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The *Rhizobium etli rpoN* Locus: DNA Sequence Analysis and Phenotypical Characterization of *rpoN*, *ptsN*, and *ptsA* Mutants

JAN MICHIELS, TOM VAN SOOM, INGE D'HOOGHE, BRUNO DOMBRECHT, TRAKI BENHASSINE,†
PETRA DE WILDE, AND JOS VANDERLEYDEN*

F. A. Janssens Laboratory of Genetics, K. U. Leuven, B-3001 Heverlee, Belgium

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The *rpoN* region of *Rhizobium etli* was isolated by using the *Bradyrhizobium japonicum rpoN1* gene as a probe. Nucleotide sequence analysis of a 5,600-bp DNA fragment of this region revealed the presence of four complete open reading frames (ORFs), ORF258, *rpoN*, ORF191, and *ptsN*, coding for proteins of 258, 520, 191, and 154 amino acids, respectively. The gene product of ORF258 is homologous to members of the ATP-binding cassette-type permeases. ORF191 and *ptsN* are homologous to conserved ORFs found downstream from *rpoN* genes in other bacterial species. Unlike in most other microorganisms, *rpoN* and ORF191 are separated by approximately 1.6 kb. The *R. etli rpoN* gene was shown to control in free-living conditions the production of melanin, the activation of *nifH*, and the metabolism of C_4-dicarboxylic acids and several nitrogen sources (ammonium, nitrate, alanine, and serine). Expression of the *rpoN* gene was negatively autoregulated and occurred independently of the nitrogen source. Inactivation of the *ptsN* gene resulted in a decrease of melanin synthesis and *nifH* expression. In a search for additional genes controlling the synthesis of melanin, an *R. etli* mutant carrying a Tn*5* insertion in *ptsA*, a gene homologous to the *Escherichia coli* gene coding for enzyme I of the phosphoenolpyruvate:sugar phosphotransferase system, was obtained. The *R. etli ptsA* mutant also displayed reduced expression of *nifH*. The *ptsN* and *ptsA* mutants also displayed increased sensitivity to the toxic effects of malate and succinate. Growth of both mutants was inhibited by these C_4-dicarboxylates at 20 mM at pH 7.0, while wild-type cells grow normally under these conditions. The effect of malate occurred independently of the nitrogen source used. Growth inhibition was decreased by lowering the pH of the growth medium. These results suggest that *ptsN* and *ptsA* are part of the same regulatory cascade, the inactivation of which renders the cells sensitive to toxic effects of elevated concentrations of malate or succinate.

Bacterial sigma (σ) factors confer promoter specificity to transcription initiated by the RNA polymerase holoenzyme ($\alpha_2\beta\beta'\sigma$). On the basis of structural and functional criteria, σ factors fall into two major classes. Most σ factors are similar to the major vegetative σ factor of *Escherichia coli*, σ^{70} (18, 26). This sigma factor recognizes sequences similar to the canonical −35/−10 type of promoter and directs transcription of many housekeeping genes. Besides σ^{70}, this family contains several alternative σ factors allowing cells to respond to many different environmental stimuli, each controlling a specific process such as sporulation, heat shock response, and flagellation (26). The second type of σ factor, σ^{54} (RpoN, NtrA, or GlnF), shows little sequence similarity to the first class and has been identified so far in many gram-negative and gram-positive bacterial species. Although it was originally recognized for its role in nitrogen metabolism, it is clear that σ^{54} also controls the expression of genes responding to many other physiological needs (24, 31). Promoters recognized by RpoN share conserved DNA sequences of which the consensus is 5′-CT**GG**C AC-N_5-TT**GC**A-3′ (−24/−12 type of promoter; the invariant dinucleotides GG/GC are in boldface) (2).

Whereas the σ^{70}-holoenzyme complex often initiates transcription in the absence of transcriptional activators, transcription from all known σ^{54}-dependent promoters has an absolute dependence on the presence of an activator protein, such as NifA or NtrC (9, 24, 49). In the latter case, activators often bind to sequences located more than 100 bp upstream from the transcriptional start site (5, 9). This difference is probably correlated with the ability of σ^{54}, but not σ^{70}, to bind (in vitro) to the promoter in the absence of RNA polymerase core enzyme (6, 12). The strong interaction of σ^{54} with its promoter keeps the σ^{54} holoenzyme-promoter complex in a closed conformation which may then require activation by another protein to induce local DNA melting and initiate transcription (38, 46).

In most bacteria in which the *rpoN* region has been analyzed, a common organization of downstream open reading frames (ORFs) is found (31). The function of the conserved ORFs has been studied in only a few bacterial species, and their role has not been clearly defined yet. These genes are thought to modulate the activity of σ^{54} (30). In *Klebsiella pneumoniae*, the inactivation of the two ORFs, ORF95 and *ptsN*, located immediately downstream from *rpoN* increases the expression from several σ^{54}-dependent promoters (30, 32), while a mutation in the fourth gene (ORF90) reduces the activity of these same promoters (32). For *Pseudomonas aeruginosa*, it was suggested that, together with *rpoN*, ORF2 functions as a coinducer of genes involved in the assimilation of glutamine (20). In *Caulobacter crescentus*, ORF159 modulates the expression from a σ^{54}-dependent flagellar promoter.

Bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Mesorhizobium*, and *Sinorhizobium* (collectively called rhizobia) elicit nitrogen-fixing nodules on the roots of their leguminous host plant. The *rpoN* genes in four rhizobial species, *Rhizobium meliloti* (43), *Bradyrhizobium japonicum* (23), *Azorhizobium caulinodans* (48), and *Rhizobium* sp. strain NGR234 (52), have been characterized. In these organisms, σ^{54} has been shown to control C_4-dicarboxylate

* Corresponding author. Mailing address: F.A. Janssens Laboratory of Genetics, K.U. Leuven, Kardinaal Mercierlaan 92, B-3001 Heverlee, Belgium. Phone: 32 16 321631. Fax: 32 16 321966. E-mail: jozef.vanderleyden@agr.kuleuven.ac.be.

† Present address: Laboratoire de Biologie Moléculaire, Institut Pasteur d'Algérie, Alger, Algeria.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Source or reference
E. coli		
DH5α		Gibco/BRL
HB101	Sm^r	45
R. etli		
CNPAF512	Nal^r, wild type	This work
FAJ1154	Nm^r *rpoN*::Ω-Km, opposite orientation	This work
FAJ1155	Nm^r *rpoN*::Ω-Km, same orientation	This work
FAJ1156	Nm^r *rpoN*::*gusA-aphII*	This work
FAJ1157	Nm^r *rpoN*::*aphII-gusA*	This work
FAJ1164	Nm^r *ptsN*::Ω-Km, same orientation	This work
FAJ1165	Nm^r *ptsN*::Ω-Km, opposite orientation	This work
FAJ1166	Nm^r *ptsA*::Tn*5-mob*	This work
R. meliloti		
2011	Wild type	
1681	Sm^r Km^r *rpoN::*Tn*5*	43
Plasmids		
pUC18	Ap^r, cloning vector	35
pJQ200-UC1	Gm^r *sacB*	40
pHP45Ω-Km	Ap^r Km^r	14
pWM6	Ap^r Nm^r *uidA2*	33
pLAFR1	Tc^r, broad-host-range vector	15
pFAJ21	Tc^r, *A. brasilense* p*nifH-gusA*	51
pFAJ1150	Tc^r, *rpoN* gene in pLAFR1	This work
pFAJ1152	Ap^r, pUC18, *rpoN* 6.5-kb *Hin*dIII fragment	This work
pFAJ302	Tc^r, fusion between *A. brasilense* ammonium transporter *amtB* and *gusA*	Anne Van Dommelen (this laboratory)

utilization (23, 43, 48), nitrate assimilation (23, 43, 48), and several symbiotic functions (23, 43, 48, 52). However, only partial DNA sequence information on the downstream regions of rhizobial *rpoN* genes is available (23, 43, 48). In addition, the role and regulation of the conserved ORFs located 3′ to *rpoN* have not been investigated yet.

