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Expression of the *putA* Gene Encoding Proline Dehydrogenase from *Rhodobacter capsulatus* Is Independent of NtrC Regulation but Requires an Lrp-Like Activator Protein

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Four *Rhodobacter capsulatus* mutants unable to grow with proline as the sole nitrogen source were isolated by random Tn*5* mutagenesis. The Tn*5* insertions were mapped within two adjacent chromosomal *Eco*RI fragments. DNA sequence analysis of this region revealed three open reading frames designated *selD*, *putR*, and *putA*. The *putA* gene codes for a protein of 1,127 amino acid residues which is homologous to PutA of *Salmonella typhimurium* and *Escherichia coli*. The central part of *R. capsulatus* PutA showed homology to proline dehydrogenase of *Saccharomyces cerevisiae* (Put1) and *Drosophila melanogaster* (SlgA). The C-terminal part of PutA exhibited homology to Put2 (pyrroline-5-carboxylate dehydrogenase) of *S. cerevisiae* and to aldehyde dehydrogenases from different organisms. Therefore, it seems likely that in *R. capsulatus*, as in enteric bacteria, both enzymatic steps for proline degradation are catalyzed by a single polypeptide (PutA). The deduced amino acid sequence of PutR (154 amino acid residues) showed homology to the small regulatory proteins Lrp, BkdR, and AsnC. The *putR* gene, which is divergently transcribed from *putA*, is essential for proline utilization and codes for an activator of *putA* expression. The expression of *putA* was induced by proline and was not affected by ammonia or other amino acids. In addition, *putA* expression was autoregulated by PutA itself. Mutations in *glnB*, *nifR1* (*ntrC*), and *nifR4* (*ntrA* encoding σ^{54}) had no influence on *put* gene expression. The open reading frame located downstream of *R. capsulatus putR* exhibited strong homology to the *E. coli selD* gene, which is involved in selenium metabolism. *R. capsulatus selD* mutants exhibited a Put$^+$ phenotype, demonstrating that *selD* is required neither for viability nor for proline utilization.

Many bacteria can use proline as a sole nitrogen source. In a general pathway known for eukaryotes (12, 49) and bacteria (28, 29), two enzymes catalyze proline degradation to glutamate: proline dehydrogenase and pyrroline-5-carboxylate (P5C) dehydrogenase.

The first step catalyzed by the proline dehydrogenase yields P5C and needs (at least in enteric bacteria) the reduction of a tightly associated flavin adenine dinucleotide cofactor (5, 38). The reoxidation of the cofactor requires the association of the enzyme with the electron transport chain, and therefore the enzyme is membrane associated (32, 38). In the second step, P5C dehydrogenase converts P5C to glutamate in an NAD^+-dependent reaction. Whereas in eukaryotes proline dehydrogenase and P5C dehydrogenase are encoded by two separate genes (*put1* and *put2* in *Saccharomyces cerevisiae* [22, 49]), in enteric bacteria both steps of proline degradation are catalyzed by a single polypeptide encoded by the *putA* gene (1, 24). The *putA* gene product is not only a bifunctional dehydrogenase but also acts as an autogenous repressor of *putA* and *putP*, a gene transcribed divergently from *putA* that encodes the major proline permease (8, 10, 36–38). In the absence of proline, PutA binds to several operator sites in the *putA-putP* intergenic region and abolishes the expression of both genes (38, 39). Besides this autoregulation by PutA, the *put* genes of enteric bacteria are catabolite repressed and nitrogen regulated (8). In *Klebsiella aerogenes* (25) and *Klebsiella pneumoniae* (46) the nitrogen-controlled expression of proline utilization genes is regulated by the Nac (nitrogen assimilation control) protein. The *nac* gene product couples the activation of σ^{70}-dependent promoters to the general nitrogen regulation system (Ntr) (for a review, see reference 4). The Ntr system of enteric bacteria includes the *glnB* gene product, NtrB-NtrC, a two-component regulatory system, and NtrA (σ^{54}), an alternative sigma factor of RNA polymerase (for a review, see reference 27).

The purple nonsulfur photosynthetic bacterium *Rhodobacter capsulatus* has in some respects a nitrogen regulation network similar to that of enteric bacteria (for a review, see reference 20). Genes encoding homologs to GlnB, NtrC, NtrB, and NtrA (σ^{54}) have been identified in *R. capsulatus* (9, 17, 21, 48). However, mutations in these genes affect only the regulation of nitrogen fixation and do not produce a typical Ntr phenotype. To obtain more information about the regulation of *R. capsulatus* genes involved in general nitrogen metabolism, we have identified and characterized genes required for proline utilization. The *put* genes of *R. capsulatus* were shown to form an autoregulatory, substrate-induced circuit, and no evidence for the involvement of a general nitrogen control system was obtained.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The *R. capsulatus* strains were grown in RCV medium as described previously (18). The screening of Tn*5* mutants for the ability to utilize different amino acids as sole nitrogen sources was carried out in RCV-N medium containing individual amino acids at a final concentration of 10 mM.

