Analysis of the *cbbXYZ* Operon in *Rhodobacter sphaeroides*

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Three genes, *cbbX*, *cbbY*, and *cbbZ* were found downstream from the form I ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) genes of *Rhodobacter sphaeroides*. As in chemoautotrophic bacteria, *cbbZ* was shown to encode phosphoglycolate phosphatase (PGP), whereas the identities of *cbbX* and *cbbY* are not known. To determine the physiological function of the *cbbXYZ* gene products, we constructed *R. sphaeroides* strains in which the genes were inactivated and characterized the resultant mutant strains according to growth phenotype and levels of RubisCO and PGP. Only a mutation in *cbbX* resulted in a discernible phenotype, namely, impaired photoautotrophic growth. No PGP activity was observed in any of the mutants, suggesting that the three genes are transcriptionally linked. Studies with a spontaneous chemoautotrophic competent derivative of the CBBX mutant suggested that the *cbbXYZ* gene products are not essential for chemoautotrophic growth. PGP activity determined in the wild-type strain grown under a variety of growth conditions, and in various strains containing mutations in Calvin-Benson-Bassham cycle structural and regulatory genes, indicated that transcription of the *cbb_I* operon influenced expression of the downstream *cbbXYZ* operon.

Purple nonsulfur photosynthetic bacteria have the capacity to grow under a diverse range of culture conditions, in which CO₂ fixation via the Calvin-Benson-Bassham (CBB) reductive pentose phosphate cycle has been shown to play varied roles. During photo- and chemoautotrophic growth, all of the cellular carbon is derived from CO₂. Accordingly, during autotrophic growth, key Calvin cycle enzymes, such as ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) and phosphoribulokinase, are maximally induced (6, 13, 32). On the other hand, during photoheterotrophic growth, CO₂ fixation functions in a different capacity, fulfilling the role of electron acceptor in the presence of excess reducing potential derived from fixed organic substrates, such as malic or butyric acid, and intermediate levels of RubisCO and phosphoribulokinase are observed (16, 32). Alternate electron acceptors, such as dimethyl sulfoxide, can replace the need for CO₂ fixation under these growth conditions, leading to repressed synthesis of CBB cycle enzymes (14, 15, 34).

In *Rhodobacter sphaeroides*, several enzymes of the CBB cycle are duplicated, and their genes are organized within two physically distinct operons, *cbb_I* and *cbb_{II}* (2, 7, 8), both of which are part of the *cbb* regulon (6, 13). Expression of genes within both *cbb* operons is positively regulated and coordinated through a common regulatory protein, CbbR, encoded by the *cbbR* gene, which is divergently transcribed from the *cbb_I* operon (12). In various *cbb* mutants, CbbR is able to mediate a compensatory expression of genes within one operon in response to disruption of the second operon, thereby maintaining induced levels of key CBB cycle enzymes. Environmental factors affecting synthesis of *cbb* genes include CO₂, O₂, and reduced carbon sources, among others (16, 34). Obviously, control of the *cbb* regulon involves an extremely complex regulatory network. In attempts to further our understanding of how CO₂ fixation is controlled in *R. sphaeroides*, we have extended analysis of the *cbb_I* operon beyond the

RubisCO coding sequences and have identified three open reading frames (ORFs), *cbbX* and *cbbY*, which to date have no assigned function, and *cbbZ*, which encodes phosphoglycolate phosphatase (PGP) (30). Although these genes have previously been demonstrated in *cbb* gene clusters in aerobic chemoautotrophic bacteria (19, 23), demonstration of a physiological function has remained elusive. The unique transcriptional arrangement of *cbbX*, *cbbY*, and *cbbZ* in *R. sphaeroides* raises intriguing questions concerning the role of the products of these genes in autotrophic metabolism. In this study, the genes were inactivated and the resultant mutants were analyzed to assess the function of these genes in CO₂ assimilation.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *R. sphaeroides* strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria broth (3) at 37°C. *E. coli* JM109 (33) and JM109 (λ *pir*) (24) were used for propagation of plasmid derivatives. Strains SM10 (31) and S17-1 (λ *pir*) (24) were used to deliver plasmids into *R. sphaeroides*. *R. sphaeroides* strains were grown chemoheterotrophically in a peptone-yeast extract medium (35), photoheterotrophically on either 0.4% malate or 0.2% butyrate, or photoautotrophically with continuous bubbling in an atmosphere of 1.5% CO₂–98.5% H₂ as described previously (16). Chemoautotrophic growth was carried out in the dark with continuous bubbling in an atmosphere of 5% CO₂–45% H₂–50% air in the same medium used for photoautotrophic growth. Photosynthetic growth on agar plates was carried out in GasPak (Scientific Products Div., McGaw Park, Ill.) anaerobic jars. Antibiotics were added to culture media, when appropriate, at the following concentrations (micrograms per milliliter): for *E. coli*, ampicillin, 25; trimethoprim, 200; kanamycin, 25; tetracycline, 25; and spectinomycin (Sp), 25; for *R. sphaeroides*, streptomycin, 25; trimethoprim, 200; spectomycin, 25; and tetracycline, 1.0.

Genetic techniques. Plasmid DNA isolations, transformations, and other routine DNA manipulations were carried out by using standard procedures (1). DNA sequencing was performed on double-stranded DNA templates by the dideoxy-chain termination method of Sanger et al. (29), using either a Sequenase kit obtained from New England Nuclear, Boston, Mass., or an ABI PRISM dye terminator cycle sequencing kit from Perkin-Elmer, Foster City, Calif. Universal and reverse M13 primers obtained commercially and custom-made primers were used to sequence approximately 2.9 kb of DNA containing the *cbbX*, *cbbY*, and *cbbZ* genes. DNA sequences were determined completely on both strands of DNA, both manually and by use of a Perkin-Elmer model 310 automatic sequencer. Southern hybridizations were performed by using the blotting and hybridization protocols supplied with Gene Screen Plus membranes (New England Nuclear). Chromosomal DNA was prepared from mid-log-phase cultures of *R. sphaeroides* grown photoheterotrophically on malate by using a previously described procedure (4).

