Expression of \textit{glnB} and a \textit{glnB}-Like Gene (\textit{glnK}) in a Ribulose Bisphosphate Carboxylase/Oxygenase-Deficient Mutant of \textit{Rhodobacter sphaeroides}

YILEI QIAN$^1$ AND F. ROBERT TABITA$^{1,2,*}$

\textbf{The Biochemistry Program$^1$ and The Department of Microbiology and Plant Molecular Biology/Biotechnology Program,$^2$ The Ohio State University, Columbus, Ohio 43210-1292}

Received 2 February 1998/Accepted 18 June 1998

In a ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO)-deficient mutant of \textit{Rhodobacter sphaeroides}, strain 16PHC, nitrogenase activity was derepressed in the presence of ammonia under photoheterotrophic growth conditions. Previous studies also showed that reintroduction of a functional RubisCO and Calvin-Benson-Bassham (CBB) pathway suppressed the deregulation of nitrogenase synthesis in this strain. In this study, the derepression of nitrogenase synthesis in the presence of ammonia in strain 16PHC was further explored by using a \textit{glnB}:\textit{dacZ} fusion, since the product of the \textit{glnB} gene is known to have a negative effect on ammonia-regulated \textit{nif} control. It was found that \textit{glnB} expression was repressed in strain 16PHC under photoheterotrophic growth conditions with either ammonia or glutamate as the nitrogen source; glutamine synthetase (GS) levels were also affected in this strain. However, when cells regained a functional CBB pathway by \textit{trans} complementation of the deleted genes, wild-type levels of GS and \textit{glnB} expression were restored. Furthermore, a \textit{glnB}-like gene, \textit{glnK}, was isolated from this organism, and its expression was found to be under tight nitrogen control in the wild type. Surprisingly, \textit{glnK} expression was found to be derepressed in strain 16PHC under photoheterotrophic conditions in the presence of ammonia.

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Strain 16 of the nonsulfur purple photosynthetic bacterium \textit{Rhodobacter sphaeroides}, a ribulose bisphosphate carboxylase/oxygenase (RubisCO)-deficient mutant, is devoid of a functional Calvin-Benson-Bassham (CBB) reductive pentose pathway (1). Under photoheterotrophic growth conditions, the CBB pathway is normally employed to balance the redox potential of the cell, with CO$_2$ serving as the major electron sink (25, 35). Thus, an alternative electron acceptor, such as dimethyl sulfoxide (DMSO), is required for phototropherotrophic growth of strain 16 in order to dispose of excess reducing equivalents generated from carbon (malate in this case) photomethabolism (25, 35). However, strain 16PHC, a spontaneous mutant derived from strain 16, exhibits the ability to grow under phototrophic conditions without the addition of DMSO. It was recently established (28) that the \textit{nifH}-encoded nitrogenase, and probably not alternative nitrogenases, is required for optimal phototrophic growth of strain 16PHC. Moreover, \textit{nifH} transcription was only partially derepressed in strain 16PHC in the presence of ammonia, with deregulation suppressed when cells regained a functional CBB pathway after \textit{trans} complementation of the missing \textit{cbb} genes. These results suggest that the mutation in strain 16PHC is of a regulatory nature and has a pleiotropic effect on cellular metabolism. It is thus proposed that there is a molecular link between the \textit{cbb} and \textit{nif} systems and that the mutation in strain 16PHC might affect the nitrogen-regulatory cascade (28).