Construction of a size-fractionated *Pst*I gene bank. Total DNA of the *R. capsulatus* wild type was digested with *Pst*I, and restriction fragments were separated by gel electrophoresis. Fragments of the appropriate sizes were cut from the agarose gel and purified with GENECLEAN (Bio 101 Inc., La Jolla, Calif.). The size-fractionated *Pst*I fragments were ligated into pUC9.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Bacterial strains		
E. coli		
S17-1	RP4-2 (Tc::Mu) (Km::Tn*7*) integrated in the chromosome	44
JM83	Host for pUC9 and pSVB plasmids	47
R. capsulatus		
B10S	Spontaneous Sm^r mutant of *R. capsulatus* B10	18
n1	*nifR1*::Tn*5*	18
KS111AII	*nifR4*::Gm	K. Schüddekopf, Bielefeld
PHU330	*glnB*::Km	P. Hübner, Basel
BK16 AI/II	Gm interposon mutant, Put^+	This work
BK16 BI/II	*selD*::Gm	This work
BK16 CI	*selD*::Gm	This work
BK16 DII	Gm interposon mutant, Put^+	This work
BK30I/II	*putR*::Gm	This work
BK12	*putA*::Sp	This work
BK13	*putA*::Sp	This work
BK15I/II	*putA*::Gm interposon mutant	This work
BK12/30	*putA*::Sp *putR*::Gm	This work
Plasmids		
pACYC184	Tc, Cm	7
pSUP2021	pSUP202::Tn*5*	44
pUC9	Ap, Lac^+	47
pSVB30	Ap, Lac^+	3
pPHU235	Broad-host-range *lacZ* fusion vector	16
pBKM4	Chromosomal *Eco*RI fragment from mutant strain BKM4 harboring the Tn*5* insertion cloned into pACYC184	This work
pBKM5	Chromosomal *Eco*RI fragment from mutant strain BKM5 harboring the Tn*5* insertion cloned into pACYC184	This work
pBKP1	2,055-bp *Pst*I fragment cloned into pUC9	This work
pBKP20	1,449-bp *Bam*HI-*Hin*dIII fragment of *R. capsulatus* cloned into pPHU235 (*putA-lacZ*)	This work
pBKP22	972-bp *Bcl*I-*Hin*dIII fragment of *R. capsulatus* cloned into pPHU235 (*putA-lacZ*)	This work
pBKP23	569-bp *Bam*HI-*Hin*dIII fragment of *R. capsulatus* cloned into pPHU235	This work
pBKP24	651-bp *Pst*I-*Hin*dIII fragment of *R. capsulatus* cloned into pPHU235	This work
pBKP28	972-bp *Hin*dIII-*Bcl*I fragment of *R. capsulatus* cloned into pPHU235 (*putR-lacZ*)	This work

DNA sequence analysis. Sequencing was performed for both DNA strands by the chain termination method (43). Overlapping deletion fragments from the *Pst*I wild-type fragment were generated with the nested deletion kit (Pharmacia). Various defined restriction fragments from the recombinant plasmids pBKM4 and pBKM5 were cloned into appropriate restriction sites of pSVB30 or pUC9. Subsequently, various deletions were introduced to generate new sequencing start points. In one region lacking appropriate restriction sites, synthetic oligonucleotides were used for DNA sequence determination. All primers used for sequencing were labeled with fluorescein for use in the A.L.F. DNA sequencer (Pharmacia LKB) according to the manufacturer's instructions.

The Staden programs were used for editing and translating DNA sequences as well as for statistical and structural predictions (45). For homology searches in sequence databases, BLAST (basic local alignment search tool [2]) was used. Sequence alignments were performed with the aid of the Clustal V program (14).

Construction of *R. capsulatus* insertion and deletion mutants. For the construction of *R. capsulatus* interposon mutants, various wild-type fragments were cloned by standard methods (42) into mobilizable narrow-host-range vector plasmids. Suitable restriction sites were subsequently used to insert a gentamicin (15) or spectinomycin resistance cassette (41). The resulting hybrid plasmids were mobilized from *Escherichia coli* S17-1 into *R. capsulatus* B10S or mutant strains by filter mating (31). Mutants were selected by the interposon-encoded resistance, and double-crossover events were identified by the loss of the vector-encoded resistance.

Construction of in-frame *put-lacZ* fusions. To analyze the expression of *putA*, the *lacZ* gene of *E. coli* was fused in frame at the *Hin*dIII site within *putA*. For this purpose four different DNA fragments (Fig. 1B) were cloned into the mobilizable vector plasmid pPHU235, resulting in hybrid plasmids pBKP20, pBKP22, pBKP24, and pBKP23. A *putR-lacZ* in-frame fusion was constructed by ligating the 972-bp *Bcl*I-*Hin*dIII fragment into the *Hin*dIII site of pPHU235 with a *Bam*HI-*Hin*dIII linker, resulting in hybrid plasmid pBKP28.