Here we describe the identification and analysis of the *rpoN* region of *Rhizobium etli*, the nodulating symbiont of the common bean plant, *Phaseolus vulgaris*. In contrast to the case for most other bacterial species, the *rpoN* gene is separated by approximately 1.6 kb from the conserved ORFs, ORF191 and *ptsN*, which are normally found immediately downstream from *rpoN*. We analyzed the regulation of *rpoN* transcription and the phenotypes of *rpoN* and *ptsN* mutant strains. Moreover, we identified an *R. etli* Tn*5* mutant strain displaying reduced melanin production. A further examination of this mutant revealed that the phenotype resulted from a mutation in *ptsA*, the gene coding for enzyme I of the phosphoenolpyruvate: sugar phosphotransferase system (PTS). Our results indicate that in *R. etli*, *ptsN* and *ptsA* may coregulate several RpoN-dependent activities, including the activation of *nifH*, the production of the pigment melanin, and the assimilation of alanine. In addition, the *ptsN* and *ptsA* mutant strains display an increased sensitivity to the toxic effects of high concentrations of malate and succinate. Therefore, *ptsN* and *ptsA* are thought to be part of the same regulatory cascade which may be involved in the sensing of C_4-dicarboxylates.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Some are also diagrammed in Fig. 1. Plasmids used merely for sequencing are not shown. The *Rhizobium* isolate used in the present study is CNPAF512 (CNPAB/EMBRAPA culture collection), a Brazilian isolate from *P. vulgaris* nodules. Based on our nucleotide sequence analysis of a 260-bp fragment of the 16S rRNA gene, amplified by PCR with the primers Y1 (5′-TGGCTCAGAACGAACGCTGGCGGC-3′) and Y2 (5′-CCCACTGCTGCCTCCCGTAGGAGT-3′), and multilocus enzyme electrophoresis of this isolate (26a), CNPAF512 should be classified as *R. etli. R. etli* strains were routinely grown in liquid TY (0.5% tryptone, 0.3% yeast extract, 7 mM $CaCl_2$) at 30°C and maintained on yeast-mannitol agar plates. *E. coli* was grown in Luria-Bertani medium at 37°C. Antibiotics supplied to the medium were at the following concentrations: nalidixic acid and neomycin, 40 μg/ml; kanamycin and gentamicin, 30 μg/ml; and ampicillin, 100 μg/ml. Tetracycline was added to a final concentration of 1 μg/ml (for *R. etli*) or 10 μg/ml (for *E. coli*).

Triparental conjugations and site-directed mutagenesis of *R. etli* were done as previously described (11).

Growth tests. Tests of growth of *R. etli* in liquid medium were carried out in acid minimal salts (AMS) medium (37) containing 1 mM $CaCl_2$. Cells were first grown overnight in TY, washed twice in 10 mM $MgSO_4$, brought to an optical density (OD) at 600 nm of 0.02 in a Perkin-Elmer lambda 2 spectrometer, and diluted 100 times in AMS medium. Carbon and nitrogen sources were added to

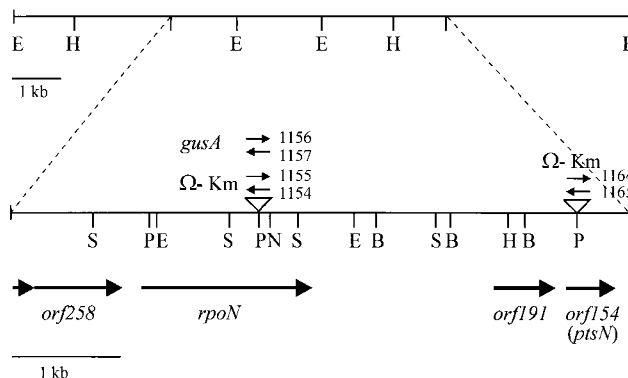

FIG. 1. Physical map of the *R. etli rpoN* region. The 1.8-, 4.6-, and 6.1-kb *Eco*RI fragments hybridizing to the *A. tumefaciens* probe are shown above the physical map of the 5.6-kb region that was sequenced. Triangles represent insertions of the Ω-Km interposon or the *uidA-aphII* cassette. The positions and orientations of the identified ORFs are indicated below the restriction map. Restriction sites are abbreviated as follows: B, *BstXI*; H, *Hin*dIII; E, *Eco*RI; N, *Not*I; P, *Pst*I.

the appropriate concentrations with a sterile concentrated stock solution at pH 7.0. Cell growth was monitored in terms of turbidity at 600 nm in a microtiter-plate reader. Carbon sources were D-(−)-mannitol, D-(+)-glucose, sucrose, L-(+)-arabinose, D-(+)-galactose, D-sorbitol, D-(−)-fructose, glycerol, trisodium citrate, *cis*-aconic acid, DL-isocitric acid, α-ketoglutaric acid, succinic acid, fumaric acid, DL-malic acid, oxaloacetic acid, and pyruvic acid. Nitrogen sources were NH_4Cl, L-alanine, L-glutamine, and KNO_3. For growth tests at various pH values, 3 (*N*-morpholino)propanesulfonic acid (pH 7.0) and 2(*N*-morpholino)ethanesulfonic acid (pH 6.5, 6.0, and 5.5) were used at a concentration of 30 mM.

For growth tests on plates, appropriate combinations of mannitol (0.2%, wt/vol), ammonium (10 mM), or amino acids (0.2%, wt/vol) were added to AMS agar (15 g/liter). Plates were supplied with (i) only the amino acid, (ii) the amino acid and ammonium, (iii) the amino acid and mannitol, or (iv) the amino acid, ammonium, and mannitol. Plates were incubated at 30°C, and colony size was monitored over a period of 3 to 7 days.

DNA methods. General DNA manipulations were performed as described previously (3, 45). DNA fragments were recovered from agarose gels by using the Nucleotrap kit (Macherey-Nagel). Southern blotting and hybridizations were carried out as described previously (3, 34, 45). DNA probes were labeled with a digoxigenin labeling and detection kit (Boehringer). To generate blunt ends to incompatible DNA fragments, DNA was incubated with Klenow or T4 DNA polymerase in the presence of the four deoxynucleoside triphosphates. Automated DNA sequencing was performed on a Pharmacia A.L.F. sequencer with fluorescein-labeled universal and synthetic oligonucleotide primers. Both strands of overlapping pUC18 subclones covering the 5,600-bp DNA fragment were read with multiple sequencing.

PCR conditions. Amplification of DNA fragments by PCR was performed in a TRIO-Thermoblock (Biometra). Twenty-five-microliter reaction mixes, containing 0.65 U of *Taq* DNA polymerase (Boehringer), each of the deoxynucleoside triphosphates at 200 μM, and each of the primers at 1 μM, were subjected to 30 cycles of incubation at 94°C for 60 s, 60°C (primers ojm065 and ojm072) for 60 s, and 72°C for 210 s.

Construction of mutants. To mutagenize the *R. etli rpoN* gene, the 1.8-kb *Eco*RI fragment of pFAJ1150 was blunt-end ligated into the *Sma*I site of pJQ200-UC1. The resulting plasmid was digested with *Pst*I. This plasmid was used in two separate reactions. First, it was blunt-end ligated to the 1.8-kb *Bam*HI fragment from pHP45Ω-Km, resulting in two constructs in which the Ω-Km fragment is inserted in opposite directions. The Ω-Km interposon contains the *aphII* gene from Tn*5* and confers resistance to kanamycin and neomycin. Second, to construct a transcriptional *rpoN-gusA* fusion, it was also blunt-end ligated to the 3.8-kb *Bam*HI fragment from pWM6. Two plasmids carrying the *gusA*-Km^r cassette in opposite orientations were obtained. These four insertional mutations (Ω-Km and *gusA*) were finally recombined into the *R. etli* CNPAF512 chromosome. Insertion of these mutations was verified by Southern blot hybridization with the appropriate probes. In this way, the following mutants were constructed: FAJ1154 (orientations of Ω-Km and *rpoN* are opposed), FAJ1155 (same orientations), FAJ1156 (orientations of *gusA* and *rpoN* are the same), and FAJ1157 (opposite orientations).

For the inactivation of *ptsN*, a 1.7-kb fragment containing *ptsN* was amplified by PCR with pFAJ1150 as template DNA and the following two sequence-specific primers: ojm065 (5′-GAGCGCGGCCGCGTGGATCGGACTGATCTC-3′) and ojm072 (5′-ACTCGCGGCCGCGCTTCCGGGTCTCCGGTTCG-3′). The amplified fragment carries *Not*I recognition sites at both ends. Following digestion with *Not*I, this fragment was inserted at the corresponding site of pJQ200-UC1. Finally, the 2.2-kb *Bam*HI fragment of pHP45Ω-Km was cloned into the *Pst*I site of the 1.7-kb insert of pJQ200-UC1, after blunting of both fragments, thereby inactivating *ptsN*. The different constructs were used to generate site-directed mutants of *R. etli* CNPAF512. In the mutants FAJ1164 and FAJ1165, *ptsN* and Ω-Km read in the same and opposite directions, respectively.

Nucleotide sequence accession number. The nucleotide sequence of the 5,600-bp DNA fragment containing *R. etli rpoN* and associated genes has been deposited with DDBJ-EMBL-GenBank under accession no. U23471.

RESULTS

Cloning of the *R. etli rpoN* gene. To detect the *rpoN* gene, *Eco*RI-restricted genomic DNA from *R. etli* CNPAF512 was hybridized with, as a probe, the 1.7-kb *Eco*RI-*Hin*dIII fragment from pRJ7694 carrying the *B. japonicum rpoN1* gene (23). One *Eco*RI fragment of 1.8 kb strongly hybridized to the probe. Also, only one hybridizing fragment was observed on *Hin*dIII- and *Sal*I-restricted genomic DNA. The same probe was therefore used to screen a gene library of *R. etli* CNPAF512 maintained in *E. coli* HB101. This library was constructed in the *Eco*RI site of pLAFR1. One hybridizing cosmid, pFAJ1150, was identified. When pFAJ1150 was digested with *Eco*RI and hybridized with a 3.5-kb *Eco*RI fragment containing *Agrobacterium tumefaciens rpoN* and flanking DNA (53), three fragments of 1.8, 4.6, and 6.1 kb hybridized strongly. A physical map of the *R. etli rpoN* region is shown in Fig. 1.

