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Nucleotide Sequence and Functional Analysis of CbbR, a Positive Regulator of the Calvin Cycle Operons of *Rhodobacter sphaeroides*

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Structural genes encoding Calvin cycle enzymes in *Rhodobacter sphaeroides* are duplicated and organized within two physically distinct transcriptional units, the form I and form II *cbb* operons. Nucleotide sequence determination of the region upstream of the form I operon revealed a divergently transcribed open reading frame, *cbbR*, that showed significant similarity to the LysR family of transcriptional regulatory proteins. Mutants containing an insertionally inactivated *cbbR* gene were impaired in photoheterotrophic growth and completely unable to grow photolithoautotrophically with CO_2 as the sole carbon source. In the *cbbR* strain, expression of genes within the form I operon was completely abolished and that of the form II operon was reduced to about 30% of the wild-type level. The cloned *cbbR* gene complemented the mutant for wild-type growth characteristics, and normal levels of ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) were observed. However, rocket immunoelectrophoresis revealed that the wild-type level of RubisCO was due to overexpression of the form II enzyme, whereas expression of the form I RubisCO was 10% of that of the wild-type strain. The *cbbR* insertional inactivation did not appear to affect aerobic expression of either CO_2 fixation operon, but preliminary evidence suggests that the constitutive expression of the form II operon observed in the cbbR strain may be subject to repression during aerobic growth.

Purple nonsulfur photosynthetic bacteria are metabolically capable of growth under a wide variety of physiological conditions. During photo- and chemolithoautotrophic growth, CO_2 fixation through the Calvin cycle is essential for providing all of the cellular carbon. On the other hand, when cells are growing photoheterotrophically, the bulk of carbon is derived from fixed carbon sources and CO_2 fixation serves predominantly as an electron sink and may be supplanted by external electron acceptors ((24, 33). In *Rhodobacter sphaeroides*, two separate sets of Calvin cycle genes have apparently evolved to accommodate the different roles that CO_2 fixation plays under diverse sets of growth conditions (6, 10, 12, 13, 17). One set of genes, which constitutes the form I operon, encodes the Calvin cycle enzymes fructose 1,6-bisphosphatase (FBPase), phosphoribulokinase (PRK), fructose 1,6-bisphosphate aldolase (FBA), and an L_8S_8 ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) encoded by the $cbbF_I$, $cbbP_I$, $cbbA_I$, and $cbbL_I$ $cbbS_I$ genes, respectively (7). The second gene cluster, the form II operon, also contains genes coding for FBPase, PRK, FBA and a functionally distinct L_4 RubisCO encoded by the $cbbF_{II}$, $cbbP_{II}$, $cbbA_{II}$, and $cbbM_{II}$ genes, respectively (6). In addition, two genes, $cbbT_{II}$ and $cbbG_{II}$, that code for transketolase and glyceraldehyde 3-phosphate dehydrogenase are inserted between $cbbP_{II}$ and $cbbA_{II}$ (2). It should be noted that the gene designation *cbb*, which stands for the Calvin-Benson-Bassham reductive pentose phosphate pathway, has recently been adopted by several groups in order to provide a uniform designation for Calvin cycle genes in bacteria (30). For *R. sphaeroides*, the subscripts denote chromosomes I or II, since the two Calvin cycle gene clusters have been localized to the two different genetic elements of this organism (28). As might be expected for such an energetically expensive pathway, the expression of Calvin cycle genes has been shown to be tightly regulated. In aerobically growing cells, in which the Calvin cycle is not required, there is virtually no expression of these genes (17, 36). During photosynthetic growth, however, the expression of genes from the two operons becomes quite complex, responding independently to a variety of environmental factors, such as the level of CO_2 and the reduction state of fixed carbon compounds (3, 8, 12, 13, 17). Photoheterotrophic growth on carbon compounds such as malate or butyrate results in varying degrees of derepression or induction of both the form I and form II operons (3, 7, 12, 13, 17). The more reduced carbon source results in higher levels of Calvin cycle enzymes (29). Generally, the form II gene products predominate during growth on fixed carbon sources, under conditions of relative carbon and electron abundance (3, 7, 12, 13, 17). Alternate electron acceptors, such as dimethyl sulfoxide, repress synthesis of both sets of Calvin cycle enzymes during photoheterotrophic growth, underscoring the primary role that CO_2 fixation plays in balancing the redox potential of the cell under these conditions (4, 12, 13, 33). Expression of the form II genes appears to be more sensitive to repression by alternate electron acceptors than that of the form I genes (12, 13, 33). During photolithoautotrophic growth, in which CO_2 is the only source of carbon, maximum expression from both operons is observed, although the form I enzymes are induced to higher levels than are their form II counterparts (3, 7, 12, 13, 17). The differential expression of the two sets of Calvin cycle genes in response to varying nutritional conditions has led to speculation that the primary roles of the functionally diverse form I and form II RubisCOs differ, such that the major function of the form II RubisCO is in maintaining the redox