Hybrid plasmids were mobilized from *E. coli* S17-1 into different *R. capsulatus* strains. Since the broad-host-range vector plasmid pPHU235 is able to replicate in *R. capsulatus*, the resulting tetracycline-resistant exconjugants harbored either the *putR-lacZ* fusion or the *putA-lacZ* fusion. The levels of β-galactosidase activity of these strains were determined by the sodium dodecyl sulfate-chloroform method (34) in late-exponential-phase *R. capsulatus* cultures grown anaerobically in RCV medium with different nitrogen sources.

Nucleotide sequence accession number. The complete nucleotide sequence of the *R. capsulatus put* gene region was determined and will appear in the EMBL nucleotide sequence database under the accession number X78346.

RESULTS

Identification and cloning of the *R. capsulatus put* gene region. To identify *R. capsulatus* genes required for proline utilization, a random transposon Tn*5* mutagenesis was carried out in the wild-type strain B10S as described previously (18). Tn*5*-induced *R. capsulatus* mutants were screened for loss of the ability to grow in RCV minimal medium with proline as the sole source of nitrogen. Among 10,000 mutants tested, four strains failed to grow with proline but retained the ability to grow with other amino acids (arginine, asparagine, aspartate, and serine), ammonia, and dinitrogen. These mutant strains were called BKM4, BKM5, BKM34, and BKM75. Southern analysis of chromosomal DNA of these mutants, digested with different restriction enzymes with Tn*5* as a probe, demonstrated that the Tn*5* insertions were located in two adjacent chromosomal *Eco*RI fragments (data not shown; Fig. 1A). The Tn*5*-containing *Eco*RI fragments of mutant strains BKM4 and BKM5 were cloned into pACYC184. The corresponding plasmids were designated pBKM4 and pBKM5, respectively. A 2-kb *Pst*I fragment overlapping the junction of the two *Eco*RI fragments was isolated from a size-fractionated *Pst*I gene bank of *R. capsulatus* wild-type DNA as described in Materials and