To ascertain that pFAJ1150 contained a functional *rpoN* gene, this plasmid was transferred to the *R. meliloti rpoN* mutant 1680 (43). In contrast to the wild-type strain 1021, mutant 1680 cannot fix atmospheric nitrogen during symbiosis with alfalfa plants and is not able to grow on C_4-dicarboxylates as carbon sources or to assimilate nitrate. Plasmid pFAJ1150 was shown to complement each of these defects in strain 1680 (data not shown).

Sequence analysis of the *rpoN* region. To further characterize the *R. etli rpoN* gene, the nucleotide sequence of a 5,600-bp DNA fragment was determined. Examination of this nucleotide sequence revealed the presence of one partial and four complete (ORFs) (Fig. 1). All of the ORFs are transcribed in the same direction as *rpoN*. The initiation codons were assigned on the basis of sequence homology with homologous ORFs in other bacterial species.

The first complete ORF, ORF258, codes for a protein of 258 amino acids with a calculated molecular mass of 28,335 Da. No obvious *E. coli*-like Shine-Dalgarno sequence was detected upstream from the ORF258 initiation codon. A DNA sequence (GATTTCAGGCC-N_5-GGCCTGAAATC) with the potential to form a hairpin loop secondary structure (ΔG [25°C] = −21.2 kcal) was detected 15 bp downstream from the termination codon. ORF258 shows homology with ORFs and partially sequenced ORFs located upstream from *rpoN* genes in other bacterial species (e.g., 81% amino acid identity with *R. meliloti*). The proteins encoded by these ORFs resemble members of the ATP-binding cassette-type permeases. ORF258 is preceded by the 3′ end of an incomplete ORF whose derived protein shows 50% amino acid identity with the corresponding *R. meliloti* protein.

The second ORF, named *R. etli rpoN*, is located 215 bp downstream from ORF258. The *rpoN* gene codes for an acidic (calculated isoelectric point, 4.32) protein of 520 amino acids with a predicted molecular mass of 57,477 Da. A putative ribosome-binding site (GGAG) is located 13 bp upstream from the *rpoN* initiation codon. Further analysis of the upstream region of *rpoN* revealed strong nucleotide sequence identity with the *R. meliloti rpoN* promoter. The *R. meliloti rpoN* start of transcription has been determined previously, and potential −35 and −10 regions have been identified (1). Sequences identical to the −35 and −10 regions of the *R. meliloti rpoN* promoter (CTTGAC-N_{17}-CAATTT) as well as the transcriptional start site (CAATTTTTGGGCC$\underline{A}$ACT [the transcriptional start is underlined]) are conserved in the *R. etli rpoN* promoter region, suggesting that these sequences might also be operative in *R. etli*. Strong conservation of amino acid residues was found between *R. etli* RpoN and all known RpoN proteins of rhizobia (*R. meliloti*, 68% amino acid identity; *Rhizobium* sp. strain NGR234, 65%; *A. caulinodans*, 53%; and *B. japonicum*, 54% with RpoN1 and 50% with RpoN2). The three regions of the *R. etli* RpoN protein, as defined for other RpoN proteins (31, 52), are the amino-terminal region I (50 amino acids), region II (108 amino acids), and the carboxy-terminal region III (362 amino acids). A conserved helix-turn-helix motif, implicated in binding of the −24/−12 promoter (10), is found in the *R. etli* RpoN protein between amino acid positions 398 and 418 (helix [N-I]-turn [K-H]-helix [E-S]). A highly conserved sequence of 10 amino acids (ARRTVAKYRE), termed the RpoN box (52), is located near the carboxy terminus of *R. etli* RpoN between amino acids 488 and 497.

A third complete ORF was located 1.6 kb downstream from

TABLE 2. Expression of a chromosomally integrated *R. etli rpoN-gusA* fusion in wild-type [FAJ1156(pFAJ1150)] and *rpoN* mutant (FAJ1156) backgrounds

Growth condition[a]	β-Glucuronidase activity (Miller units)[b] in:	
	FAJ1156	FAJ1156(pFAJ1150)
Ammonium and mannitol	82 (37)	19 (13)
Nitrate and mannitol	84 (37)	11 (6)
Ammonium and succinate	112 (20)	19 (13)
TY, aerobic	64 (12)	20 (8)

[a] Cells were grown in TY or AMS medium containing the indicated combinations of ammonium, nitrate, and succinate (each at 10 mM) and mannitol (10 g/liter). Microaerobic inductions were carried out with 0.5% oxygen as detailed in Materials and Methods.

[b] Data are the means for four replicates. Standard deviations are given in parentheses.

rpoN. This ORF is predicted to encode a protein of 191 amino acids with a calculated molecular mass of 21,172 Da. ORF191 is preceded by a putative ribosome-binding site, AGAAGG, located 8 bp upstream from the presumptive initiation codon. The protein sequence derived from ORF191 is homologous to sequences of proteins encoded by genes that are normally found immediately downstream from *rpoN* (e.g., *E. coli* ORF95). Eleven of these genes in various bacterial species have been sequenced (39); nine of them were completely sequenced. The number of amino acids encoded by these genes typically ranges from 95 to 130, with the exception of *B. japonicum* ORF203 and *C. crescentus* ORF208, which code for 203- and 208- amino-acid proteins, respectively. These proteins were shown to be homologous to an *E. coli* protein encoded upstream from *pheA* (SwissProt accession no. P11285 [31]).

The fourth ORF is located 76 bp downstream from ORF191 and codes for a protein of 154 amino acids (PtsN) with a predicted molecular mass of 16,675 Da. A putative ribosome-binding site (AGAAGG) is located 8 bp upstream from the initiation ATG. A DNA sequence (AAAAAAGGCGCCTG-N_6-CAGGCGCCTTTTTT) with the potential to form a stable hairpin loop secondary structure (ΔG [25°C] = −28.2 kcal) was detected 19 bp downstream from the termination codon. This sequence may function as a *rho*-independent terminator (GC-rich stem loop followed by several Ts). This ORF is homologous to previously identified *ptsN* genes located at the same position in other bacterial species. This ORF encodes a protein that is similar to the enzyme IIA protein of the PTS, and in particular to enzyme IIA proteins specific for the transport of mannitol and fructose (39). No ORF with homology to *E. coli* ORF284 or ORF90 (encoding the Hpr-like protein Npr), located 3′ of the *ptsN* gene, was found downstream from *ptsN* (data not shown).

Expression of *R. etli rpoN*. To monitor expression of *R. etli rpoN*, an *rpoN-gusA* (*gusA* in both orientations) fusion was inserted into the chromosome by site-directed mutagenesis, thereby inactivating the *rpoN* gene. The resulting strains are FAJ1156 (correct orientation of *gusA*) and FAJ1157 (opposite orientation). *R. etli* FAJ1156 was also complemented by using pFAJ1150. FAJ1156(pFAJ1150) did not differ from the wild type with respect to growth on nitrate or succinate, melanin production, and symbiotic properties (data not shown). Expression of *rpoN* was assayed in different media (Table 2). No difference between the expression levels in these media was observed, except that in FAJ1156 the expression level of *rpoN* was slightly lower when the strain was cultured in TY than when it was cultured in AMS medium containing ammonia and succinate. Also, expression was unaffected by the ammonia concentration (from 1 to 10 mM) (data not shown). However, under all free-living conditions tested, expression of *rpoN-gusA* was higher in FAJ1156 than in the complemented strain (Table 2). No β-glucuronidase activity was measured in the control strain FAJ1157. From these data it appears that the *rpoN* gene is expressed independently of the nitrogen concentration and is negatively autoregulated.

Growth of *R. etli rpoN* mutants. To investigate the function of *rpoN*, the Ω-Km interposon was inserted in both orientations in the *R. etli rpoN* gene. These mutations were recombined into the chromosome by site-specific mutagenesis, yielding mutants FAJ1154 and FAJ1155 (Fig. 1 and Table 1). Insertion of the interposon at the correct site was confirmed by Southern hybridization. The *rpoN* mutants were tested for growth on defined media containing amino acids, ammonium, or KNO_3 as a nitrogen source and mannitol or succinate as a carbon source.

First, growth of the *rpoN* mutant FAJ1154 was tested on different amino acids. Therefore, cells were grown on solid AMS minimal medium containing only the amino acid and compared with the parental strain CNPAF512. To evaluate possible toxic effects of the amino acid, mannitol (carbon source) or ammonium (nitrogen source) was additionally supplied to the plates (Table 3). No difference was observed for media containing lysine, glutamine, glutamate, proline, leucine, or isoleucine (Table 3). However, growth of the mutants was strongly inhibited on medium containing alanine or serine, in the presence or absence of ammonium or mannitol (Table

TABLE 3. Growth of *R. etli* wild-type strain CNPAF512 and *rpoN* mutant FAJ1154 on defined media[a]

Amino acid(s)	Growth[b] with: Amino acid(s)		Amino acid(s) and ammonium		Amino acid(s) and mannitol		Amino acid(s), ammonium, and mannitol	
	Wild type	*rpoN* mutant	Wild type	*rpoN* mutant	Wild type	*rpoN* mutant	Wild type	*rpoN* mutant
Gln	+	+	+	+	+++	+++	+++	+++
Pro	++	++	++	++	+++	+++	+++	+++
Ala	+	−	+	−	+++	−	+++	−
Ser	+	+/−	+	+/−	+++	+/−	+++	+/−
Ala and Gln	+	+	+	+	+++	+++	+++	+++
Ser and Gln	+	+	+	+	+++	++	+++	++

[a] Growth tests were carried out on AMS plates containing the indicated combinations of amino acid(s) (0.2%), ammonium (10 mM), and mannitol (10 g/liter) (see Materials and Methods). Colony size was determined after 3 days of incubation at 30°C. Growth on Glu, Lys, Ile, and Leu was similar to that on Gln.

