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The Poor Growth of *Rhodospirillum rubrum* Mutants Lacking RubisCO Is Due to the Accumulation of Ribulose-1,5-Bisphosphate▽

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Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) catalyzes the first step of CO_2 fixation in the Calvin-Benson-Bassham (CBB) cycle. Besides its function in fixing CO_2 to support photoautotrophic growth, the CBB cycle is also important under photoheterotrophic growth conditions in purple nonsulfur photosynthetic bacteria. It has been assumed that the poor photoheterotrophic growth of RubisCO-deficient strains was due to the accumulation of excess intracellular reductant, which implied that the CBB cycle is important for maintaining the redox balance under these conditions. However, we present analyses of *cbbM* mutants in *Rhodospirillum rubrum* that indicate that toxicity is the result of an elevated intracellular pool of ribulose-1,5-bisphosphate (RuBP). There is a redox effect on growth, but it is apparently an indirect effect on the accumulation of RuBP, perhaps by the regulation of the activities of enzymes involved in RuBP regeneration. Our studies also show that the CBB cycle is not essential for *R. rubrum* to grow under photoheterotrophic conditions and that its role in controlling the redox balance needs to be further elucidated. Finally, we also show that CbbR is a positive transcriptional regulator of the *cbb* operon (*cbbEFPT*) in *R. rubrum*, as seen with related organisms, and define the transcriptional organization of the *cbb* genes.

The purple nonsulfur photosynthetic bacterium *Rhodospirillum rubrum* is metabolically diverse and can grow under photoautotrophic, photoheterotrophic, and chemoheterotrophic conditions. It can use different kinds of nitrogen and carbon sources, including N_2 and CO_2, through effective metabolic systems such as the nitrogenase and the Calvin-Benson-Bassham (CBB) cycles (2, 17, 21, 30). Both the nitrogenase system and the CBB cycle are very energy-demanding processes and are therefore usually tightly regulated and very sensitive to environmental signals (12, 20, 38, 40, 47).

The main role of the CBB cycle is to fix CO_2 into organic carbon under photoautotrophic conditions where CO_2 serves as the sole carbon source. Thus, most *cbb* genes have their highest expression levels under these conditions (2, 15, 28, 36, 47). However, under photoheterotrophic conditions in the presence of organic carbon such as malate or acetate, the CBB cycle also functions as a major electron sink in many photosynthetic bacteria, and it is assumed that this property maintains the redox balance in the cell (18, 24, 34, 57, 61, 62, 67). Recently, it was shown that the CBB cycle acts as an electron-accepting process to recycle the excess reduced cofactors under non-N_2-fixing conditions in *Rhodopseudomonas palustris* (39). Thus, an *R. rubrum cbbM* mutant in which the CBB cycle is blocked by the elimination of its key enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (encoded by *cbbM*), not only fails to grow under photoautotrophic conditions but also grows poorly under photoheterotrophic conditions (66). Similar phenotypes have been seen for RubisCO-deficient strains of *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* unless an alternate electron acceptor, such as dimethyl sulfoxide (DMSO), is supplied in the medium (18, 43, 62). It was hypothesized that the blockage of the CBB cycle causes an accumulation of excess reductant and that this redox imbalance perturbs cell growth (62). However, the actual mechanism behind the poor photoheterotrophic growth of *cbbM* mutants is unknown for any of these organisms.

The regulation of *cbb* operons has been extensively studied for *R. sphaeroides*, *R. capsulatus*, and *R. palustris* (6, 16, 22, 26, 46, 57, 64). These organisms all have two forms of RubisCO, form I and form II, encoded by *cbbLS* and *cbbM*, which are located in the cbb_I and cbb_{II} operons, respectively (32). The transcription of these *cbb* operons is activated by a LysR-type transcriptional regulator, CbbR (22, 43), which might need coinducers like ribulose-1,5-bisphosphate (RuBP) or its derivatives for its regulation (7, 13, 54, 63). In addition, *cbb* expression is also regulated by a two-component global regulatory system, RegB/RegA, which is also responsible for the regulation of nitrogen fixation, hydrogen metabolism, and energy generation in *R. capsulatus* and *R. sphaeroides* (10, 12, 14, 15, 27, 44, 51, 64). Models for the complex formation of RegA and CbbR at a *cbb* promoter were proposed for *R. capsulatus* and *R. sphaeroides* (8), and the phosphorylation of RegA is crucial for the transcription of *cbb* operons (11, 64). In *R. palustris* there is no RegB/RegA regulatory system. Instead, a three-

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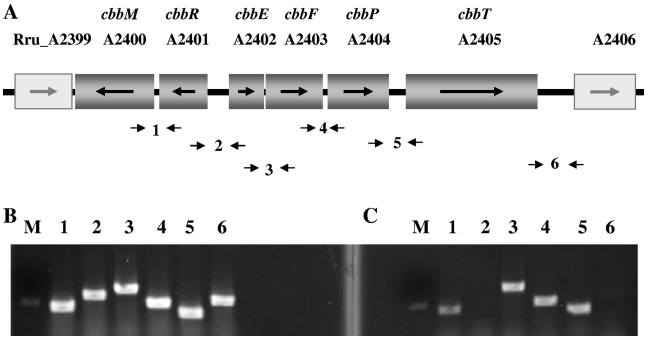

FIG. 1. *cbb* gene cluster and flanking genes of *R. rubrum* and identification of transcripts of *cbb* operons by reverse transcription-PCR (RT-PCR). (A) Gene organization based on genome information from the National Center for Biotechnology Information (NCBI) database, where the genes shown at either end are not involved in the *cbb* regulon. The small arrows below the genetic map indicate the locations and directions of primers used for the regular PCR and RT-PCR, as described below. The bottom two panels show the results of the transcription analysis of the *cbb* operons in *R. rubrum* by RT-PCR. (B and C) Data for the intergenic spaces of *cbbM-cbbR* (lane 1), *cbbR-cbbE* (lane 2), *cbbE-cbbF* (lane 3), *cbbF-cbbP* (lane 4), *cbbP-cbbT* (lane 5), and *cbbT*-Rru_A2406 (lane 6) amplified by using genomic DNA of the wild type (UR2) as the PCR template (B) and cDNA of UR2 as the RT-PCR template (C). Lane M has a DNA marker indicating 550 bp.

protein CbbRRS system acts as a signal transduction system that regulates the transcription of *cbb* operons (46).

R. rubrum lacks both the RegB/RegA and the CbbRRS systems, and the mechanism of the regulation of its *cbb* operons is unknown. In addition, the CBB cycle of *R. rubrum* also has several features that distinguish it from those of other related photosynthetic bacteria: (i) *R. rubrum* has only one form of RubisCO (form II, encoded by *cbbM*), while many other organisms have two (32), and (ii) in *R. rubrum*, the *cbbM* gene is not located in the same operon with other major *cbb* genes as seen in other organisms (12, 42, 52). Instead, it is located at the 3′ end of *cbbR*, which encodes a putative regulatory protein. These genes are transcribed in the opposite direction from the main *cbb* gene cluster, which includes *cbbE*, *cbbF*, *cbbP*, and *cbbT* (Fig. 1A) (17). The linkage of *cbbR* with *cbbM* raises a question about the role of CbbR in the regulation of the expression of *cbbM* and other *cbb* operons.

Some insight into the basis of the poor photoheterotrophic growth of *cbbM* mutants was gained by seeking suppressors of that phenotype. A spontaneous mutant of an *R. sphaeroides* RubisCO-deficient strain (*cbbM cbbLS*) restored photoheterotrophic growth and also produced active nitrogenase in the presence of ammonia, and the expression of nitrogenase in this RubisCO-deficient strain presumably consumed excess reductant and allowed cell growth (27, 62, 63). A suppressor of an *R. rubrum cbbM* mutant that gained the ability to grow photoheterotrophically also had an altered regulation of nitrogenase activity, consistent with the redox balance hypothesis (66). These results strongly suggested a linkage between the CBB cycle and the nitrogenase system.