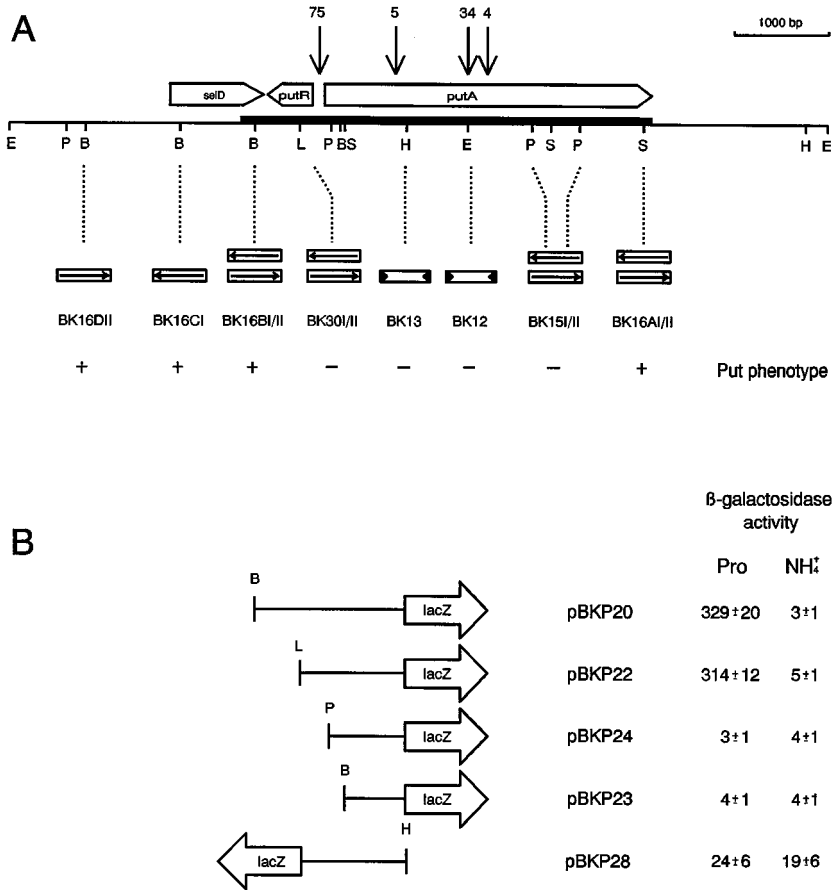

FIG. 1. Physical and genetic map of the *R. capsulatus put* gene region and analysis of in-frame *put*::*lacZ* fusions. (A) Vertical arrows indicate the locations of Tn*5* insertions resulting in a Put phenotype. The sequenced region is marked by a heavy line, and the localizations of ORFs are given by thick open arrows carrying their respective gene designations. Below the physical map the locations of interposon insertions are shown. The directions of transcription of the gentamicin resistance gene are symbolized by arrows in boxes, indicating polar and nonpolar insertion mutations. The spectinomycin cassettes used for the construction of mutant strains BK12 and BK13 are symbolized by open rectangles and the transcription and translation termination signals on both ends of the interposon are marked with arrowheads. The ability of the corresponding *R. capsulatus* mutant strains to grow with proline as the sole nitrogen source is indicated by + or −. (B) DNA fragments fused to the reporter gene *lacZ* are shown. The β-galactosidase activities of *R. capsulatus* strains carrying the corresponding hybrid plasmids were analyzed in cells grown in RCV medium with proline (Pro) or ammonia as the nitrogen source as indicated. Mean values and standard deviations were calculated from three independent measurements. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; L, *Bcl*I; P, *Pst*I; S, *Sal*I.

Methods with plasmids pBKM4 and pBKM5 as probes. The physical map of the *R. capsulatus put* gene region and the locations of the four *put*::Tn*5* mutations are shown in Fig. 1A.

Mutational analysis of the *R. capsulatus put* gene region. To rule out the possibility that the Put phenotype of the Tn*5*-containing mutants was not linked to the Tn*5* insertions but resulted from secondary mutations, cassettes encoding spectinomycin resistance were cloned into the *Hin*dIII and *Eco*RI sites of the *put* gene region (Fig. 1A). These mutations were introduced into wild-type *R. capsulatus* by homogenotization, and the resulting strains BK12 and BK13 were tested for proline utilization. The Put phenotype of these mutant strains demonstrated that the Tn*5* insertions in the *put* gene region were indeed responsible for the Put phenotype.

The four Tn*5* insertions in the mutant strains BKM4, BKM5, BKM34, and BKM75 were located in close proximity to each other in a 1.7-kb region of the *R. capsulatus* genome. To determine whether adjacent DNA fragments were also involved in proline utilization, defined insertion mutations were constructed. For this purpose an interposon encoding gentamicin resistance was inserted into different restriction sites as shown in Fig. 1A. The corresponding *R. capsulatus* mutant strains were tested for their ability to use proline as a nitrogen source. The Put$^+$ phenotypes of the mutants BK16B I/II and BK16A I/II delimited the *R. capsulatus put* gene region to about 4 kb.

Nucleotide sequence analysis of the *R. capsulatus put* gene region. Sequence analysis of the *R. capsulatus put* gene region revealed two open reading frames (ORFs) preceded by typical ribosome-binding sites, which are transcribed in opposite directions (Fig. 2). The larger ORF encoded a putative protein of 1,127 amino acid residues with a deduced molecular weight of 117,000. The corresponding gene was termed *R. capsulatus putA* since the predicted amino acid sequence showed 44% identity to the *putA* gene product of *Salmonella typhimurium* (1, 24).

As shown in Fig. 3A, the N-terminal part of PutA, encompassing 86 amino acid residues which are highly conserved among *S. typhimurium*, *E. coli*, and *K. pneumoniae* PutA proteins (8), is not present in *R. capsulatus* PutA. The central parts of *R. capsulatus* and *S. typhimurium* PutA proteins exhibited homology to the C-terminal part of *S. cerevisiae* Put1 and *Drosophila melanogaster* SlgA (12, 49), the proline dehydrogenases of these eukaryotes (Fig. 3A). The C-terminal parts of the bacterial *putA* gene products are homologous not only to

