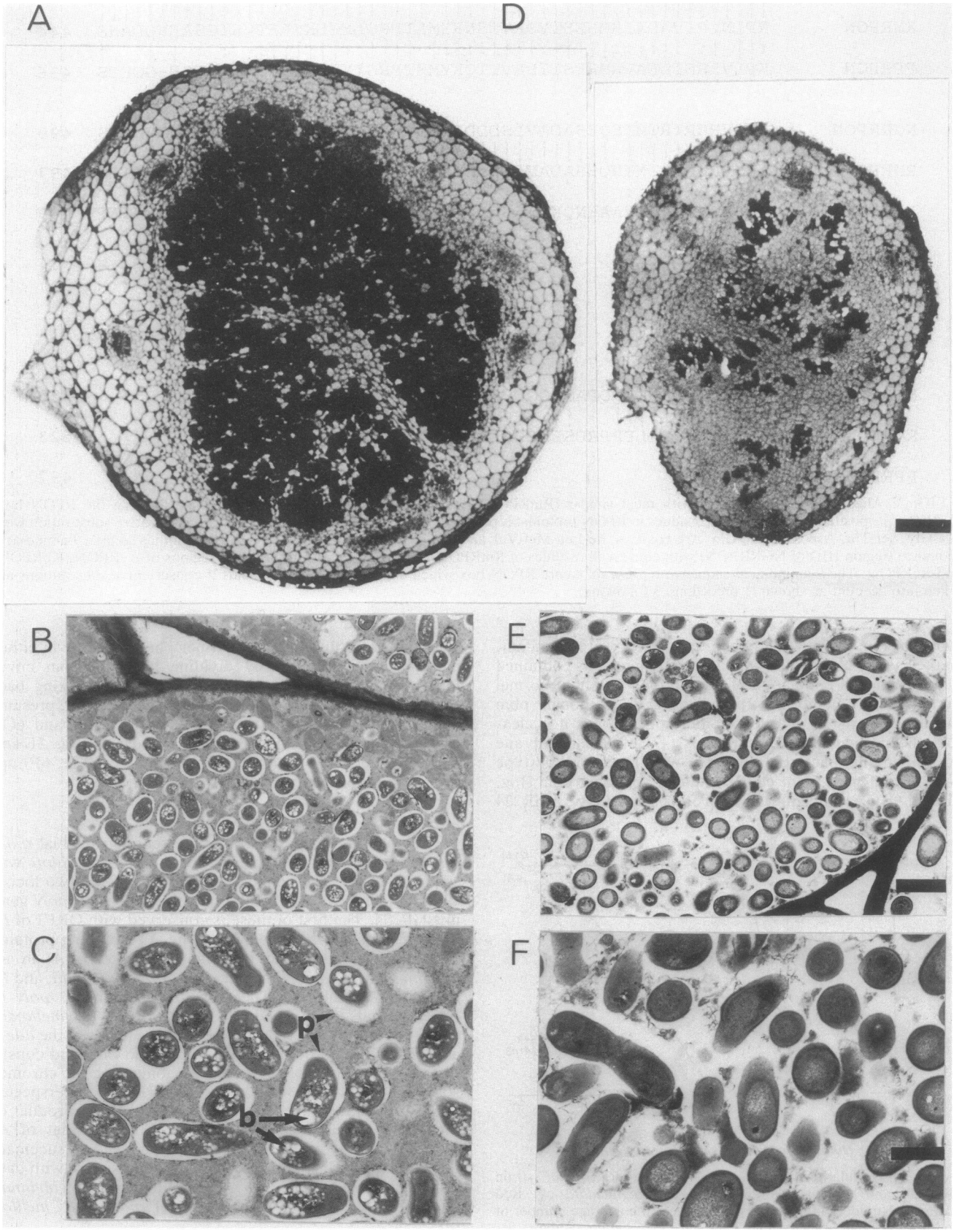


FIG. 4. Nodulation kinetics of wild-type *Rhizobium* strain NGR234 and mutant NGR234*rn3* on *V. unguiculata* cv. Red Caloona. Numbers in parentheses represent the average number of nodules per plant at each time. The figure is based on 25 plants per strain (1 plant per growth pouch).

