Differential Regulation of *Rhizobium etli rpoN2* Gene Expression during Symbiosis and Free-Living Growth

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The *Rhizobium etli rpoN1* gene, encoding the alternative sigma factor σ^{54} (RpoN), was recently characterized and shown to be involved in the assimilation of several nitrogen and carbon sources during free-living aerobic growth (J. Michiels, T. Van Soom, I. D'hooghe, B. Dombrecht, T. Benhassine, P. de Wilde, and J. Vanderleyden, J. Bacteriol. 180:1729–1740, 1998). We identified a second *rpoN* gene copy in *R. etli*, *rpoN2*, encoding a 54.0-kDa protein which displays 59% amino acid identity with the *R. etli* RpoN1 protein. The *rpoN2* gene is cotranscribed with a short open reading frame, *orf180*, which codes for a protein with a size of 20.1 kDa that is homologous to several prokaryotic and eukaryotic proteins of similar size. In contrast to the *R. etli rpoN1* mutant strain, inactivation of the *rpoN2* gene did not produce any phenotypic defects during free-living growth. However, symbiotic nitrogen fixation was reduced by approximately 90% in the *rpoN2* mutant, whereas wild-type levels of nitrogen fixation were observed in the *rpoN1* mutant strain. Nitrogen fixation was completely abolished in the *rpoN1 rpoN2* double mutant. Expression of *rpoN1* was negatively autoregulated during aerobic growth and was reduced during microaerobiosis and symbiosis. In contrast, *rpoN2-gusA* and *orf180-gusA* fusions were not expressed aerobically but were strongly induced at low oxygen tensions or in bacteroids. Expression of *rpoN2* and *orf180* was abolished in *R. etli rpoN1 rpoN2* and *nifA* mutants under all conditions tested. Under free-living microaerobic conditions, transcription of *rpoN2* and *orf180* required the RpoN1 protein. In symbiosis, expression of *rpoN2* and *orf180* occurred independently of the *rpoN1* gene, suggesting the existence of an alternative symbiosis-specific mechanism of transcription activation.

Bacteria are able to activate or switch off specific sets of genes as they face changing environmental conditions. This can be achieved through the activities of RNA polymerases containing alternate σ factors and their cognate regulatory proteins. RNA polymerases containing the alternative sigma factor σ^{54} (σ^N [RpoN]) recognize a conserved sequence motif centered at -24 and -12 nucleotides from the transcriptional start site (5'-TGGCAC-N$_5$-TTGCA/T-3' [see reference 9]). Transcription initiation from these promoters depends on the presence of specific activators which typically bind to DNA sequences located over 100 nucleotides upstream from the transcriptional start site (4). The activity of these activator proteins has been shown in several cases to be regulated by phosphorylation in response to physiological signals. In addition, evidence is accumulating that gene transcription by the σ^{54} RNA polymerase holoenzyme not only is regulated by the activator protein but also is regulated under certain conditions by σ^{54} abundance (3). In *Caulobacter crescentus*, transcription of *rpoN* is temporally regulated during the cell cycle and is 10-fold induced at the transition from swarmer to stalked cell (3). Up-regulation of *rpoN* coincides with the onset of stalk formation and occurs before the σ^{54}-dependent increase in flagellar gene expression. The *rpoN* gene from *Rhodobacter capsulatus* is organized in a *nifU₂-rpoN* superoperon, and transcription of *rpoN* is under the control of two promoters. One promoter upstream of *nifU₂* is constitutively expressed with respect to nitrogen; a secondary nitrogen-dependent promoter is autoactivated by RpoN and NifA (5). Expression from the *R. capsulatus rpoN* secondary promoter is required for growth under

certain stress conditions (5). *Bradyrhizobium japonicum* is presently the only bacterium in which two differentially regulated *rpoN* genes have been identified (16). Expression of the first copy, *rpoN1*, is activated in microaerobiosis by the FixLJ-FixK2 regulatory cascade, while the *rpoN2* gene is negatively autoregulated (9, 16). In *Azorhizobium caulinodans*, one of the two genes coding for σ^{54}, *rpoF*, is required for high-level *nifA* expression (19, 33). Indirect genetic evidence suggests that *Rhodobacter sphaeroides* (20) also contains a second *rpoN* gene.

Rhizobium etli is the nodulating symbiont of the common bean plant, *Phaseolus vulgaris*. It possesses at least two different regulatory cascades of nitrogen fixation genes. The first cascade is dependent on the transcriptional regulator *nifA* and controls the expression of both *nifHDK* operons, the third *nifH* gene, which is not linked to other *nif* genes, and the production of the black pigment melanin (23, 24, 34). Although the *R. etli nifA* gene is transcribed under aerobic and microaerobic conditions, NifA-dependent gene activation is only operative at low oxygen tensions (24). The second cascade is controlled by the *R. etli fixLJ* genes (6). Unlike the FixL protein from *Sinorhizobium meliloti* (14), *R. etli* FixL is not a hemoprotein (7). The physiological signal sensed by *R. etli* FixL is presently unknown. We have recently identified the *R. etli rpoN* gene (hereafter called *rpoN1* [26]). Under free-living conditions, *rpoN1* controls growth on C$_4$-dicarboxylic acids and on several nitrogen sources. In addition, inactivation of *rpoN1* abolishes microaerobic expression of *nifH* and the production of the black pigment melanin under free-living conditions (26).

Here, we describe the isolation of a second *rpoN* gene of *R. etli* CNPAF512, named *rpoN2*. This copy is located near a cluster of nitrogen fixation genes. Mutant analysis reveals that the *rpoN1* and *rpoN2* genes are both active, but under different physiological conditions. The *rpoN1* gene is essential during free-living growth, while the second copy, *rpoN2*, is required

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Source or reference
Strains		
E. coli		
DH5α		Gibco BRL
HB101	Sm^r	30
R. etli		
CNPAF512	Nal^r, wild type	26
FAJ1154	Nm^r *rpoN1*::Ω-Km	26
RpFAJ1002	Nm^r *fixL*::Ω-Km	6
Rp1000	Nm^r *nifA*::*aphII*	24
FAJ1169	Nm^r *rpoN2*::Ω-Km	This work
FAJ1170	Nm^r Sp^r *rpoN1*::Ω-Sp *rpoN2*::Ω-Km	This work
FAJ1172	Nm^r Sp^r *rpoN1*::Ω-Sp *rpoN2*::pJQ200-UC1-Km	This work
FAJ1173	Nm^r *orf180*::Ω-Km	This work
FAJ1175	Sp^r *rpoN1*::Ω-Sp	This work
FAJ1176	Nm^r Sp^r *fixL*::Ω-Km *rpoN1*::Ω-Sp	This work
CNPAF512::pFAJ1187	Nm^r *rpoN2*::pFAJ1187	This work
CNPAF512::pFAJ1188	Nm^r *orf180*::pFAJ1188	This work
Plasmids		
pUC18	Ap^r, cloning vector	27
pUC18Not	Ap^r, cloning vector	15
pCR2.1	Ap^r, PCR TA cloning vector	Invitrogen
pJQ200-UC1	Gm^r *sacB*	29
pHP45Ω-Km	Ap^r Km^r	8
pHP45Ω-Sp	Ap^r Sp^r	8
pWM6	Ap^r Nm^r *uidA2*	22
pLAFR1	Tc^r, broad-host-range vector	12
pLAFR3	Tc^r, broad-host-range vector	32
pFAJ21	Tc^r, *A. brasilense pnifH-gusA*	35
pFAJ1172	Tc^r, *rpoN2* gene in pLAFR1	This work
pFAJ1173	Tc^r, *rpoN2* gene in pLAFR1	This work
pFAJ1174	Ap^r, 4.2-kb *Not*I fragment containing *rpoN2* and *orf180* cloned in pUC18Not	This work
pFAJ1175	Tc^r, *rpoN2-gusA* fusion in pLAFR3	This work
pFAJ1176	Tc^r, *orf180-gusA* fusion in pLAFR3	This work
pFAJ1180	Tc^r, *rpoN2-gusA Sma*I promoter deletion in pLAFR3	This work
pFAJ1181	Tc^r, *orf180-gusA Sma*I promoter deletion in pLAFR3	This work
pFAJ1183	Gm^r Km^r, pJQ200-UC1 containing *rpoN2*::Ω-Km	This work
pFAJ1184	Gm^r Km^r, pJQ200-UC1 containing *orf180*::Ω-Km	This work
pFAJ1185	Ap^r, pCR2.1 containing a 0.6-kb internal PCR fragment from *rpoN2*	This work
pFAJ1186	Gm^r Km^r, pJQ200-UC1 containing *rpoN1*::Ω-Sp	This work
pFAJ1187	Gm^r Km^r, pJQ200-UC1 containing *rpoN2*::*gusA*	This work
pFAJ1188	Gm^r Km^r, pJQ200-UC1 containing *orf180*::*gusA*	This work