The \textit{P}$_{II}$ protein, the product of the \textit{glnB} gene, plays a central role in the signal transduction cascade of nitrogen-regulatory systems in prokaryotes (9, 15, 17, 20). In \textit{Escherichia coli}, signals influencing the cellular N status are transmitted through the \textit{P}$_{II}$-adenyltransferase-glutamine synthetase pathway to rapidly influence glutamine synthetase (GS) enzyme activity by adenylylation or deaminylation of the protein (9). In addition, the \textit{P}$_{II}$-NtrBC cascade regulates the synthesis of GS (reference 34 and references therein). In some diazotrophic organisms, signal transduction through \textit{P}$_{II}$ could also be demonstrated at the level of \textit{nif} transcription. However, in \textit{Azospirillum brasilense}, a \textit{glnB} null mutation caused a Nif$^-$ phenotype (7, 22), while in \textit{Rhizobium leguminosarum}, a \textit{glnB} mutation did not seem to have any effect on \textit{nif} expression yet the \textit{nrt} system was influenced (1). In another purple nonsulfur photosynthetic bacterium, \textit{Rhodospirillum rubrum}, derepression of nitrogenase synthesis was observed, even though high concentrations of extracellular ammonia were present (19, 28). Therefore, it is believed that the reduction of protons by the nitrogenase enzyme complex under phototrophic conditions in the presence of ammonia serves as an alternative redox-balancing mechanism in the CBB pathway under phototrophic growth conditions. This strain produces copious hydrogen gas when grown under these conditions. In this and similar CBB pathway-defective mutants derived from \textit{Rhodobacter capsulatus} (26) and \textit{Rhodospirillum rubrum} (19), derepression of nitrogenase synthesis was observed, even though high concentrations of extracellular ammonia were present (19, 28). Therefore, it is believed that the reduction of protons by the nitrogenase enzyme complex under phototrophic conditions in the presence of ammonia serves as an alternative redox-balancing mechanism in strain 16PHC. The acquisition of the capacity for hydrogen evolution presumably renders this strain capable of phototropherotrophic growth in the absence of a functional CBB pathway and precludes the need for exogenous electron acceptors, such as DMSO. It was recently established (28) that the \textit{nif}-encoded nitrogenase, and probably not alternative nitrogenases, is required for optimal phototrophic growth of strain 16PHC. Moreover, \textit{nifH} transcription was only partially derepressed in strain 16PHC in the presence of ammonia, with deregulation suppressed when cells regained a functional CBB pathway after \textit{trans} complementation of the missing \textit{cbb} genes. These results suggest that the mutation in strain 16PHC is of a regulatory nature and has a pleiotropic effect on cellular metabolism. It is thus proposed that there is a molecular link between the \textit{cbb} and \textit{nif} systems and that the mutation in strain 16PHC might affect the nitrogen-regulatory cascade (28).

* Corresponding author. Mailing address: Department of Microbiology, The Ohio State University, 484 West 12th Ave., Columbus, OH 43210-1292. Phone: (614) 292-4297. Fax: (614) 292-6337. E-mail: Tabita.1@osu.edu.

Previous studies of the \textit{R. sphaeroides} \textit{glnBA} operon led to
the proposal that there is only one glnB gene (39) in the R. sphaeroides genome. It was apparent that the CBB pathway to be complete in strain 16PHC (14). In the wild-type strain, HR, a high level of glnB::lacZ expression was obtained when ammonia was used as the nitrogen source (Fig. 2), but a further threefold increase was observed under glutamate growth conditions. Thus, glnB expression appears to be somewhat constitutive in this organism, although there is still a modicum of nitrogen control, which is similar to results reported for R. capsulatus (13) and Rhodospirillum rubrum (18). However, there is no direct evidence as to whether glnB expression is controlled by the nitrogen status of the cell even if the expression of glnB is essential for normal cell division in R. sphaeroides. Because of the key role played by the PII protein in nitrogen regulation (9, 15, 17, 20), an R. sphaeroides glnB::lacZ transcriptional fusion was constructed to facilitate the analysis of glnB expression in R. sphaeroides strains grown with different nitrogen sources. It was apparent that glnB regulation in the wild type differed from that in the RubisCO-deficient strain 16PHC. In addition, a glnB homolog, glnK, was isolated from R. sphaeroides and was also shown to be differentially controlled in the wild type and strain 16PHC.

MATERIALS AND METHODS

R. sphaeroides strains and growth conditions. The R. sphaeroides strains and plasmids used in this study are listed in Table 1. Phototrophotrophic growth, with either 30 mM ammonia or 5 mM glutamate as the nitrogen source, was described previously (28).