DISCUSSION

The *rpoN* locus of NGR234 is the second rhizobial *rpoN* (*ntrA*) gene to be characterized; that of *R. meliloti* was sequenced (36). The genetic organization of the two loci is similar. One ORF was found on each side of the *rpoN* gene in NGR234. The first of these is conserved with ORF1 of *R. meliloti*, which is homologous to the ATP-binding proteins (2). This gene is found upstream of *rpoN* (*ntrA*) in *K. pneumoniae*, *Salmonella typhimurium*, *A. vinelandii*, and *P. putida* (2, 21, 24, 25). The ORF downstream of *rpoN* in NGR234 is also conserved in *K. pneumoniae*, *A. vinelandii*, and *R. meliloti* (25). The *rpoN* locus maps between the *ade-1* and *his-2* markers on the NGR234 chromosome and constitutes a useful point of reference for comparing the chromosomal maps of NGR234 and *R. meliloti* (12, 32). Interspecies complementation experiments indicated that the product of the NGR234 *rpoN* gene could substitute for that of *A. vinelandii* to drive expression of RPON-dependent succinate catabolism or transport or both. This is consistent with data showing that the cloned *ntrA* (*rpoN*) locus of *A. vinelandii* complemented the Dct$^-$ phenotype of *rpoN* in *R. meliloti* (9).

The potential ribosome-binding site and putative promoter


A
D
B
E
C
F
p
b

region are conserved between NGR234 and *R. meliloti*; transcript mapping (2) located the active *rpoN* promoter in the *R. meliloti* sequence homologous to nucleotides 420 to 425 of NGR234. Low-level constitutive expression of *rpoN* was reported for both *R. meliloti* (36) and *K. pneumoniae* (26), and we concur that our NGR234 sequence suggests a weak promoter. The phenotype of pSD21::ΩS1 indicated that readthrough from ORF1 was not required for *rpoN* expression in NGR234. This is consistent with the transcript analysis made for the corresponding *R. meliloti* locus (2). We did not find any regulatory elements in the intergenic region between *rpoN* and ORF3 of NGR234. ORF3 may therefore be cotranscribed with *rpoN* but translated from its own potential ribosome-binding site.

Our analysis of the deduced amino acid sequence confirms that the NGR234 *rpoN* gene encodes an alternative sigma factor of the σ^{54} family defined for enteric bacteria (18, 25). The protein NGRRPON is large and acidic and contains three major regions (Fig. 2). Divergences between NGRRPON and other RPON proteins are found primarily in the second of these regions, which is probably less essential for function. Within the third region we confirmed the existence of subregions, three of which were first defined for AvRPON (25) and which we here term IIIA, IIIB, IIIC, and IIID. These subregions constitute a fingerprint of the RPON family (although RcRPON is divergent) and may play a role in the protein-protein and protein-DNA interaction between RPON and RNAP, activators, and the promoter. We found one clear helix-turn-helix motif which we have termed subregion IIIC. It has been proposed that this structure, typical of DNA-binding proteins, participates in promoter recognition (25, 33, 41). *Bacillus subtilis* σ^{29}, SPO1 gp28, and SPO1 gp34 also contain one such sequence (15). We also found another subregion, IIID, a block of identical amino acids (core box of 9 residues and maximum box of 13 residues) conserved between NGRRPON and five other RPON proteins, even where low overall homology exists, as between NGR234 and either *R. capsulatus* or *P. putida*. By analogy, a conserved sequence of 13 amino acids in the principal sigma factor, RPOD (RPOD box), of *E. coli*, *B. subtilis*, and *Streptomyces coelicolor* is presumed to have an intrinsic function in principal sigma factors (43). Following this example, we term the conserved sequence of RPON an RPON box. Further molecular investigation could be made of potential interactions between subregions IIIC and IIID of RPON and the target promoter (−24 to −12 region) in DNA.