for symbiosis. Transcription of the *R. etli rpoN2* gene is highly induced under free-living microaerobic conditions by the RpoN1 and NifA proteins. In contrast, expression of *rpoN2* in bacteroids occurs independently of the *rpoN1* gene. We therefore propose that the activity of *rpoN2* is regulated, in addition to oxygen, by a symbiosis-specific signal.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this work are listed in Table 1 or schematically presented in Fig. 1. *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium. *R. etli*

strains were cultured in liquid TY (0.5% tryptone, 0.3% yeast extract, 7 mM CaCl$_2$) medium at 30°C or maintained on yeast-mannitol (YM) agar plates. Antibiotics were supplied to the medium to maintain selection for plasmids or to select recombinant strains at the following concentrations: 60 μg of nalidixic acid, spectinomycin, and neomycin per ml; 30 μg of kanamycin and gentamicin per ml; 100 μg of ampicillin per ml; and 1 μg (*R. etli*) or 10 μg (*E. coli*) of tetracycline per ml. Growth tests of *R. etli* on plates were carried out with acid minimal salts (AMS) medium (28) containing 1 mM CaCl$_2$ and the carbon and nitrogen sources indicated in the text. Plates were incubated at 30°C under aerobic or microaerobic conditions (0.5% oxygen), and colony size was monitored over a period of 3 to 7 days. Triparental conjugations and site-directed mutagenesis of *R. etli* were done as described previously (6).

DNA methods. DNA preparation and recombinant DNA techniques were performed according to standard procedures (2, 30). DNA fragments were recovered from agarose gels with the Nucleotrap kit (Macherey-Nagel). Hybridizations were carried out with digoxigenin-labeled probes as described by the manufacturer (Boehringer Mannheim). To generate blunt ends to incompatible DNA fragments, DNA was incubated with Klenow or T4 DNA polymerases in the presence of the four deoxynucleoside triphosphates.

DNA sequencing. Automated DNA sequencing was performed with an A.L.F. sequencer (Pharmacia Biotech) with fluorescein-labeled oligonucleotide primers. Sequence data analysis was performed with the PC/Gene software package (IntelliGenetics, Inc.). Homology searches were carried out with the BLAST server at the National Center for Biotechnology Information.

PCR-mediated isolation of cosmid clones carrying the *R. etli rpoN2* gene. A 0.6-kb fragment from the *R. etli rpoN2* gene was amplified with the primers OJM081 (5′-CTGAGAATTCGACGCGGCTGACGGTGGATTCGTGC-3′) and OJM082 (5′-CTGAGAATTCGATCCGCCGGGTGTTTTCGC-3′) and genomic DNA from the *R. etli rpoN1* mutant strain FAJ1154 as a template (26). Amplification of DNA fragments by PCR was performed as described previously (26), except that the annealing temperature was 50°C. The primers were chosen in conserved regions of *rpoN* genes and annealed at each site of the *Pst*I restriction site of the *rpoN1* gene used to insert the Ω-Km interposon, thereby avoiding preferential amplification of the *rpoN1* gene. The 0.6-kb PCR product was subsequently cloned into the pCR2.1 TA cloning vector (Invitrogen). The nucleotide sequence of this fragment was different from that of *rpoN1*, but was clearly homologous to the *rpoN1* and *rpoN* genes from other bacterial species.

Because we were unable to isolate a cosmid clone containing *rpoN2* by colony PCR with OJM081 and OJM082, new primers perfectly matching the *rpoN2* sequence were designed (OJM093, 5′-GAATCCATCATACCCGACGTC-3′; and OJM094, 5′-TGGTGGCGCACGATTTCGGTC-3′) to amplify a 240-bp fragment. These primers were used to screen a pLAFR1 cosmid library of *R. etli* CNPAF512, maintained in *E. coli* HB101 and containing approximately 2,000 clones (6), for plasmids carrying the *R. etli rpoN2* gene. PCRs were first carried out with cell mixtures of 16 different clones. In a second step, pools of 16 clones yielding a fragment of the expected size were each analyzed separately. Two positive overlapping cosmid clones, pFAJ1172 and pFAJ1173, were selected for further analysis.

Expression analysis of *gusA* fusions and melanin production. Precultures were grown overnight in TY medium and diluted 20-fold in AMS medium containing 10 mM NH$_4$Cl and 10 mM succinate. Microaerobic induction of the different strains was carried out overnight at 0.3% oxygen as described previously (24). β-Glucuronidase activity and melanin production were quantified as described previously (26).

Plant culture and bacteroid isolation. Seeds of *Phaseolus vulgaris* cv. Limburgse vroege were sterilized, and seedlings were inoculated as previously described (25). Bean plants were grown in 250-ml cylindrical flasks in a Sanyo Gallenkamp Fitotron plant growth room with a 12-h photoperiod (day/night temperature, 22°C/18°C; day/night relative humidity, 65%/75%). Acetylene reduction activities (ARAs) were determined 4 weeks after inoculation on a Hewlett-Packard 5890A gas chromatograph equipped with a "PLOT fused silica" column. Ethylene production was quantified with propane as an internal standard.

To determine symbiotic expression of the different *gusA* fusion plasmids, bacteroids were isolated from the nodules of 4-week-old plants. For this, all of the nodules of one plant were collected and treated essentially as described by Leyva et al. (18). The nodules were crushed on ice in 15-ml plastic tubes in the presence of 0.5 g of polyvinylpolypyrrolidone and 2 ml of Mg-phosphate buffer (2.5 mM MgCl$_2$, 50 mM potassium phosphate [pH 6.8]). Next, the paste was diluted with 10 ml of ice-cold Mg-phosphate buffer. The tubes were centrifuged at 121 × g for 1 min to remove plant cell material. This step was repeated with the supernatant. Bacteroids were finally precipitated at 4°C by centrifugation at 4,500 × g for 5 min and resuspended in 100 μl to 1 ml of Mg-phosphate buffer. Dilutions of the bacteroids were immediately assayed for glucuronidase activity.

Construction of mutants. To construct *rpoN2* and *orf180* mutants, a 4.2-kb fragment containing the entire *rpoN2* gene, except the amino terminus, and the complete *orf180* gene was amplified by PCR with primers OJM104 (5′-CTGAG GATCCGCGGCCGCGCCTGTTTCCTTGAGCTTG-3′) and OJM106 (5′-CT GAGGATCCGCGGCCGCTTTGTCCTGAATGTCAGTTC-3′) annealing near the 3′ end of *rpoN2* and approximately 2 kb upstream from *orf180*, respectively. Both primers contain *Not*I recognition sites at their 5′ ends. The 4.2-kb DNA fragment was cloned into the *Not*I site of pUC18Not (15). The resulting
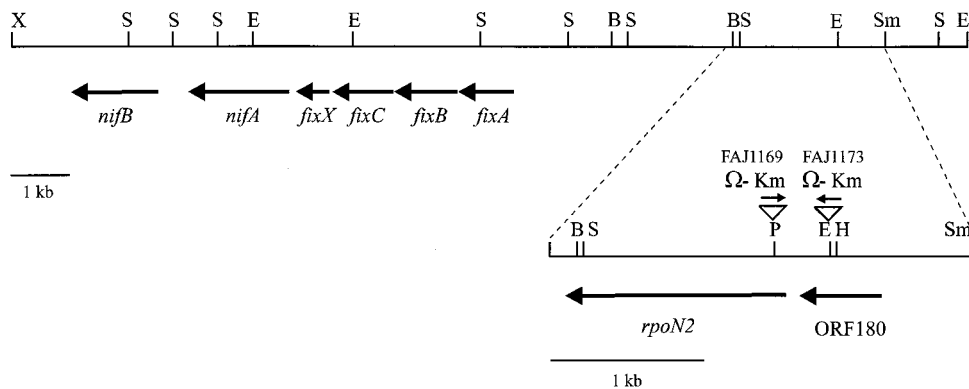
FIG. 1. Physical map of the *R. etli nif* region containing the *rpoN2* gene. The physical map of the 2.8-kb fragment that was sequenced is given below. The positions and orientations of the different ORFs are presented below the restriction maps. Triangles represent genomic insertions of the Ω-Km interposon or the *gusA* gene. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; X, *Xba*I.

plasmid, pFAJ1174, was digested with *Pst*I (*rpoN2*) or *Eco*RI (*orf180*) and blunt end ligated to the 1.8-kb *Bam*HI fragment from pHP45Ω-Km, thereby inactivating the *rpoN2* gene or *orf180*, respectively. In both plasmids, the orientations of Ω-Km and *rpoN2* or *orf180* are opposed and the same, respectively (Fig. 1). Finally, the 6-kb *Not*I fragments from the resulting plasmids were cloned into the *Not*I site of the *Rhizobium* suicide plasmid pJQ200-UC1, yielding pFAJ1183 (*rpoN2*::ΩKm) and pFAJ1184 (*orf180*::ΩKm). These two mutations were finally recombined into the genome of *R. etli* CNPAF512. Insertion of the Ω-Km cassette at the correct site was confirmed by Southern hybridization with the appropriate DNA probes. The *R. etli rpoN2* mutant was named FAJ1169, and the *R. etli orf180* mutant was named FAJ1173.

