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The *Rhodobacter capsulatus glnB* Gene Is Regulated by NtrC at Tandem *rpoN*-Independent Promoters

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The protein encoded by *glnB* of *Rhodobacter capsulatus* is part of a nitrogen-sensing cascade which regulates the expression of nitrogen fixation genes (*nif*). The expression of *glnB* was studied by using *lacZ* fusions, primer extension analysis, and in vitro DNase I footprinting. Our results suggest that *glnB* is transcribed from two promoters, one of which requires the *R. capsulatus ntrC* gene but is *rpoN* independent. Another promoter upstream of *glnB* is repressed by NtrC; purified *R. capsulatus* NtrC binds to sites that overlap this distal promoter region.

Most free-living nitrogen-fixing organisms regulate the synthesis of nitrogenase, the enzyme which reduces atmospheric nitrogen to ammonia, in response to environmental oxygen and fixed nitrogen levels. In the photosynthetic bacterium *Rhodobacter capsulatus*, this signal transduction cascade is mediated by the products of at least five regulatory genes: *glnB*, *ntrB*, *ntrC*, *rpoN*, *nifA1*, and *nifA2* (for reviews, see references 19 and 20). In the absence of both fixed nitrogen and oxygen, transcription of the genes encoding the nitrogenase polypeptides (*nifHDK*) and other *nif* genes required for the synthesis of nitrogenase is activated by NifA1 or NifA2 (25) at promoters recognized by the alternative sigma factor RpoN (σ^{54}, σ^{N}, or NtrA). Induction of *nifA1* and *nifA2* occurs when fixed nitrogen is limited and requires *ntrC* (*nifR1*) but not *rpoN* (*nifR4*) (14, 29). *R. capsulatus glnB* is proposed to be a negative regulator of *nif* gene transcription, since *glnB* mutants express nitrogenase constitutively with respect to fixed nitrogen although oxygen repression is still present (22).

In *R. capsulatus*, *glnB* was proposed to be organized in a *glnBA* operon. Expression of a *glnBA-lacZ* fusion was reduced approximately 50% in an *ntrC* mutant (22), suggesting that the *glnB* gene could be regulated by the *R. capsulatus ntrC* product. NtrC (NR_I) is a member of a family of procaryotic enhancer-binding proteins which characteristically activate RpoN-dependent promoters (reviewed in references 23, 27, and 36). To extend our studies of NtrC activation in *R. capsulatus*, we have analyzed the *glnB* promoters in vivo by primer extension analysis and by in vitro DNase I footprinting with purified *R. capsulatus* NtrC (RcNtrC). Our results suggest that the *R. capsulatus glnB* gene is transcribed from tandem promoters, one repressed (*glnBp1*) and the other activated (*glnBp2*) by NtrC. Like the promoters for *nifA1* and *nifA2* in *R. capsulatus*, *rpoN* is not required for transcription of *glnBp2*. Moreover, *glnBp2* does not possess an RpoN-type promoter sequence. By comparing sequences protected from DNase I in the *nifA1*, *nifA2*, and *glnB* promoter regions, we have identified a potential recognition site for RcNtrC.

Expression of a *glnB-lacZ* fusion. An *R. capsulatus* strain which contains a Tn*5* element in *glnB* is a glutamine auxotroph (22). The polarity of this mutation indicated that the *glnBA* genes are cotranscribed, although a second possibility is that the transposon is located in a *glnA* promoter element within the *glnB* gene (as discussed below). To confirm that a *glnB* promoter(s) is regulated by *ntrC*, we constructed a *lacZ* fusion (pRKR5) that is dependent solely on *glnB* promoter function (see the Fig. 1 legend). β-Galactosidase assays were performed essentially as described previously (22), and the results are shown in Fig. 1. The expression of pRKR5 in the wild-type strain was reduced approximately 40% when cells were induced in media containing ammonia as a nitrogen source. In media lacking ammonia, the expression of pRKR5 in an *ntrC* strain (J61) was significantly lower (25%) than in the wild-type strain. These results support the idea that *glnB* is regulated by *ntrC*.