Cloning of glnB and construction of the glnB::lacZ transcriptional fusion. Primers glnBF (5’-GAGGGCATCATTGCGGTTC-3’) and glnBR (5’-GGCTCCAGGCAGGATGCG-3’) were designed according to the previously published 5’ and 3’ nucleotide sequences of the glnB coding region of R. sphaeroides 2R (39). Subsequently, an approximately 340-bp glnB-specific PCR product was synthesized by these two primers was used as a homologous probe to screen a genomic library of strain HR. The positively hybridizing library clone pVKD8 was isolated, and a 4.0-kb EcoRI fragment, containing the glnB region, was subcloned into pUC19 to generate pUC840E (Fig. 1A). The 0.88-kb BamHI-BglII fragment containing the glnB upstream region and part of the glnB coding region was cloned into the low-copy-number IncQ vector pHRP309 to construct the glnB::lacZ transcriptional fusion plasmid pHRPglnB (Fig. 1B), which is compatible with IncP plasmid pGJ106.

Cloning of glnK and construction of glnK::lacZ transcriptional fusions from strains HR and 16PHC. When a genomic library of strain 16PHC (29) was examined with the same glnB probe, a library clone, pRK3D11, was found to hybridize to a moderate extent; this was subsequently isolated and shown to contain the glnK gene (Fig. 1B). A 2.2-kb EcoRI-BglII fragment was subcloned into pUC19 to generate pUCEB2.2 (Fig. 1B), and the 1.1-kb HindIII-BglII fragment containing the glnK upstream region and part of the glnK coding region was cloned into pHRP309 to construct glnK::lacZ transcriptional fusion plasmid pHRPglnK(PHC) (Fig. 1B). The same 1.1-kb HindIII-BglII fragment was also isolated from strain HR via PCR procedures, and a glnK::lacZ fusion plasmid, pHRPglnK(HR), was similarly constructed from this strain.

Enzyme assays. GS levels were determined by the γ-glutamyltransferase assay at pH 7.5 and 30°C with Mn2+ as the divalent cation, as previously described (16, 31) except that the reaction time was 15 min. This time was shown to be well within the linear time response for these assays. β-Galactosidase activities were measured as previously described (28).

Nucleotide sequence accession number. The sequences for the glnB and glnK regions from R. sphaeroides HR and 16PHC, respectively, have been submitted to the GenBank-EMBL data bank under accession no. AF032116 and AF023909, respectively.

RESULTS

glnB4 operon from R. sphaeroides HR. Nucleotide sequence analysis of the promoter and coding region of the glnB gene from R. sphaeroides HR showed 98.8% identity to the R. sphaeroides glnB sequence previously determined for a different strain, 2R (39) (accession no. X71659). When the deduced amino acid sequences were compared, 82.3% identity between these two sequences was observed, with differences at only two residues: the amino acids at positions 50 and 81 in strain HR are glutamate and alanine, respectively, while in strain 2R they are alanine and serine, respectively. These differences are each due to a single base change. The Glu-50 and Ala-81 residues of the strain HR GlnB protein are conserved among other PII-proteins and products of glnB-like genes, such as glnK of E. coli (33) and glnZ of A. brasilense (6). A putative NtrC-binding site, 5′-TGCAACAAAAATCGGCCGGC-3′, located at nucleotides 443 to 459 in the glnB (strain HR) sequence, was also found at the same position upstream of the glnB coding region of strain 2R, with only one base pair difference (5′-TGCAACAAAAATCGGGCGC-3′) (39).