The nitrogenase system consists of nitrogenase (MoFe protein) and dinitrogenase reductase (Fe protein), whose expression and activity are regulated by NH_4^+ at both the transcriptional and posttranslational levels in *R. rubrum* (5, 38, 40, 74). The transcription of the *nif* genes requires active NifA (73). Under nitrogen-limiting conditions, NifA is activated by GlnB-UMP, one of the P_{II} proteins in *R. rubrum* (72, 73, 76). The posttranslational regulation of nitrogenase is the result of the reversible ADP-ribosylation of dinitrogenase reductase in response to the addition of NH_4^+ or the depletion of energy (37, 70, 72). Two enzymes catalyze this reversible process. Dinitrogenase reductase ADP-ribosyl transferase (referred to as DRAT, the gene product of *draT*) carries out the transfer of the ADP-ribose from NAD to dinitrogenase reductase, inactivating that enzyme. The dinitrogenase reductase-activating glycohydrolase (referred to as DRAG, the gene product of *draG*) removes the ADP-ribose group attached to dinitrogenase reductase, thus restoring nitrogenase activity (38, 40). We have previously obtained mutants altered in *nifA* (named *nifA**, encoding NifA-M173V) and *draT* in which the nitrogenase functions even in the presence of negative stimuli such as NH_4^+ (37, 77). When these *nifA** and *draT* mutations were introduced into the *R. rubrum cbbM* mutant, the constitutive expression of active nitrogenase restored normal growth, which we interpreted to be due to the dissipation of the excess reductant caused by the *cbbM* mutation (66). Indeed, we also showed that the previously reported *cbbM* mutant of *R. rubrum* (strain I-19) also had a similar *nifA* mutation, which had not been recognized (66). However, the mechanisms of the effects of the reductant on the regulation of nitrogenase activity and cell growth are still unknown for *R. rubrum*.

In this paper, we further investigate the basis for the poor growth of *R. rubrum cbbM* mutants under photoheterotrophic conditions. We have performed Tn*5* random mutagenesis and identified suppressor mutants that are able to restore a normal-growth phenotype to an *R. rubrum cbbM* mutant. To our surprise, the analysis of these suppressors suggests that the causative property for the poor growth is not a redox imbalance, as has been supposed, but instead the accumulation of the substrate of RubisCO, RuBP, or its derivatives. The results are potentially of broad significance to similar mutant phenotypes reported for other organisms. In the course of this analysis we have better defined the relevant gene products in the CBB pathway, the transcriptional organization of *cbb* operons, and the role of the apparent regulator, CbbR, in *R. rubrum*.

MATERIALS AND METHODS

Bacterial strains. The *R. rubrum* strains used in this study are listed in Table 1. Antibiotics were used at the following concentrations: ampicillin (Ap) at 100 mg/liter, kanamycin (Km) at 25 mg/liter, gentamicin (Gm) at 5 mg/liter, chloramphenicol (Cm) at 25 mg/liter, and tetracycline (Tc) at 12.5 mg/liter for *Escherichia coli* and streptomycin (Sm) at 100 mg/liter, Km at 12.5 mg/liter, nalidixic acid (Nx) at 6 mg/liter, Tc at 1 mg/liter, Gm at 10 mg/liter, and Cm at 5 mg/liter for *R. rubrum*.

Growth conditions. *E. coli* cells were grown in LC medium (similar to Luria-Bertani medium but with 5 g/liter NaCl). *R. rubrum* was routinely grown in rich SMN (supplemented malate-ammonium) medium aerobically (19). To monitor cell growth under photoheterotrophic or photoautotrophic conditions, *R. rubrum* cultures were first grown in 5-ml aliquots of SMN medium aerobically without illumination at 30°C for 2 to 3 days. For photoheterotrophic growth, SMN cultures were diluted 60-fold into 25-ml anaerobic tubes that were almost completely filled with MG medium (a nitrogen-limiting, malate-glutamate minimal medium with glutamate as a nitrogen source) (41). These cultures had an initial optical density at 600 nm (OD_{600}) of 0.04 to 0.05 and were incubated at 30°C

TABLE 1. *R. rubrum* strains and their genotypes

Strain	Relevant genotype and/or description[a]	Reference
UR2	Wild type	19
UR5251	Δ*cbbM1*::*aacC1*; same orientation	66
UR5253	UR5251 with a pRK404 derivative carrying *cbbM* and its own promoter region	This work
UR5311	Δ*cbbR2*::*aacC1*; opposite orientation	This work
UR2553	Δ*cbbM1*::*aacC1 draT3 nifA12* (encoding NifA-M173V)	66
UR2555	Δ*cbbM1*::*aacC1* Δ*cbbF1*::*kan*	This work
UR2557	Δ*cbbM1*::*aacC1* Δ*cbbP1*::*kan*	This work
UR2562	Δ*cbbM1*:: *aacC1 nifA12* (encoding NifA-M173V)	66
UR2564	Δ*cbbF1*::*kan*; opposite orientation	This work
UR2565	Δ*cbbP1*::*kan*; opposite orientation	This work
UR2633	ΔRru_A0595::*aacC1*	This work
UR2651	Δ*cbbT1*::*kan*; same orientation	This work
UR2653	Δ*cbbM1*::*aacC1* Δ*cbbT1*::*kan*	This work
UR2654	P_{aacC1}-*cbbR* integrated in the chromosome of the wild type (UR2) at the *nifH draT* region	This work
UR2657	Δ*cbbRM1*::*aacC1*; same orientation	This work
UR2658	Δ*cbbRM2*::*aacC1*; opposite orientation	This work

[a] "Same orientation" refers to the case where the direction of transcription of the inserted *kan* or *aacC1* gene is the same as that of the mutated *cbb* gene, while "opposite orientation" refers to the transcription of the inserted gene being in the opposite direction.

under illumination. One milliliter of culture was taken out every 24 h to measure the optical density at 600 nm for 6 days. For photoautotrophic growth, SMN cultures were diluted 60-fold into 10 ml of Ormerod's minimal medium (41) supplemented with 0.1% NH_4Cl and 0.05% yeast extract in a 100-ml serum bottle. After evacuation and replacement of the gas phase with CO_2-H_2 (2:98 [vol/vol] ratio), the cultures were incubated at 30°C under illumination on an orbital shaker with a speed of 120 rpm. Subsequently, extra CO_2 gas was added into the gas phase of well-grown cultures every 24 h to keep the CO_2 concentration at 1.5 to 3%, which was reported previously to result in the highest level of RubisCO activity (47).

Tn*5* random mutagenesis and screen for suppressors of a *cbbM* mutation. Plasmid pRL27 carries a hyperactive Tn*5* transposase gene (*tnp*), a mini-Tn*5* element containing both kanamycin resistance (Km^r) and the origin of replication from plasmid R6K (*ori*R6K) (33). This plasmid was transferred into an *R. rubrum cbbM* mutant (UR5251) through biparental conjugation as described previously (37). Transconjugants were enriched in SMN liquid medium with Nx, Gm, and Km aerobically and then inoculated into MG medium for anaerobic photoheterotrophic growth, where the parental *cbbM* mutant (UR5251) strain could not grow well (66). After incubation for 3 to 5 days, well-grown cultures in tubes with a pink or red color were plated onto SMN medium with Nx, Gm, and Km after a serial dilution. Nx^r Gm^r Km^r colonies were purified on SMN selection plates and then grown in MG medium again to verify their photoheterotrophic growth. The sites of the Tn*5* insertions in 10 transconjugants were identified by DNA sequencing, as described previously (33, 71).