Alternative sigma factors such as the heat shock factor σ^{32} of *E. coli* permit selective transcription of genes whose products engender a common physiological response, whereas σ^{54} is required to transcribe genes encoding proteins with diverse physiological roles (23). In *E. coli* and *K. pneumoniae*, σ^{54} is required for the use of arginine, histidine, and proline as nitrogen sources (6). Again, an *rpoN* mutant of *P. putida* (22) could not utilize the uncharged amino acids alanine, glycine, isoleucine, leucine, and serine as nitrogen sources. In NGR234 we found no effect of the *rpoN* mutation on ability to use as nitrogen source either of the nonoverlapping groups of amino acids (histidine, arginine, and proline or uncharged amino acids) whose uptake and degradation requires RPON in enteric bacteria or *Pseudomonas* species. On the other hand, the various phenotypes of *rpoN* mutation in NGR234 are unified by their common effect on symbiosis. For instance, *rpoN* mutation abolishes the ability of NGR234 to grow on 0.5 to 5 mM KNO_3. An *rpoN*-dependent phenotype of assimilatory nitrate reductase is coregulated by NTRC in *K. pneumoniae* (5) and *A. vinelandii* (44) but does not operate solely within a given range of nitrate concentrations. We note that since the inhibitory effect of soil nitrate on tropical legume nodulation occurs above 5 mM (48), the RPON requirement for the Nas (assimilatory nitrate reductase) phenotype of NGR234 exists only within the range of soil nitrate concentrations permissive for legume nodulation. It is not relevant under bacterial growth conditions which preclude plant infection.

The *rn3* mutation delayed nodulation of *V. unguiculata*, and this delay was quantified by the growth pouch method. The data in Fig. 4 are consistent with our previous finding that expression of a *nodC-lacZ* fusion was reduced by two-thirds in the *rn1* background (40). An *rpoN* mutant of *R. meliloti* is affected in the ex planta expression of *nod-lacZ* fusions (8). This mutant also had a delayed nodulation phenotype on its host plant, *Medicago sativa*, though the kinetics are less marked than those seen in Fig. 4 for NGR234*rn3* or NGR234R on *V. unguiculata*. In this context, Dusha et al. noted an interesting partial homology of the sequence TTGCA (found at nucleotides − 20 to − 25 relative to the transcriptional start sites of *nodABC* and the regulatory gene *nodD3* of *R. meliloti*) with the consensus promoters of *rpoN*-dependent genes.

The acetylene reduction activities of diverse legume hosts indicate that the *rn3* mutation abolished endosymbiotic nitrogen fixation (Fix^-) regardless of the plant host; i.e., there was no host plant-specific effect. Root nodule morphology is classified into two types. Indeterminate nodules typical of temperate-zone legumes such as *Medicago sativa* remain meristematic and are cylindrical. Determinate nodules formed by tropical legumes such as *V. unguiculata*, *M. atropurpureum*, and *Glycine max* are spherical at maturity and do not retain a meristematic zone. Fischer et al. (14) showed that the *nif* activator protein, NIFA, of *Bradyrhizobium japonicum* is required for normal development of determinate nodules on soybean; gross nodule morphology and bacteroid persistence were found to be *nifA* dependent. Our microscopic analysis indicated that although NGR234*rn3* formed true determinate nodules on *V. unguiculata* and *M. atropurpureum*, these nodules contained fewer infected plant cells and within the cells was a uniform population of *rn3* bacteroids apparently lacking pbm sacs. Whether this general major pbm defect is due to nonformation, instability, or premature senescence of the plant-generated membrane should be tested by immunocytochemical analysis. Our conclusion is that determinate nodules formed by the *rpoN* mutant lacked the critical membrane interface of "late" symbiotic nodule function, reflecting