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TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Relevant characteristic(s)	Reference
Plasmids		
pUC9	Ap^r	32
pUC1318	Ap^r	18
pVK102	Tc^r Km^r, broad-host-range vector	20
pRK415	Tc^r, broad-host-range vector	19
pSUP202	Tc^r Ap^r Cm^r Mob^+	26
pUC1889Δ*Eco*RI	Ap^r Tp^r, in which the *Eco*RI site within the Tp^r cartridge was deleted	4
pJG336	Tc^r, pVK102 containing the form I Calvin cycle gene cluster	9
pJG106	Tc^r, pVK102 containing the form II Calvin cycle gene cluster	9
pUC12EH	Ap^r, pUC9 containing a 1.8-kb *Eco*RI-*Hin*dIII fragment of pJG336 which carries *cbbR*	This study
pUC1312	Ap^r, pUC1318 containing the 1.8-kb *Eco*RI-*Hin*dIII fragment of pUC12EH	This study
pUC1312Tp	Ap^r Tp^r, pUC1312 containing the Tp^r gene inserted into the *Xho*I site of *cbbR*	This study
pSUP1312	Ap^r Tc^r Tp^r, pSUP202 containing the 4.5-kb fragment of pUC1312	This study
pVK12	Ap^r Tc^r Mob^+, pVK102 containing pUC12EH inserted into the *Hin*dIII site	This study
pRPS429	Tc^r, pRK404 containing *R. sphaeroides cbbA cbbL cbbS* under control of the *R. rubrum* promoter	4
pRK411	Tc^r, pRK415 containing a 2.7-kbp *Xho*I fragment which carries $cbbF_I$ and $cbbP_I$	This study
R. sphaeroides strains		
HR	Wild type, Sm^r	34
1312	*cbbr*::Tp^r	This study
16	$cbbL_I$ $cbbS_I$::Km^r $cbbM_{II}$::Tp^r	4
$cbbM_{II}$ mutant	$cbbM_{II}$::Tp^r	4
1884	$cbbF_I$::Tp^r	7

poise of the cell, whereas that of the form I RubisCO is to provide cellular carbon (12, 13, 17, 33).

Although the form I and form II genes are subject to independent regulation, when expression of genes from either cluster is disrupted by insertional mutagenesis, synthesis of the gene products from the unperturbed operon is elevated so that enzyme levels reach the same or in some cases highly exceed the level observed in wild-type cells (3, 7, 12, 13). This compensatory effect suggested the existence of a common regulatory element that allows for communication between the duplicate sets of structural genes, and, on a broader scale, between the two chromosomes. A good candidate for mediating the coordinate regulation of Calvin cycle genes in *R. sphaeroides* would be a transcriptional regulatory protein capable of differentially affecting expression of genes within the two operons in response to a common inducer. In this regard, it is interesting to note that in several other bacterial systems in which enzymes of CO_2 assimilation have been studied, genes have been identified on the basis of sequence similarity with the LysR family of transcriptional regulatory proteins, which are divergently transcribed from Calvin cycle structural gene operons (21, 23, 31, 35). In one of these bacteria, *Alcaligenes eutrophus*, a physiological role of the putative regulatory protein has been demonstrated (35). The findings reported in this communication show that a single regulatory protein appears to be involved in positive regulation of genes from both the form I and form II operons of *R. sphaeroides*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmids and *R. sphaeroides* strains used in this study are listed in Table 1. *Escherichia coli* JM107 was used for maintenance and propagation of all pUC plasmid derivatives (32). *E. coli* SM10 (26) was used to mobilize pSUP202 constructs into *R. sphaeroides* HR (34), and *E. coli* MM294, carrying plasmid pRK2013, was used in triparental matings for mobilizing plasmid derivatives into *R. sphaeroides* for complementation analyses (5). *E. coli* strains were routinely grown in Luria-Bertani broth at 37°C. *R. sphaeroides* strains were grown aerobically in a peptone-yeast extract medium (34), photoheterotrophically on 0.4% malate in cultures bubbled with argon, or photolithoautotrophically in an atmosphere of 1.5% CO_2–98.5% H_2, as described previously (17). Photosynthetic growth on agar plates was carried out in GasPak (Scientific Products Division, McGaw Park, Ill.) anaerobic jars. Butyric acid was supplied at a final concentration of 0.2% and sodium bicarbonate was supplied at 0.1% when the organism was grown on solid media. Antibiotics were added to media as required at the following concentrations (micrograms per milliliter): for *E. coli*, ampicillin, 100; tetracycline, 25; trimethoprim, 200; and kanamycin, 25; for *R. sphaeroides*, streptomycin, 25; tetracycline, 12.5 or 1.0; and trimethoprim, 200.