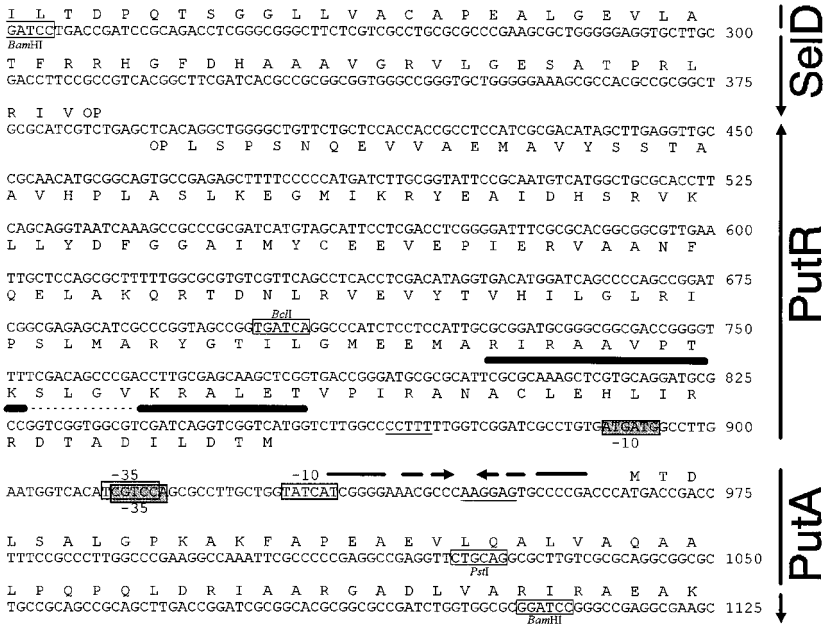

FIG. 2. Nucleotide sequence of the *R. capsulatus putR* gene region. The predicted amino acid sequences of *putR*, the 5′ end of *putA*, and the 3′ end of *selD* are in the single-letter code. Relevant restriction sites and putative −10 and −35 sequences of the *putR* and *putA* promoters are in boxes. To discriminate between the promoter elements of *putA* and *putR*, the −10 and −35 sequences of *putA* are shown in open boxes and those of *putR* are shown in shaded boxes. Putative Shine-Dalgarno sequences are underlined. A putative PutA-binding site is indicated by broken horizontal arrows. The helix-turn-helix motif within PutR is marked by heavy lines. Numbering corresponds to the complete DNA sequence of the *R. capsulatus put* gene region, which will appear in the EMBL nucleotide sequence database under the accession number X78346.

the P5C dehydrogenase (Put2) of *S. cerevisiae* (22) but also to a large number of aldehyde dehydrogenases from different species. This homology includes not only the conserved glycine motif ($GXGX_3GX_{13}$FTGST [Fig. 3A]) postulated to be involved in NAD binding but also the conserved Glu and Cys residues (Fig. 3A) of the putative active center (13).

The small ORF transcribed divergently from *putA* (Fig. 1) was designated *putR* (for *put* regulation), because *putR* is absolutely required for *putA* expression as shown below. On the basis of the predicted amino acid sequence, the *putR* gene encodes a polypeptide of 154 amino acid residues with a calculated molecular weight of 17,000. A comparison of *R. capsulatus* PutR with the leucine-responsive regulatory protein Lrp of *E. coli* (50), the branched-chain keto acid dehydrogenase regulator BkdR of *Pseudomonas putida* (26), and AsnC from *E. coli* (19) is shown in Fig. 3B. These regulatory proteins are characterized by a DNA-binding helix-turn-helix motif (11) in the N terminus, which is also highly conserved in *R. capsulatus* PutR.

Downstream of *putR* the 3′ end of another ORF transcribed in the opposite direction was identified. This putative gene is homologous to *E. coli selD*, which encodes a protein involved in selenocysteinyl-tRNA synthesis (23). Figure 3C shows the amino acid sequence alignment of the C-terminal parts of *R. capsulatus* and *E. coli* SelD. Preliminary sequence data indicated that SelD of *R. capsulatus* is approximately the same length as the *E. coli* protein, and the homology spans the whole sequence (data not shown). The Put^+ phenotype of the mutant strains BK16B and BK16C demonstrated that *selD* was required neither for the viability of *R. capsulatus* nor for proline utilization.

The DNA sequence data revealed that *putA* and *putR* are divergently transcribed. It is therefore likely that the promoters of these two genes are located within the intergenic region of 110 bp between the two start codons. As shown in Fig. 2, two overlapping elements with homology to the canonical −10 and −35 promoter were found in this region. Only 30 bp upstream of the *putA* start codon we found a well conserved −10 motif (TATcAT) which was preceded by a less conserved putative −35 motif (TcGtCc). Upstream of *putR* a putative −10 motif (cATcAT) and −35 motif (TgGACg) were detected. In addition, the intergenic region between *putR* and *putA* contains a palindromic sequence of 27 bp overlapping the putative −10 region of the *putA* promoter and the putative Shine-Dalgarno sequence of the *putA* gene (Fig. 2).

Expression of *put-lacZ* fusions in a wild-type background. As shown in Fig. 1B, different translational *put-lacZ* fusions were constructed as described in Materials and Methods. The *putA-lacZ* fusions pBKP20, pBKP22, pBKP23, and pBKP24 were fused at the *Hin*dIII site within the *putA* coding region. As expected from the sequence data, pBKP23 and pBKP24 showed no significant β-galactosidase activity, since these fusions lack the *putA* promoter. The fusions pBKP20 and pBKP22 exhibited no significant β-galactosidase activity in cells grown with NH_4^+ as the nitrogen source (Fig. 1B) nor did they show any significant expression in the presence of serine or N_2 as the nitrogen source (data not shown). However, the expression of these *putA-lacZ* fusions was markedly induced in the presence of proline (Fig. 1B). This induction by proline was only poorly impaired if ammonium and proline were added simultaneously to the growth medium (data not shown).

The *putR* gene was fused to *lacZ* at the *Bcl*I site within the *putR* coding region. Compared with *putA* expression, *putR* itself is expressed at a lower level (~15-fold) (Fig. 1). The expression of *putR-lacZ* was tested in media with proline, other amino acids, NH_4^+, and N_2 as nitrogen sources, but no significant differences in the expression rates could be observed.