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>R. sphaeroides</i>		
HR	Sm ^r , wild type	35
CAC	Sm ^r spontaneous chemoautotrophic competent variant of wild-type strain HR	25
CBBX	Sm ^r , <i>cbbX</i> ::Sp ^r	This study
CBBYZ	Sm ^r , <i>cbbYZ</i> ::Sp ^r	This study
CBBZ	Sm ^r , <i>cbbZ</i> ::Tp ^r	This study
XCAC	Sm ^r , Sp ^R , spontaneous chemoautotrophic competent variant of strain CBBX	This study
CBBAI	Sm ^r , <i>cbbA</i> ::Tp ^r , previously designated <i>cfxA</i>	8
1312	Sm ^r , <i>cbbR</i> ::Tp ^r	12
1884	Sm ^r , <i>cbbF</i> ::Tp ^r	8
310	Sm ^R , <i>cbbF</i> ::Tn5Km ^r	7
Plasmids		
pUC18	Ap ^r	33
pUC1318	Ap ^r , pUC vector with hybrid cloning site	17
pSUP202	Cm ^r , Tc ^r , Ap ^r , Tra ⁻ , Mob ⁺ , pBR325 replicon	31
pJP5603	Km ^r , Mob ⁺ , R6K replicon	26
pHP45Ω	Ap ^r , containing Ω cassette encoding Sp ^r /Sm ^r	27
pUC1889ΔE	Ap ^r , Tp ^r , pUC18-89 with <i>EcoRI</i> site filled in	8
pJG336	Tc ^r , pVK102 containing <i>cbbR</i> , <i>cbbI</i> , and <i>cbbXYZ</i> within 24-kb insert	10
pJG106	Tc ^r , pVK102 containing <i>cbbII</i> genes within 26-kb insert	10
pJG45	Ap ^r , pUC8 containing <i>cbbA</i> ₁ <i>L</i> ₁ <i>S</i> ₁ <i>X</i> within 4-kb <i>SmaI</i> insert	10
pVK12	Tc ^r , Ap ^r , pVK102 containing <i>cbbR</i>	12
pUC232E	Ap ^r , pUC18 containing 2.3-kb <i>EcoRI</i> insert of pJG336	This study
pUC45S	Ap ^r , pUC8 containing 1.5-kb <i>Sall-SmaI</i> fragment of pJG45	This study
pUC45S::Sp	Ap ^r , Sp ^r , pUC45S containing Sp ^r Ω cartridge in <i>BamHI</i> site within <i>cbbX</i>	This study
pSUP45	pSUP202 containing <i>EcoRI</i> fragment of pUC45S::Sp	This study
pUC1323	Ap ^r , 2.3-kb <i>EcoRI</i> insert of pUC232E in pUC1318	This study
pUC1323::Sp	Ap ^r , Sp ^r , pUC1323 containing <i>BamHI</i> deletion and <i>cbbYZ</i> Sp ^r cartridge insertion	This study
pUC1323::Tp	Ap ^r , Tp ^r , pUC1323 containing Tp ^r cartridge in <i>PstI</i> site within <i>cbbZ</i>	This study
pJP1323::Sp	Km ^r , Sp ^r , pJP5603 containing <i>EcoRI</i> fragment of pUC1323::Sp	This study
pJP1323::Tp	Km ^r , Tp ^r , pJP5603 containing <i>EcoRI</i> fragment of pUC1323::Tp	This study

Insertional mutagenesis. Inactivation of *cbbX* was accomplished by insertion of the spectinomycin omega cartridge into a unique *BamHI* site that lies within the *cbbX* coding sequence in plasmid pUC45S (Fig. 1). The resultant plasmid was linearized with *EcoRI* and ligated to pSUP202. The suicide plasmid derivative pSUP45 was mobilized into *R. sphaeroides* via *E. coli* SM10 in a diparental mating as described previously (8), and spectinomycin-resistant transconjugants were screened for plasmid loss on agar plates containing tetracycline. Southern hybridization confirmed that gene replacement had occurred. The 2.3-kb *EcoRI* fragment (Fig. 1), containing the *cbbY* and *cbbZ* sequences, was subcloned into pUC1318 to eliminate vector *PstI* and *BamHI* sites. An internal 0.6-kb *BamHI* fragment was then replaced with the spectinomycin resistance cartridge, resulting in the interruption of both the *cbbY* and the *cbbZ* sequences. The same *EcoRI* construct, pUC1323, was used to insert a trimethoprim resistance gene into a unique *PstI* site which is located at the 3' end of *cbbZ*. In each case, the *EcoRI* fragment containing the disrupted gene(s) was ligated into the *EcoRI* site of pJP5603, mobilized into *R. sphaeroides* via S17-1 (*λ pir*), and screened for kanamycin sensitivity to indicate loss of vector sequences. Southern hybridization was used to confirm the double crossover events and replacement of wild-type sequences.

Enzyme assays and immunological techniques. Cell extracts were prepared from 20 ml of cells that were harvested, washed with TEM (25 mM Tris-Cl, 1 mM EDTA, 5 mM 2-mercaptoethanol), and suspended in 1.0 ml of TEM. Cells were broken by sonication and centrifuged for 10 min in a microcentrifuge to remove unbroken cells and debris. The supernatant fraction was used to assay RubisCO (9) and PGP (30). Protein was measured according to the modified Lowry protocol described by Markwell et al. (22). To quantify the amount of RubisCO protein, rocket immunoelectrophoresis was performed as previously described (16), using antibodies directed against form I and form II RubisCO.

Nucleotide sequence accession number. The GenBank nucleotide accession number for the sequence data presented herein is U67781.