Identification of the 5′ ends of *glnB* mRNA by primer extension analysis. We decided to initially analyze the basis of the *ntrC* control at *glnB* by determining the transcription start site(s). RNA was isolated from *R. capsulatus* strains essentially as described previously (14). Strains were first cultured in the light in minimal medium plus 7.5 mM NH_4^+ and then induced anaerobically overnight in minimal medium containing either NH_4^+ (7.5 mM), glutamate (10 mM), or no fixed nitrogen. Glutamate is a poor nitrogen source for *R. capsulatus* and only slightly represses the expression of *nif* genes. In each experiment, the ^{32}P-end-labelled oligodeoxynucleotide CTTCCTT CACTTCATCGAGCTTGAACGGCT was annealed to RNA and extended with avian myeloblastosis virus reverse transcriptase (Life Sciences) as described previously (14). The results are shown in Fig. 2.

A single transcript, P2, was detected when the reaction contained template RNA from wild-type cells induced in medium with no fixed nitrogen (Fig. 2, lane 1). However, when the template RNA was from the same strain induced in medium containing NH_4^+, an additional minor transcript, P1, was observed (lane 2). The P2 product was also present in reactions of wild-type cells induced in glutamate minimal medium (lane 3). In contrast, reaction mixtures containing RNA from two different *R. capsulatus* $NtrC^-$ strains also induced in glutamate minimal medium, 102-C4 (lane 4) and KGR3BC (lane 5), showed only the second transcript, P1. The band corresponding to the P1 transcript was also the only transcript detected in the $NtrC^-$ strain J61 induced in both glutamate and NH_4^+ media (data not shown). These results indicate that two promoters are present upstream of the *R. capsulatus glnB* gene. Since no transcript corresponding to P2

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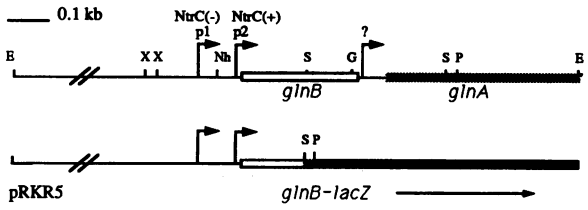

Strain	genotype	ß-galactosidase assays	
		-NH_4^+	+NH_4^+
SB1003 (pRKR5)	wt	2336	1521
J61 (pRKR5)	*ntrC*	593	692

FIG. 1. Map of the *glnBA* region and β-galactosidase expression of a *glnB-lacZ* fusion. A *glnB-lacZ* fusion was constructed by using the *R. capsulatus glnB* plasmid pRGK1218 (22) and the *lacZYA* translational fusion vector pSKS105 (6). These were restricted and ligated together such that the *glnB* open reading frame up to the *SalI* site was in frame with the *lacZ* coding region. (This fusion also contains 27 bp encoding 9 amino acid residues of the *glnA* open reading frame—from *SalI* to *PstI*.) The *glnB-lacZYA* fragment was then cloned into the mobilizable, broad-host-range vector pUCA6 to create pRKR5. The wild type (wt; SB1003 [38]) and an *ntrC* strain (J61 [35]) harboring pRKR5 were grown in RCV minimal medium (described in reference 3) containing ammonia as the nitrogen source and then induced anaerobically overnight in the light in RCV with (7.5 mM) and without ammonia essentially as described previously (20). Activity values shown are in nanomoles of *o*-nitrophenol per minute per milligram of protein and are the averages of four independent assays; the pattern of expression of one strain compared with another was the same in each case. The angled arrows indicate transcriptional start sites, including a possible site within the *glnB* gene (?). The letters refer to restriction enzyme sites as follows: E, *Eco*RI; X, *Xho*I; Nh, *Nhe*I; S, *Sal*I; G, *Bgl*II; and P, *Pst*I.