FIG. 5. Structure of *V. unguiculata* determinate nodules formed by *Rhizobium* strain NGR234 or NGR234*rn3*. Tissue maps (A and D) (light microscopy; magnification, ×50; bar, 200 μm) and electron microscopy (B and E [magnification, ×4,500; bar, 2 μm] and C and F [magnification, ×10,000; bar, 1 μm]) of nodules are shown. Nodules were harvested 21 days (NGR234) or 30 days (NGR234*rn3*) after plant inoculation. A, Wild-type Fix^+ nodule composed of a mosaic of infected cells (dark areas) and uninfected cells (light areas); D, Fix^- NGR234*rn3* nodule, smaller and with fewer infected cells; B through F, interiors of infected cells. In panels B and C (NGR234), bacteroids are surrounded by pbm (p) and contain prominent granules of polyhydroxybutyrate (b). The plant infected-cell cytosol is dark (metabolically active), and peribacteroid spaces are white. In panels E and F (NGR234*rn3*), pbm is not seen and bacteroids contain no polyhydroxybutyrate.

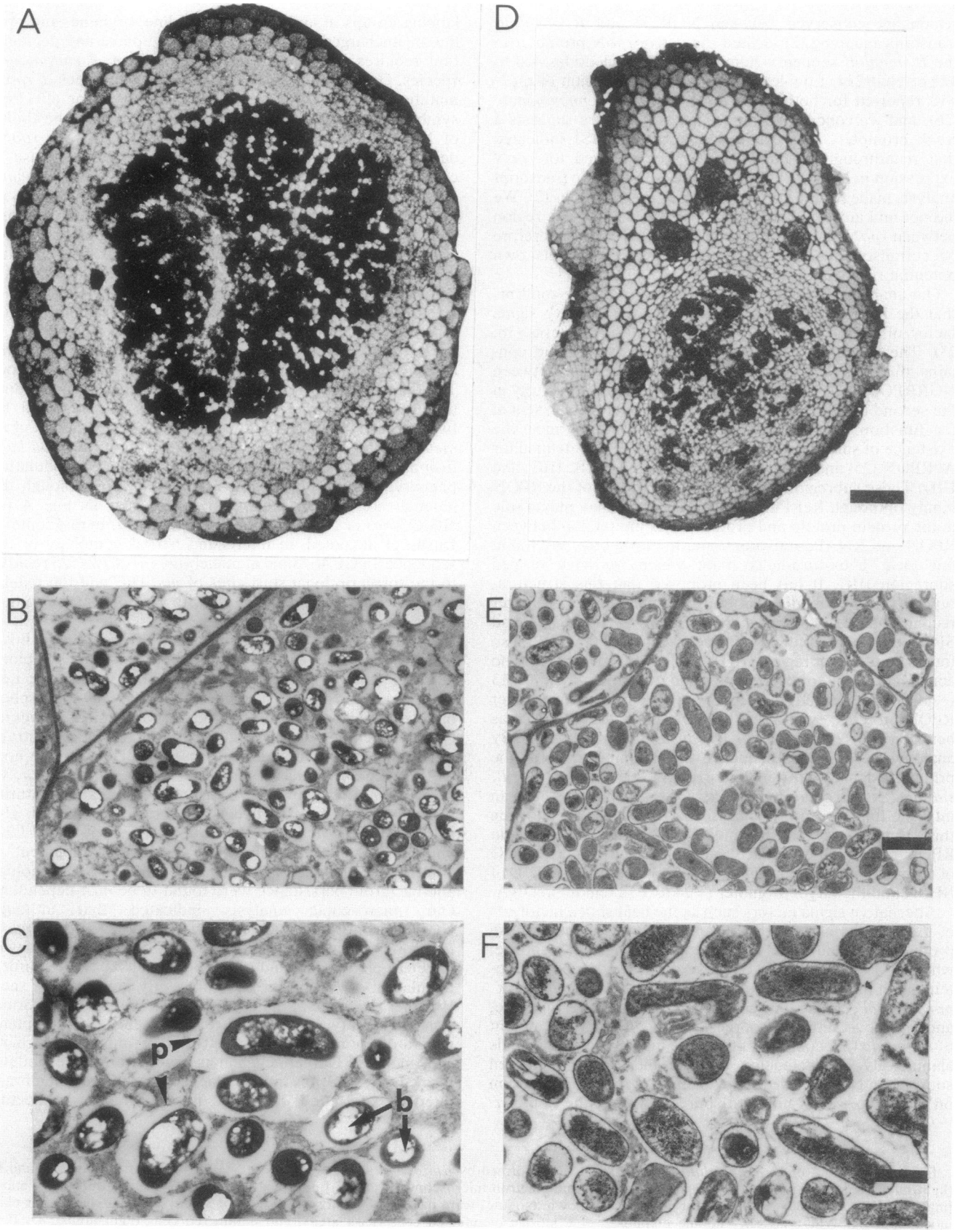


FIG. 6. Analysis of *M. atropurpureum* determinate root nodules formed by *Rhizobium* strain NGR234 or NGR234*rn3*. Magnifications, values, and labeling are like those given for Fig. 5, and similar histological features are seen in each comparison between NGR234 (Fix^+ nodule) and NGR234*rn3* (Fix^- nodule).

aberrant organogenesis. Since the bacteria are not partitioned outside plant cell cytoplasm, the association becomes parasitic rather than endosymbiotic. It will be of interest to examine such nodules for induction of host defense responses, such as phytoalexin synthesis (47).

The *rn3* phenotype is different from published phenotypes associated with mutations in the RPON-dependent genes *dctA* and *nifH*. All described *dct* mutants (*R. meliloti*, *Rhizobium trifolii*, or *Rhizobium leguminosarum*) have been shown to form normal bacteroid pbm sacs. *R. trifolii dct* mutant CR7098 (35) formed pleiomorphic bacteroids with normal pbm in clover nodules, as did wild-type bacteria. Electron microscopic pictures of *dct* mutant GF31 of *R. leguminosarum* (13) showed normal bacteroid