RESULTS

Nucleotide sequence of *cbbX*, *cbbY*, and *cbbZ*. The nucleotide sequence for both strands of approximately 2.9 kb of DNA downstream of *cbbS*₁ was determined and is shown in Fig. 2. Previously, the sequence up to position 294 was reported as

FIG. 1. Organization and insertional mutagenesis of *cbbX*, *cbbY*, and *cbbZ*. (A) Genetic map of the *cbb1* and *cbbXYZ* gene clusters. Arrows denote direction and size of potential transcripts. (B) Physical map of the *cbbXYZ* cluster and constructs used for insertional mutagenesis. Vector DNA is not shown. Restriction sites: B, *BamHI*; E, *EcoRI*; P, *PstI*; S, *SmaI*; Sm, *SmaI*. For the CBBYZ strain, the hatched area denotes DNA that was deleted in construction of the mutant. The growth phenotype for each strain is indicated for photoheterotrophic growth on malate (PH) or photoautotrophic growth on 1.5% CO₂-98.5% H₂ (PA).

GCCGCTCTCCACGGATCGCGGACGCGGCCAAGGCCGGGGCTTCGCGCCCCCGGACCCCGCGGGATATTGACCCAGAGTGAGAGGGGGACTGCGACGAATGGCTGGCTTGAC 120
 cbbX ->
 CGGCGCTGCGCGGGCTACTCTCTCAACGACAGCTATCGGAGTTTCGCGATGACCGACGCGCAACGGCCCCACCTCGATCGACCTGCGCGCCGAATATGAGGGATCGGGCGCAAGGA 240
 M T D A A T A P T S I D L R A E Y E G S G A K E
 GGTGCTCGAGGAGCTCGACCGCGAGCTGATCGGGCTGAAGCCGGTCAAGGACCGGATCGCGAGACGCGCGCTTCTTCTTGTCGAGCGGGCGCGCAGAAGCTGGGCTTCCCCATGA 360
 V L E E L D R E L I G L K P V K D R I R E T A A L L L V E R A R Q K L G L A H E
 GACGCCACGCTGACATGAGCTTACCGGCAATCCCGGACCGGCAAGACCGCTCGCGCTGAAGATCGCGGGCTGCTGCACCGCTCGGCTATGTCGCAAGGGACATCTGGTCAG 480
 T P T L H M S F T G N P G T G K T T V A L K M A G L L H R L G Y V R K G H L V S
 CGTCACGCGCGAGCTGCTGGTGGGGCAGTATATCGGCCATACCGGCCCCAAGACGAAGGAGGTTCTCAAGCGCGCATGGGCGGCTGCTCTTCATCGACAGGCTATTACCTCTACAG 600
 V T R D D L V G Q Y I G H T A P K T K E V L K R A M G G V L F I D E A Y Y L Y R
 GCCCGACAACGAGCGGACTACGGGCGAGGAAGCCATCGAGATCTGCTTCAGGTGATGGAGAACAACCGCGACGACCTCGTGGTGTATCTTGCAGGATACGCGGATCGGATGAGAAATTT 720
 P D N E R D Y G Q E A I E I L L Q V M E N N R D D L V V I L A G Y A D R M E N F
 CTTCAGTGAATCCCGCTTCGCTTCGCGCATCGCGCATCACATGAATTCGCGACTACAGCGACGAAGAGCTGTTCGAGATCGCAGGACACATGCTGGACGACGAGAATATCAGAT 840
 F Q S N P G F R S R I A H H I E F P D Y S D E E L F S I A G H M L D D Q N Y Q M
 GACCCCGAGGCGGAGACCGCGCTGCGCGCTATATCGGCTGCGCGCAACAGCGCATTTCCGCAATGCCGCTCGATCCGCAATGCGCTCGACCCCGCGCGCTCGCGCAGGCCAA 960
 T P E A E T A L R A Y I G L R N Q P H P A N A R S I R N A L D R A R L R Q A N
 CCGGCTGTTCACGCTCTGTCGGGGCGCTCGACGCGCGCGCTCTCGACCATGGCCGAGGAAGACATCCGCGCGAGCGGGTGTTCAGGGCGGGCTCGACAGCGAGCGCGCGCGC 1080
 R L F T A S S G P L D A R A C C L T C S T M A E E D I R A S R V F K G G L D S E R R A A
 cbbY ->
 GGAGGCTCTGGCCAGATGACGAGGCGATCTTTTCGACGTGGACGGGACGCTCGCGGAGACCGAGGAGCTGCACCGCGCGCTTTAACGAGACCTTCGCGCGCTGGGGTGGACTGG 1200
 M I E A I L F D V D G T L A E T E E L H R R A F N E T F A A L G V D W
 E A L A R *
 TTCTGGGACCGCGAGGAATACCGCGAGCTTCTGACCAACACGGGCGGCAAGGACGCGCATCGCGCGGTCTCTGCGCCATCAGAAGGGCGATCCGGCGCGCTGCCCATATCCAC 1320
 F D R E E Y R L T T T G G K E R I A L F L H Q K G D P A P L P I A I H
 CGCGCAAGACCGAGCGGTTCTGTCGCTGATGGCCGAGGGCGAGATCGCGCTGCGCGCGGATCGCGGACCTGATCGCGGAGCGAAGCGCGCGCGGATCGCGCTCGCGCTGGCCACC 1440
 R A K T E R F V A L M A E G E I A L R P G I A D L I A E A K R A G I R L A V A T
 ACCACGAGCTTCCCAATGTCGAAGCCCTGTGCGGGGCTGTTTCGCGCATCCGCGCGCGAGATCTTCGACGTGATCGCGGCGGAGACATGTCGCGGAGAAAGCCCTCGCGCGGAC 1560
 T T S L P N V E A L C R A C F G H P A R E I F D V I A A G D M V A E K K P S P D
 ATCTACCGGCTTGCCTGCGGAGCTCGACGTGCGCGGAGCGGGCGCTCGCGCTGGAGGATTGCTCAACGCTCTTCGGGCGCGCAAGGGGCGGCTCGCGCTCGCTCGCGCT 1680
 I Y R L A L R E L D V P P E R A V A L E D S L N G L R A A K G A G L R C I V S P
 cbbZ ->
 GGCTTACACCCGCGACGAGGAGTTTCGCGGGGCGGACCGGCTCTCGACAGTTTCGCGGAGCTCGCGGGCTGCGCGGCTCGACCTGACGCGCGCGTGGCTGCGCGGAGTTCG 1800
 G F Y T R H E E F A G A D R L L D S F A E L G G L A G L D L T A P V A *
 M P G V
 TCTTCGATCTGACGGGACCTCGTCCATTCGCGACCGACATCCATGCGCGCTCAACAAGCGCTGCGCGAGGAGGAGGCGCCCTTCACGCTTCCGAGATCACCGGCTTCATCG 1920
 V F D L D G G T L V H S A P D I H A A V N K A L A F L E E G G A P F T L A F I T G F I
 GCAACGCGGTGCGGCTGCTGATCCAGCGCTGCTGCGCGCGGGGCGAGGCGCGGATGCGCACCGCCAGGCTGAGCTTCAGGGCGGTTTATGCGCGATTACGAGGCGGATCCGCGGA 2040
 G N G V P V L I Q R V L A A R G E A P D A H R Q A E L Q G R F M A H Y E A D' P A
 CGCTACCGAGCTCTATCCCGGTGCGGAGGCGGCGATCCGCGACCTGCGTGAAGGCGGCTGCGGCTCGCGCTTCGACCAACAAGCGGCTGGGCGGCTCTCGCGAGATCTGTCGCTCT 2160
 T L T S Y P V G A E A I R H L R A E G W R I G L C T N K P V G A S R Q I L S L
 TCGGCTGCTCGAGCTGTTGATGCCATCATCGCGCGGAGAGCTGCCCCGAGCGCAAGCCAGACCCGCGCGCTCGCGCGGAGCGCGCGCTGAAAGGAGGCTCTGCTCTATG 2280
 F G L L E L F D A I I G G E S L P Q R K P D P A P L R A T A A A L N E E V V L Y
 TGGGCGACAGCGAGTGGATCCGCCACCGCGGAGGCGCGGGCTGCGCTTTCGCTCTTTCACGAGGCTATCGCCATGCGCGGCTGATGAGTTGCGCGACCGAGGCTCTTCAGAC 2400
 V C D S E V D A A T A E A A G L R P A L F T E G Y R H A P V H E L P H H G L F S
 ATCAGCAGAGCTGCGAGCTCTTCGAGCGGCTTTCGCGTGAAGGCGGCGGCTTATCTTTCGCGAATCATCGCGGAGGCGCGCATGGCTGGAGCGGACCGAGGACCGGA 2520
 H H D E L Q D L L R L L A *
 CTGGGTGGAGCGGCGCGGCGACCGGCTGTGGCTTCTGGATCAGGCGCGGGGCTGTTTCGACCTTTCGACCGCGCTGATCCGCGCGGCGCTTTCATCGCTTCGACCTGCGCGGCC 2640
 AGCGCTGGGCGGCGCGGCGGCGAGGCTGCTGATCCACGACAGCGCGCATGCTCCATGTTACACGCTCGCGCGGAGCTGGGGCTGCGCGCGCGGACCGGATGATCGACCAOG 2760
 GGATCGAGAGCTCTGTTCCCGCCACCGGATCCCGGATGCGCGCTATGCTGGAGCTTCCATCCCGACGGGCGGTTGAACGCTCGAAGCAGGCTTACGCGCATGCTTCTGCTCC 2880
 TGGCGCGGCTTCGCGCCCG 2900