Construction of *cbb* mutants. To construct Δ*cbbP* mutants, two 1.5-kb fragments immediately 5′ and 3′ of *cbbP*, respectively, were amplified by PCR with the chromosomal DNA of *R. rubrum* strain UR2 (wild type) as a template. These fragments have SalI/BamHI sites or BamHI/HindIII sites at both ends and were cloned into the SalI and HindIII sites of pSUP202 (53). *kan* (encoding Km^r) from pUC4K (65) was inserted in both orientations into a BamHI site between these two fragments, yielding pUX2918 and pUX2919. These plasmids were then transferred into *R. rubrum* UR2 by biparental conjugation as described previously (37). Sm^r Km^r *R. rubrum* colonies were selected and replica printed to identify Cm-sensitive (Cm^s) colonies resulting from a double-crossover recombination event (Cm^r is encoded by pSUP202). Two Δ*cbbP* mutants were obtained with *kan* transcribed in either the same or the opposite direction as *cbbP*. Because these mutants behaved identically in subsequent studies, only the data for one of the mutants, UR2565 (Δ*cbbP1*::*kan*, with *kan* inserted in the opposite direction of the original *cbbP*), are reported in this study. To construct the *cbbM cbbP* double mutants, pUX2918 and pUX2919 were transferred into *cbbM* mutant strain UR5251. Sm^r Gm^r Km^r Cm^s *R. rubrum* colonies resulting from a double-crossover recombination event were screened. Similarly, two double mutants were obtained, and only UR2557 (Δ*cbbM1*::*aacC1* Δ*cbbP1*::*kan*) is reported in this study.

All other single and double mutants listed in Table 1 were constructed in the same way as described above, except for the single deletions/insertions lacking both *cbbR* and *cbbM*. These mutants were constructed by the deletion of the entire *cbbRM* region and its replacement by an *aacC1* gene fragment from pUCGM (50), resulting in UR2657 (Δ*cbbRM1*::*aacC1*) and UR2658 (Δ*cbbRM2*::*aacC1*), which have different orientations of the *aacC1* insertion. Unlike other constructed mutants, the insertion orientation affected the phenotypes of these two *cbbRM* mutants, so the data for both mutants are reported. All mutations were verified by PCR.

Complementation of a *cbbM* mutant with *cbbM* expressed from its own promoter between the *cbbR* and *cbbM* genes. To study the expression of *cbbM* from its own promoter between the *cbbR* and *cbbM* genes, *cbbM* and 118 bp of the immediately adjacent 5′ region were cloned into the broad-host-range vector pRK404 (9) at BamHI and HindIII sites in the opposite orientation of the *lac* promoter on pRK404 to eliminate the possibility of *cbbM* expression from this promoter. This pRK404 vector carrying *cbbM* and its 5′ region was then introduced into a *cbbM* mutant strain (UR5251) by triparental conjugation as described previously (23), yielding UR5253.

Overexpression of *cbbR* in wild-type *R. rubrum*. To overexpress *cbbR* in *R. rubrum*, *cbbR* was amplified by PCR with a pair of primers with NdeI and XhoI sites at the 5′ and 3′ ends, respectively, and was then cloned into plasmid pUX2519, which has the promoter of *aacC1* cloned into pBSKS (Stratagene, La Jolla, CA), yielding pUX3197. The fragment including the *aacC1* promoter and the *cbbR* gene was digested with BamHI and XhoI and then cloned into plasmid pYPZ261, which is a pUX19 derivative carrying a 2-kb *nifH draT* region of *R. rubrum*, yielding pUX3198. This plasmid was then integrated into the chromosome of wild-type *R. rubrum* (UR2) through biparental conjugation. Sm^r Km^r colonies were isolated and verified by PCR, indicating that a new copy of *cbbR* expressed from the *aacC1* promoter was integrated into the chromosome of UR2 at the *nifH draT* region. The otherwise wild-type strain with the plasmid-carried *cbbR* was designated UR2654.

Quantitation of intracellular levels of RuBP. Phenylhydrazine was used for the derivatization of RuBP and other sugars for better separation and detection by high-performance liquid chromatography (HPLC), as described previously (56). RuBP was then quantitated by HPLC using a method reported previously, with some modifications (45). *R. rubrum* strains were grown in MG medium under photoheterotrophic conditions for 2 to 3 days or until the OD_{600} reached 1. A 3-ml sample of the MG culture was collected by centrifugation at 20,000 × *g* for 1 min. The pellets were resuspended in 60 μl of 10% acetic acid to break the cells and extract RuBP, followed by another centrifugation at 20,000 × *g* for 5 min. The supernatant of each sample was then transferred into a new tube, and freshly made phenylhydrazine derivatization buffer (0.5% phenylhydrazine and 10% acetic acid) was added at a 10% (vol/vol) final concentration. The derivatization reaction was carried out at 80°C for 15 min.

A C_{18} Intersil HPLC column (10-μm particle size, ODS-4, 4.6 by 150 mm; GL Sciences Inc., Torrance, CA) was used with the Shimadzu CTO-20A HPLC system (Shimadzu Scientific Instruments, Columbia, MD). The mobile phase containing methanol, HPLC-grade water, and acetic acid at a 60:38.5:1.5 ratio and 5 mM tetrabutylammonium hydrogen sulfate was set at a flow rate of 1 ml per min. The HPLC oven temperature was set at 40°C, and detection was carried out at 325 nm. Twenty microliters of the derivatized samples and RuBP standards were injected into HPLC columns for detection and quantitation.