An *R. etli rpoN1 rpoN2* double mutant was constructed as follows. A pJQ200-UC1 derivative containing the 1.8-kb *Eco*RI fragment from pFAJ1150 carrying part of the *R. etli rpoN1* gene (26) was digested with *Pst*I and blunt end ligated with the 1.7-kb Ω-Sp cassette from pHP45Ω-Sp, thereby inactivating the gene. The construct carrying *rpoN1* with the Ω-Sp cassette in the opposite orientation (pFAJ1186) was used to mutagenize FAJ1169. The resulting mutants were finally tested for growth on AMS plates containing 10 g of mannitol and 2 g of alanine per liter. *rpoN1* mutants are unable to grow on alanine (26). Appropriate insertion of the resistance cassette was verified by Southern hybridization. The *R. etli rpoN1 rpoN2* double mutant was named FAJ1170.

To confirm the results obtained with FAJ1170, we also constructed a second, independent *R. etli rpoN1 rpoN2* double mutant. *R. etli* CNPAF512 was mutagenized with plasmid pFAJ1186, yielding the *R. etli rpoN1* mutant FAJ1175. Next, the *rpoN2* internal 0.6-kb PCR fragment, amplified with primers OJM081 and OJM082 (described above), was cloned into pCR2.1. The resulting vector was digested with *Bam*HI and ligated to the 3.8-kb *Bam*HI fragment from pWM6, thereby inserting a Nm^r gene in a site flanking the 0.6-kb insert. The 4.4-kb *Kpn*I-*Not*I insert from this construct was finally ligated into *Sma*I-digested pJQ200-UCI, after the ends of the insert fragment had been blunted. This plasmid was used to mutagenize *R. etli* FAJ1175. In the resulting *R. etli rpoN1 rpoN2* strain, FAJ1172, the *rpoN2* gene is disrupted by insertion of the entire plasmid into the gene.

Finally, to determine whether the *R. etli rpoN2* and *fixLJ* genes belong to the same regulatory cascade, a *R. etli fixL rpoN1* double mutant was generated. For this, the *R. etli fixL* mutant RpFAJ1002 (6) was mutagenized with pFAJ1186. The resulting strain was named FAJ1176.

Construction of *rpoN2-gusA* and *orf180-gusA* plasmid and chromosomally integrated fusions. To construct transcriptional *rpoN2-gusA* and *orf180-gusA* fusions, pFAJ1174 was digested with *Pst*I (*rpoN2*) or *Eco*RI (*orf180*) and blunt end ligated to the 3.8-kb *Bam*HI fragment from pWM6. The two 8-kb *Not*I fragments carrying the *gusA* gene insertion in the right orientation were cloned, after being blunted, into *Bam*HI-digested pLAFR3 or into the *Not*I site of pJQ200-UC1. The resulting plasmids were named pFAJ1175 (pLAFR3-*rpoN2-gusA*), pFAJ1176 (pLAFR3-*orf180-gusA*), pFAJ1187 (pJQ200-UC1-*rpoN2-gusA*), and pFAJ1188 (pJQ200-UC1-*orf180-gusA*). To construct chromosomally integrated *rpoN2-gusA* and *orf180-gusA* fusions, pFAJ1187 and pFAJ1188 were integrated at the homologous site into the *R. etli* CNPAF512 genome, yielding CNPAF512::pFAJ1187 and CNPAF512::pFAJ1188. Promoter deletions of pFAJ1175 and pFAJ1176 were constructed as follows (also see Fig. 1). In pFAJ1180, the 6.3-kb *Sma*I fragment from pFAJ1175 was blunt end ligated into the *Bam*HI site of pLAFR3. Similarly, to generate pFAJ1181, the 6.3-kb *Sma*I fragment from pFAJ1176 was blunt end ligated into the *Bam*HI site of pLAFR3.

Nucleotide sequence accession number. The nucleotide sequence of the *R. etli rpoN2* gene locus has been deposited in the DDBJ-EMBL-GenBank nucleotide sequence databases under accession no. AJ005696.

RESULTS

Isolation and localization of the *R. etli rpoN2* gene. We have recently characterized the *R. etli rpoN1* gene (26). Mutant analysis indicated that although the *R. etli rpoN1* mutant had phenotypic defects under free-living conditions (26), symbiotic nitrogen fixation was not affected, suggesting the existence of a second *rpoN* copy. Therefore, based on the DNA sequence of *R. etli rpoN1*, several PCR primers were designed such that they annealed to conserved regions of *rpoN* genes belonging to different organisms. The primers were chosen to anneal at both sites of the *rpoN1* Ω-Km insertion, making it possible to distinguish between PCR fragments originating from the *rpoN1* and *rpoN2* genes, because PCR was performed with genomic DNA isolated from the *R. etli rpoN1* mutant, FAJ1154. A 0.6-kb internal fragment of the *rpoN2* gene was amplified with one of these primer combinations (see also Materials and Methods). Based on the DNA sequence of this fragment, new primers, specific for the *rpoN2* gene, were designed and used to screen a pLAFR1 genomic library of *R. etli* CNPAF512 (6), as detailed in Materials and Methods. Two overlapping cosmid clones, pFAJ1172 and pFAJ1173, were isolated. Partial DNA sequence analysis and restriction mapping of the DNA fragments common to both clones confirmed the identity of *rpoN2* and allowed us to localize the *rpoN2* gene approximately 3.5 kb from the previously identified *nifB-nifA-fixABCX* cluster of nitrogen fixation genes (23, 24). A physical map of this DNA region is presented in Fig. 1.

Sequence analysis of *R. etli rpoN2*. To further characterize the *rpoN* homologous DNA region, the DNA sequence of a 2,739-bp fragment was established (Fig. 2). Two putative open reading frames (ORFs) were identified (Fig. 1 and 2). The first ORF codes for a protein that displays 59% amino acid identity with the previously characterized *R. etli* RpoN1 protein and was therefore named *rpoN2*. RpoN2 contains 483 amino acids and has a calculated molecular mass of 54,013 Da and a pI of 5.71, which is higher than that of RpoN1 (pI, 4.32). A putative ribosome-binding site (AGGAG) is located 13 bp upstream from the *rpoN2* initiation codon. An alignment was made between *R. etli* RpoN1 and RpoN2 and the RpoN proteins from *S. meliloti* (1), *B. japonicum* (16), *A. caulinodans* (33), and *Rhizobium* sp. strain NGR234 (36). The three domains (as defined by van Slooten et al. [36]) of RpoN2 are amino-terminal glutamine-rich region I (M1 to E50), acidic region II (E50 to E118), and carboxy-terminal region III (E118 to G483). The latter domain contains a conserved helix-turn-helix motif involved in