FIG. 2. Nucleotide and deduced amino acid sequences of the *R. sphaeroides* *cbbXYZ* genes. The predicted amino acid sequence is given below the nucleotide sequence. Putative ribosome binding sites are indicated by a double underline, and stop codons are indicated by asterisks. ORFs are identified above the start site of the respective gene.

part of the *cbb*₁ cluster (8). However, this region, which was beyond the area fully studied, was analyzed only cursorily in this earlier report. Several discrepancies have been discovered and corrections made in this region in this study, and analysis of the sequence including and beyond position 294 for potential protein coding sequences revealed three closely linked ORFs, beginning 170 nucleotides downstream from *cbbS*₁, that were oriented in the same direction as the genes in the *cbb*₁ operon. All three ORFs show G+C codon bias in the third position typical for *R. sphaeroides* genes.

A computer search of the data banks revealed significant similarity of the three deduced amino acid sequences to three previously described coding sequences associated with aerobic

chemoautotrophic bacterial *cbb* operons (19, 23). The first ORF in the gene cluster extends 927 nucleotides from an ATG start codon at position 170 to a TGA stop codon at position 1096. A search for amino acid homology with other proteins showed this protein to be similar to CbbX of chemoautotrophic bacteria. The *R. sphaeroides* CbbX shares 73 and 65% identical amino acid residues with the coding sequences from *Xanthobacter flavus* (23) and *Alcaligenes eutrophus* (19), respectively. As noted previously, bacterial *cbbX* has a eucaryotic counterpart in the chloroplast of a red alga, *Antithamnion* sp. (18), found some distance downstream from RubisCO coding sequences. Database searches revealed two more *cbbX* homologs, *cfxQ* (accession number ZC7753) and ORF301 (acces-

sion number U38804) from the nongreen algae *Odontella sinensis* and *Porphyra purpurea*, respectively. The deduced amino acid sequences of the algal genes have approximately 65% identity with the bacterial coding sequences. Although both algal sequences are plastid encoded, only the *Porphyra cbbX* analog is found immediately downstream from the RubisCO genes, as are all of the bacterial *cbbX* genes thus far described. In *Odontella*, *cfxQ* is situated 12 kb upstream and oriented in the opposite direction of the RubisCO coding sequences. Significant similarity was also noted between *spoVJ* of *Bacillus subtilis* (5) and a partial sequence of a gene adjacent to *glgB* in *Butyrivibrio fibrisolvens* (28). Alignment of the deduced amino acid sequences revealed several regions of invariant residues among the CbbX-like sequences, including a putative ATP binding site situated at position 75 in the *R. sphaeroides* sequence (Fig. 3A).