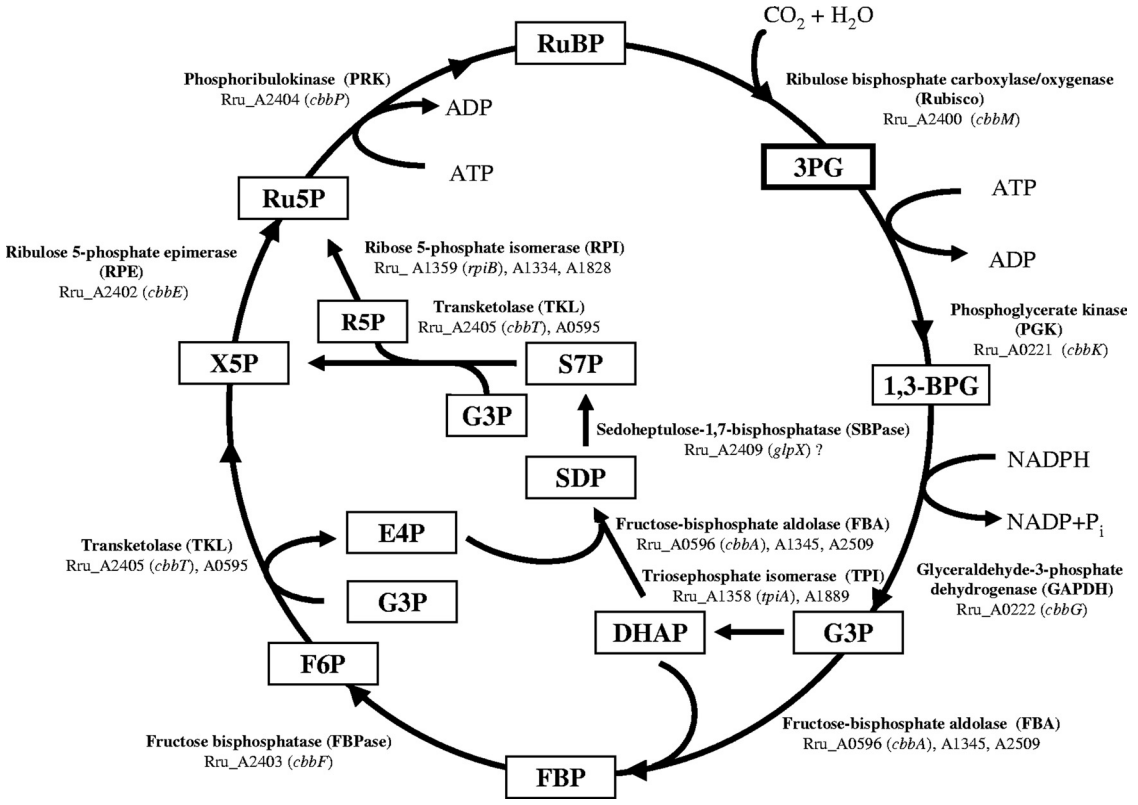

FIG. 2. Calvin-Benson-Bassham cycle in *R. rubrum*. Abbreviations: 3PG, 3-phosphoglycerate; 1,3-BPG, 1,3-bisphosphoglycerate; G3P, glyceraldehyde-3-phosphate; DHAP, dihydroxy-acetone-phosphate; SDP, sedoheptulose-1,7-bisphosphate; S7P, sedoheptulose-7-phosphate; E4P, erythrose-4-phosphate; FBP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; R5P, ribose-5-phosphate; X5P, xylulose-5-phosphate; Ru5P, ribulose-5-phosphate; RuBP, ribulose-1,5-bisphosphate. All enzymes and their putative encoding gene(s) in *R. rubrum* are also indicated.

RNA isolation and reverse transcription. Total RNA was prepared from harvested cells as previously described (60) and was then treated with RNase-free DNase I (Invitrogen, Carlsbad, CA) and purified by using an RNeasy minikit (Qiagen, Valencia, CA) as described by the manufacturer. The RNA concentration was quantitated with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE).

The reverse transcription (RT) reaction was performed with the GoScript reverse transcription system (Promega, Madison, WI) according to the manufacturer's instructions. The random hexamer primers were synthesized by Integrated DNA Technologies (Coralville, IA). The cDNA was cleaned up by using a QIAquick PCR purification kit (Qiagen) after the reverse transcription reaction. The cDNA concentration was quantitated by using a NanoDrop ND-1000 spectrophotometer.

RT-PCR. Six pairs of primers were designed to amplify the interspaces between the genes Rru_A2400 to Rru_A2406 in *R. rubrum*, which were tested by regular PCR using the genomic DNA of *R. rubrum* wild-type strain UR2 as a template. RT-PCR was performed by using cDNA as templates.

Quantitative real-time PCR. Primers were designed with Primer3 (http://primer3.sourceforge.net/) to amplify 100- to 150-bp PCR products. GoTaq quantitative real-time PCR (qPCR) master mix (Promega) was used for qPCRs, which were performed with an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The PCR was accomplished with a 2-min denaturation step at 95°C, followed by 40 cycles of 20 s at 95°C, 20 s at 50°C, and 40 s at 72°C. Primer dimers were evaluated by a melting-curve analysis reaction for 15 s at 95°C, 1 min at 60°C, and 15 s at 95°C.

rpoD, encoding the σ^{70} transcription factor, is considered to be stable in *R. rubrum* and many other bacteria (31, 49) and was used as a housekeeping gene control in qPCRs to normalize the results of relative comparisons of target gene expression levels. Because different PCR efficiencies (E) were obtained from each PCR, we calculated the relative expression ratio with the correction of efficiency (E) by the following formula, as described previously (48): relative expression ratio = $(1 + E_{\text{target}})^{-\Delta C_T \text{ target (sample } - \text{ control)}}/(1 + E_{rpoD})^{-\Delta C_T \, rpoD \text{ (sample } - \text{ control)}}$.

cDNA from the wild type (UR2) was used as the control, and cDNA from the *cbbR* mutant (UR5311) was used as the sample for qPCR.

RESULTS AND DISCUSSION

Identification of genes and their encoded enzymes in the CBB pathway in *R. rubrum*. In collaboration with the U.S. Department of Energy Joint Genome Institute, the genome of *R. rubrum* ATCC 11170 was sequenced in 2004. Based on the genomic sequence information available at the NCBI website under accession number NC_007643 and primary pathway mapping by the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg-bin/show_pathway?rru00710), we were able to identify the putative CBB cycle in *R. rubrum* (Fig. 2). The cycle is similar to that found in many other organisms, except no gene for sedoheptulose-1,7-bisphosphatase was found in *R. rubrum*. However, in many organisms the fructose-1,6-bisphosphatases, especially of the class II type, also have sedoheptulose-1,7-bisphosphatase activity (1, 3, 55, 59, 69), and a *cbbF* homolog in *R. rubrum*, Rru_A2409 (*glpX*), is predicted to be of this type.

As mentioned in the introduction, several *cbb* genes are located in a cluster in *R. rubrum*. *cbbM* is adjacent to *cbbR*, and these genes are transcribed in the opposite direction from the other *cbb* genes, *cbbEFPT* (Fig. 1A). Although *cbbA*, encoding fructose-bisphosphate aldolase, is typically found in the *cbb* gene cluster, this is not the case in *R. rubrum*. The gene 3′ of *cbbT* in *R. rubrum*, Rru_A2406, appears unrelated to *cbb* and encodes a diguanylate cyclase/phosphodiesterase with a PAS (Per-Arnt-Sim)/PAC (PAS-associated C-terminal motif) sen-

TABLE 2. Locations of Tn*5* suppressors of the *R. rubrum cbbM* mutation (UR5251)

Strain	Locus of Tn*5* insertion (positions, orientation)[a]	Encoded enzyme[b]
UR5281	*cbbR* (61–62, +)	LysR-type transcription regulator (CbbR)
UR5282	*cbbR* (360–361, +)	
UR5283	*cbbR* (744–745, +)	
UR5284	*cbbF* (215–216, −)	Fructose-1,6-bisphosphatase (FBPase or CbbF)
UR5285	*cbbF* (418–419, +)	
UR5286	*cbbF* (334–335, +)	
UR5287	*cbbP* (33–34, +)	Phosphoribulokinase (PRK or CbbP)
UR5288	*cbbP* (95–96, −)	
UR5289	Rru_B0037 (2789–2790, −)	Hemolysin-type calcium-binding region
UR5290	Rru_A3737 (928–929, +)	KAP-P loop

[a] The Tn*5* insertion position is indicated as the number of nucleotides from the start codon of the structural gene to the Tn*5* insertion site, including the 9-bp duplication. "+" indicates the same orientation of the gene and the inserted *kan* gene, while "−" indicates the opposite orientation.

[b] The locations of Tn*5* inserted into open reading frames (ORFs) were identified by BLAST searches at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

sor(s), and it is separated from *cbbT* by a 325-bp gap. Instead, three *cbbA* homologs (Fig. 2, bottom right), including one (Rru_A0596) adjacent to another *cbbT* homolog (Rru_A0595), lie elsewhere in the *R. rubrum* genome. Multiple copies of *rpiB* (predicted to encode ribose 5-phosphate isomerase) and *tpiA* (predicted to encode triosephosphate isomerase) were also found in *R. rubrum* (Fig. 2), although the physiological roles of their products are unknown.

Mutations in several other *cbb* genes can suppress the poor growth of a *cbbM* mutant. As reported previously, *R. rubrum cbbM* mutants grow poorly in MG medium (malate-glutamate minimal medium with glutamate as a poor nitrogen source) and MN medium (minimal malate-ammonium medium) under photosynthetic, anaerobic conditions (66). To gain insight into the cause of this growth defect, we sought mutations that suppress this phenotype. We randomly mutagenized a *cbbM* mutant (UR5251) with the Tn*5* transposon and selected for fast-growing mutants in MG liquid medium under anaerobic conditions. The sites of the Tn*5* insertion in 10 suppressors were identified by DNA sequencing as described previously (33, 71), and 8 out of 10 Tn*5* insertions were found to be located in different sites in *cbbR*, *cbbF*, or *cbbP* (Table 2). This result strongly suggests that the disruption of other *cbb* genes in the CBB cycle can restore normal growth to a *cbbM* mutant under these anaerobic, photoheterotrophic growth conditions. We will focus on the *cbbF* and *cbbP* mutations first and discuss *cbbR* in a later section.