[b] Symbols: −, no growth; +/−, colony size of <0.5 mm; +, colony size of 0.5 to 1 mm; ++, colony size of 1 to 2 mm; +++, colony size of >2 mm.

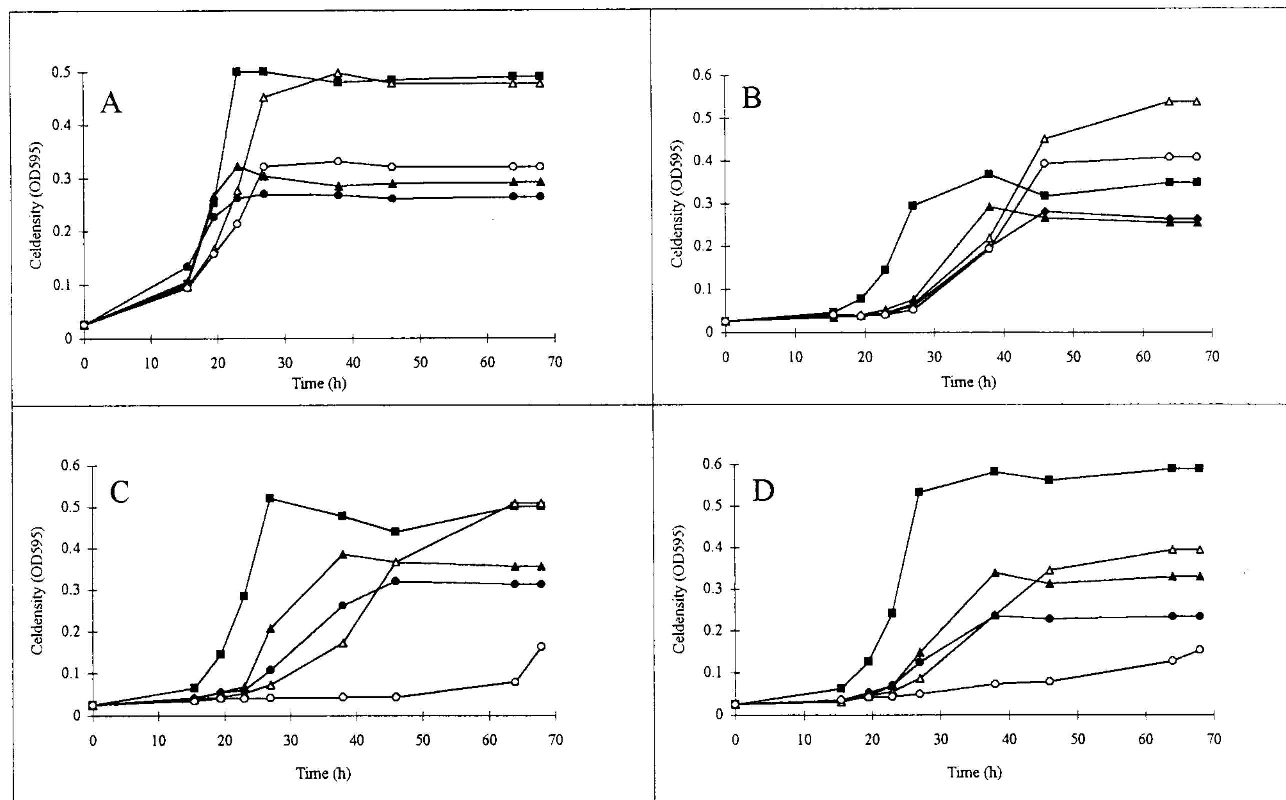

FIG. 2. Effects of malate and succinate concentrations on the growth curves of the *R. etli* wild-type strain CNPAF512 (A) and *rpoN* (B), *ptsN* (C), and *ptsA* (D) mutants. Cultures were grown in AMS medium containing NH_4Cl (20 mM) as a nitrogen source and mannitol (20 mM) (■), succinate (10 mM [▲] or 20 mM [△]), or malate (10 mM [●] or 20 mM [○]) as a carbon source. OD595, OD at 595 nm.

3). These results indicate that the *rpoN* gene is required for the metabolism of alanine and serine. In addition, since growth of the *rpoN* mutant is also restricted on media containing, in addition to alanine or serine, ammonium and mannitol, these amino acids are likely to be toxic to the cell or to inhibit the assimilation of ammonium or mannitol. To discriminate between these possibilities, glutamine was additionally supplied to the plates. Glutamine is a good nitrogen source but a poor carbon source in wild-type *R. etli* and the *rpoN* mutant (Table 3). Upon the addition of glutamine to these media, growth was restored to the wild-type level. Therefore, alanine and serine probably inhibit the assimilation of ammonia in the *rpoN* mutant.

Second, growth of the *rpoN* mutants FAJ1154 and FAJ1155 in liquid AMS medium containing 10 mM NH_4Cl or KNO_3 (nitrogen source) and 10 mM mannitol, succinate, fumarate, or malate (carbon source) was monitored. When grown in the presence of ammonium and mannitol, both *rpoN* mutants have an extended lag phase which is approximately 10 h longer than that of the wild type (Fig. 2A and B). An additional delay of 10 h was observed when succinate, fumarate, or malate was used instead of mannitol (Fig. 2B). Finally, in comparison with that of the wild type, the lag phases of the *rpoN* mutants were extended for 30 h on mannitol and KNO_3 and for 60 h on medium containing both succinate and KNO_3 (data not shown).

Growth of *R. etli ptsN* mutants. Insertion mutants of *ptsN* (FAJ1164 and FAJ1165) were constructed as detailed in Materials and Methods. The insertion of the Ω-Km interposon in both mutants was controlled by Southern hybridization with the appropriate probes.

In a preliminary experiment, growth of each of the *ptsN* mutants was assayed on AMS plates containing alanine. When compared with that of the parental strain, growth of the *R. etli ptsN* mutants was impaired on medium containing mannitol as a carbon source and alanine as a nitrogen source. This observation led us to analyze the effect of these mutations on the metabolism of two other carbon sources, glucose and succinate. Therefore, strains were grown on AMS plates containing alanine, serine, glutamine, or ammonium in combination with mannitol, glucose, or succinate. From Table 4 it can be seen that the inactivation of *ptsN* clearly reduces growth on medium containing succinate in the presence of alanine, serine, ammonium, or, to a lesser extent, glutamine. Such a strong effect was not observed with medium devoid of succinate but containing alanine or serine in the absence or presence of ammonium (data not shown), indicating that growth inhibition of the *ptsN* mutant is mediated primarily by the carbon source (see below).

In addition to tests with mannitol and glucose, colony size of the *ptsN* mutants was also evaluated on AMS plates containing NH_4Cl, alanine, or glutamine as a nitrogen source and sucrose, arabinose, galactose, sorbitol, fructose, or glycerol as a carbon source. No difference or only a small decrease in colony size between the wild type and the *ptsN* mutants was observed (data not shown).

Production of melanin and expression of p*nifH-gusA* in *R. etli rpoN* and *ptsN* mutants. For *R. etli* CNPAF512, the production of the black pigment melanin was previously demon-

TABLE 4. Growth of wild-type *R. etli* and mutants on defined media containing Ala, Ser, Gln, or ammonium in combination with mannitol, glucose, or succinate[a]

Strain	Growth with:											
	Mannitol and:				Glucose and:				Succinate and:			
	Ala	Ser	Gln	Am	Ala	Ser	Gln	Am	Ala	Ser	Gln	Am
CNPAF512	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
FAJ1154	−	−	+++	+	−	+/−	+++	++	−	−	++	+/−
FAJ1165	++	+++	+++	+++	+++	++	+++	+++	+/−	+	++	+
FAJ1166	+	++	+	+	+	++	+	+	+/−	+/−	+/−	+/−

[a] Growth tests were carried out on AMS plates containing the indicated combinations of amino acids (0.2%) or ammonium (Am) (10 mM) and sugars (0.2%). Growth conditions and symbols are as defined in the footnotes to Table 3.

strated to depend on the *nifA* gene (34). In addition, when reconstituted in *E. coli*, this phenotype was shown to be RpoN dependent (17). We therefore tested melanin synthesis in the *rpoN* mutant. These experiments were also performed with the *ptsN* mutant, since this gene was previously demonstrated to affect the expression of RpoN-dependent genes (30). Melanin production was first assayed on TY plates containing tyrosine and $CuSO_4$ (17). As a control, we used the *nifA* mutant strain Rp1000, which is Mel^- (34). Both *rpoN* mutant strains were unable to produce the black pigment melanin, while no difference between the wild-type strain and *ptsN* mutants was observed. However, since minor differences in the production of melanin cannot readily be detected by this method, melanin synthesis was also determined quantitatively on media containing different carbon sources (Fig. 3A and B). These quantified data clearly show a reduction of melanin synthesis in all mu-