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CCCGGGTGCCGGTCGGCATCAGCCGATCGATATTGGCGGCTGCGAGCTCTTCACGCCCCC    60
AACCTGCGGATATCCGCCATCGTCGTCTTCTGTGACGAAATTATCGGAGCCGTTTGCGTT    120
TCACCATTTTTCGATGCGCGGTCCGAGCCTGGCGCCCCCTTTTGTCGGACCTCAAACTTT    180
CTTGTGCAGTTTCAAACAAGAAAACGTGCGCGGGTAGACATACGCACGAGCAGAACCTCA    240
AGCCCCGTGACCCGCGCCTTCCCGCCCGCGACCGGACTGGCGGGGCAGTATGCTAAGGTTCT    300
AAAAGGCGACAGAGAAACCTTGATCGAGATGCGGTCGGAAGTAGCCGAAACAGCGCCCGG    360
CCGTCAGCGCGGCAGCAACGGCTTCACAATTCGCTCGCCCGCACGAGCAGCGCACGAGTG    420
CGGCGACGAAGCCCTGTGCTGACCGCAAGTCCATCTCCTTCAGGCCAAATGGCATGACGC    480
TTGCTTGCCCTGGCGTGTGGGCCGAGTCGGATTCGTGTTAGCCGTGACTGAGAGGCCGCA    540
AATAAATTTTCCTAGAGGAGCTTCATTGATGACCATGGAAAAGCAGGTCCCTATTGTCAC    600
                    (orf180)  M  T  M  E  K  Q  V  P  I  V  T
CTTTCGCACGCGGGTTCGCGACGAGTCTATATCCGGTCCCAACCCCTACCGCTGGAAGAG    660
F  R  T  R  V  R  D  E  S  I  S  G  P  N  P  Y  R  W  K  S
CAAGACGACGACCGACGACTATTTCAGCGGTAAGCGGGTAATCCTTTTTTCGCTGCCGGG    720
K  T  T  T  D  D  Y  F  S  G  K  R  V  I  L  F  S  L  P  G
CGCCTTCACGCCAATCTGCTCCACCTTCCAACTGCCTGATTTCGAAAGTCTTTATGTGGA    780
A  F  T  P  I  C  S  T  F  Q  L  P  D  F  E  S  L  Y  V  E
GTTTAGGAAGAATGGCATTGACGACATCTACTGTCTCTCCGGTCAACGATGCCTTCGTCAT    840
F  R  K  N  G  I  D  D  I  Y  C  L  S  V  N  D  A  F  V  M
GAATGGTTGGGGCAAGTCTCAGGGGTTGAAAAATGTCAAGCTTATCCCGGACGGTTCGGG    900
N  A  W  G  K  S  Q  G  L  K  N  V  K  L  I  P  D  G  S  G
AGAATTCACTCGGAAAATGGGTATGCTCGTCGCCAAGGACAATCTCGGTTTCGGTCTGCG    960
E  F  T  R  K  M  G  M  L  V  A  K  D  N  L  G  F  G  L  R
CTCCTGGCGCTACGCTGCCGTGATCAATAATGGCGTCGTGGAGGGGTGGTTCGAGGAAGA    1020
S  W  R  Y  A  A  V  I  N  N  G  V  V  E  G  W  F  E  E
AGGCTTTGGCGACAACTGCGCGACCGATCCGTATGGCGTTTCTTCGCCGCAAAACATTTT    1080
G  F  G  D  N  C  A  T  D  P  Y  G  V  S  S  P  Q  N  I  L
GAAATGCCTTAAGGCCCCAGCCTTCGTATGAGCATTGAAGCCAACGACCAGCAGCTACGA    1140
K  C  L  K  A  P  A  F  V  -
CTTGTCGTGCACACCTGCGCCGAATGATAGGAGTTTTGCTCGAACCATGAAGTCCTTTAC    1200
                              (RPON2)  M  K  S  F  T
AAGCCTTCATCAGCGTCAGCAGCAATCGCTGGTTATTACGCCCCAGCTCATCGCGTCTAT    1260
S  L  H  Q  R  Q  Q  Q  S  L  V  I  T  P  Q  L  I  A  S  I
TCGCTTGCTGCAGCTGGCGCACACCGAACTGAATCAGTTCGTGGAACAGGAAATCGAGAA    1320
R  L  L  Q  L  A  H  T  E  L  N  Q  F  V  E  Q  E  I  E  K
GAACCCATTTCTCGACCTGGCGTCGAATGACGGCGGGGCTTCTGGCGTATCGCCAACTGC    1380
N  P  F  L  D  L  A  S  N  D  G  G  A  S  G  V  S  P  T  A
GGCGGATGATCCAAATATCAGCGCACGCGAAGACCACGTTGATGGACCGGCCAGGACGGA    1440
A  D  D  P  N  I  S  A  R  E  D  H  V  D  G  P  A  R  T  D
CATGCCTGAGCTGTCTAATCCCTGGAAATCAATCCGTGACACAAGGAACCTTTCGCCGGG    1500
M  P  E  L  S  N  P  W  K  S  I  R  D  T  R  N  L  S  P  G
GGAAAAGGCTTCCCTGGATGAGTTCGCCGCCTCAACTGAGACATTGCACGAACATGTCGG    1560
E  K  A  S  L  D  E  F  A  A  S  T  E  T  L  H  E  H  V  A
CCATCAGATCGCCCTCACTGGCTTCACAACCCAGGAGCGGCTGATTGCTGGCGCGCTTGC    1620
H  Q  I  A  L  T  G  F  T  T  Q  E  R  L  I  A  G  A  L  A
CGACAATCTGGATGACACTGGGTATCTGCAAGTGAACCTTCAGGAGCTAGCAGAGAGGCT    1680
D  N  L  D  D  T  G  Y  L  Q  V  N  L  Q  E  L  A  E  R  L
AAATAGCCCTGAGGCTGGAGTGAAGCGGGTTCTTGAGGCTTTGCAGCTATTTGACCCGCC    1740
N  S  P  E  A  G  V  K  R  V  L  E  A  L  Q  L  F  D  P  P
GGGCATATTTGCGCGAAGTCTGGGCGAATGCCTCGAAATCTCGAGTTCAGTTGCGCCAACGAGACCG    1800
G  I  F  A  R  S  L  G  E  C  L  E  I  Q  L  R  Q  R  D  R
GTTCGACCCGGCCATGGCAGCTCTGGTCCGCAAATCTCGAGTTGCTTGCGCGACGCGATTT    1860
F  D  P  A  M  A  A  L  V  A  N  L  E  L  L  A  R  R  D  F
TCGAGCACTGAAGCGACGTTGCCGGGTCGATGAAGACGACCTTCTCGATATGTTGAACGA    1920
R  A  L  K  R  R  C  R  V  D  E  D  D  L  L  D  M  L  N  E
AATTCGTGCACTTGACCCAAAGCCTGGAAACCAGTTCCAGTCCGCAAGGTCGGAATCCAT    1980
I  R  A  L  D  P  K  P  G  N  Q  F  Q  S  A  R  S  E  S  I
CATACCCGACGTCATCGTCTAACCTCGCCGGGAGGCGGATGGCACGTCGAGCTTAATCG    2040
I  P  D  V  I  V  L  T  S  P  G  G  W  H  V  E  L  N  R
GGAGATTTTGCCCAAGGTACTGATCAACCAAACTTATTTTGCGCAAGTTACTCGCCTAAC    2100
E  I  L  P  K  V  L  I  N  Q  T  Y  F  A  Q  V  T  R  L  T
CGCCCAAAGCTCGAAAGATCAGTCATTCCTCAACGAATGCTTCCAGAGCGCGAGCTGGTT    2160
A  Q  S  S  K  D  Q  S  F  L  N  E  C  F  Q  S  A  S  W  L
GGTTCGGAGTCTCGATCAGCGCGCCACGACGATCCTTAAGGTGGCGACCGAAATCGTGCG    2220
V  R  S  L  D  Q  R  A  T  T  I  L  K  V  A  T  E  I  V  R
CCACCAGGGATGTCTTTTTGGAAGATGGCATTGCTCATCTTCGCCCTCTCAATCTTAAGCAC    2280
H  Q  D  V  F  L  E  D  G  I  A  H  L  R  L  N  L  K  T
CATCGCTGACGCGATCGACATGCACGAGTCCACTGTAAGTCGGGTAACGTCGAACAAATA    2340
I  A  D  A  I  D  M  H  E  S  T  V  S  R  V  T  S  N  K  Y
CATTCTCACGCCCCGCGGCGTGTTCGAGCTCAAGTATTTTTTCACCGTCGCGATCCCGTC    2400
I  L  T  P  R  G  V  F  E  L  K  Y  F  F  T  V  A  I  P  S
CTCACAAGGCGGTGACGCGCACTCGGCCGAAGCTGTGCGCCATCAGATAAGGGCACTTAT    2460
S  Q  G  G  D  A  H  S  A  E  A  V  R  H  Q  I  R  A  L  I
TGCCGCAGAGACGCTCCATGATGTGCTGTCCGACGATGCATCGTTGCCAAGCTCAAGGA    2520
A  A  E  T  L  H  D  V  L  S  D  D  G  I  V  A  K  L  K  E
AACAGGCGTCGACATTGCTCGCCGCACAGTCGCGGGAGAAACGCCTGCGCAGAGGATAAGT    2580
T  G  V  D  I  A  R  R  T  V  A  K  Y  R  E  A  M  R  I  P
CTCGTCCACGCAACGGCGGCGGGAGAAACGTTGGCTGGCGGCCTGCGGAGGGATAAGT    2640
S  S  T  Q  R  R  R  E  K  R  L  Y  W  R  P  A  E  G  -
CCTTTGTACCGTAGTGATGCCCTTTTCCCAAAAACCGCGGAACTGAGGCTATGTGACATT    2700
GTGCTTAGTCCTCTCCATGGAGGATCGACTTCATATGGG    2739
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FIG. 2. DNA sequence of the 2,739-bp DNA fragment containing *R. etli* *rpoN2* and *orf180*. The deduced amino acid sequence of these genes is indicated below the DNA sequence. Putative ribosome-binding sites are underlined. Sequences resembling the consensus of a −24/−12 promoter and a NifA-binding site are marked with a double line and with dots, respectively. The *Sma*I, *Eco*RI, and *Pst*I restriction sites are indicated in boldface.