To confirm that the loss of some other *cbb* functions could suppress a *cbbM* mutation, we constructed *cbbM cbbF* and *cbbM cbbP* double mutants. We used previously constructed *cbbM*::*aacC1* mutants (UR5251 and UR5252, which have two different orientations of the *aacC1* insertion) as host strains (66). We used the *kan* cassette from pUC4K (65) to replace deleted sections of *cbbF* or *cbbP* as described in Materials and Methods. With all double mutants, both orientations of the *kan* and *aacC1* insertions were constructed and analyzed in all experiments described below. All mutants with different insertion orientations behaved identically, and only the data for one orientation are reported. As in previous experiments, a fairly heavy inoculum of the SMN-grown culture was used, which allowed cell growth in MG medium to reach an OD_{600} of approximately 0.2 to 0.3 (after 1 day) before the growth defects of *cbbM* mutants were apparent (66). The *cbbM* single mutant grew poorly in MG medium under heterotrophic anaerobic conditions, as seen previously (66), but the *cbbM cbbP* and *cbbM cbbF* double mutants grew much better than did the *cbbM* single mutant (Fig. 3B). These results confirm that the loss of either fructose-1,6-bisphosphatase (CbbF) or phosphoribulokinase (CbbP) activities can suppress a *cbbM* mutation. This demonstrates that a functional CBB pathway is not necessary for photoheterotrophic growth and suggests that the growth defect seen for *cbbM* mutants is likely caused by the accumulation of some pathway intermediate rather than a redox imbalance.

Photoheterotrophic and photoautotrophic growths of various *cbb* mutants. Because we detected suppressor mutations in only some *cbb* genes, we wanted to determine the phenotypes of loss-of-function mutations in other *cbb* genes. We were able to construct Δ*cbbT* (Rru_A2405), Δ*cbbF* (Rru_A2403), and Δ*cbbP* (Rru_A2404) single mutants but not a Δ*cbbE* (Rru_A2402) mutant.

As shown in Fig. 3A, unlike the *cbbM* mutant, the *cbbF* (UR2564), *cbbP* (UR2565), and *cbbT* (UR2651) single mutants grew as well as the wild type in MG medium. Similar to the *cbbM* mutant, the *cbbF* (UR2564) and *cbbP* (UR2565) single mutants failed to grow under photoautotrophic conditions (Fig. 4A), indicating that the CBB cycle is blocked in these mutants. As expected, all double mutants containing a *cbbM* mutation failed to grow under photoautotrophic conditions (Fig. 4B). The inability of the *cbbF* single mutant to grow photoautotrophically indicates that the potential *cbbF* homolog (Rru_A2409 [*glpX*], encoding a putative class II type of fructose bisphosphatase) cannot fully replace the *cbbF* product in the CBB cycle. Presumably, GlpX functions as a sedoheptulose-1,7-bisphosphatase, as mentioned above. However, the *cbbT* mutant (UR2651) grew as well as the wild type under the photoautotrophic conditions, implying that the product of Rru_A0595, which has 53% amino acid sequence identity with CbbT (Rru_A2405), has sufficient transketolase activity to maintain a functional CBB cycle. Interestingly, we were unable to construct the *cbbT* Rru_A0595 double mutant under any of several growth conditions tested, indicating that some level of transketolase is essential in *R. rubrum* under these conditions.

We were unable to obtain a *cbbE* (Rru_A2402) mutant under any growth condition tested, including photoheterotrophic growth in MG or SMN rich medium or aerobic growth in SMN rich medium. We were also unable to delete the entire *cbbEFPT* region, even though *cbbFPT* were separately eliminated in the strains described above, suggesting that ribulose 5-phosphate epimerase (encoded by *cbbE*) is essential and might be involved in some other critical metabolic pathways in *R. rubrum*. Note that the lethality of a *cbbE* mutation is apparently not the result of an accumulation of its precursor, xylulose-5-phosphate (X5P) (Fig. 2), since the *cbbEFPT* deletion would not accumulate that molecule.

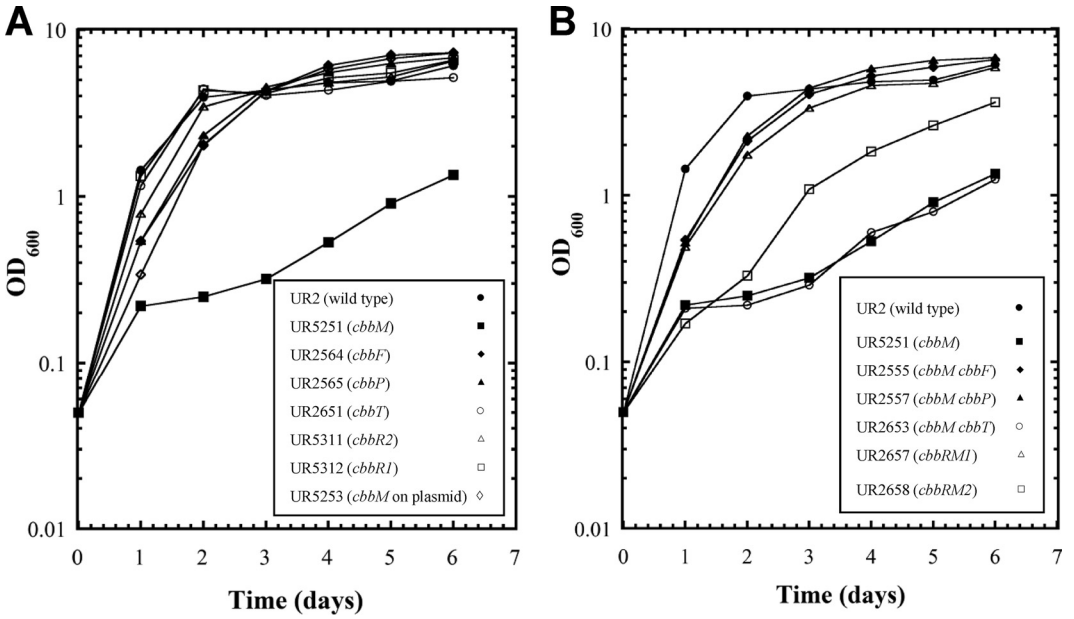

FIG. 3. Photoheterotrophic growth of the *R. rubrum* wild type (UR2), the *cbbM* mutant (UR5251), and other *cbb* mutants. A culture grown in SMN medium (0.4 ml) was used to inoculate 25 ml of MG medium and then grown under phototrophic and anaerobic conditions. This fairly heavy inoculum of the culture grown in SMN medium allows growth in MG medium to an OD_{600} of approximately 0.2 to 0.3 (after 1 day) before growth defects of *cbbM* mutants are apparent. All single mutants are listed in A, and all double mutants are shown in B, while the wild type (UR2) and the *cbbM* mutant (UR5251) are shown in both panels for comparison.