Influence of *putA* and *putR* mutations on *put* gene expres-

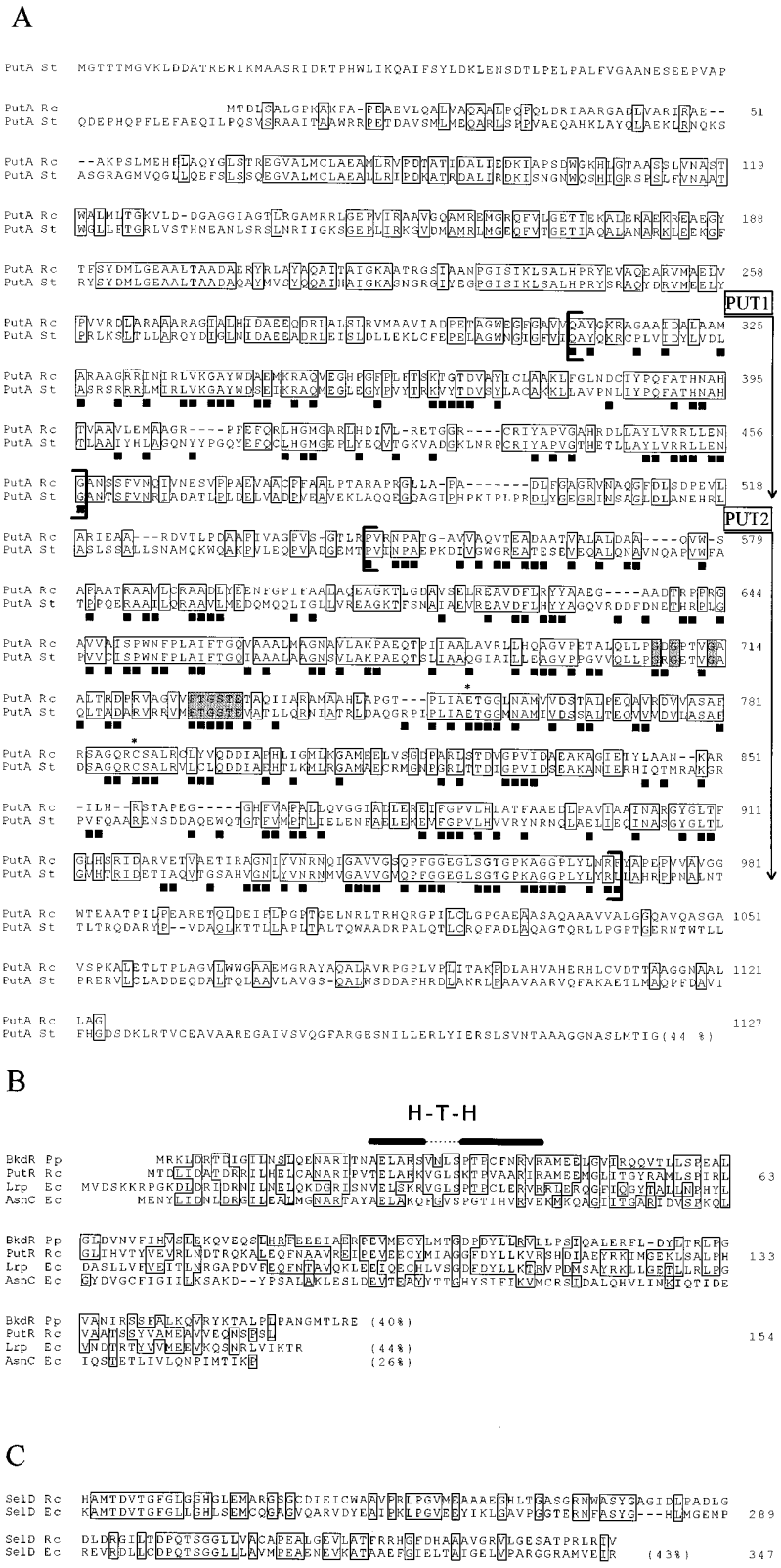

FIG. 3. Alignment of the deduced amino acid sequences of *R. capsulatus* PutA, PutR, and SelD with homologous proteins from other organisms. The sequences were aligned for maximum matching by using the Clustal V program. Identical amino acid residues are in boxes, and the percentages of identity to the *R. capsulatus* proteins are given at the end of each sequence. (A) Two regions in the PutA sequences of *R. capsulatus* and *S. typhimurium* marked by brackets are homologous to Put1 and Put2 from *S. cerevisiae*, respectively. Amino acid residues identical to those of *S. cerevisiae* and at least one of the PutA sequences are marked by black squares. The conserved and presumably functionally important E and C residues are marked with asterisks, and the conserved motif of a putative NAD-binding site is emphasized with shading. (B) Alignment of the predicted amino acid sequences of BkdR (*P. putida*), PutR (*R. capsulatus*), Lrp (*E. coli*), and AsnC (*E. coli*). A putative DNA-binding helix-turn-helix motif (H-T-H) is marked. (C) Comparison of the C-terminal parts of the deduced amino acid sequences of*selD* from *R. capsulatus* and *E. coli.*