Expression of the glnB::lacZ fusion in R. sphaeroides. The glnB::lacZ fusion plasmid pHRPglnB was introduced into strains HR, 16PHC, and 16PHC(pGJ106). Plasmid pGJ106 contains an intact cbbM gene (encoding form II RubisCO), which allows the CBB pathway to be complete in strain 16PHC (14). In the wild-type strain, HR, a high level of glnB::lacZ expression was obtained when ammonia was used as the nitrogen source (Fig. 2), but a further threefold increase was observed under glutamate growth conditions. Thus, glnB transcription appears to be somewhat constitutive in this organism, although there is still a modicum of nitrogen control, which is similar to results reported for R. capsulatus (13) and Rhodospirillum rubrum (18); however, another glnB::lacZ fusion study indicated that glnB expression is constitutive in R. capsulatus (4). In strain 16PHC, glnB expression was extremely low when either ammonia or glutamate was used as the nitrogen source (Fig. 2), but a further threefold increase was observed under glutamate growth conditions. Thus, glnB expression appears to be somewhat constitutive in this organism, although there is still a modicum of nitrogen control even at these low levels of activity. Interestingly, for strain 16PHC(pGJ106), which regained a functional CBB pathway, glnB::lacZ expression was restored to wild-type levels (or slightly higher) under both ammonia and glutamate growth conditions. These results indicate that glnB expression was repressed (or not activated) in strain 16PHC and that the presence of a functional CBB pathway suppresses this effect and restores glnB expression to the level observed in the wild type.
Levels of GS in *R. sphaeroides*. Since there is evidence indicating that *glnA* is most likely cotranscribed with *glnB* in *R. sphaeroides* (39), GS levels were examined in the different *R. sphaeroides* strains. In the wild-type strain, HR, the levels of GS were lower in the presence of ammonia, with a fivefold increase observed when glutamate was used as the nitrogen source (Fig. 3). These results parallel the *glnB* expression pattern observed for the wild-type strain. However, for strain 16PHC, although the levels of GS were comparable to that for strain HR in the presence of ammonia, GS activity did not increase further under glutamate growth conditions. When plasmid pJG106 was introduced into strain 16PHC, high levels of GS activity were obtained when glutamate was used as the nitrogen source for growth. Since the genes *glnB* and *glnA* (encoding GS) are cotranscribed in *R. sphaeroides* (39) and *glnB* is not expressed in the absence of a functional CBB pathway (Fig. 2), it is possible that *glnA* expression was affected under N-limiting conditions in strain 16PHC. Lower derepression levels of GS activity in glutamate-grown cells were also observed in CBB pathway-deficient strains of *R. capsulatus* and *Rhodospirillum rubrum* than in their wild-type CBB pathway.
positive parent strains (data not shown). Immunoblot analysis with antisera raised against *Rhodospirillum rubrum* P_{II} protein (17) and GS did not yield good cross-reactivity with *R. sphaeroides* crude cell extracts.

Sequence of the *glnK* gene from *R. sphaeroides*. During the cloning of the *glnB*/*A* cluster from *R. sphaeroides*, a *glnB*-like gene, *glnK*, was isolated from a genomic library of strain 16PHC. The gene showed similarity to the deduced sequences of *glnB* and *glnB*-like genes from various organisms. The deduced amino acid sequence of *R. sphaeroides* GlnK showed 64% identity with GlnZ from *A. brasilense* (6); 62% identity with GlnB from *Rhodospirillum rubrum* (17) and GS did not yield good cross-reactivity with *R. sphaeroides* crude cell extracts.

Expression of *glnK*::*lacZ* fusions in *R. sphaeroides*. *glnK*::*lacZ* transcription fusions were constructed from DNA fragments isolated from strains HR and 16PHC, and the expression levels were examined. In strain HR, a very low level of *glnK* expression was observed in the presence of ammonia, and it was highly derepressed when glutamate was used as the nitrogen source under nitrogen-limiting growth conditions (Fig. 4A). These results indicated that *glnK* expression is under tight nitrogen control in wild-type *R. sphaeroides*, much like *nifH* expression (28). In strain 16PHC, this tight nitrogen control was abolished and there was a significant level of *glnK* derepression in the presence of ammonia. Although this depression was significant, the level of *glnK* expression did not reach the levels attained in this strain under glutamate growth conditions. Strain 16PHC (pJG106), which possesses a functional CBB pathway, suppressed the derepression of *glnK* expression that was observed in strain 16PHC in the presence of ammonia. Moreover, with glutamate as the nitrogen source, high levels of *glnK* expression were achieved, even somewhat higher than in the wild type. The fusion plasmid pHRPglnK(PHC), which was constructed from sequences derived from strain 16PHC, yielded approximately the same pattern of gene expression (Fig. 4B), the only difference being a lower level of derepression in strain 16PHC in the presence of ammonia than that with plasmid pHR-PglnK(HR). Nevertheless, it is unlikely that the derepression of *glnK* was caused by a cis mutation in the *glnK* promoter region in strain 16PHC, since *glnK*::*lacZ* fusions from both strains yielded similar results. These findings were quite similar to the pattern of *nifH* expression in *R. sphaeroides* strains (28).
DISCUSSION