DNA binding and the RpoN box (36). All rhizobial RpoN proteins have a domain II of 98 to 110 amino acids, with two notable exceptions in which the acidic domain is considerably shorter, *B. japonicum* RpoN1 (62 amino acids [E50 to T112]) and *R. etli* RpoN2 (68 amino acids). Two hydrophobic heptad repeats have been proposed to form an intramolecular leucine zipper in the *E. coli* RpoN protein (31). Similar structures were found in rhizobial RpoN proteins and have the consensus L21-L28-L35-E42 (amino acid numbering refers to *R. etli* RpoN1) and (L/I)198-L205-V212-(L/I)219. An alignment of RpoN proteins indicated that while *B. japonicum* RpoN1 and RpoN2 are highly similar (87% amino acid identity), the RpoN proteins

from *R. etli* are more divergent. The *R. etli* RpoN1 protein displays higher amino acid identity with the RpoN proteins of *S. meliloti* (68%) and *Rhizobium* sp. strain NGR234 (65%) compared to the *R. etli* RpoN2 protein. Also at the level of DNA sequence, the G+C contents of both *R. etli* rpoN genes differ (*rpoN1*, 64%; *rpoN2*, 56%). It is therefore likely that the *rpoN2* gene did not arise by gene duplication but by lateral transfer.

Sequence analysis of *R. etli* orf180. Upstream from the *R. etli* *rpoN2* gene, a second ORF was identified (Fig. 1 and 2). This ORF codes for a putative protein of 180 amino acids with a predicted molecular mass of 20,180 Da and a pI of 7.82. *orf180* is located 78 bp upstream from *rpoN2* and might therefore be cotranscribed with the *rpoN2* gene. A putative Shine-Dalgarno sequence (AGGAG) is found 8 bp upstream from *orf180*. In addition, putative regulatory sequences were identified in the 5′ region of *orf180*. A sequence (TGGCA-N6-TTGCT [underlined in Fig. 2]) resembling the consensus of a −24/−12 promoter (9) was located 84 nucleotides upstream from the putative ATG initiation codon. Approximately 270 bp upstream from this −24/−12 type of promoter, a possible NifA-binding (TGT-N10-ACA) site was found. Homology searches in databases revealed homology between the gene product of *R. etli* *orf180* and a protein encoded by the *y4vd* ORF located on the symbiotic plasmid of *Rhizobium* sp. strain NGR234 (74% amino acid identity [Q53212]), a protein from *Haemophilus influenzae* (44% identity [P44758]), and a putative membrane protein from *Synechocystis* sp. strain PCC6803 (60% identity [D90909]). An alignment of these proteins is shown in Fig. 3. In addition, lower homology was detected with yeast peroxisomal proteins from *Lipomyces kononenkoae* (26% identity [U11244]), *Candida boidinii* (26% identity [P14292] and 25% identity [P14293]), *Schizosaccharomyces pombe* (35% identity [G2598045]), and an unknown protein from *Arabidopsis thaliana* (32% identity [AC002292]). Homology is found over the entire protein length, except for the amino terminus (the first 44 amino acids in the *R. etli* protein encoded by *orf180*). Amino acids conserved between the prokaryotic proteins and the yeast peroxisomal proteins are underlined in Fig. 3. The conserved amino acids are also found in the *A. thaliana* protein, with the exception of the strongly conserved PGAFTP(T/I/P)C motif, in which only the G and F residues are maintained. The positive and negative charges in the protein encoded by *orf180* are evenly distributed throughout the protein, with the exception of a stretch of 17 amino acids (overlined in Fig. 3) predicted to form a putative transmembrane segment (17). Interestingly, this hydrophobic segment is conserved among the prokaryotic and eukaryotic (except *A. thaliana*) proteins and contains the PGAFTP(T/I/P)C motif.

Analysis of *R. etli* rpoN mutants. To further characterize the *rpoN* genes of *R. etli*, a phenotypical analysis of *R. etli* *rpoN1* (FAJ1154), *rpoN2* (FAJ1169), *rpoN1 rpoN2* (FAJ1170 and FAJ1172), and *rpoN1 fixL* (FAJ1176) mutants was carried out under free-living and symbiotic conditions. First, growth of these mutants was tested under aerobic and microaerobic conditions on defined media containing ammonium, nitrate, and alanine as nitrogen sources (Table 2). These compounds were previously shown to reduce growth of an *R. etli* rpoN1 mutant during aerobic growth (26). In contrast to the wild type, growth of FAJ1154 was clearly reduced on alanine and mannitol both aerobically and microaerobically, while growth was not affected on the complex TY medium and on AMS minimal medium containing mannitol (carbon source) and glutamine (nitrogen source) (Table 2). Similar phenotypes were observed with the double mutants *rpoN1 rpoN2* (FAJ1170 and FAJ1172) and *rpoN1 fixL* (FAJ1176). However, the *R. etli* rpoN2 mutant displayed wild-type growth on each of the different media tested

```
RE       MTMEKQVPIVTFRTRVRDESISGPNPYRWKSKTTTDDYFSGKRVILFSLPGAFTPICSTFQLPDFESLYVEFRKNGIDDIYC       82
NGR234   MPVKKRVPFVAFRTRVRDETIGGPNPYRWEVRTT-EDYFSGKRVVLFSLPGAFTPTCSTQQLPDFERLYDEFGKVGIEAVYC       81
SYN       MTPERVPSVVFKTRVRDESVPGPNPYRWEDKTT-EQIFGGKKVVLFSLPGAFTPTCSSNHLPRYEQLFEEFQALGVDDIIC       80
HI       MSSMEGKKVPQVTFRTRQGD--------KW-VDVTTSELFDNKTVIVFSLPGAFTPTCSSSHLPRYNELAPVFKKYGVDDILV       74
         .** *.*.**  *         .*   .* .. *..* *..*********.**. .** .. *   *  *....
```

```
RE       LSVNDAFVMNAWGKSQGLKNVKLIPDGSGEFTRKMGMLVAKDNLGFGLRSWRYAAVINNGVVEGWFEEEGFGDNCATDPYGVSS   166
NGR234   LSVNDAFVMNAWGKALGLEKVRLIPDGSGEFTRKMGMLVAKDNLGFGMRSWRYAAVVNDSVVEQWFEEEGFSDNCESDPYWASS   165
SYN      LSVNDAFVMFQWGKQIGADKVKLLPDGNGEFTRKMGMLVEKSNLGFGMRSWRYSMFVNDGKIEKMFIEPEFGDNCPVDPFECSD   164
HI       VSVNDTFVMNAWKEDEKSENISFIPDGNGEFTEGMGMLVGKEDLGFGKSRWRYSMLVKNGVVEKMFIEP----NEPGDPFKVSD   154
         .****.*** .*.     ......***.**** *****.*..**** *****.  .... .*  *  *      *   **. .*.
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```
RE       PQNILKCLKAPAFV                                                                       180
NGR234   PQNILETLRTFDTARLGRVPIKF                                                              188
SYN      ADTMLAYLKGAEAPGVSEPVKAFVG                                                            189
HI       ADTMLKYLAPQHQVQESISIFTKPGCPFCAKAKQLLHDKGLSFEEIILGHDATIVSVRAVSGRTTVPQVFIGGKHIGGSDDLEKYFA   241
          ....* *
```

FIG. 3. Alignment of the prokaryotic protein sequences homologous to the gene product of *R. etli orf180*. RE, protein encoded by *R. etli orf180*; NGR234, *Rhizobium* sp. strain NGR234 21.4-kDa protein translated from the ORF *y4vd* (Q53212); SYN, *Synechocystis* protein encoded by ORF sll1621 (D90909); HI, *H. influenzae* 26.7-kDa protein (P44758). Identical and similar amino acids (S-T-A, L-V-I-M, K-R, Q-N, and F-Y-W) are indicated below the protein sequences by stars and dots, respectively. Residues that are also conserved in the yeast proteins from *Lipomyces kononenkoae* (U11244), *C. boidinii* (P14292 and P14293), and *S. pombe* (G2598045) are underlined. The putative transmembrane hydrophobic domain in the protein encoded by *R. etli orf180* is overlined.

under aerobic as well as microaerobic conditions. Growth phenotypes were different when nitrate or ammonium was used as an N source and mannitol was used as a C source (data not shown). Aerobic growth and microaerobic growth of the wild type and the *rpoN2* mutant were indistinguishable when nitrate or ammonium was used as an N source. Also, the growth rates of the *rpoN1*, *rpoN1 fixL*, and *rpoN* double mutants were identical both aerobically and microaerobically on these media. Growth of these mutants was clearly reduced compared to that of the wild type during aerobic growth. Under microaerobic conditions, these mutants produced increased amounts of exopolysaccharides compared to the *rpoN2* mutant and the wildtype strain. Excessive production of exopolysaccharides by the *rpoN1* mutants may indicate a low intracellular concentration of fixed nitrogen, as can be expected from nitrate assimilatory mutants. These results indicate that under free-living conditions, the phenotypes of the *R. etli* wild type and the *rpoN2* mutant are similar, and also the growth rates of the *rpoN1* and *rpoN* double mutant strains are indistinguishable from each other on the different media tested.