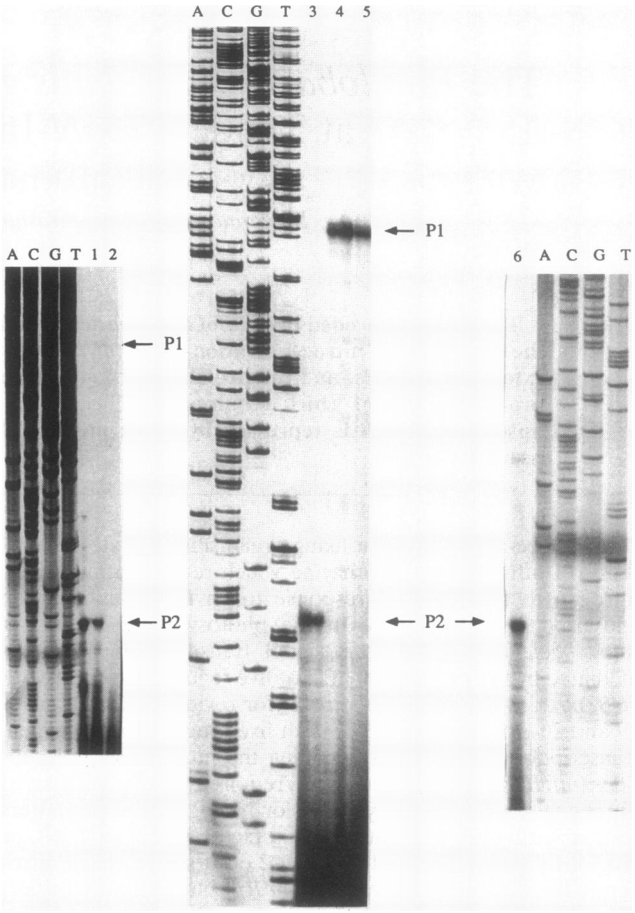

FIG. 2. Mapping the 5′ ends of *glnB* by primer extension analysis. Cells were initially incubated in the light in minimal medium plus 7.5 mM NH_4^+ and then induced anaerobically overnight in minimal medium without added fixed nitrogen (lane 1), with 7.5 mM NH_4^+ (lane 2), or with 10 mM glutamate (lanes 3 to 6). The extension products were separated on 6 or 7% sequencing gels next to dideoxy sequencing ladders (lanes A, C, G, and T) of the noncoding strand generated with the same oligodeoxynucleotide primer. Lane 1, approximately 20 μg of SB1003 (wild-type) RNA; lane 2, approximately 30 μg of SB1003 RNA; lane 3, 50 μg of SB1003 RNA; lane 4, 50 μg of 102-C4 (*ntrC* [3]) RNA; lane 5, 50 μg of KGR3BC (Δ*nifR3 ntrBC* [13]) RNA; lane 6, 55 μg of Δ40 (*rpoN* [9]) RNA. Two reverse transcriptase products were detected and are marked with arrows (P1 and P2).

was observed in any $NtrC^-$ background, the *glnBp2* promoter may be activated by NtrC. In order to test whether the NtrC-activated *glnBp2* is dependent on the RpoN sigma factor, primer extension analysis using RNA from an *R. capsulatus rpoN* deletion strain (lane 6) was performed. The P2 transcript is easily detected in this strain, Δ40, demonstrating that, like the *nifA1* and *nifA2* promoters, *glnBp2* requires NtrC but not RpoN for activation. Since the P1 transcript was observed only in the wild-type strain when cells were grown in high nitrogen conditions (+NH_4^+) or in an $NtrC^-$ background, *glnBp1* may be repressed by NtrC.

DNase I protection studies. On the basis of the in vivo primer extension results, we predicted that RcNtrC would bind upstream of *glnBp2*, possibly in a position to repress *glnBp1*. To test this prediction, end-labelled DNA fragments containing the *glnB* promoter region were incubated with several concentrations of purified RcNtrC and subjected to DNase I footprint analysis (15). RcNtrC concentrations of 40 to 80 nM protected several regions of the lower-strand probe from DNase I digestion, approximately from positions −127 to −76 (Fig. 3A, lanes 5C and 6C); Fig. 3B, lanes 5C and 6C, shows that similar concentrations of RcNtrC protected regions from positions −124 to −90 of the upper-strand probe. (Numbering is relative to the *glnBp2* start site.) Several sites with increased DNase I sensitivity are marked with arrows. Since the RcNtrC was purified from an *Escherichia coli* overexpression system, extracts from *E. coli* subjected to the same steps of purification were added as a control to lanes 8P. In a comparison of lanes 1 and 8P, the patterns of DNase I digestion were essentially the same with the control extract as with no added protein. With both probes, high concentrations of protein (~180 nM) protected additional regions of the promoter approximately from positions −180 to −132 (lower strand) and −155 to −125 (upper strand).