The second ORF in the *R. sphaeroides* cluster begins at an ATG codon at position 1096 that overlaps the TGA stop codon of *cbbX* (Fig. 2). Database searches found the ORF to have 31% identity with the *cbbY* coding sequence of *A. eutrophus* (19).

Database searches with the third ORF in the sequence revealed 37% identity with *cbbZ*, encoding PGP in *A. eutrophus* (30). A potential start site of *cbbZ*, based on alignment with *cbbZ* of *A. eutrophus*, is an ATG codon that overlaps the TGA stop codon of *cbbY*. Recently, a *cbbZ* gene has been identified in the cyanobacterium *Synechococcus* sp. strain 7942 (20). The cyanobacterial gene has 29% identity with the purple bacteria counterparts and is not linked to any other *cbb* sequences. Interestingly, *cbbY* and *cbbZ* show significant similarity (30). The *R. sphaeroides* CbbY has 26% identity with CbbZ. In Fig. 3B, alignments of CbbZ predicted amino acid sequences are shown. The *R. sphaeroides* CbbY sequence is included to illustrate the relatedness of the two sequences. Moreover, database searches have revealed hypothetical ORFs with significant similarity to *cbbY* and *cbbZ* in organisms that do not possess a CBB cycle. In *E. coli*, a gene product within the *dam*-containing operon has actually been shown to possess PGP activity (21). Examples of other bacteria in which *cbbY*-*cbbZ* homologs have been identified include *Pseudomonas putida* (accession number P42509), *Pseudomonas syringae* (accession number 42510), *Haemophilus influenzae* (accession number P44755), and *Lactobacillus casei* (accession number P35924).

Analysis of the nucleotide sequence downstream of the *cbbXYZ* cluster revealed a partial coding sequence that begins at an ATG codon at position 2492 and is preceded by a reasonable ribosome binding site. No similarities were found to any sequence in the database. However, a partial ORF that begins at an ATG codon at position 2694 has 30% identity with a hypothetical protein (accession number P32140) encoded in the region between *glnA* and *fdhE* in *E. coli*. Further work is in progress to determine whether this sequence is in any way involved in CO₂ fixation.

Insertional mutagenesis of *cbbXYZ*. Initial attempts at uncovering the role of the *cbbX*, *cbbY*, and *cbbZ* gene products in *R. sphaeroides* were based on analysis of mutants in which these genes had been disrupted. Three mutants, CBBX, CBBXY, and CBBZ, in which *cbbX*, *cbbYcbbZ*, and *cbbZ*, respectively, were inactivated by insertional mutagenesis (Fig. 1B), were initially screened for the ability to grow under conditions that require CO₂ fixation. In cultures cultured photoheterotrophically, containing malate or butyrate as the electron donor, growth of all three mutant strains was indistinguishable from that of wild-type strain HR. However, upon examination of the mutants on agar plates that had been incubated under photoautotrophic conditions, strain CBBX was found to be impaired

in growth, whereas strains CbbYZ and CbbZ showed growth characteristics of the wild-type strain. In photoautotrophic liquid cultures grown under a gas atmosphere of 1.5% CO₂–98.5% H₂, the CBBX strain routinely exhibited a lag that lasted 5 to 10 days before growth occurred. The ability to grow after the extended lag period was the result of a stable alteration, since subsequent transfer of the culture resulted in growth with no lag, essentially paralleling the growth of the wild type (data not shown). Complementation of the CBBX strain was achieved with plasmid pJG336, which contains the *cbb_I* operon in addition to the *cbbX*, *cbbY*, and *cbbZ* genes. Plasmid pJG106, which carries the *cbb_{II}* operon, was unable to complement the CbbX mutant to photoautotrophic competence.

RubisCO and PGP activities in *cbbXYZ* mutants. RubisCO activity in the *cbbXYZ* mutants was similar to that determined for the wild-type strain HR both in absolute activity and in the degree of induction observed between photoheterotrophic and photoautotrophic growth conditions (Table 2). RubisCO activity measured for strain CBBZ after photoautotrophic growth was routinely higher than that measured for strain HR, although the relative levels of the form I and form II RubisCO proteins were identical, as determined by rocket immunoelectrophoresis (data not shown). No PGP activity could be detected in any of the mutants, suggesting the three genes form part of an operon (Table 2).

Since the substrate of PGP, phosphoglycolate, is the product of the oxygenase reaction of RubisCO, it was of obvious interest to determine whether the *cbbX*, *cbbY*, and *cbbZ* gene products were necessary for chemoautotrophic growth. *R. sphaeroides* is normally not capable of chemoautotrophic growth, although the closely related organism *R. capsulatus* is able to grow well under these conditions. Recently, however, it was found that cultures of *R. sphaeroides* could stably adapt to chemoautotrophic growth after a prolonged incubation of several weeks (25). Interestingly, strain CBBX was found to eventually grow chemoautotrophically following incubation for a similar prolonged period of time. The resultant chemoautotrophic growth of this strain paralleled that of the chemoautotrophic competent wild-type strain, CAC; however no PGP activity was detected. The RubisCO activity measured in strain XCAC, the chemoautotrophic competent variant of strain CBBX, grown chemoautotrophically (Table 2) was comparable to the RubisCO activity in strain CAC grown under the same conditions (Table 3).

Expression of RubisCO and PGP in the wild-type strain HR.