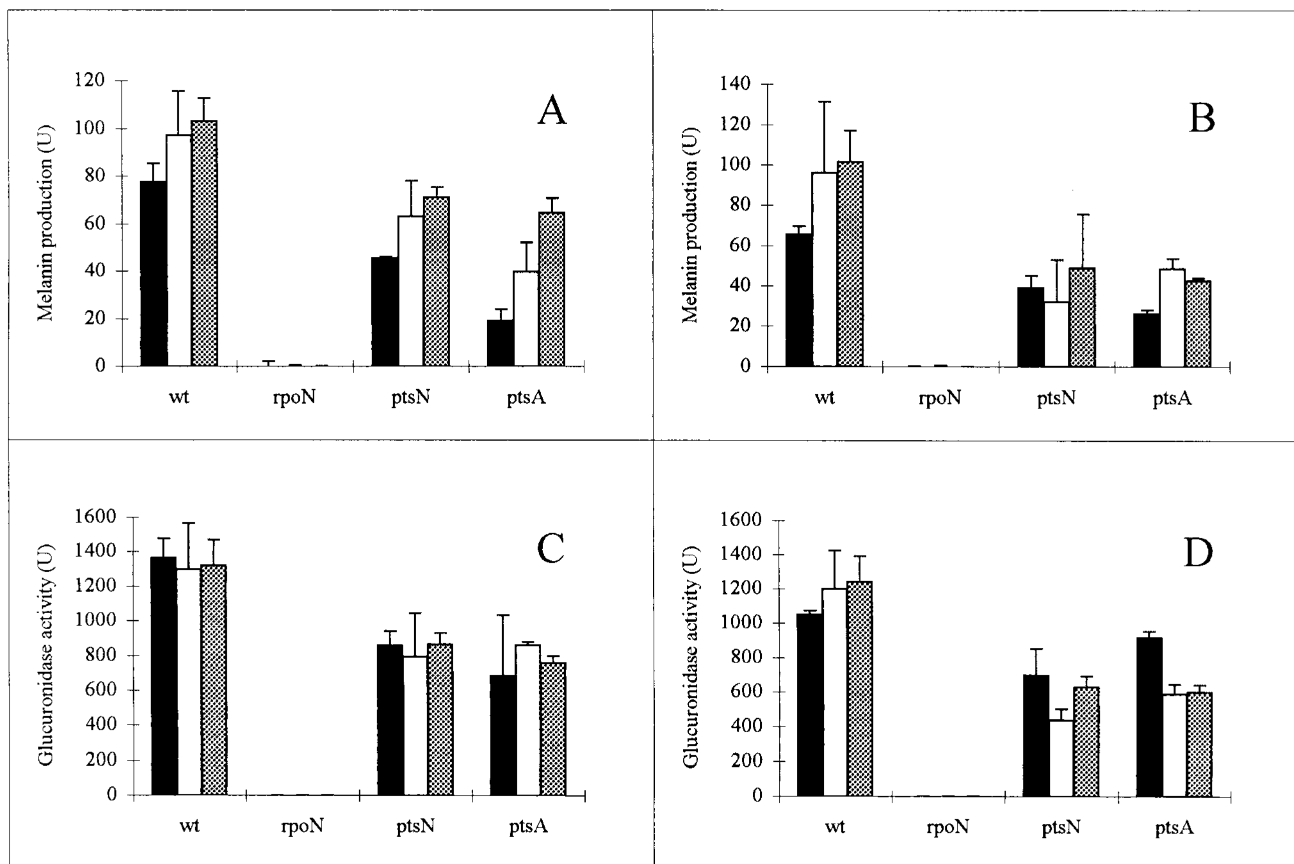

FIG. 3. Melanin production (A and B) and expression of *nifH* (C and D) in wild-type *R. etli* (wt) and *rpoN* (FAJ1154), *ptsN* (FAJ1165), and *ptsA* (FAJ1166) mutants. All data are the means from four independent replicates. Error bars denote the standard deviations. Precultures were grown overnight in TY medium at 30°C, diluted 20-fold in the different media, and incubated overnight with 0.5% oxygen (34). The nitrogen source used was alanine (20 mM). The carbon sources were mannitol (black bars), succinate (stippled bars), and malate (white bars) at 5 mM (A and C) and 20 mM (B and D). To quantify melanin production, cultures were lysed at 37°C in the presence of a solution containing sodium dodecyl sulfate (1%), $CuSO_4$ (10 μg/ml), and tyrosine (30 μg/ml). The OD of the culture after lysis was measured at 340 nm in a microplate reader after 60 and 120 min of incubation. The difference between the ODs at 340 nm was used to calculate the units. Units are expressed as the ratio of the change in OD at 340 nm to the OD at 595 nm. β-Glucuronidase activities of the translational p*nifH-gusA* fusion plasmid pFAJ21 are expressed as Miller units.

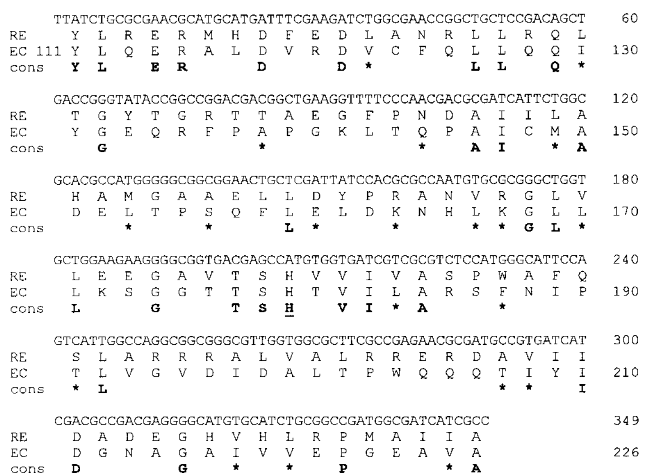

FIG. 4. DNA sequence flanking the Tn*5-mob* insertion of *R. etli* FAJ1166. The deduced amino acid sequence (RE) is compared to that of the *E. coli* (EC) phosphoenolpyruvate-protein phosphotransferase PtsA (enzyme I; accession no. P32670) from amino acid 111 to 226. The conserved phosphorylation site (H189) is underlined. Identical and similar (S-T-A, L-V-I-M, K-R, Q-N, and F-Y-W) residues are indicated below the amino acid sequences (cons). Stars denote similar residues.

tants tested. Pigment production was abolished in the *rpoN* mutants and reduced two- to threefold in the *ptsN* mutant, depending on the composition of the growth medium.

Finally, we analyzed the induction of the p*nifH-gusA* fusion plasmid pFAJ21 (Fig. 3C and D). Similar to the case for the production of melanin, expression of pFAJ21 is abolished in both *rpoN* mutants and is reduced by 30 to 70% in the *ptsN* mutant compared to the wild type.

The decreased melanin synthesis or *nifH* expression is not caused by a reduction of *nifA* transcription. No difference in the expression level of a p*nifA-gusA* fusion was observed for the different mutant strains (data not shown). Also, expression of the p*amtB-gusA* fusion plasmid pFAJ302 was tested under the same conditions. The *amtB* gene is regulated by the RpoN and NtrC proteins. As expected, this fusion plasmid was expressed at a low level in the *rpoN* mutant strain. However, no reduction of the activity of this fusion was observed in the *ptsN* mutant compared to the wild type under the same conditions as tested in Fig. 3 (data not shown). The *nifH* and *amtB* genes, used to construct the fusions pFAJ21 and pFAJ302, were originally isolated from *Azospirillum brasilense*. Expression of pFAJ21 is RpoN/NifA dependent, while that of pFAJ302 is RpoN/NtrC dependent. These fusions have been clearly demonstrated to be regulated similarly in *Azospirillum* and *Rhizobium* and are therefore suitable for the expression studies detailed above.

Analysis of the *R. etli* Mel⁻ strain FAJ1166. In an attempt to isolate regulatory mutants affected in the production of melanin, a library of Tn*5-mob* mutants of *R. etli* CNPAF512 was previously screened on plates. Several mutants with a reduced level of melanin production were isolated. One of these mutants, FAJ1166 (which has the lowest level of melanin production among the isolated mutants), was further characterized. By the quantitative assay, FAJ1166 was shown to have a two- to fivefold reduction in melanin synthesis (Fig. 3A and B). Also, expression of pFAJ21 was reduced approximately twofold (Fig. 3C and D). These phenotypes were not caused by reduced expression of the *nifA* gene as demonstrated by the analysis of a p*nifA-gusA* fusion plasmid (data not shown). Also, under the same growth conditions, expression of pFAJ302 was not reduced in this mutant (data not shown). To further analyze this mutant, chromosomal DNA was partially digested with *Eco*RI and ligated to the cosmid vector pSUP205 (47). The ligation mixture was packaged into phage heads and used to infect *E. coli* HB101 cells. Cosmids containing the Tn*5* insert were selected on kanamycin. Next, a fragment of approximately 14 kb carrying the Tn*5* transposon was subcloned into pUC18. By using a primer derived from the sequence of the Tn*5* inverted repeat (5′-GGTTCCGTTCAGGACGCT-3′), the DNA region of one site of the Tn*5*-flanking DNA was sequenced. This sequence coded for a peptide with homology to *E. coli* enzyme I of the PTS system (Fig. 4). Further analysis indicated that growth of mutant FAJ1166 was considerably impaired on all solid media tested, although the effect was strongest on medium containing succinate (Tables 3 and 4). However, growth of this mutant did not depend solely on the carbon source used. Higher growth rates were observed when serine instead of alanine, glutamine, or ammonia was used in combination with mannitol or glucose. Finally, in contrast to the wild type or the *ptsN* mutants, FAJ1166 had lost its mucoid morphology on all media tested.