In enteric bacteria, NtrB phosphorylates NtrC at a conserved aspartate residue (see references 32 and 36 for examples). Although the phosphorylated form of NtrC is required to activate transcription, unphosphorylated NtrC is capable of specifically binding to the promoter (see references 2 and 28 for examples). The concentrations of RcNtrC (dimer) required to protect the *glnB* promoter in this study are within the range used previously to detect specific binding of the nonphosphorylated *E. coli* NtrC at the *glnA* promoters (28) or the *Klebsiella pneumoniae* NtrC at the *nifL* promoter (26). Phosphorylation of NtrC increases the cooperativity of activator bound at the promoter (see references 26 and 37 for examples). Since *R. capsulatus ntrB* is required for nitrogen fixation (13, 21) and the

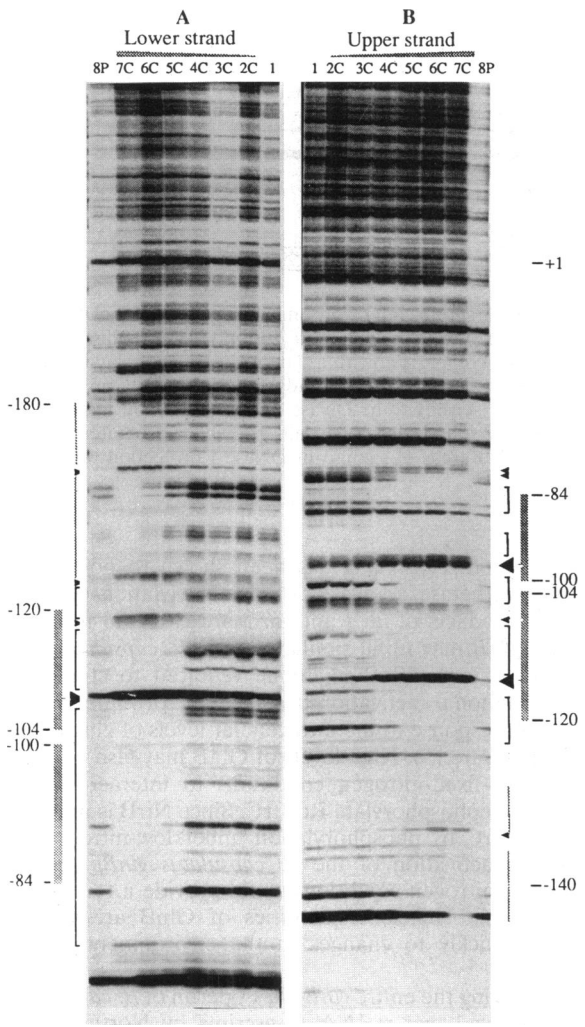

FIG. 3. DNase I footprinting of the *glnB* promoter region with a lower-strand probe (A) and an upper-strand probe (B). The 379-bp *Ava*II-*Nhe*I lower-strand probe (positions −418 to −39), and the 479-bp *Xho*I-*Bgl*II upper-strand probe (−185 to +294) were generated from pRGK1218 (22). (Numbers refer to the start of *glnBp2*.) ^{32}P-end-labelled probes were incubated with increasing concentrations of purified RcNtrC and subjected to limited DNase I digestion. NtrC concentrations were as follows: lanes 1, no added protein; lanes 2C, 5 nM; lanes 3C, 10 nM; lanes 4C, 20 nM; lanes 5C, 40 nM; and lanes 6C, 80 nM. Lanes 7C and 8P each contained 1 μg (approximately 180 nM) of a partially purified 0.5 M heparin-agarose fraction of RcNtrC (lane 7C) or an *E. coli* control extract purified in the same way (lane 8P). Regions of increased sensitivity to DNase I (large and small arrowheads), high-affinity regions of protection seen at 80 nM RcNtrC (brackets), and weaker areas of protection seen at approximately 180 nM (wavy lines) are marked; numbers refer to the distance (in base pairs) from the *glnBp2* transcription start site; and potential RcNtrC binding sites as described in the text are indicated by broad stippled bars.

site of RcNtrC phosphorylation is conserved (18), RcNtrC is also likely to be phosphorylated in vivo under conditions of limiting nitrogen.