Another approach that we have taken toward understanding the physiological role of the *cbbX*, *cbbY*, and *cbbZ* gene products in CO₂ fixation is to examine the expression pattern of the genes in the wild-type strain HR. Since the three genes appear to be cotranscribed, PGP activity was used to monitor transcription of the operon. The levels of PGP and RubisCO were measured in wild-type strain HR cultured under conditions known to affect *cbb* expression and the level of CBB cycle enzymes (Table 3). For the most part, the PGP activity pattern paralleled that observed for RubisCO. In chemoheterotrophic cultures, PGP and RubisCO were present at very low levels, whereas photoheterotrophic growth in the presence of malate resulted in intermediate levels of both enzymes (Table 3). PGP and RubisCO were maximally expressed during photoautotrophic growth under an atmosphere of 1.5% CO₂–98.5% H₂. Also included are results obtained for CAC, a spontaneous variant of strain HR that is capable of chemoautotrophic growth with O₂ as the terminal electron acceptor (25). Since phosphoglycolate is the product of the RubisCO oxygenase reaction, it was of interest to determine whether O₂ affected

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RsMTDAATAPTSIDLRAE.....YEGSGAK.EVLEE 50
 XfMLDVATSAFSAALPAEAAEGRDLGALFTFSEVP.EFLAE
 AeMSAPETAPLQPPAAQAASLPGLAESLA.SGITSELLAQ
 OsMSSMDTSVNLQEEYNKTEIA.KILNL
 PpMTQLTNIIVSNDTLVNLQEEYDKTQIQ.EVLNE
 Bs MLERAVTYKNNGGINIILNGQKQVLTNAEAEAYQAALQKNEAKHGILKE
 *
 Rs LDRE...LIGLKPVKDRIRETAALLLVERARQKLGLAHETPTLHMSFTGN 100
 Xf LDRE...LIGLKPVKRRIRETAALLVIGRAREKLGTSGLTHMAFTGN
 Ae LDRE...LIGLKPVKARIRDTAALLVLDKLAARGFSAAGASLHMCFTGN
 Os LNEE...LVGLVPKSRIRETAALLLIDKLRKNGITAGSPGLHMSFTGS
 Pp LNQE...LIGLVPVKTRIRETAALLLIDLRRLKLELVGNSPGLHMSFTGS
 Bs IEKMSALVGMEMKRNKIKIYAWIFVFNQKRAEQGLKVGKQALHMMFKGN
 * * * * *
 Rs PGTGKTTVALKMAGLLHRLGVYVRGHLVSVTRDDLVGQYIGHTAPKTKEV 150
 Xf PGTGKTTVALKMAQILHRLGVYVRGHLVSVTRDDLVGQYIGHTAPKTKEI
 Ae PGTGKTTVAMRMAQILHRLGVYVRGHLVAVTRDDLVGQYIGHTAPKTKEI
 Os PGTGKTTVGLKMSDILFQGLYIKKGHLVTRDDLVGQYIGHTAPKTKEV
 Pp PGTGKTTVAMKADILHRLGVYIKKGHLVTRDDLVGQYIGHTAPKTKEV
 Bs PGTGKTTVARLIGLKFEMNVLSKGHLIAERADLVGEYIGHTAQKTRDL
 * * * * *
 Rs LKRAMGGVLFIDEAYLYRPDNERDYGQEAIEILLQVMENNRDDLVLVLA 200
 Xf LKRAMGGVLFIDEAYLYRPDNERDYGQEAIEILLQVMENNRDDLVLVLA
 Ae LKALGGVLFIDEAYLYRPDNERDYGQEAIEILLQVMENNRDDLVLVLA
 Os LKRAMGGVLFIDEAYLYKPNEDRGYSAIEILLQVMENNRDDLVLVLA
 Pp LKQAMGGVLFIDEAYLYKADNERDYGSAIEILLQVMENNRDDLVLVLA
 Bs IKKSLGGVLFIDEAYLSLAR.GGEEKDFKEADITLVKHMEDKQHEFILLVA
 * * * * *
 Rs GYADRMENEFQSNPGRSRIAHHIEFPDYSDIEELFETAGHMLDDQYQMT 250
 Xf GYKDRMDRFFESNPGFRSRIAHHIDFPDYDEAEIVETAKTMAADADYTF
 Ae GYKDRMDRFFESNPGMSRVAHVDFPDYQDLDELQIADLMLAEMQYRFD
 Os GYKPMKDFYESNPGLSRIAHHIDFPDYVEELKISKMLLEEQQYQMT
 Pp GYKDRMEKFYESNPGLSRVAHVDFPDYTSSELLQAKMIEEQYQCLT
 Bs GYSREMDHFLSLNPGQSRFPISIDFPDYSVTQLMETAKRMIDEREYQLS
 * * * * *
 Rs PEAEATLRAVYIGLRNQP...HFANARSIRNALDRARLRQANRLFTASSG 300
 Xf PEAEVAIEEYVAKRRIQF...NFANARSIRNALDRMLRQSLRL.F.BSGG
 Ae DESRAVFAVYALARRMAQF...HFANARSIRNALDRARLRHARLLDAGT
 Os TOAEVALTOYIARRKKEK...LFANARSIRNALDRARLRQANRIFDSRGQ
 Pp EADKTLLEYTERMRKQF...YFANARSIRNALDRARLRQANRIFASGSK
 Bs QEAEWKLDYLMVTKSTSPKIFNGRFRVNVIEKSIIRAQANRLMGDQY
 * * * * *