The normal growth of the *cbbP* and *cbbF* single mutants under photoheterotrophic conditions has the following implications. (i) It disproves the hypothesis that the poor growth of *cbbM* mutants is caused by the accumulation of excess reductant, since these single mutants are also compromised in the CBB cycle. (ii) It also clearly shows that the entire CBB cycle is not critical for cell growth under photoheterotrophic conditions. (iii) Unlike the *cbbM* mutant, the blockage of the CBB cycle at other steps does not cause significant growth defects under photoheterotrophic conditions, strongly suggesting that

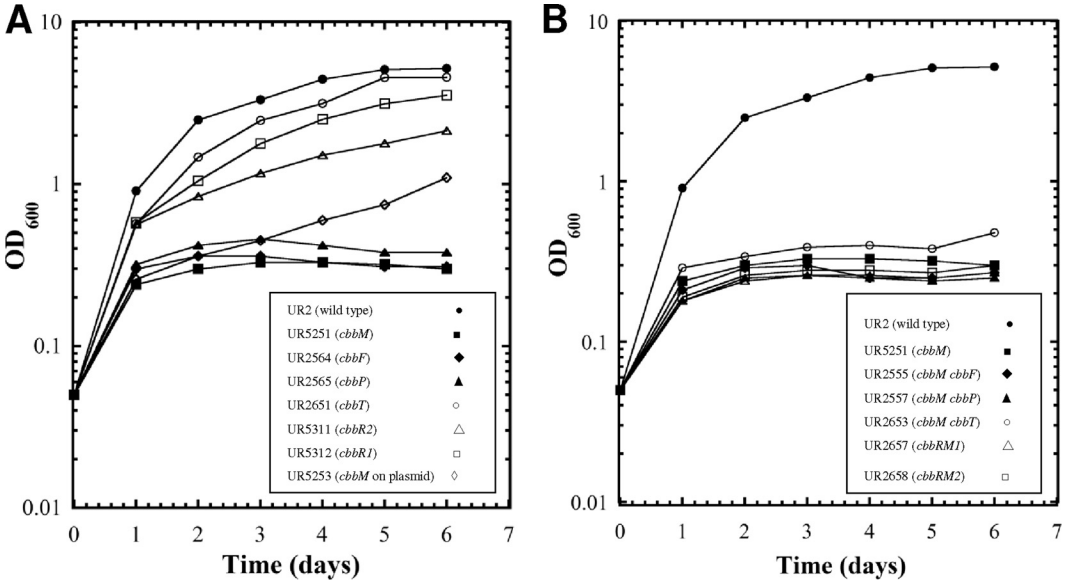

FIG. 4. Photoautotrophic growth of the *R. rubrum* wild type (UR2), the *cbbM* mutant (UR5251), and other *cbb* mutants. A culture grown in SMN medium (0.2 ml) was used to inoculate 10 ml of CO_2-H_2-NH_4^+ minimal medium supplemented with 0.05% yeast extract and then grown under phototrophic and anaerobic conditions. This fairly heavy inoculum of the culture grown in SMN medium and a small amount of yeast extract allows growth in CO_2-H_2-NH_4^+ minimal medium to an OD_{600} of approximately 0.2 to 0.3 (after 1 day) before growth defects of *cbbM* mutants are apparent. All single mutants are listed in A, and all double mutants are shown in B, while the wild type (UR2) and the *cbbM* mutant (UR5251) are shown in both panels for comparison.

TABLE 3. RuBP levels in *R. rubrum* cultures grown in MG medium

Strain	Genotype	Growth rate[a]	Mean RuBP level[b] (nmol per mg dry wt) ± SD
UR2	Wild type	Fast	0.5 ± 0.2
UR5251	Δ*cbbM1*::*aacC1*	Very slow	55.2 ± 1.2
UR5311	Δ*cbbR2*::*aacC1*	Fast	1.0 ± 0.4
UR2564	Δ*cbbF1*::*kan*	Fast	1.5 ± 0.6
UR2565	Δ*cbbP1*::*kan*	Fast	0.7 ± 0.3
UR2651	Δ*cbbT1*::*aacC1*	Fast	0.7 ± 0.2
UR2657	Δ*cbbRM1*::*aacC1*; same orientation	Fast	1.2 ± 0.8
UR2658	Δ*cbbRM2*::*aacC1*; opposite orientation	Slow	10.2 ± 0.1
UR2555	Δ*cbbM1*::*aacC1* Δ*cbbF1*::*kan*	Fast	1.3 ± 0.5
UR2557	Δ*cbbM1*::*aacC1* Δ*cbbP1*::*kan*	Fast	1.7 ± 0.2
UR2653	Δ*cbbM1*::*aacC1* Δ*cbbT1*::*aacC1*	Very slow	64.2 ± 1.8
UR2562	*cbbM nifA*(M173V)* mutant	Fast	1.8 ± 0.4
UR2553	*cbbM draT nifA*(M173V)* mutant	Fast	0.9 ± 0.2
UR2654	P_{aacC1}-*cbbR*	Slow	25.5 ± 0.6

[a] Growth rate refers to the growth rate on day 1 (when the initial nutrients have been exhausted) and was estimated based on data in Fig. 3, previously published data (66), or data not shown. Fast, a doubling time of 8 to 12 h; slow, a doubling time of 20 to 24 h; very slow, a doubling time of greater than 36 h.

[b] The detection limit of RuBP by this HPLC method is 0.15 nmol, which is ~0.5 nmol RuBP per mg dry weight.

the growth defect of *cbbM* mutants is likely caused by the accumulation of some pathway intermediates.

The poor growth of the *cbbM* mutant is caused by the accumulation of RuBP in the cell. The obvious intermediate that would accumulate in a *cbbM* mutant but be absent in the *cbbM cbbP* and *cbbM cbbF* double mutants is RuBP, the substrate of RubisCO (the *cbbM* product). It is well known that the accumulation of phosphorylated intermediates in the galactose pathway is also toxic for the cell (29, 68). Indeed, a recent report showed that this intermediate, UDP-galactose, might also serve as a stress signal under some conditions (35). It was also reported previously that an *E. coli* strain in which spinach phosphoribulokinase was overproduced had a high level of RuBP in the cells and grew very poorly (25). As shown in Fig. 2, RuBP is the substrate of RubisCO and the product of phosphoribulokinase (encoded by *cbbP*). Ribulose-5-phosphate 3-epimerase (encoded by *cbbE*), fructose-1,6-bisphosphatase (encoded by *cbbF*), and transketolase (encoded by *cbbT*) provide the substrate ribulose-5-phosphate (Ru5P) for the regeneration of RuBP. The mutational elimination of RubisCO in a *cbbM* mutant would lead to the accumulation of RuBP, while the disruption of *cbbP* or *cbbF* should block this and would explain why the *cbbM cbbP* and *cbbM cbbF* double mutants grow well in MG medium.

To test the hypothesis that the growth problem of *cbbM* mutants is caused by the accumulation of RuBP, we used an HPLC method to determine intracellular RuBP levels. As shown in Table 3, the *cbbM* mutant (UR5251) had more than a 30-fold-higher level of intracellular RuBP than did the wild type and all suppressor mutants displaying better growth, including the *cbbM cbbF* (UR2555) and *cbbM cbbP* (UR2557) double mutants as well as the *cbbF* (UR2564), *cbbP* (UR2565), and *cbbT* (UR2651) single mutants. These results strongly suggest that the poor growth of the *cbbM* mutants is caused by the accumulation of RuBP in the cell.

Unlike the *cbbM cbbF* and *cbbM cbbP* double mutants, the *cbbM cbbT* double mutant (UR2653) grew poorly in MG medium (Fig. 3B) and also had a much higher level of RuBP than did the wild type (Table 3). This again implies that the other *cbbT* homolog (Rru_A0595) can provide the transketolase function to produce inhibitory levels of RuBP in the absence of *cbbT* (Rru_A2405).