The different mutant strains were also inoculated on *P. vulgaris*, the common bean plant, and tested for nodulation and symbiotic nitrogen fixation activity (Table 3). The shape and size of the nodules induced by the wild type and the different mutant strains were similar. Also, nodule dry weight and nodule number of plants inoculated with the *rpoN2* and *rpoN1*

rpoN2 mutants were not different from those of plants nodulated by the wild type. In contrast, the nodule number was significantly higher on plants inoculated with the *nifA* mutant, although the nodule dry weight was not statistically different. A marked difference in symbiotic nitrogen fixation activity of both *rpoN* mutants was observed. While the *rpoN1* mutant shows wild-type ARA, nitrogen fixation is reduced by more than 90% in the *rpoN2* mutant strain FAJ1169 and completely abolished in the *rpoN1 rpoN2* mutants FAJ1170 and FAJ1172. From these results, it can be concluded that the *rpoN2* gene is essential for nitrogen fixation while *rpoN1* is dispensable. In addition, ARA of an *rpoN1 fixL* double mutant is not abolished, indicating that the *R. etli fixL* gene is not required for *rpoN2* transcription during symbiosis. Taken together, the freeliving and symbiotic data indicate that the activity of both *R. etli rpoN* genes is required under distinct physiological conditions. While *R. etli rpoN1* controls the assimilation of several nitrogen and carbon sources during free-living growth, *rpoN2* function is essential for symbiotic nitrogen fixation.

Analysis of an *R. etli orf180* mutant. Immediately upstream from *R. etli rpoN2*, an ORF coding for a 180-amino-acid protein was identified. To determine its function, *orf180* was inactivated by insertion of the Ω-Km cassette into the *Eco*RI restriction site of the gene, yielding strain FAJ1173 (Fig. 1). This mutant was tested for growth on several nitrogen sources under aerobic and microaerobic conditions (Table 2). No dif-

TABLE 2. Growth of *R. etli rpoN* mutants under free-living aerobic and microaerobic conditions

Strain[a]	Relevant genotype	Result for growth on[b]:					
		Glutamine + mannitol		TY medium		Alanine + mannitol	
		Aerobic	Microaerobic	Aerobic	Microaerobic	Aerobic	Microaerobic
CNPAF512	Wild type	+++	++	++	+	+++	+
FAJ1154	*rpoN1*	+++	++	++	+	+/−	+/−
FAJ1169	*rpoN2*	+++	++	++	+	+++	+
FAJ1170	*rpoN1 rpoN2*	+++	++	++	+	+/−	+/−
FAJ1172	*rpoN1 rpoN2*	+++	++	++	+	+/−	+/−
FAJ1173	*orf180*	+++	++	++	+	+++	+
FAJ1176	*fixL rpoN1*	+++	++	++	+	+/−	+/−

[a] *R. etli* strains CNPAF512 (wild type), FAJ1154 (*rpoN1*), FAJ1169 (*rpoN2*), FAJ1170 and FAJ1172 (*rpoN1 rpoN2*), FAJ1173 (*orf180* mutant), and FAJ1176 (*rpoN1 fixL*).
[b] Growth tests were carried out on TY or AMS plates containing glutamine or alanine at 2 g/liter and mannitol at 10 g/liter. Plates were incubated aerobically or microaerobically (0.5% oxygen), and colony size was determined after 5 days of incubation at 30°C. +/−, colony size <0.5 mm; +, size 0.5 to 1 mm; ++, size 1 to 2 mm; +++, size >2 mm.

TABLE 3. Nitrogenase activity and nodulation of
R. etli wild type and *rpoN* mutants

Strain	Relevant genotype	ARA (μmol of ethylene/ plant/h)[a]	No. of nodules/ plant[b]	Nodule dry wt/plant (mg)[b]
CNPAF512	Wild type	3.2 (1.3)	170 (35)	48 (19)
Rp1000	*nifA*	0	212 (45)	60 (24)
FAJ1154	*rpoN1*	2.8 (0.7)	143 (53)	41 (23)
FAJ1169	*rpoN2*	0.3 (0.3)	149 (41)	38 (19)
FAJ1170	*rpoN1 rpoN2*	0	188 (47)	63 (14)
FAJ1176	*rpoN1 fixL*	3.5 (1.1)	ND[c]	ND
FAJ1173	*orf180*	0.6 (0.5)	ND	ND
FAJ1173/ pFAJ1175		0.9 (0.3)	ND	ND

[a] Data are the means of at least five plants. Standard deviations are given in parentheses.

[b] Data are the means of 11 (FAJ1169), 10 (CNPAF512 and Rp1000), and 8 (FAJ1170) plants. Standard deviations are given in parentheses.

[c] ND, not determined.

ference between the wild type and mutant FAJ1173 was observed on these media. In addition, the symbiotic phenotype of this mutant strain was determined (Table 3). The nodulation phenotypes of the wild type and mutant were indistinguishable. However, nitrogen fixation activity was strongly reduced compared with the wild-type level and was not different from that of the *R. etli rpoN2* mutant. The phenotypes of the *R. etli orf180* mutant and the *rpoN2* mutant, as shown in Tables 2 and 3, are identical. Since the Ω-Km interposon is known to create polar mutations, it is possible that the observed phenotypes of FAJ1173 are due to absence of transcription of the *rpoN2* gene, which is located downstream from *orf180*. This hypothesis was tested by complementation of the *R. etli orf180* mutant strain, FAJ1173, with plasmid pFAJ1175 containing *R. etli orf180* and an inactivated *rpoN2* gene (Table 3). No complementation of the symbiotic phenotype of FAJ1173 was observed, indicating that *orf180* and *rpoN2* are probably transcribed from a promoter located upstream from *orf180*.

Expression analysis of *nifH-gusA* and production of melanin. The effect of mutations introduced into the *rpoN1* and *rpoN2* genes and *orf180* on the RpoN/NifA-dependent expression of *nifH* and production of melanin was tested under free-living aerobic and microaerobic (0.3% oxygen) conditions and in bacteroids (Table 4). Expression of *nifH* was monitored by using the *Azospirillum brasilense* translational *nifH-gusA* fusion plasmid, pFAJ21 (35). The production of the black pigment melanin in *R. etli* was previously shown to be dependent on the

presence of NifA and RpoN1 (24, 26). From Table 4, it can be seen that as expected, expression of the *nifH* gene and the synthesis of melanin are abolished under aerobic conditions in all strains tested but are highly induced under microaerobic conditions in the wild type. No expression of pFAJ21 under microaerobic conditions or production of melanin during microaerobiosis or symbiosis is observed in the *R. etli nifA* mutant and in both *rpoN1 rpoN2* double mutants. However, levels of β-glucuronidase activity of pFAJ21 and melanin production during microaerobic growth were identical in the *rpoN2* mutant, the *orf180* mutant, and the wild type. These results indicate that *rpoN1*, but not *rpoN2*, is required for free-living expression of the *A. brasilense nifH-gusA* fusion and for the production of melanin. During symbiosis, melanin production was abolished only in the *nifA* mutant and in the *rpoN* double mutant strain, suggesting that both *rpoN* genes can substitute for each other with respect to synthesis of melanin. Induction of pFAJ21 was also assayed in complex TY medium and in AMS medium containing mannitol as a carbon source and NH_4Cl or nitrate as a nitrogen source (data not shown). In contrast to the wild type, no activity of the fusion could be observed in the *rpoN1* mutant strain under any of the growth conditions tested.

Expression analysis of *R. etli rpoN1, rpoN2,* and *orf180*. From the data concerning growth and symbiotic phenotypes, expression of *nifH*, and production of melanin in single and double *R. etli rpoN* mutant strains, it appears that both *rpoN* genes are active under different physiological conditions. The *rpoN1* gene is essential during free-living growth, and the presence of *rpoN2* is required for an effective symbiosis. We tested whether these observations could be explained by differential expression of both genes. First, the expression of genomic *gusA* fusions, integrated at their homologous site, was monitored (Table 5). Expression of the *R. etli rpoN1* gene was previously shown to occur independently of the nitrogen concentration (26). In addition, activity of a chromosomally integrated *rpoN1-gusA* fusion was shown to be negatively autoregulated. Here, we observed that the level of expression of *rpoN1-gusA* under free-living microaerobic and symbiotic conditions in a wild-type background was reduced approximately fourfold compared to free-living aerobic expression (Table 5). On the other hand, microaerobic expression of *rpoN1-gusA* in the *rpoN1* mutant strain FAJ1156 was only slightly reduced (approximately 40%) compared to the aerobic expression level. In contrast to the expression of *rpoN1-gusA*, wild-type genomic *orf180-gusA* and *rpoN2-gusA* fusions were not expressed aerobically but were strongly induced during microaerobic growth and symbiosis (Table 5). From this expression analysis, it can be concluded

TABLE 4. Expression of the *nifH-gusA* fusion plasmid pFAJ21 and production of melanin in *R. etli* wild type and mutants

Strain	Relevant genotype	β-Glucuronidase activity of pFAJ21 (Miller units)[a]		Melanin production[b]		
		Aerobic	Microaerobic	Aerobic	Microaerobic	Symbiotic
CNPAF512	Wild type	19 (6)	1,841 (327)	0 (2)	93 (4)	114 (56)
Rp1000	*nifA*	0 (1)	0 (2)	0 (5)	0 (2)	4 (7)
RpFAJ1002	*fixL*	9 (7)	1,778 (125)	ND[c]	ND	104 (14)
FAJ1154	*rpoN1*	0 (10)	0 (14)	0 (5)	10 (11)	118 (66)
FAJ1169	*rpoN2*	54 (14)	2,149 (243)	0 (2)	82 (21)	121 (69)
FAJ1170	*rpoN1 rpoN2*	0 (17)	0 (21)	0 (3)	15 (21)	11 (11)
FAJ1172	*rpoN1 rpoN2*	0 (6)	0 (11)	0 (5)	14 (19)	8 (9)
FAJ1173	*orf180*	23 (6)	2,107 (167)	0 (1)	89 (12)	122 (28)

[a] β-Glucuronidase activities are the means of at least four replicates. Standard deviations are given within parentheses.