B

RsZMPG VVFDLDGLTV HSAPDIIHAHV NKALAEEGGA PF.TLAEITG 50
 AeZ MATVSMPCTA VLIDDLDGLTV DSAPDIEVAA NRMLADFGSP AL.PDFTVAG
 EcZ .MNKFEDIRG VAFDLDGLTV DSAPGLAAAV DMALYALELP VA.GEERVIT
 SyZLQA IIFDLDGLTV DSLPTVVAIA NAHAPDFGYD PI.DERYAQ
 RsYMIEA LFDVDGLTGA ETEELHRRAP NETFAALGVD WFWWDREYRE
 * * * * *
 RsZ FIGNGVPLVI QRV.LAARGE APDAHQRAEL QG.....R 100
 AeZ FIGRGVPLV RRV.LETAGL TPVRV.AAEA VA.....M
 EcZ WIGNGADVLM ERA.LTWARQ ERATQKTMG KPPVDDDIPA EQQVRLRLK
 SyZ LRQWSRTIV RRAQSPWQQ ARLLQR.....
 RsY LLTTTGK... ..ERLAFR LRHQKGDPA LPDIADIRAK
 * * * * *
 RsZ FMAHYEADPA TLTSYVPGA AAIHRLRAEG WRIGLCTNKP VGASRQLLSL 150
 AeZ FHRHYAETNG RLGSVFPGE AGLEALRRQG YRLACVTNKP RALAVPLAL
 EcZ FDRYGEVAE EGTFLFPHVA DTLGALQAKG LPLGLVTNKP TPFVAPLLEA
 SyZ VQRQLGDCLP ALQ.LFPQVA DLLAQLSRS LCLGILSSNS RQNIAPLQR
 RsY TERFVALMAE GEALRPGIA DLIAEAKRAG IRLAVATTS LPNVLEACRA
 * * * * *
 RsZ ...FGLLELF DAIIGGESLP QRKPDPAFLR ATAAALNEEV .VLVVGDS 200
 AeZ ...TGLSQYL EVLVAGDSIA QMKPDPEPLR HACNLLDVT AQGLVVGDS
 EcZ ...LDIAKYF SVVIGGDDVQ NKKPHDPPLL LVAERMGIAP QQMLFVGDS
 SyZ ...QGLRSIF SVVQAGTPII SKRR...ALS QLVAREGWOP AAVMFDGDET
 RsY CFHGPAREIF DVIAAGDMVA EKKSPDIYR LALRELDVPP ERAVALEDL
 * * * * *
 RsZ VDAATAEAAAG LRFALFTEGY .RHAPVHELP HHGLFSHHDE LQDLRL..RL 250
 AeZ VDVAARAAG IPVCLVRYGY AGPGGPAALG ADALLDSLEA LPALLTPARL
 EcZ NDIQAARAAG CPVGLTYGY NYGEAIDLQ PDVIYQSDND LLPALGLPHS
 SyZ RDVEAARQVG LIAVAVTWGF NDRQSLVAC PDWLLETSPD LQAVTQLMR
 RsY NGLRAAKGAG LRCIVSPGY TRHEFA..G ADRLDSFAE LGGLAGLDLT
 * * * * *
 RsZ LA*.....
 AeZ APAA.....
 EcZ ENQESKND
 SyZ Q*.....
 RsY APVA*.....

FIG. 3. Predicted amino acid sequences of the *cbbX* and *cbbZ* gene products and alignments with related sequences from various organisms. (A) The derived amino acid sequence of *cbbX* from *R. sphaeroides* (Rs) aligned with CbbX sequences from *X. flavus* (Xf) (23), *A. eutrophus* (Ae) (19), *O. sinensis* (Os) (GenBank accession number Z67753), and *P. purpurea* (Pp) (GenBank accession number U38804) and the SpoVJ sequence from *B. subtilis* (Bs) (5). Shaded areas represent residues that are invariant within all CbbX sequences. Residues within the SpoVJ coding sequence that have identity with CbbX are also shaded. Asterisks indicate residues that comprise a consensus ATP binding site. Dots

TABLE 2. RubisCO and PGP activities in *cbbXYZ* mutant strains

Strain	Growth condition ^a	Activity (nmol/min/mg) ^b	
		RubisCO	PGP
HR	PH	48	23
	PA	295	89
CBBX	PH	47	ND
	PA	40	ND
CBBYZ	PH	263	ND
	PA	45	ND
CBBZ	PH	352	ND
	PA	124	ND
XCAC	PH	48	23
	PA	295	89

^a PH, photoheterotrophic growth on malate; PA, photoautotrophic growth on 1.5% CO₂-98.5% H₂; CA, chemoautotrophic growth on 5% CO₂-50% H₂-45% air.

^b Average of three independent cultures. In separate experiments, the absolute value for RubisCO varied as much as twofold and that for PGP varied up to fourfold; however, the pattern of activity relative to the wild-type strain was always the same. ND, not detected.

the levels of PGP. At least under the growth conditions described, the presence of O₂ did not result in levels of PGP higher than those observed under anaerobic conditions, nor did the presence of O₂ cause a substantial diminution (less than twofold) in the level of RubisCO (Table 3).

Analysis of PGP activity in *cbb* mutant backgrounds. PGP activity was also measured in several mutant strains that show altered CBB cycle gene expression. Expression of the *cbb_I* and *cbb_{II}* operons in *R. sphaeroides* is coordinated through a common regulator, CbbR (12). Mutations in the promoter-proximal gene, *cbbF* (encoding fructose 1,6/sedoheptulose 1,7-bisphosphatase), of the *cbb_I* and *cbb_{II}* gene clusters abolish transcription of the entire operon and cause a CbbR-dependent overexpression of the unaffected operon such that RubisCO levels appear normal. As shown in Table 4, RubisCO activity in the *cbbF* mutants grown under photoheterotrophic conditions is actually higher than that observed in the wild-type strain although only one form of RubisCO is synthesized in each strain. During photoheterotrophic growth, PGP activity does not seem to be affected by mutations in either the *cbb_I* or the *cbb_{II}* operon. PGP activity in the *cbbF_I* mutant implies the presence of a promoter separate from that directing expression of *cbb_I* genes.

Since the *cbbX*, *cbbY*, and *cbbZ* genes appear to be transcribed from a new promoter, it was of interest to determine whether this promoter was subject to regulation by CbbR. The insertion within the *cbbR* gene in strain 1312 in some way interferes with transcription of the *cbb_I* operon such that the observed complementation of strain 1312 with the *cbbR* gene supplied in *trans* is the result of overexpression of the *cbb_{II}* genes; the *cbb_I* genes are barely expressed (12). In contrast, when strain 1312 is complemented to photoautotrophic growth with a cosmid containing the *cbb_{II}* operon, no synthesis of the *cbb_I*-encoded enzymes is observed and the *cbb_{II}* genes are not overexpressed in the absence of a functional copy of CbbR. PGP activity measured in the two photoheterotrophically grown and complemented 1312 strains did not seem to be

represent gaps introduced to maintain alignment. (B) The deduced amino acid sequence of the *R. sphaeroides* *cbbZ* gene product (RsZ) aligned with related sequences: AeZ, *A. eutrophus* CbbZ (30); EcZ, *E. coli* CbbZ (21); SyZ, *Synechococcus* sp. strain 7942 CbbZ (20); and RsY, *R. sphaeroides* CbbY. Asterisks indicate residues shared by at least four of the sequences, and dots represent gaps necessary for alignment. Alignments in both panels were generated by using the PILEUP program of the Genetics Computer Group package.