TABLE 2. Expression of *putA-lacZ* and *putR-lacZ* in-frame fusions in the *R. capsulatus* wild-type strain and different mutant strains

Strain	Relevant characteristic	β-Galactosidase activity (Miller units)[a] in the expression of:			
		putA-lacZ[b] with:		*putR-lacZ* with:	
		Proline	NH_4^+	Proline	NH_4^+
B10S	Wild type	329 ± 20	3 ± 1	24 ± 6	19 ± 6
BK12	*putA* mutant	1,189 ± 34	207 ± 33	19 ± 4	13 ± 4
BK30	*putR* mutant	0 ± 0	2 ± 0	142 ± 9	87 ± 15
BK12/30	*putA putR* mutant	5 ± 2	6 ± 2	111 ± 2	88 ± 8
n1	*ntrC* mutant	260 ± 55	3 ± 1	13 ± 1	15 ± 3
KS111AII	*ntrA* mutant	376 ± 37	3 ± 1	19 ± 1	10 ± 1
PHU330	*glnB* mutant	359 ± 17	4 ± 3	11 ± 3	17 ± 2

[a] β-Galactosidase activity was determined by the method described by Miller (34). *R. capsulatus* cultures were grown photoheterotrophically in RCV medium containing either proline or NH_4^+ as the sole nitrogen source. The background level of the wild-type strain B10S containing the vector plasmid pPHU235 was about 1 ± 1. Mean values and standard deviations were calculated from three independent measurements.

[b] The *putA-lacZ* expression was determined with the reporter plasmid pBKP20. To analyze the expression of *putA* in a *putR* background, the plasmid pBKP22 was used, because pBKP20 encodes the complete *putR* gene and complements this mutant to the wild-type level. In the wild type the reporter plasmids pBKP20 and pBKP22 show the same expression pattern (data not shown).

sion. To study the influences of *putA* and *putR* on the expression of these genes, the corresponding *lacZ* fusions were analyzed in different mutant backgrounds (Table 2). In a *putA* mutant background, the level of *putA-lacZ* expression was markedly higher than that of the wild type (by ~3.6-fold), indicating negative autoregulation by PutA. Even in the absence of proline, a high level of expression could be detected in this mutant. However, the proline inducibility of *putA* expression was retained in the *putA* mutant background. In *putR* mutants, *putA* expression decreased to background levels. Even in a *putR putA* double mutant, no significant *putA* expression could be detected.

The analysis of a *putR-lacZ* fusion demonstrated that *putA* mutations did not affect *putR* expression. However, a *putR* mutation increased *putR* expression approximately four- to fivefold, indicating that *putR* is also negatively autoregulated (Table 2). In contrast to the expression of *putA*, which was induced by the presence of proline, no significant effect of proline was observed for *putR* expression.

To test whether the Ntr system of *R. capsulatus* affected *put* gene expression, the *putA*- and *putR-lacZ* fusions were introduced into strains carrying mutations in *ntrC* (*nifR1*), *ntrA* (*nifR4*), and *glnB*. The expression patterns of the *putA-lacZ* and *putR-lacZ* fusions revealed no differences between these mutants and the wild type.

DISCUSSION

In enteric bacteria, genes involved in proline utilization are organized in a region consisting of the two divergently transcribed genes *putA* and *putP* encoding proline dehydrogenase and the major proline permease, respectively (8). The *putA* gene product is a bifunctional dehydrogenase (proline dehydrogenase and P5C dehydrogenase) which catalyzes both enzymatic steps of proline degradation to glutamate (1, 24, 32, 33). In contrast to enteric bacteria, in eukaryotes like *S. cerevisiae* and *D. melanogaster*, the two enzymatic activities of proline dehydrogenase are encoded by separated genes (12, 22, 49). The analysis of proline dehydrogenase from the phototrophic purple bacterium *R. capsulatus*, a member of the α subdivision of proteobacteria, revealed a domain structure similar to that of enteric bacteria in which both steps of proline degradation are catalyzed by a single polypeptide. Despite the high degree of homology found between the structural gene of proline dehydrogenase from enteric bacteria and that of *R. capsulatus*, their genetic organizations differ significantly in these species. In contrast to enteric bacteria, the gene encoding proline permease of *R. capsulatus* is not located close to *putA*. Instead, a regulatory gene designated *putR*, which was shown to be absolutely required for the expression of proline dehydrogenase, is located immediately upstream of *putA* in *R. capsulatus*. The deduced amino acid sequence of *R. capsulatus* PutR exhibited strong similarities to the regulatory proteins Lrp and AsnC of *E. coli* and to BkdR of *P. putida*. Whereas Lrp is a general regulatory protein responsible for the leucine-dependent control of several dozen operons in *E. coli* (for a review, see reference 6), PutR of *R. capsulatus* seems to be responsible only for the substrate-dependent activation of genes involved in proline utilization. Like *bkdR* and *asnC*, *R. capsulatus putR* is divergently transcribed from the gene which is the target of PutR regulation. In addition, the regulatory proteins of the Lrp family, including *R. capsulatus* PutR, were shown to be negatively autoregulated. A conserved helix-turn-helix motif located in the N-terminal part of Lrp-like regulatory proteins was also identified in *R. capsulatus* PutR (Fig. 3B), indicating that PutR may act directly by DNA binding.