Growth on TCA cycle acids. As observed on solid medium, growth of *ptsN* and *ptsA* mutants is impaired in the presence of succinate (Table 4). Therefore growth of both mutants was also tested in liquid AMS medium containing mannitol or the C_4-dicarboxylate succinate, fumarate, or malate as a carbon source and NH_4Cl, alanine, or glutamine as a nitrogen source. Growth in the presence of NH_4Cl is presented in Fig. 2. Growth of the *ptsN* and *ptsA* mutants was inhibited on malate and succinate. Inhibition was shown to depend on the concentration of the dicarboxylate. Complete growth inhibition of the *ptsN* and *ptsA* mutants, but not the wild-type strain, occurred in the presence of 20 mM malate (Fig. 2) or 30 mM succinate but not 30 mM fumarate (data not shown). Mannitol at concentrations of up to 30 mM or malate and succinate at a concentration of 5 mM did not interfere with growth (data not shown). Growth inhibition of these mutants decreased upon lowering of the concentration of malate or succinate. These observations were independent of the nitrogen source used.

Since cyclic AMP (cAMP) has been shown to play a central role in signalling of the PTS in *E. coli*, we tested whether this compound could modulate the inhibitory activity of malate. Therefore, cells of the wild-type, *ptsN*, *ptsA*, and *rpoN* strains were cultured in AMS medium containing alanine as the nitrogen source and mannitol, malate, succinate, or fumarate as a carbon source, either in the absence or in the presence of 0.5 mM cAMP. No effect of cAMP on the growth of these strains in the different media could be observed.

We also examined growth of the wild type and the *ptsN* or *ptsA* mutant on the other tricarboxylic acid (TCA) cycle intermediates, i.e., citrate, aconitate, isocitrate, α-ketoglutarate, oxaloacetate, and pyruvate. The organic acids were each at 20 mM, with the exception of oxaloacetate, which was used at 2 mM. Alanine was used as the nitrogen source. *R. etli* cells did not grow on citrate or isocitrate, while no obvious growth differences compared to the wild type were observed with the other acids.

When malate was added to the growth medium in combination with mannitol or subinhibitory concentrations of succinate, growth of the *ptsN* and *ptsA* mutants was also strongly inhibited (Fig. 5). Inhibition was not observed in the presence of the same concentrations of fumarate, aspartate, or asparagine (data not shown). These data clearly indicate that, even in the presence of carbon sources that are normally metabolized

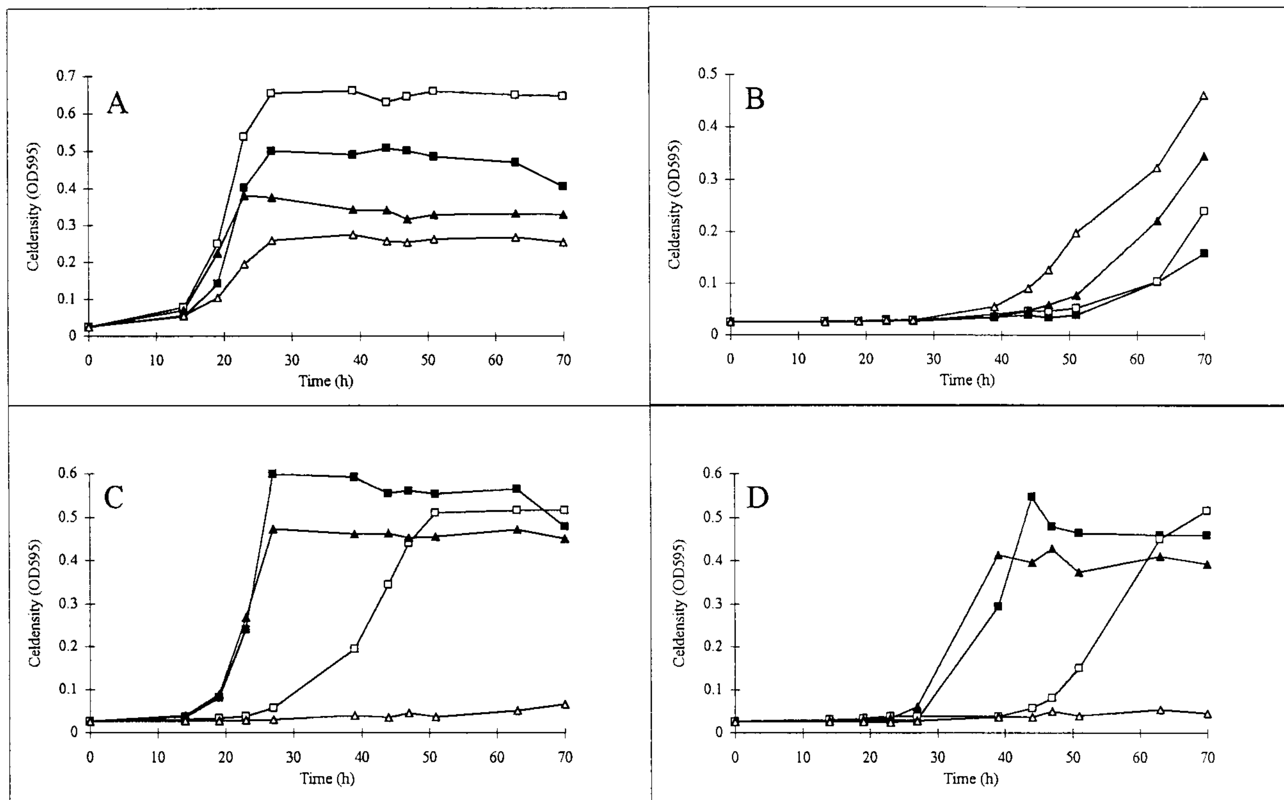

FIG. 5. Toxic effects of malate on cell growth in the presence of mannitol and succinate. The strains tested are the *R. etli* wild-type strain CNPAF512 (A) and *rpoN* (B), *ptsN* (C), and *ptsA* (D) mutants. Cultures were grown in AMS medium containing alanine (20 mM) as a nitrogen source and mannitol (20 mM) (■) or succinate (10 mM) (▲) alone or in combination with 20 mM malate (□, mannitol and malate; △, succinate and malate). OD595, OD at 595 nm.

by *ptsN* and *ptsA* mutants, malate inhibits growth and therefore probably affects an essential cellular function.

The degree of ionization of dicarboxylic acids depends on the pH of the medium. In order to test whether the pH affects the toxicity of the C_4-dicarboxylic acids, cell cultures were grown at various external pH values in the presence of either malate, succinate, or fumarate at 5 and 20 mM. The effect of malate is presented in Fig. 6. In contrast to the case for the wild type, the external pH affects the toxicity of malate and, to a lesser extent, succinate in the *ptsN* mutant at a concentration of 20 mM but not 5 mM (Fig. 6 and data not shown). Growth inhibition was highest at pH 7.0 and 6.5 but was absent at pH 6.0 or pH 5.5. Similar results were obtained with the *ptsA* mutant. No effect of pH on the assimilation of fumarate was observed (data not shown). Also, in the *rpoN* mutant at 20 mM malate, growth was inhibited by increasing the pH (Fig. 6). No effect of external pH values of between 5.5 and 7.0 on the assimilation of succinate in the *rpoN* mutant was found (data not shown).