The locations of the predicted start sites for *glnBp1* and *glnBp2* and the region of DNase I protection are depicted in Fig. 4. The solid brackets in Fig. 4 mark sites protected from DNase I cleavage at 40 to 80 nM RcNtrC. Stippled brackets mark sequences which were protected from DNase I cleavage only at RcNtrC concentrations of at least 180 nM. The sequence immediately preceding the transcription start for *glnBp1* is AT rich and contains several motifs homologous to the σ^{70} −10 and −35 promoter regions although the spacing with respect to the start site is not conserved. Two potential high-affinity RcNtrC binding sites (Fig. 4, solid bars; see the description below) are located within the protected region at positions −31 to +6 relative to the start of *glnBp1* and could act as a repressor of this promoter, possibly by excluding RNA polymerase (8).

As predicted by *glnB* primer extension in an RpoN$^-$ background, no sequence with significant similarity to the −12 and −24 RpoN (σ^{54}) promoter consensus (4, 23) is present upstream of the *glnBp2* transcription start (Fig. 4; as discussed below). RcNtrC dimers bound upstream at sites centered at positions −112 and −92 relative to the start of *glnBp2* could also activate transcription of *glnBp2*, consistent with the observation that cDNA products corresponding to transcriptions at *glnBp2* (P2) were completely absent in NtrC$^-$ strains. Thus, the location of DNase I protection at *glnB* supports the primer extension data that RcNtrC could act as both a repressor and an activator of transcription. It is also important to note that the location of the RcNtrC binding sites relative to *glnBp2* are distal to the promoter (>90 bp) as is characteristic for the NtrC class of enhancer-binding proteins. This simultaneous repression and activation by NtrC is reminiscent of the *glnAp1* and *glnAp2* promoters in enteric bacteria (for one example, see reference 12).

Definition of a potential RcNtrC recognition site. The promoter regions of *nifA1*, *nifA2*, and *glnB* protected from DNase I digestion by RcNtrC contain palindromic sequences (shown in Fig. 5 by arrows) and were aligned at the centers of symmetry. In five of six cases, the base at position 4 of the boxed region is hypersensitive to DNase I attack when RcNtrC contacts the DNA, indicating torsional stress. In almost every case, the corresponding base on the opposite strand (position 14) is also hypersensitive. When the six sequences are compared, the left half site CGCC is highly conserved. The C at position 15 is invariant, although the right half site GC is conserved in only four of six cases. Particularly striking are the AT-rich sequences which are common for procaryotic regulatory binding sites but uncommon in the 65% G+C genome of *R. capsulatus*. The extended consensus recognition site for the enteric NtrC is shown below the *R. capsulatus* sequence in Fig. 5. Three bases in each half site are conserved with the RcNtrC proposed binding site along with the AT-rich core. When compared with the enteric NtrC, the predicted amino acid sequence of RcNtrC (18) has three substitutions in the proposed solvent-exposed face of the recognition helix of the DNA binding motif, the helix-turn-helix motif. It is possible that these changes account for differences in the proposed RcNtrC recognition site.