Previous studies demonstrated that nitrogenase derepression occurs when the RubisCO-deficient strain 16PHC is grown in the presence of ammonia, with nitrogenase presumably serving to substitute for the CBB pathway as the means of achieving redox balancing under photoheterotrophic growth conditions (19). The NifA phenotype of strain 16PHC is unique in that nitrogenase synthesis is only partially derepressed; however, derepression is reversible and suppressed when a functional CBB pathway is introduced by the addition of a RubisCO gene in trans. This suggests that the mutation that caused nif derepression is somehow linked to the nif regulatory system of *R. sphaeroides*, yet the basis of the NifA phenotype is undoubtedly different from those of other, previously described NifA mutants, which are unrelated to CBB function (21, 39, 40). Since a glnB mutation of *R. capsulatus* resulted in a NifA phenotype and the PII protein had been previously proposed to be a negative regulator of nif regulation in this organism (20), we examined glnB expression in strain 16PHC. Our results clearly indicated that glnB expression was affected in strain 16PHC. Despite the fact that a previous study showed the presence of a σ70 promoter motif upstream of the glnB coding region in *R. sphaeroides* (39), glnB expression was nevertheless affected by the nitrogen source supplied to this organism; e.g., the high level of glnB expression found in cells grown in the presence of ammonia was increased threefold when glutamate was used as the nitrogen source. In *R. sphaeroides* 16PHC, however, there was little or no glnB: lacZ fusion activity when either ammonia or glutamate was used as the nitrogen source. Since it is possible that the PII protein might also function as a nif repressor in *R. sphaeroides*, the absence of glnB expression in strain 16PHC might lead to the derepression of nitrogenase synthesis in the presence of ammonia. However, repression of glnB expression was relieved by the introduction of a gene which yields a functional CBB pathway, leading to a concomitant suppression of nitrogenase synthesis in the presence of ammonia (19, 28). These results are compatible with a suggested role of a functional CBB pathway in nif expression in strain 16PHC, presumably via the activation and/or inactivation of PII protein synthesis.

It has been shown for many bacteria that the glnB and glnA genes are cotranscribed and are part of a two-gene operon controlled by a glnB promoter(s) (1, 13, 18, 24). Evidence that a third promoter might also be used to specifically regulate glnA expression has been presented (1, 7, 13, 18). In strain 16PHC, GS activity was extremely low and levels were comparable to those obtained from the wild-type strain, HR, in the presence of ammonia. This low, yet constitutive, level of GS activity in strain 16PHC is probably due to the existence of a glnA promoter which may not be controlled by the nitrogen source used for growth. The fact that GS activity is detected in the absence of glnB expression in strain 16PHC might explain why glutamine auxotrophy was not observed in this strain. Similar results were obtained for *Rhizobium leguminosarum* (1), in which a glnB mutation seemed to affect GS expression only under glutamate growth conditions. In the same study, a glnA: lacZ fusion which was not sensitive to the nitrogen source for growth was constructed. In addition, glnA: lacZ activities were not affected by a glnB mutation. It is thus reasonable to believe that in R. sphaeroides, like *Rhizobium leguminosarum*, if transcription from a glnB promoter(s) is not possible, glnA may be transcribed from its own promoter, a promoter which is not regulated by the nitrogen source used for growth.