DISCUSSION

The *rpoN* locus of *R. etli* was isolated by using the *B. japonicum rpoN1* gene as a probe. The nucleotide sequence of this region was determined, and four complete ORFs (ORF258, *rpoN*, ORF191, and *ptsN*) were identified. In most organisms analyzed so far, the organization of ORFs linked to the *rpoN* gene is remarkably well conserved (31). Exceptions are *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, in which *rpoN* is cotranscribed with nitrogen fixation genes (27, 28). The products of the conserved ORFs located upstream from *rpoN* genes, including *R. etli* ORF258, share features with the family of the ATP-binding cassette-type permeases. However, unlike those in other bacterial species, the two conserved downstream ORFs in *R. etli* (ORF191 and *ptsN*) are not adjacent to the *rpoN* gene but are separated by approximately 1.6 kb. Also, in *C. crescentus* homologs of these two genes are located 0.8 kb downstream from *rpoN* (19). The *ptsN* gene is homologous to genes coding for the enzyme IIA protein of the PTS (42). In bacteria, the PTS facilitates the uptake of many sugars (44) (see below). In *E. coli* and *K. pneumoniae*, sequence analysis of the DNA region downstream from *ptsN* has revealed the presence of two additional ORFs: ORF284 (also partially sequenced in *Pseudomonas putida* and *P. aeruginosa* [20]) and ORF90 (21, 32, 39). The product of the latter gene, NPr, is homologous to Hpr-like proteins of the PTS. No homologs of these two genes were detected in the DNA region downstream from *R. etli ptsN*. In addition, a *rho*-independent termination sequence was found 19 bp downstream from *ptsN*, suggesting that transcription did not extend further downstream. For *E. coli*, it was suggested that the NPr protein modulates the phosphorylation status of PtsN and as a result determines the activity of the protein (39) (see below). It is therefore possible that mechanisms of fine tuning of PtsN activity in *R. etli* are different from those in *E. coli.*

Expression of *rpoN* genes in *E. coli* and *K. pneumoniae* is at a low constitutive level (7, 29). In contrast, expression of the *R. etli rpoN* gene is increased in an *rpoN* mutant background. A similar situation exists in *B. japonicum* (23) and *P. putida* (22), where the *rpoN* gene (*rpoN2* of *B. japonicum*) is also negatively

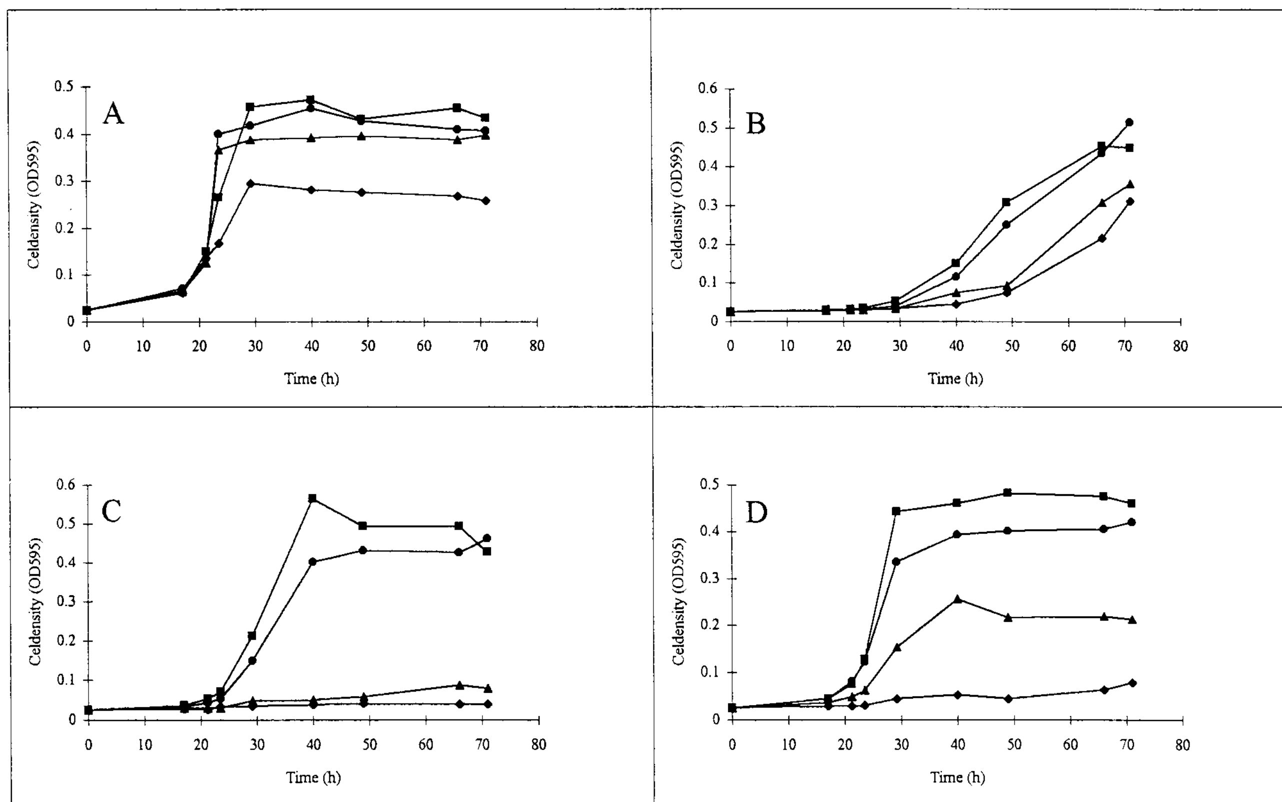

FIG. 6. Influence of pH on growth of *R. etli* wild-type strain CNPAF512 (A) and *rpoN* (B), *ptsN* (C), and *ptsA* (D) mutants. Alanine (20 mM) was used as a nitrogen source. Malate was at 20 mM. Symbols: ■, pH 5.5; ●, pH 6.0; ▲, pH 6.5; ◆, pH 7.0. OD595, OD at 595 nm.

autoregulated. Inspection of the *R. etli rpoN* promoter region revealed the presence of an RpoN consensus binding site, reading in the leftward direction (on the template strand). On the basis of sequence conservation between the *R. meliloti* and *R. etli rpoN* promoter regions (the *R. etli rpoN* gene can also functionally substitute for *R. meliloti rpoN*), this RpoN-binding site was shown to overlap the −10 promoter region and the transcription start site, as determined for *R. meliloti* (Fig. 7). Moreover, we observed that the promoter sequences and the oppositely oriented RpoN-binding sites were also conserved in

	−35 −10 ↓
Rm	GCTTGACCAAATTCCAGTAATAAGCAATTTTTGGGCCAACT
Re	GCTTGACCAAATGAGATAACAAAGCAATTTTTGGGCCAACT
NGR234	GCTTGACCAAATCCCGGTAATAAGCAATTTTT GGCCAACT
BjrpoN2	ACATCAGGCTCAGACTAGGATAAGCAAAAATCGGACCAACT
Cons -24/-12	T**GC**AA GTG**CC**AG
Ec -35/-10	TTGACA TATAAT

FIG. 7. Alignment of promoter regions from rhizobial *rpoN* genes. The aligned *rpoN* sequences are from *R. meliloti* (Rm), *R. etli* (Re), *Rhizobium* sp. strain NGR234, and *B. japonicum rpoN2* (BjrpoN2). The −10 and −35 regions from the *R. meliloti rpoN* promoter are overlined, and the transcription start site is indicated by an arrow (43). The inverse/complement of the consensus (Cons) sequence of a −24/−12 promoter is indicated; conserved GC and CC residues are in boldface. Nucleotides identical to the consensus −24/−12 sequence are underlined. Ec, *E. coli.*

the promoter regions of *Rhizobium* sp. strain NGR234 *rpoN* (one nucleotide missing) and *B. japonicum rpoN2* (Fig. 7). We could not detect such a binding site in the 5′ region of the *E. coli* and *K. pneumoniae rpoN* genes, which are not autoregulated but are transcribed at a low constitutive level (7, 29). In *P. putida*, a putative RpoN-binding sequence also was identified three nucleotides downstream from the −10 region (22). However, this sequence was located on the noncoding strand. Also, in the *Acinetobacter calcoaceticus rpoN* gene, which is negatively autoregulated, a −24/−12 promoter consensus sequence is found on the noncoding strand near the transcriptional start site (13). The RpoN protein is able to bind in vitro to −24/−12-type promoters in the absence of the RNA polymerase core enzyme or of an activator protein (6, 11). It is therefore possible that negative autoregulation of *R. etli* and *B. japonicum rpoN* genes occurs by direct interference with $E\sigma^{70}$ functioning through the binding of σ^{54} (or $E\sigma^{54}$) to the −10 to +1 promoter region of the *rpoN* gene, thereby inhibiting transcription initiation. Negative regulation of σ^{70} promoters by binding of the repressor to the −10 to +1 region of the promoter is well documented for *E. coli* (16).

R. etli rpoN mutants were constructed by insertion of the Ω-Km interposon in both directions in the gene. These mutations are polar and may thus affect the functioning of the genes located downstream from and belonging to the same operon as *rpoN*. However, the phenotypes of an *R. etli* strain carrying a mutation immediately downstream from the *rpoN* gene (data not shown) and of the *ptsN* mutants were different from that of the *rpoN* mutants. In addition, insertion of a *gusA-aphII* cassette into the *rpoN* gene, giving rise to nonpolar mutations

(33), produced the same phenotypic defects as in the interposon mutants. Therefore, the phenotype of the *rpoN* mutants is not caused by a polar effect on downstream genes but can be attributed to inactivation of *rpoN*.