A model of *put* gene regulation in *R. capsulatus* based on studies of *putA*- and *putR-lacZ* fusions in wild-type and different mutant strains is presented in Fig. 4. The *putR* gene is constitutively transcribed at a low level, and the *putR* gene product negatively autoregulates its own transcription, since the level of expression of a *putR-lacZ* fusion in a *putR* mutant strain was more than four times greater than that in the wild type (Table 2). In the absence of proline, PutR activates the expression of *putA* to a low level. However, under these conditions the negative autoregulation of the proline dehydrogenase itself, demonstrated by a significant increase in levels of *putA-lacZ* expression in *putA* mutants, results in a decrease of *putA* expression to background levels. This negative autoregulation by PutA was also found in enteric bacteria (30), and it was shown that PutA binds to a DNA sequence of dyad symmetry in front of *putA* (10, 38, 39). An inverted-repeat structure overlapping the putative *putA* promoter was also found in *R. capsulatus* (Fig. 2), suggesting that a similar mechanism of negative regulation by PutA occurs in this bacterium. Therefore, the proline dehydrogenases of *R. capsulatus* and enteric bacteria are not only bifunctional dehydrogenases but might also be DNA-binding regulatory proteins. However, in the presence of proline, the expression of *putA* is enhanced more than a 100-fold but is still subjected to negative autoregulation by PutA. Since a *putA* mutant retains this proline inducibility, we assume that proline causes a conformational change in PutR, altering the affinity for its target promoter as was shown for the leucine response of Lrp (40).

In enteric bacteria, the expression of *put* genes is under the control of the general nitrogen regulatory system and therefore expression is repressed in the presence of ammonia (25, 27, 46). In contrast, the expression of *putA* in *R. capsulatus* is activated by its specific regulatory protein, PutR, only in the presence of proline and no influences of other nitrogen sources could be observed. This is in line with the absence of σ^{54} (NtrA)-dependent −24 and −12 promoters in the intergenic region between *putR* and *putA*. In addition, the regulation of *putA* expression was not influenced by mutations in *nifR1* (*ntrC*), *glnB*, or *nifR4* (*ntrA*) (Table 2).

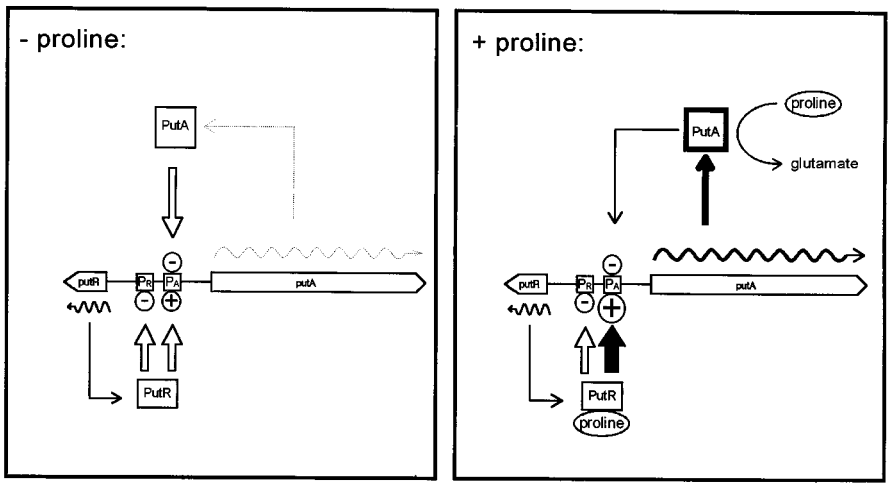

FIG. 4. Model of *put* gene regulation in *R. capsulatus*. Transcription is symbolized by wavy arrows. In the presence of proline, activation by PutR is stimulated (solid arrow), resulting in large amounts of proline dehydrogenase (thickly outlined box). Genes and the intergenic region are not drawn to scale. For details, see Discussion. P_R, promoter of *putR*; P_A, promoter of *putA*.

The analysis of *R. capsulatus putA* demonstrated that the expression of proline dehydrogenase is regulated in this phototrophic purple bacterium only by the presence of its substrate via a regulatory protein belonging to the class of Lrp-like activator proteins, and no indication of general nitrogen control could be observed. *R. capsulatus* arginase, another enzyme involved in amino acid utilization, was also shown to be only substrate regulated (35). Therefore, it is likely that the regulation of general nitrogen metabolism in *R. capsulatus* is not controlled by a hierarchic Ntr system as found in enteric bacteria and in most other diazotrophs but instead by parallel regulatory networks.

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