TABLE 3. Activities of RubisCO and PGP under different growth conditions

Growth condition ^a	Strain	Activity (nmol/min/mg) ^b	
		RubisCO	PGP
CH	HR	0.4	4.9
PH	HR	22	14
PA	HR	295	89
PA	CAC	189	110
CA	CAC	106	50

^a CH, chemoheterotrophic growth on malate; PH, photoheterotrophic growth on malate; PA, photoautotrophic growth on 1.5% CO₂-98.5% H₂; CA, chemoautotrophic growth on 5% CO₂-50% H₂-45% air.

^b Average of three independent cultures (see Table 2), footnote b.

affected by the presence of CbbR (Table 4). However, measurement of PGP activity in the same mutants grown photoautotrophically revealed an interesting feature of PGP expression (Table 4). In this growth mode, in which both PGP and RubisCO activities are maximally expressed in the wild-type strain HR, PGP activity appeared to be affected not by CbbR directly but by transcription of the *cbbI* operon. In the mutants that have little or no transcription of *cbbI* genes (strains 1884 and 1312), regardless of the presence of CbbR, the PGP activity is threefold lower than that observed in the wild-type strain and is actually higher in the *cbbF_{II}* mutant in which the *cbbI* genes are overexpressed (Table 4).

DISCUSSION

The *cbbX*, *cbbY*, and *cbbZ* genes were identified by sequencing the region situated immediately downstream from the *cbbI* operon of *R. sphaeroides*. The identities of the *cbbX* and *cbbY* gene products are not known, whereas *cbbZ* has been shown to encode PGP (30). Although the function is not known, *cbbX* shows a rather broad distribution in CBB cycle gene clusters, insofar as a related sequence has been identified downstream from a chloroplast RubisCO coding sequence in a red alga (18) in addition to its presence in chemoautotrophic bacterial gene clusters (19, 23). Interestingly, *cbbX* also has considerable sequence similarity with genes in unrelated systems, e.g., *spoVJ*, which is involved in some unknown way in sporulation in *B. subtilis* (5), and a gene adjacent to a glycogen-branching enzyme gene cluster in *Butyrovibrio fibrisolvens* (28). Although the functions of the gene products in these two systems are

unknown, a role specific to CBB cycle function can reasonably be ruled out. Also intriguing is the presence of *cbbY* and *cbbZ* homologs in other bacteria lacking the CBB cycle.

Characterization of the mutant strains in which *cbbX*, *cbbYZ*, or *cbbZ* were insertionally inactivated showed that all three strains exhibited wild-type growth characteristics during photoheterotrophic growth on malate and butyrate. However, the *cbbX* mutant was impaired in photoautotrophic growth on 1.5% CO₂-98.5% H₂; the *cbbYZ* and *cbbZ* mutants exhibited no discernible phenotype under these conditions. Based on these results, the growth defect is due to *cbbX*, and not *cbbY* or *cbbZ*.

No detectable PGP activity was observed in the *cbbX* mutant, presumably because of polarity on expression of the downstream gene *cbbZ*, although the possibility that CbbX is required for transcription of *cbbY* and *cbbZ* cannot be ruled out. However, the overlapping gene arrangement of *cbbX*, *cbbY*, and *cbbZ* is suggestive of an operon. In addition, the fact that PGP activity was observed in strains containing insertions upstream from *cbbX* implies the presence of a promoter distinct from that driving expression of the previously characterized *cbbI* operon. In spite of the presence of a separate promoter, the expression of PGP activity followed a pattern similar to that for RubisCO under the growth conditions examined, although regulation does not seem to be directly mediated by CbbR. Instead, an indirect effect of CbbR is apparently transmitted via readthrough transcription of the *cbbI* genes; this is most pronounced during autotrophic growth. The gene arrangement and control of expression of *cbbX*, *cbbY*, and *cbbZ* with respect to other CBB cycle genes in *R. sphaeroides* is distinct from that observed in the aerobic chemoautotrophic bacterium *A. eutrophus*. In *A. eutrophus*, *cbbX* and *cbbY* are linked and are positioned immediately downstream from RubisCO coding sequences (19), whereas *cbbZ* is located much further downstream within the *cbb* gene cluster (30). All three genes in *A. eutrophus* appear to be transcribed from a single promoter which is situated upstream of *cbbL* and therefore subject to similar control.

Although thus far it has not been possible to assign functions to the gene products of *cbbX* and *cbbY*, the transcriptional association with *cbbZ* in *R. sphaeroides* suggests a possible role concerning O₂, which could come into play either during chemoautotrophic growth in which the CBB cycle is functioning in the presence of O₂ or during transitions from photosynthetic conditions into oxygenic environments, either of which might be expected to lead to the deleterious accumulation of phosphoglycolate from the oxygenase reaction of RubisCO. Future experiments will explore these possibilities.

TABLE 4. RubisCO and PGP activity levels in *cbb* mutants

Strain	Plasmid	Growth conditions ^a	Activity (nmol/min/mg) ^b	
			RubisCO	PGP
HR	— ^c	PH	22	14
		PA	295	89
1884 (<i>cbbF_I</i>)	—	PH	40	13
		PA	304	30
310 (<i>cbbF_{II}</i>)	—	PH	69	13
		PA	310	133
1312 (<i>cbbR</i>)	pJG106	PH	57	10
		PA	51	31
1312 (<i>cbbR</i>)	pVK12	PH	28	19
		PA	209	27

^a PH, photoheterotrophic growth on malate; PA, photoautotrophic growth on 1.5% CO₂-98.5% H₂.

^b Average of three independent cultures (see Table 2), footnote b.

^c —, not present.

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