A glnB-like gene, glnK, was also isolated from *R. sphaeroides*, but glnK expression appeared to be controlled differently from glnB expression in that it was highly regulated by the nitrogen source used for growth. Indeed, glnK appeared to be expressed only under N-limiting growth conditions, e.g., with glutamate as the nitrogen source. Similar results were also obtained from studies of glnB-like genes from *E. coli* (33) and *Bacillus subtilis* (38), although the expression of the glnB-like gene glnZ in *A. brasilense* seemed to be insensitive to nitrogen sources (6). In strain 16PHC, this tight nitrogen control was lost and glnK expression was partially derepressed in the presence of ammonia, similar to nitrogenase synthesis in this strain, suggesting that perhaps nifH and glnK expression might have common regulatory elements. However, in a recent study (28), it was found that the same upstream activation and −12/−24-type promoter sequences that were present in the nifH promoter region did not appear to be present in the upstream region of glnK. Another fact to be considered in the future is that many organisms contain two different types of GS (e.g., in *Synechocystis* sp. strain PCC 6803 [30] and *Rhizobium leguminosarum* [1]), which are differentially regulated by nitrogen availability. Why certain organisms possess two such different systems is unknown at this point; however, such findings might relate to potential efficient ways to control nitrogen metabolism under the widest possible range of environmental conditions. It should be noted that glnB-like genes have also been reported for *H. seropedicae* (3), *B. subtilis* (argB) (38), *A. brasilense* (glnZ) (6), and *E. coli* (glnK) (33). Although the syntheses of GlnB and GlnK are differentially regulated in *R. sphaeroides*, these two proteins are both predicted to be composed of 112 amino acids and have very similar sequences, including a conserved uridylylation site, Tyr-51. It has been shown that purified GlnK from *E. coli* (33) can activate adenylylation of GS in vitro in the presence of ammonia (even though GlnK is not synthesized under such conditions in vivo). GlnK can also be modified by uridylylation under nitrogen-limiting conditions in vitro, suggesting that GlnK, the PII homolog, might function similarly to the PII protein itself and interact with regulatory proteins in the cell. Therefore, it is possible that the GlnK protein, which is presumably synthesized in the presence of ammonia in strain 16PHC, takes on the role of the PII protein, which presumably is not synthesized in this strain. Thus, if GlnB acts as a negative regulator for nif derepression by binding NtrB, subsequently leading to dephosphorylation of NtrC-Pi, perhaps GlnK is not as efficient as GlnB in binding to NtrB. Perhaps, then, partial derepression of nitrogenase synthesis in the absence of PII is related to the efficiency of GlnK-NtrB binding and/or its relative influence on NtrC dephosphorylation. It appears that the NifA phenotype of strain 16PHC is different from the phenotypes of other NifA mutants, because the mutation in strain 16PHC caused a pleiotropic effect on gene expression, including the repression of glnB expression and derepression of glnK and nitrogenase synthesis in the presence of ammonia. It will thus be important to determine if glnK derepression in the presence of ammonia is caused by the absence of PII synthesis in strain 16PHC.

Although the control of nitrogen metabolism and the complicated regulatory cascade have been studied extensively for prokaryotes, our results indicate that carbon metabolism also plays a significant role in regulating nitrogen metabolism. It is apparent that the CBB pathway is linked to PII expression in strain 16PHC. A regulatory link between carbon metabolism and nitrogen metabolism is also suggested for the cyanobacterium *Synechococcus* sp. strain PCC 7942, in which the rate of CO2 fixation through the CBB pathway affects phosphorylation of the PII protein (11, 12). In cyanobacteria, modification by phosphorylation seemingly is the equivalent of uridylylation of the PII protein observed in other prokaryotes (5). The precise
mechanism by which the CBB pathway affects gene expression and enzyme activity within the nitrogen assimilatory system remains to be determined. However, it seems apparent that further probing of the link between the two major biosynthetic processes of carbon assimilation and nitrogen assimilation will lead to definitive answers.

ACKNOWLEDGMENTS

We thank Stefan Nordlund and Paul Ludden for the gifts of antisera to PII protein and GS, respectively. This study was supported by Public Health Service grant GM45404 from the National Institutes of Health and by Department of Energy grant DE-FG02-91ER 20033.

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