Growth defects of the *R. etli rpoN* mutants on different C and N sources were observed. When growth was tested on plates containing different amino acids as carbon and/or nitrogen sources, growth of the mutant, but not that of the wild-type strain, was inhibited on plates containing either serine or alanine as the sole C and N source. Growth defects may be attributed to the inability of the *rpoN* mutant strain to catabolize serine and alanine or to assimilate its degradation products. In *E. coli*, the metabolism of serine and that of alanine involve different genes (41). Both amino acids are degraded to pyruvate and ammonium. The ammonia produced is used to synthesize glutamate and glutamine. The corresponding *R. etli* genes may also be subject to an RpoN-type regulation. However, serine and alanine also inhibit growth of the *R. etli rpoN* mutants on medium containing ammonium and mannitol. The supplementary addition of glutamine to these media relieved growth inhibition. In *E. coli*, alanine and serine inhibit the activity of glutamine synthetase. A similar inhibitory action on the glutamine synthetase enzyme in *R. etli* might explain the observed phenotypes. The analysis of ammonia assimilation in *R. etli* is complicated by the presence of three different glutamine synthetase genes, *glnA* (coding for GSI), *glnII* (GSII), and *glnT* (GSIII). *Rhizobium* GSI enzymes are similar to the GSI of *E. coli* and might therefore also be inhibited by alanine and serine. GSII is similar to eukaryotic glutamine synthetases. In *R. etli*, transcription of *glnII* is RpoN dependent (36), while that of the *glnA* gene is not (8). Possibly, the wild-type strain synthesizes GSI and/or GSII, while the *rpoN* mutant produces only GSI. The observed phenotypes may therefore be attributed to the inability of the *rpoN* mutant to assimilate ammonia produced by the degradation of alanine and serine. A defect in the assimilation of ammonia by the *rpoN* mutant strain was also observed in liquid medium containing ammonium as the sole nitrogen source. The presence of a second glutamine synthetase, encoded by *glnII*, may thus confer a selective advantage under those conditions where GSI activity is inhibited. It is not known whether *Rhizobium* is subject to such conditions during its life cycle.

Growth of the *rpoN* mutants on succinate, fumarate, and malate was delayed compared to that of the parental strain. Therefore, in *R. etli*, an RpoN-dependent system for the uptake of C_4-dicarboxylates is likely to exist. However, after an extended lag phase, the mutants grew as fast as the wild type, indicating that in the absence of RpoN, the activation of alternative control mechanisms of the RpoN-dependent C_4-dicarboxylate transport (*dct*) system or the existence of a second, inducible, *dct* system may account for the observed growth kinetics. This is in contrast to the case for *R. meliloti*, *Rhizobium* sp. strain NGR234, and *A. caulinodans* (43, 48, 52). *rpoN* mutants of these bacteria cannot grow on succinate as the sole carbon source. Alternatively, a *B. japonicum rpoN* double mutant is not impaired in the assimilation of dicarboxylates. For this species, the existence of an RpoN-independent dicarboxylate uptake system has been suggested (23).

In *R. etli*, interposon mutagenesis of *ptsN* results in reduced expression of p*nifH* and decreased production of the pigment melanin. Due to the presence of a putative *rho*-independent terminator, the *ptsN* gene is probably not cotranscribed with downstream genes, and the observed phenotype is likely due to its inactivation. Growth of the *ptsN* mutant in media containing mannitol, glucose, and dicarboxylic acids in combination with different nitrogen sources (alanine, serine, glutamine, and ammonium) was tested. Although growth inhibition of the mutant on plates containing alanine and serine in the presence of mannitol and glucose was observed, the strongest inhibition resulted from the presence of the C_4-dicarboxylates. Also, in liquid medium, growth of the *ptsN* mutant was impeded in the presence of malate or succinate but not fumarate or mannitol, independently of the nitrogen source (alanine, ammonia, or glutamine) used. This growth inhibition was dependent on the concentration of the dicarboxylic acid. Malate was inhibitory at lower concentrations than succinate. Also, a clear effect of pH on the toxicity of malate was observed; toxicity was greatly reduced upon lowering of the pH of the growth medium. Finally, the inhibitory activity of malate is dominant over the assimilation of additional carbon sources that are normally metabolized (mannitol or low concentrations of succinate). These observations indicate that malate inhibits an essential cellular function or metabolic activity.

Independently of the analysis of the *rpoN* locus, we identified a Tn*5*-induced *R. etli* mutant with a reduced production of the black pigment melanin. Partial sequence analysis indicated that the mutated gene corresponds to a homolog of the *E. coli* gene coding for enzyme I of the PTS. Interestingly, this mutant also displayed reduced expression of p*nifH*. In addition, growth of this mutant was inhibited in the presence of malate or succinate, similar to what was observed with the *ptsN* mutant. Presently, we cannot exclude the possibility that the phenotype of FAJ1166 may be due to polarity of the Tn*5* transposon on a thus-far-unidentified downstream gene.

From these observations, two conclusions can be drawn. First, the *rpoN*, *ptsN*, and *ptsA* mutants display several similar phenotypes. The expression of *nifH* or the production of melanin is abolished in the *rpoN* mutant and reduced in the *ptsN* and *ptsA* mutant strains. Also, depending on the carbon source used, growth of the *ptsN* mutant is impaired in the presence of alanine and serine, as observed in the *rpoN* mutant. These data indicate that PtsN and PtsA may act as coregulators of RpoN-dependent genes. However, no effect of these mutations on the activation of the RpoN-dependent *amtB* promoter was observed, indicating that not all of the RpoN-regulated genes are also controlled by PtsN and PtsA. Analysis of the conserved ORFs located downstream from *rpoN* genes has been carried out with only a few bacterial species. In *K. pneumoniae*, mutations in the two ORFs immediately downstream from *rpoN* (ORF95 and *ptsN*) increase the expression from the σ^{54}-dependent p*nifH*, p*nifL*, and p*glnAp2* (30). However, inactivation of the fourth gene (encoding an HPr-like product) results in decreased expression from these same promoters (32). In *P. aeruginosa*, *ptsN* positively controls genes involved in the assimilation of glutamine and does not influence pilin or flagellin genes (20). In *E. coli*, induction of the *glnAp2* promoter is not dependent on *ptsN*, although *ptsN* is required for maximal growth on alanine or adenosine as the sole nitrogen source. In addition, *ptsN* facilitates the use of these compounds as organic nitrogen sources in the presence of additional carbon sources, especially TCA cycle intermediates. Finally, *ptsN* suppresses mutations in the GTPase Era (39). In *C. crescentus*, *ptsN* is involved in fine tuning the expression from the *fljK* promoter but not from those of other σ^{54}-dependent flagellar genes (19). Clearly, the phenotypes of mutations in the *rpoN*-linked genes largely differ between different species and between the experimental systems used. Merrick and Coppard (30) proposed that the ORF95 and PtsN proteins control the stability of either the $E\sigma^{54}$ complex or the $E\sigma^{54}$-DNA complex. Alternatively, it was recently suggested that these downstream ORFs may also control the level of reinitiation of transcription by affecting the rate of release or conformational change of the

σ^{54} protein bound at the −24/−12 promoter after transcription has initiated (50). In both models, since the primary sequence of the promoter affects the stability of σ^{54} binding, this sequence may also account for the differential effects of mutations in the downstream ORFs on the expression of these genes.

Second, it is striking that both the *rpoN* mutant and the *ptsN* (or *ptsA*) mutant have growth defects on C_4-dicarboxylates. However, these defects clearly result from two different mechanisms. The *ptsN* and *ptsA* mutant strains have wild-type growth on low concentrations of malate and succinate, while these concentrations are strongly inhibitory for the *rpoN* mutant. In addition, increasing concentrations of these dicarboxylates stimulate growth of the *rpoN* mutant strain (Fig. 2 and 5) but repress growth of the *ptsN* and *ptsA* mutants. The defects of the *rpoN* mutant are probably due to the absence of a high-affinity *dct* system (see above). Growth inhibition of the *ptsN* and *ptsA* mutants is relieved by low pH. Assuming that the deprotonation of C_4-dicarboxylates increases their permease-mediated uptake, it is likely that at pH 7.0, the toxic effects of both dicarboxylates are caused by their increased cellular concentrations. Possibly, the input of larger amounts of dicarboxylates can affect the TCA cycle activities, resulting in growth inhibition. In *R. etli*, the catabolism of glucose is arrested in the presence of dicarboxylates (25). Therefore, a sensing system for dicarboxylates is likely to be operative. This system might signal to the cell the concentration of C_4-dicarboxylates in the growth medium. Depending on the concentration of these compounds, cells may, for example, have to decrease their uptake. Given the well-described role of the PTS in signalling in other microorganisms and since toxicity of malate and succinate is strongly increased in *ptsN* and *ptsA* mutants, this system might involve *ptsN* and *ptsA*. In other microorganisms, the PTS consists of two general proteins, enzyme I and Hpr, and the sugar-specific permease enzyme II. Enzyme II comprises up to four different polypeptides or domains (IIA, IIB, IIC, and IID). A phosphoryl group is sequentially transferred from phosphoenolpyruvate to enzyme I, Hpr, enzyme II, and, concomitant with its uptake, the sugar (44). PtsN, which is homologous to the enzyme IIA subunit, from *E. coli* is phosphorylated in vitro by the standard PTS phosphorelay involving phosphoenolpyruvate, enzyme I, and Hpr (39). Similar results were obtained with the *K. pneumoniae* ORF162 product PtsN (4, 32). Since *ptsA* and *ptsN* mutants have similar phenotypes, the phosphotransfer cascade of the PTS system in *R. etli* possibly controls the phosphorylation of the PtsN protein, thereby modulating its activity.

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