Relationship of RuBP and reductant levels in the *cbb* mutants. Although we found a correlation between poor growth and high levels of RuBP, previous studies suggested that the poor growth of *cbbM* mutants of *R. rubrum* and other related bacteria might be due to excess reductant (18, 27, 62, 67). Although this appears inconsistent with our results with the single *cbb* mutants noted above, we have reported a similar observation (66). Specifically, we showed that a constitutively active nitrogenase is able to suppress the photoheterotrophic growth defect of a *cbbM* mutant, and we interpreted this as a diversion of excess reductant to H_2 production by nitrogenase (66). The constitutive nitrogenase activity in this strain was created by a mutation in *nifA* (termed *nifA**), the positive transcriptional regulator of the *nif* genes, and another in *draT*, a posttranslational regulator of nitrogenase activity. Note that neither of these mutations has a direct effect on the *cbb* pathway. Under the hypothesis that the RuBP level is the key issue affecting growth in a *cbbM* mutant, we predicted that the RuBP level is also altered in the strains with constitutively active nitrogenase. The results in Table 3 show this to be the case. The *cbbM nifA** (UR2562) and *cbbM nifA* draT* (UR2553) mutants both had low RuBP levels in the cell. Although this result does not disprove the original hypothesis that excess reductant is the direct cause of the growth defect, there is no longer any reason to support it. Instead, the simpler model of high RuBP levels in the cell being the direct cause of poor growth is more plausible.

Although the mechanism for the observed effect of nitrogenase, and therefore of the reductant, on RuBP levels is unknown, we can imagine two possibilities. One possibility is that one or more of the RuBP-regenerating enzymes are active only under strongly reducing conditions in *R. rubrum* and that expressing the nitrogenase system in *cbbM nifA** mutants could make the conditions less reducing and thereby lower the enzyme activities in the CBB cycle for RuBP regeneration. Consistent with this idea, it was previously reported that the phosphoribulokinase (CbbP) and fructose bisphosphatase (CbbF) activities in plants are regulated by the redox state through a ferredoxin-thioredoxin system (4). Alternative models, such as differential RuBP stability under reducing conditions, cannot be ruled out, however.

There is another curiosity that should be explained. As noted above, we have shown previously that a *cbbM* mutant with the constitutive expression of active nitrogenase allows normal growth and that there is nitrogenase activity in a *nif*$^+$ background in MG medium, so why is this activity not sufficient to allow good growth if there is a *cbbM* mutation? We assume that the explanation is one of timing. For unknown reasons, the level of nitrogenase activity of the wild type in MG medium is low until the culture density is above 1 OD unit, so we imagine that high levels of RuBP arise in a *cbbM* strain before nitrogenase activity can address the problem. In contrast, a *nifA* draT* strain has high levels of nitrogenase activity initially, which presumably prevents the appearance of RuBP even with a *cbbM* mutation.

Transcriptional organization of the *cbb* operon in *R. rubrum*. As shown in Fig. 1, *cbbR* and *cbbM* in *R. rubrum* are transcribed in the opposite direction from that of the other *cbb* genes, including *cbbE*, *cbbF*, *cbbP*, and *cbbT*. This is quite different from the organization of *cbb* operons seen for other related photosynthetic bacteria, such as *R. sphaeroides*, *R. capsulatus*, and *R. palustris* (13, 32, 46), in which *cbbM* is adjacent to other *cbb* genes and *cbbR* is transcribed separately in the opposite direction. We therefore wished to define the transcriptional organization of these *cbb* genes in *R. rubrum*. First, we determined the transcriptional organization of *cbbM*, *cbbR*, *cbbE*, *cbbF*, *cbbP*, and *cbbT* by reverse transcription-PCR (RT-PCR), and the results are shown in Fig. 1B and C. Six pairs of primers were designed for amplifying the intergenic regions between *cbbM* and Rru_A2406: *cbbM-cbbR*, *cbbR-cbbE*, *cbbE-cbbF*, *cbbF-cbbP*, *cbbP-cbbT*, and *cbbT*-Rru_A2406 (Fig. 1A). When genomic DNA was used as a template, PCR products with the expected sizes were obtained with all corresponding pairs of primers (Fig. 1B), confirming that all primers annealed correctly. When cDNA synthesized from mRNA of the wild type (UR2) was used as the template, similarly sized PCR products were obtained for the gaps of *cbbM-cbbR*, *cbbE-cbbF*, *cbbF-cbbP*, and *cbbP-cbbT* (Fig. 1C). As expected, no PCR product was obtained for the gap of *cbbR-cbbE*, since these two genes are transcribed in opposite directions. This result also showed that there is no contamination of DNA in the cDNA sample. A negligible amount of PCR product was seen for the gap of *cbbT*-Rru_A2406, indicating that Rru_A2406 is not part of the *cbb* operon. These results indicate that *cbbR* and *cbbM* are cotranscribed, at least to some extent, and that *cbbE cbbF*, *cbbP*, and *cbbT* are cotranscribed in the same operon, in the opposite direction from the *cbbRM* operon.

The apparent cotranscription of *cbbR* and *cbbM* is surprising, since CbbR is predicted to be a regulatory protein (analyzed below) and would be expected to be synthesized at much lower levels than RubisCO, the product of *cbbM*. Indeed, previous studies using S1 mapping suggested that the initial site of transcription of *R. rubrum cbbM* is located in the space between *cbbM* and *cbbR*, and the size of the *cbbM* transcript determined by Northern blots was consistent with a monocistronic transcript (36). To examine the relative importance of the promoter 5′ of *cbbR* for *cbbM* expression, we created a clone that certainly lacks this promoter. As described in Materials and Methods, *cbbM* and 118 bp of the immediate 5′ region were cloned into the broad-host-range vector pRK404 and then introduced into a *cbbM* mutant (UR5251), yielding UR5253.

As noted previously and as described above, the *cbbM* mutant (UR5251) grew poorly under photoheterotrophic conditions (Fig. 3A) (66). Conversely, the strain carrying *cbbM* and its immediately proximal 5′ region (UR5253) grew well (Fig. 3A), suggesting the presence of a promoter between *cbbR* and *cbbM*. However, under photoautotrophic conditions, where high levels of RubisCO activity are required, *cbbM* harbored by the plasmid could not restore the normal growth of the *cbbM* mutant (Fig. 4A). Thus, while there does appear to be a promoter immediately 5′ of *cbbM*, it does not seem to be sufficient for the level of expression necessary for photoautotrophic growth.

As reported previously, Falcone and Tabita were unable to complement an *R. rubrum cbbM* mutant with various lengths of the *cbbM* region, including one fragment with *cbbMR* and *cbbEFPT* (17). Those authors interpreted this result with the model that the whole set of enzymes in the CBB pathway has to be "coordinately expressed" (17). In fact, that observation is better explained with the model that the reduced level of expression of *cbbM* relative to that of the *cbbEFPT* operon leads to an accumulation of RuBP and toxicity, even under heterotrophic conditions, as shown above.