[b] Melanin production was quantified essentially as described by Michiels et al. (26). Units are expressed as the ratio of the change in optical density at 340 nm (ΔOD_{340})/OD_{595} (cell density)/time (minutes). Standard deviations are given in parentheses.

[c] ND, not determined.

TABLE 5. Expression of genomic *rpoN1-gusA*, *rpoN2-gusA*, and *orf180-gusA* fusions

Strain	Relevant genotype	β-Glucuronidase activity (Miller units)[a]		
		Aerobic	Microaerobic	Symbiotic
FAJ1156	*rpoN1*, *rpoN1-gusA*	185 (46)	116 (63)	8 (1)
FAJ1156/pFAJ1150	Wild type, *rpoN1-gusA*	27 (10)	7 (10)	7 (1)
CNPAF512::pFAJ1187	Wild type, *rpoN2-gusA*	0 (2)	674 (42)	1,487 (366)
CNPAF512::pFAJ1188	Wild type, *orf180-gusA*	0 (2)	67 (14)	503 (169)

[a] β-Glucuronidase activities are the means of at least eight replicates. Standard deviations are given within parentheses.

that in the *R. etli* wild type, *rpoN1* but not *rpoN2* is expressed during aerobic growth, while the *rpoN2* gene, but not *rpoN1*, is strongly transcribed in bacteroids.

To investigate in more detail the regulation of *rpoN2* and *orf180*, plasmid-borne *gusA* fusions were constructed with both genes as detailed in Materials and Methods. Plasmid pFAJ1175 carries a 2.6-kb *rpoN2* promoter fragment, and pFAJ1176 carries a 1.9-kb DNA fragment from the *orf180* promoter. The *gusA* gene was inserted into the *Pst*I site of *rpoN2* at 28 codons from the presumptive 5′ terminus of the gene. In pFAJ1176, the *gusA* cassette was cloned in the *Eco*RI site of *orf180* at 111 codons from the proposed ATG initiation codon. Expression of *rpoN2-gusA* (pFAJ1175) and *orf180-gusA* (pFAJ1176) was monitored in the CNPAF512 wild type and in *R. etli rpoN1* (FAJ1154), *rpoN2* (FAJ1169), *rpoN1 rpoN2* (FAJ1170 and FAJ1172), *orf180* (FAJ1173), *nifA* (Rp1000), and *fixL* (RpFAJ1002) mutant strains under free-living aerobic and microaerobic conditions and in bacteroids. From Table 6, it can be seen that both fusion plasmids are not expressed aerobically and are strongly induced in the wild-type strain upon incubation at 0.3% oxygen and during symbiosis. These data are in accordance with the results obtained in strains CNPAF512::FAJ1187 and CNPAF512::FAJ1188 carrying the *rpoN2* and *orf180 gusA* genomic fusions. Wild-type expression levels are also observed in the *fixL* mutant strain RpFAJ1002, confirming the observation that nitrogen fixation is not abolished in the *R. etli rpoN1 fixL* double mutant, FAJ1176. No expression under any condition is obtained in the *R. etli nifA* mutant and in the *rpoN* double mutants, indicating that transcription of *rpoN2* and *orf180* is controlled by RpoN and NifA during free-living growth and symbiosis. No *gusA* activity of pFAJ1175 and pFAJ1176 was found in the *rpoN1* mutant under free-living microaerobic conditions, whereas wild-type activity was obtained in bacteroids. On the other hand, the inactivation of the *rpoN2* gene or *orf180* had no effect on free-living or symbiotic expression of both *rpoN2-gusA* and *orf180-gusA* fusions. To confirm these results, two additional *gusA* fusion plasmids derived from pFAJ1175 and pFAJ1176 were constructed. These plasmids carry a deletion of approximately 1.5 kb from the *Sma*I site (Fig. 1). The putative −24/−12 promoter and the NifA-binding site in the promoters of *rpoN2* and *orf180* were not deleted in these plasmids. These fusion plasmids were pFAJ1180 (*rpoN2*) and pFAJ1181 (*orf180*). Similar expression patterns to those obtained with pFAJ1175 and pFAJ1176 were observed in the different mutant strains during free-living growth (Table 6).

Several conclusions can be drawn from these observations. First, the expression patterns of *rpoN2* and *orf180* are identical, suggesting coordinate expression of both genes. Second, transcription of *rpoN2* can be highly induced under free-living microaerobic conditions and depends on the presence of a functional *rpoN1* gene and on NifA. Under these conditions, no effect of *rpoN2* inactivation on the expression level of *rpoN2* is observed. Third, *rpoN2* is highly expressed during symbiosis. Transcription is abolished in *nifA* and *rpoN* double mutant strains, but not in *R. etli rpoN1* or *rpoN2* single mutants, indicating that both *rpoN* genes can functionally substitute for each other to activate *rpoN2* and *orf180* expression during symbiosis. Finally, expression of *rpoN1* is negatively autoregulated and reduced in a wild-type background during microaerobic growth and in bacteroids. It is possible that in addition to RpoN1, RpoN2 negatively regulates *rpoN1* expression. First, compared with aerobic conditions, *rpoN1* expression is strongly reduced microaerobically in the wild type, but not in an *rpoN1* mutant strain. Second, the *rpoN2* gene is expressed microaerobically in the wild type, but not in the *R. etli rpoN1* mutant.

DISCUSSION

We have isolated the second *R. etli rpoN* gene, *rpoN2*. This *rpoN* copy is clustered with nitrogen fixation genes and is located approximately 3.5 kb upstream from the *R. etli fixA* gene (23). Kullik et al. (16) have previously demonstrated that *B. japonicum* contains two differentially regulated *rpoN* genes. None of the genes maps closely to one of the previously identified *nif*

TABLE 6. Expression of *rpoN2-gusA* and *orf180-gusA* fusions in *R. etli* wild-type and mutant strains

Strain[a]	β-Glucuronidase activity (Miller units)[b]									
	rpoN2-gusA					*orf180-gusA*				
	pFAJ1175			pFAJ1179		pFAJ1176			pFAJ1181	
	Aerobic	Microaerobic	Symbiotic	Aerobic	Microaerobic	Aerobic	Microaerobic	Symbiotic	Aerobic	Microaerobic
CNPAF512	16 (7)	1,686 (507)	1,638 (401)	202 (27)	897 (160)	15 (2)	217 (31)	290 (92)	0 (5)	267 (166)
Rp1000	0 (19)	32 (21)	0 (0)	177 (8)	175 (17)	9 (4)	4 (13)	0 (21)	8 (7)	0 (6)
RpFAJ1002	11 (1)	1,320 (105)	2,454 (878)	141 (14)	694 (35)	4 (6)	181 (21)	395 (215)	4 (4)	188 (36)
FAJ1154	0 (14)	0 (35)	1,836 (655)	205 (37)	189 (30)	11 (6)	0 (18)	409 (133)	0 (20)	0 (18)
FAJ1169	32 (4)	1,195 (105)	1,724 (667)	213 (56)	875 (86)	1 (1)	322 (166)	ND[c]	0 (1)	111 (14)
FAJ1170	20 (22)	0 (23)	51 (37)	103 (49)	125 (56)	0 (17)	0 (18)	ND	0 (0)	0 (33)
FAJ1172	0 (8)	0 (6)	0 (0)	140 (63)	190 (54)	0 (4)	0 (25)	ND	2 (9)	0 (14)
FAJ1173	32 (1)	1,194 (288)	1,483 (601)	190 (44)	731 (161)	0 (1)	343 (146)	ND	2 (5)	194 (23)

[a] *R. etli* strains CNPAF512 (wild type), Rp1000 (*nifA*), RpFAJ1002 (*fixL*), FAJ1154 (*rpoN1*), FAJ1169 (*rpoN2*), FAJ1170 and FAJ1172 (*rpoN1 rpoN2*), and FAJ1173 (*orf180* mutant).
[b] Expression was determined as defined in footnote *a* of Table 4. Standard deviations are given in parentheses.
[c] ND, not determined.

gene regions. The *B. japonicum rpoN2* gene, but not *rpoN1*, is flanked by ORFs that are also conserved in other species (21). In *A. caulinodans*, the *rpoN* gene is flanked by conserved ORFs (33). A second *rpoN* copy, named *rpoF*, was shown to control the expression of the regulatory *nifA* gene (19). On the basis of indirect evidence, a second *rpoN* gene has also been suggested to exist in the photosynthetic bacteria *R. sphaeroides* (20) and *R. capsulatus* (10). In *R. sphaeroides*, the identified *rpoN* gene is not linked to conserved ORFs but is adjacent to the 3′ end of the *nifUSVW* gene cluster and is probably cotranscribed with the latter genes (20). Because the inactivation of the *rpoN* gene in *R. sphaeroides* does not impair diazotrophic growth, the existence of a second copy was postulated.