Regulation of *glnBA*. Expression of the *glnB-glnA* operons in *Bradyrhizobium japonicum*, *Rhizobium leguminosarum* biovar viciae, and *Azospirillum brasilense* has been investigated with respect to levels of fixed nitrogen (7, 10, 11, 24). In both *B. japonicum* and *A. brasilense*, two oppositely regulated promoters are located upstream of *glnB*. The most distal (*glnBp1*) is proposed to be a σ^{70} type which is repressed under conditions of low nitrogen. Downstream of *glnBp1* is an RpoN-type promoter (*glnBp2*) which is induced under low-nitrogen conditions. In *B. japonicum*, the *glnB* promoters are regulated differentially by NtrC, which is proposed to act as a repressor of *glnBp1* and as an activator of *glnBp2* under low-nitrogen conditions (24). In *A. brasilense*, the activator-repressor of

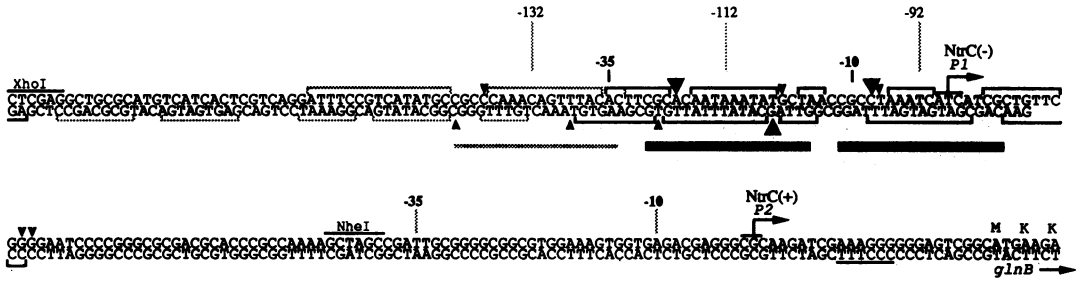

FIG. 4. Nucleotide sequence of the *glnB* promoter region (20). Potential start sites for *glnB* as inferred from the 5′ ends of the cDNA products in Fig. 2 are represented by the angled arrows. Numbers over solid vertical lines (*glnBp1*) or wavy vertical lines (*glnBp2*) mark the distance (in base pairs) from the transcription start sites. Areas of DNase I protection were localized by comparison with a dideoxy sequencing ladder generated with the synthetic oligodeoxynucleotide TCGAGGCTGCGCATGTCATC and with labelled DNA size standards. High-affinity or lower-affinity binding is indicated by solid or stippled brackets, respectively. Regions of increased sensitivity to DNase I are marked by large and small arrowheads. Potential RcNtrC binding sites (as described in the text) are denoted by solid bars. A partially protected promoter region with sequence homology to the high-affinity regions is marked with a broad stippled bar. No areas of significant DNase I protection were observed downstream of position −76 (relative to the *glnBp2* promoter).

transcription is unknown, although NtrC is probably not involved (11).

In contrast to both *B. japonicum* and *A. brasilense* genes, the *glnB* gene of *R. leguminosarum* is transcribed from a single RpoN-type promoter and the transcriptional activator is unknown (7). Our data suggest that *R. capsulatus glnB* expression is most similar to the NtrC-regulated *glnBp1* and *glnBp2* promoters of *B. japonicum*. One important difference is that in *R. capsulatus glnBp2* does not have a consensus RpoN promoter sequence (4, 23), which was confirmed by the expression of P2 from the *glnBp2* promoter in an RpoN$^-$ background (discussed below).

The physiological significance of NtrC regulation at the *R. capsulatus glnB* gene is unclear. Two different promoters may be required to ensure significant basal levels of GlnB (P_{II}) yet retain some nitrogen control. In enteric bacteria, GlnB plays a dual role in nitrogen regulation: (i) to elicit the regulated phosphatase activity of NtrB, which in turn controls the levels of phosphoryled NtrC, and (ii) to modify the activity of glutamine synthetase in conjunction with adenylyl transferase