CbbR is a positive transcriptional regulator for the *cbb* operon in *R. rubrum*. In our original suppressor selection, another putative Tn*5* suppressor in the *cbb* region was found in *cbbR* (Table 2). In *R. sphaeroides*, CbbR was shown previously to positively regulate the expression of the *cbb* operon, together with the two-component regulatory system RegB/RegA (10, 27, 44, 51). Although there are no RegB/RegA homologs in *R. rubrum*, and the mechanism for the regulation of the *cbb* operon is unknown, our Tn*5* mutagenesis results are consistent with the idea that CbbR in *R. rubrum* positively regulates the transcription of *cbbEFPT*. A mutation in *cbbR* should decrease the expression levels of these genes and prevent the accumulation of RuBP. To test this hypothesis, two deletion mutants lacking both *cbbR* and *cbbM* were constructed with both orientations of the *aacC1* insertion (UR2657 and UR2658), as described in Materials and Methods. These two mutants had quite different phenotypes: the *cbbRM* mutant (UR2658) with *aacC1* inserted in the orientation opposite of that of *cbbRM* grew slowly in MG medium, while another *cbbR cbbM* mutant (UR2657) with *aacC1* oriented in the same orientation as that of *cbbRM* transcription restored normal growth (Fig. 3B). Interestingly, all three of the *cbbR* Tn*5* insertions originally isolated as suppressors had their *kan* genes transcribed in the same orientation as that of *cbbRM* (Table 2). Analysis of RuBP levels in these strains (UR2657 and UR2658) showed that poor growth again correlated with elevated RuBP levels (Table 3). We suppose that in the slow-growing *cbbRM* mutant (UR2658), transcription from the *aacC1* insert drives sufficient *cbbEFPT* expression to allow some accumulation of RuBP. In contrast, because the orientation of the inserts in the Tn*5* suppressors and in the rapidly growing *cbbR cbbM* mutant (UR2657) is directed away from *cbbEFPT*, there is no significant transcription into these genes and, therefore, no elevated RuBP level.

Because at least one of the Tn*5* mutations in *cbbR* had the same suppression phenotype as that of Tn*5* insertions in *cbbF* and *cbbP*, we predicted that the function of CbbR in *R. rubrum* is to positively regulate the transcription of the *cbbF* and *cbbP* genes. To test this hypothesis, we constructed two Δ*cbbR* single mutants with different orientations of the *aacC1* insertion (UR5311 and UR5312), as described in Materials and Methods, and then employed quantitative real-time PCR (qPCR) to quantitate the mRNA levels of *cbb* genes in the wild type and the *cbbR* mutants. Both *cbbR* single mutants grew well in MG medium and were also able to grow under photoautotrophic conditions but less well than the wild type (Fig. 4A). This implies that some expression of the *cbb* genes is independent of CbbR and that this level is sufficient for moderate photoautotrophic growth. The following results of qPCR experiments are consistent with this.

The mRNA samples were extracted from photoautotrophi-

TABLE 4. Relative expression levels of *cbb* genes in the wild type (UR2) and the *cbbR* mutant (UR5311) based on qPCR data

Gene	cDNA sample[a]	Mean C_T ± SD[c]	Relative expression[b,c] (%)
cbbM	UR2-auto	16.2 ± 0.16	100
	UR5311-auto	25.2 ± 0.02	1.4
	UR2-hetero	24.5 ± 0.06	0.8
cbbE	UR2-auto	18.7 ± 0.04	100
	UR5311-auto	22.0 ± 0.01	9.5
	UR2-hetero	22.8 ± 0.03	2.3
cbbP	UR2-auto	21.4 ± 0.07	100
	UR5311-auto	25.5 ± 0.03	6.7
	UR2-hetero	25.0 ± 0.12	3.7

[a] cDNAs are from the *R. rubrum* wild type (UR2) and *cbbR* mutant (UR5311) grown under photoautotrophic growth conditions (auto) or photoheterotrophic conditions (hetero). The expressions of *cbbM*, *cbbE*, and *cbbP* in these two strains are compared.

[b] Relative expression was calculated as described in Materials and Methods and then normalized by using the UR2-auto sample as a control for 100% expression.

[c] The rank orders of samples for each of the genes in the "mean C_T" (threshold cycle) and the "relative expression" analyses differ because the value for the reference gene, *rpoD*, varied slightly between heterotrophic and autotrophic growth conditions.

cally grown cells, since all genes in the CBB cycle should have the highest expression levels under these conditions (2, 28, 58). As shown in Table 4, there is a much higher level of mRNA accumulation, and, therefore, presumably gene expression, of *cbbM*, *cbbE*, and *cbbP* in the wild type (UR2) under photoautotrophic conditions than under photoheterotrophic conditions. This is consistent with a greater need for CBB proteins under the former conditions. As shown in Table 4, under photoautotrophic conditions, UR5311 (Δ*cbbR*2::*aacC*1) had a much lower level of accumulation of *cbbE* and *cbbP* mRNAs than did the wild type, consistent with CbbR acting as a positive transcriptional regulator of the *cbbEFPT* operon in *R. rubrum* albeit over a modest basal level of expression. A low level of accumulation of *cbbM* in *cbbR* mutants was also seen (Table 4), suggesting that the internal promoter located between *cbbR* and *cbbM* is not a very strong one or that it is also activated by CbbR. The modest photoautotrophic growth of the *cbbR* mutants also indicates that the low level of expression of *cbbM* and other *cbb* genes does not preclude even autotrophic growth, as long as the production and enzymatic activity of other CBB enzymes are balanced with those of CbbM.

We then asked if the overproduction of CbbR in an otherwise wild-type background would provide some insight into the regulatory system. A second copy of *cbbR* was integrated into the chromosome of the wild type at a site away from the *cbb* gene cluster, as described in Materials and Methods. This second copy of *cbbR* was expressed from the *aacC1* promoter (50), which is a constitutive, relatively strong promoter in *R. rubrum* (75). The resulting strain (UR2654) grew slowly under photoheterotrophic conditions (data not shown), with a high level of RuBP accumulation (Table 3). This strain failed to grow under photoautotrophic conditions (data not shown). The simplest hypothesis for this result is that elevated CbbR levels lead to disproportionately higher levels of the CbbEFPT proteins than of CbbM, since CbbR is a positive regulator for the expression of the *cbbEFPT* operon. The fact that poor growth again correlates with RuBP levels (Table 3), albeit by a completely different genetic mechanism, further supports the causative nature of elevated RuBP levels for that phenotype.

These results and those for the suppressor mutations show that the *cbb* genes are expressed under photoheterotrophic conditions, but it remains unclear why this should be the case. The normal growth of the *cbbP* and *cbbF* single mutants under photoheterotrophic conditions (Fig. 3A) clearly showed that the CBB cycle is not critical for growth under this condition. The qPCR data suggested that the CBB cycle in *R. rubrum* is not tightly regulated by CbbR. Our results indicate that although a functional CBB cycle is not essential for *R. rubrum*, some enzymes in this cycle, including ribulose 5-phosphate epimerase (encoded by Rru_A2405 [*cbbE*]) and transketolase (encoded by Rru_A2405 or Rru_A0595 [*cbbT*]), are critical so that cells should always have sufficient amounts of *cbb* expression to provide the necessary levels of these two enzymes. We also believe that the reducing conditions used for cells grown in MG medium likely keep many enzymes, such as RubisCO, CbbP, and CbbF, in active forms, since these enzymes in plants are induced to be active under reducing conditions and lost activities when the environment became oxidized (4, 25). Another possibility for the heterotrophic expression of the *cbb* operon is that a small amount of CO_2 could be produced under photoheterotrophic conditions, which might induce the expression of *cbb* genes.

In summary, despite the plausibility of the model that growth defects in *cbbM* mutants are the result of a redox imbalance, we show that this does not appear to be the case for *R. rubrum*. Instead, we show that the high level of RuBP is the direct cause of the poor growth of these mutants, although we cannot discount the possibility that a derivative of RuBP is the toxic compound. Surprisingly, the accumulation of RuBP can apparently be affected by the reductant level in the cell, since a constitutively active nitrogenase can help reduce the RuBP level and suppress the poor growth of a *cbbM* mutant under photoheterotrophic conditions. We presume that the RuBP-regenerating enzymes may be active only under strongly reducing conditions in *R. rubrum*, but this needs further analysis. We cannot conclude that RuBP accumulation is also the cause of cell growth defects in other related organisms, since the organization and regulation of the *cbb* operons in these organisms are quite different, but this important possibility needs to be tested. We also confirmed that CbbR is a positive transcriptional regulator of the *cbbEFPT* operon and defined the transcriptional organization of the main *cbb* region in *R. rubrum*.

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