The *R. etli rpoN2* gene product displays 59% amino acid identity with the RpoN1 protein. This is considerably lower than the RpoN proteins from *B. japonicum*, which are 87% identical to each other. Also, *R. etli* RpoN1 is more closely related to the RpoN proteins from *S. meliloti* and *Rhizobium* sp. strain NGR234 than to the *R. etli* RpoN2 protein. In addition, the G+C contents of the *R. etli rpoN1* and *rpoN2* genes differ considerably. It is therefore likely that the *R. etli rpoN2* gene did not arise by duplication of a common ancestral *rpoN* gene, but may be the result of lateral transfer.

The protein encoded by *orf180* is homologous to several prokaryotic (*Rhizobium* sp. strain NGR234, *H. influenzae*, and *Synechocystis* sp. strain PCC6803) and eukaryotic (yeast and *A. thaliana*) proteins. The *H. influenzae* protein possesses an additional C-terminal glutaredoxin domain and is therefore assumed to participate in redox reactions. The highest similarity is found with the gene product of *Rhizobium* sp. strain NGR234 *y4vd*, which maps on the symbiotic plasmid pNGR234a approximately 1.4 kb upstream from *fixA* (11). The promoter region of *y4vd* contains a consensus −24/−12 promoter sequence and a NifA-binding site, as observed in the *R. etli orf180* promoter region. In *C. boidinii*, two PMP20-like peroxisomal proteins are also homologous to the *R. etli orf180*-encoded protein. These proteins are abundantly synthesized when methanol is used as the sole carbon source and are believed to be associated with the membrane of peroxisomes (13). The function of *orf180* in *R. etli* is unclear, but because it is coregulated with nitrogen fixation genes, it is tempting to speculate that it is involved in processes related to symbiotic nitrogen fixation.

R. etli orf180 is probably cotranscribed with the *rpoN2* gene. First, no recognizable terminator structure is found in the 78-bp intergenic region between *orf180* and *rpoN2*. Second, under free-living conditions, expression of both genes is controlled by NifA and RpoN1, in agreement with the presence of RpoN- and NifA-binding sites in the promoter region of *orf180*. Finally, the reduced nitrogen fixation activity of an *R. etli orf180* insertion mutant cannot be restored to the wild-type level by a plasmid carrying only *orf180*.

During aerobic growth, the *R. etli rpoN1* gene was shown to be expressed at a constant level independently of the nitrogen source and was negatively autoregulated. Under these conditions, the *rpoN2* gene is not expressed. The *R. etli rpoN1* mutant strain displays growth defects on C_4-dicarboxylic acids and on the nitrogen sources ammonium, nitrate, alanine, and serine (26).

When cells were cultured under free-living microaerobic conditions, expression of the *R. etli rpoN1* gene was strongly decreased and transcription of *rpoN2* was activated. The latter process was strictly dependent on the presence of RpoN1 and NifA proteins. Therefore, also under microaerobic conditions, no growth of the *rpoN1* mutant strain was observed when alanine was used as a nitrogen source. Similarly, microaerobic production of melanin or expression of a *nifH-gusA* fusion

plasmid was abolished in the *rpoN1* mutant strain. Therefore, no active RpoN2 protein is formed under free-living aerobic or microaerobic conditions in the *rpoN1* mutant. In *B. japonicum*, both *rpoN* genes are functional during free-living growth. The *rpoN2* gene is required aerobically for growth on nitrate, while both copies are active under microaerobic conditions (16).

We have previously proposed that the consensus of a −24/−12 promoter overlapping the −10 promoter region and the transcription start site of *rpoN1* is responsible for the observed negative autoregulation (26). RpoN1 may bind to this sequence and thereby inhibit transcription initiation from the −35/−10 promoter of the *rpoN1* gene. If active RpoN2 protein is formed during microaerobiosis in the wild-type strain, this protein could account for the strongly decreased expression level of *rpoN1* by directly interacting with the *rpoN1* promoter. This is not the case in *B. japonicum*, because repression of *rpoN2* transcription has been specifically attributed to the presence of RpoN2 and not RpoN1 (16).

Microaerobic expression of *rpoN* genes has been reported in two other organisms. In *B. japonicum*, *rpoN1* is activated by an Fnr-like protein, FixK2. The expression of the *fixK2* gene is controlled by the oxygen sensor proteins FixLJ (9, 16). The *R. capsulatus rpoN* gene is organized in a *nifU2-rpoN* superoperon structure (5). A primary promoter upstream from *rpoN* is expressed constitutively. The secondary promoter, which is NifA and RpoN dependent, accounts for increased induction under low-nitrogen and low-oxygen conditions. *R. etli rpoN2* constitutes the second example of a NifA-controlled *rpoN* gene.

Expression of the *R. etli rpoN1* gene is shut off during symbiosis. The RpoN2 protein could account for this effect, because it is strongly expressed under these conditions. In addition, an *R. etli rpoN2* mutant strain still produces wild-type levels of melanin and activates *rpoN2-gusA* and *orf180-gusA* fusions, indicating that in this mutant, the *rpoN1* gene is no longer repressed. In contrast to *rpoN1*, transcription of *rpoN2* is highly activated during symbiosis. Transcription activation of *rpoN2* requires the NifA protein, but in contrast to free-living conditions, is not abolished in an *R. etli rpoN1* mutant strain. Therefore, symbiotic expression of *rpoN2* involves a symbiosis-specific mechanism, as suggested for other *Rhizobium* genes (37). It is possible that this mechanism accounts for a low basal level of expression of the *rpoN2* gene. After initial transcription, autoactivation by NifA/RpoN2 may then amplify *rpoN2* transcription. The mechanism involved is at present unclear, but it does not involve the *R. etli fixLJ* genes, because an *rpoN1 fixL* double mutant still fixes nitrogen.

Nitrogenase activity of nodules induced by the *rpoN1* mutant strain is not different from that of the wild type. On the other hand, nitrogen fixation is reduced by approximately 90% in the *rpoN2* mutant. At present, it is unclear whether the difference between the symbiotic phenotypes of *rpoN1* and *rpoN2* mutants is due to a difference in expression level of the *rpoN* genes. The *rpoN2* gene is highly induced in bacteroids, while *rpoN1* is negatively autoregulated and can therefore not reach the high expression levels that may be required during symbiosis to activate nitrogen fixation genes. Alternatively, the RpoN1 and RpoN2 proteins may have distinct affinities for different −24/−12 promoters, depending on their DNA sequence, as was previously reported for *A. caulinodans* (19).

Transcriptional regulation of sigma factors ensures that bacteria express specific sets of genes during specific physiological conditions (38). In recent years, it has become clear that expression levels of σ^{54} may vary according to the physiological need. Organisms seem to have evolved two different mechanisms. One unique *rpoN* gene may be subject to different control mechanisms. In *C. crescentus*, expression of the *rpoN* gene

is increased at the swarmer-to-stalked-cell transition, where σ^{54} is required for stalk biosynthesis and transcription of flagellar genes (3). Expression of the *rpoN* gene from *R. capsulatus* is controlled by two promoters (5). The first promoter is expressed constitutively and allows cells to grow under normal nitrogen-limiting conditions. The secondary promoter is autoactivated and is required for growth on nitrogen-limiting high-salt or low-iron medium. A second mechanism that bacteria use to cope with an increased demand of σ^{54} is a differential regulation of two *rpoN* gene copies. In *B. japonicum*, *rpoN2* is expressed under all conditions tested. In contrast, transcription of *rpoN1* is activated during microaerobiosis and symbiosis, allowing *rpoN2* to boost its expression during nitrogen fixation (16). *A. caulinodans* also possesses two genes coding for σ^{54} (19, 33). Their regulation has not yet been studied. Also in *R. etli*, two differentially regulated *rpoN* genes were identified. The first copy, *rpoN1*, is required for housekeeping functions and allows cells to grow on several nitrogen and carbon sources under free-living conditions. The second copy is essential for symbiotic nitrogen fixation.

ACKNOWLEDGMENTS

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