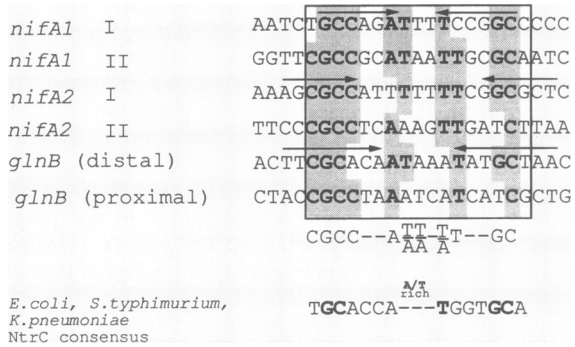

FIG. 5. Proposed recognition site for RcNtrC. DNase I-protected regions from the promoters of *nifA1* and *nifA2* (13a) and *glnB* were aligned by the presence of a large hypersensitive site at position 4 of the boxed area and by the centers of symmetry. Homologous sequences are shaded; arrows mark palindromic sequences. Nucleotides depicted below the box are present in five of six cases except for the G at position 15. The extended consensus sequence for the enteric NtrC recognition site (1, 12, 31) is shown; nucleotides conserved within the RcNtrC sites are in boldface type. *S. typhimurium*, *Salmonella typhimurium*.

(34). Since *R. capsulatus* has been shown to possess a glutamine synthetase adenylylation-deadenylylation system (17) and the products of *ntrB* and *ntrC* are essential for nitrogen fixation, *glnB* may fulfill similar roles in *R. capsulatus* (13, 20, 21). By analogy, GlnB might be required to stimulate the posttranslational activation-deactivation of glutamine synthetase in response to the intracellular levels of glutamine and 2-ketoglutarate (5). High levels of GlnB may also be required under high-fixed-nitrogen conditions to interact with NtrB (NR_{II}) to dephosphorylate RcNtrC. Since NtrB is predicted to activate NtrC by phosphorylation under low-nitrogen conditions, the activation of the *R. capsulatus glnBp2* promoter under low-nitrogen conditions could provide a feedback loop ensuring that sufficient quantities of GlnB are present to respond quickly to changes in the environmental nitrogen status.

Considering the entire *glnB-glnA* operon of *R. capsulatus*, we have observed two mRNA transcripts by Northern (RNA) analysis, one containing *glnA* and one containing *glnBA* (our unpublished data). This would suggest that either processing of a *glnBA* transcript occurs or there is an independent start site upstream of *glnA*. Since a *glnA-lacZ* fusion with an upstream junction in the *Bgl*II site (in *glnB*; see Fig. 1) shows significant levels of expression (approximately 1,000 U), we prefer the second possibility. Taken together, the data indicate that *glnBA* may form a superoperon with multiple promoters: two upstream of *glnB* and another upstream of *glnA* in *glnB*. In both *A. brasilense* (10) and *R. leguminosarum* (7), *glnA* is transcribed from an independent promoter.

Concluding remarks. The present study adds another promoter (*glnBp2*) to a short list of promoters (*nifA1* and *nifA2*) that require RcNtrC for activation. Each of these is RpoN independent, and none exhibits significant homology with the consensus position −12 and −24 promoters recognized by RpoN. At position −22 to −9, the *glnBp2* promoter contains the sequence GG-N_{10}-GA. Although this deviation from the RpoN consensus is present in the *Rhizobium meliloti nifN* (1), *Rhizobium phaseoli nifH* (30), and *Rhizobium trifolii nifH* (33) promoters, these three also contain adjacent nucleotides that are highly conserved within the RpoN class of promoters. *R. capsulatus glnBp2* does not contain these extended homologies, and the GG-N_{10}-GA (or GG-N_{10}-GC) pattern can be found very frequently in the high-GC (65%) genome of *R. capsulatus*. Accordingly, it seems unlikely that *glnBp2* represents a recognition site for RpoN. It has recently come to our attention that

the −10 and −35 sequences of *nifA1* could be considered similar to the −10 and −35 consensus of *E. coli* (16). However, the −10 regions of *nifA2* and *glnBp2* do not contain more than 2
of 6 bases of the −10 consensus TAtaaT (20). Until the housekeeping promoters in photosynthetic bacteria are clearly defined, it is premature to speculate on the RNA polymerase holoenzyme(s) used at these promoters. Results with the RcNtrC in this photosynthetic bacterium indicate that another level of versatility, that is, the activation of different RNA polymerase holoenzymes, can probably be added to this large class of bacterial enhancer-binding proteins. RcNtrC is unique, since NtrC and other enhancer-binding proteins characteristically activate the RpoN-RNA polymerase holoenzyme.

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