membranes and pbm. Engelke et al. (10) observed occasional premature senescence of bacteroids of their *dctA* mutant of *R. meliloti* but saw no evidence of bacteroids without pbm sacs. In the case of *nifH* mutants of *B. japonicum* on their determinate-nodule host, *G. max*, bacteroids in normal pbm were formed, and the only observable difference from wild type was "massive PHBA [polyhydroxybutyric acid] accumulation" (16). In *R. meliloti*, *nifH* mutant bacteroids were also enclosed in pbm sacs. Differences from wild-type bacteroid pbm were minor (17).

A precedent exists for coregulation of essential symbiotic genes by RPON and an activator. For example, NIFA is an activator of genes such as *nifH* which are coregulated by RPON (30). Since a *dctA-lacZY* fusion is not expressed in alfalfa nodules formed by a *nifA* mutant of *R. meliloti* (46) and since *dctA* has 5′ elements for RPON and NIFA regulation (10, 19), this gene belongs to the same class. We propose that the class includes uncharacterized determinants of bacterial molecular signals for pbm synthesis or stability. The nonexpression of such RPON-dependent genes could account for the observed intracellular phenotype of NGR234*rn3*. By analogy, *R. meliloti* symbiotic plasmid genes influence catabolism of plant-derived substrates and competition for nodulation. Both are NIFA dependent (31, 38), and the former has a consensus RPON promoter (31). More than 20 fragments in the total DNA of *R. meliloti* hybridized to a probe for a 200-amino-acid region of the activators DCTD, NIFA, and NTRC which is implicated in interaction with RNAP-RPON (19). These authors suggest that these fragments correspond to a gene family encoding RPON-dependent transcriptional activators. Our data suggest that some such genes would be implicated in the signaling mechanisms necessary for determinate nodule organogenesis. In conclusion, we suggest the working hypothesis that RPON is a primary coregulator of the endosymbiotic life-style of members of the genus *Rhizobium*.

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