DNA manipulations and sequence analysis. Plasmid DNA isolations, transformations, and other routine DNA manipulations were carried out by standard procedures (1). Conjugative crosses for complementation analysis were performed as described previously (3).

DNA sequencing was performed on double-stranded DNA templates by the dideoxy chain termination method of Sanger et al. (25) with the Sequenase kit obtained from U.S. Biochemical, Cleveland, Ohio, and ^{35}S-dATP obtained from New England Nuclear, Boston, Mass. Universal and reverse M13 primers obtained commercially and custom-made primers were used to sequence a 0.9-kbp *Xho*I-*Hin*dIII restriction fragment of pJG336. DNA sequences were determined completely on both strands of DNA. The DNA sequence upstream of the *Xho*I site at position 337 has been reported previously and is shown here to illustrate the position of *cbbR* relative to *fbpA* as well as the intergenic region. The nucleotide sequence data were compiled and analyzed with the Genetics Computer Group sequence analysis software package (Genetics Computer Group, Madison, Wis.).

Enzyme assays and immunological techniques. Cell extracts for enzyme assays were prepared as described previously

(17). Total protein was determined by the method described by Markwell et al. (22). RubisCO activity was measured in crude extracts according to the procedure described by Gibson and Tabita (8). Rocket immunoelectrophoresis was performed as previously described (17), with antibodies directed against form I and form II RubisCO. Western immunoblot analysis using antiserum against form I PRK was described previously (7).

Southern hybridization. Chromosomal DNA to be used in Southern hybridization analysis was prepared from photoheterotrophically grown cultures of *R. sphaeroides* as previously described (3). Chromosomal DNA was digested with *Eco*RI and *Hin*dIII and separated by electrophoresis in a 0.6% agarose gel. Southern blots were prepared and hybridized under stringent conditions according to the procedures supplied with GeneScreen Plus membranes (New England Nuclear). The DNA probe that was used to verify the insertion into *cbbR* was a 1.8-kb *Eco*RI-*Hin*dIII fragment isolated from pUC12EH by elution from an agarose gel. The DNA probe was labeled with [^{32}P]dCTP (New England Nuclear) by random-prime labeling with a kit obtained from Boehringer Mannheim, Indianapolis, Ind.

Insertional mutagenesis of *cbbR*. The *Eco*RI and *Hin*dIII sites of the 1.8-kbp *Eco*RI-*Hin*dIII restriction fragment isolated from pUC12EH were filled in with the Klenow fragment of DNA polymerase and blunt end ligated into the *Sma*I site of pUC1318, generating plasmid pUC1312, resulting in *Eco*RI sites flanking both ends of the insert. A 2.7-kbp *Sal*I fragment containing a trimethoprim resistance gene was isolated from pUC1889Δ*Eco*RI and cloned into pUC1312 within the unique *Xho*I site in pUC1312 that lies within the *cbbR* coding region. The resulting 4.5-kbp *Eco*RI fragment containing the disrupted *cbbR* sequence was cloned into the *Eco*RI site of pSUP202 and mobilized into *R. sphaeroides* with *E. coli* SM10. Trimethoprim-resistant transconjugants were screened for loss of the plasmid by testing resistance to tetracycline at a concentration of 1.0 μg/ml. Chromosomal DNA isolated from tetracycline-sensitive colonies was subjected to Southern hybridization analysis to verify that gene replacement had occurred.

Nucleotide sequence accession number. The sequence reported in this article has been entered into GenBank under accession number L20695.

RESULTS

Nucleotide sequence. Previous studies had shown promoter activity to be associated with approximately 1 kbp of DNA upstream of the form I Calvin cycle gene cluster in *R. sphaeroides* (4). Therefore, the nucleotide sequence of this region was analyzed for elements that might be involved in regulation and expression of genes required for CO_2 fixation. Nucleotide sequence analysis revealed the presence of a divergently transcribed open reading frame situated 89 bp upstream of the initiation codon of *cbbF*$_I$. The nucleotide sequence of this open reading frame is shown with the deduced amino acid sequence in Fig. 1. The open reading frame, which will be referred to as *cbbR*, extends 933 nucleotides from an ATG codon at position 121 to a TGA stop codon at position 1054 and is preceded by a Shine-Dalgarno-like sequence, GGAA, situated 7 bp upstream of the presumptive start codon. The coding region is capable of encoding a protein of 310 amino acids with a molecular weight of 33,462.

Amino acid sequence comparisons. When the deduced amino acid sequence of *cbbR* was employed in protein data

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                                    cbbF_I
GGCATCGGGGTGGGTGGGAAAGGGCTTCACTGGCATCTCCTCCGCGACGTGTGCCCAGCT   60
 A  D  P  H  T  P  F  P  K  M

TGCCGCGGATCGGATTTTATGGAATTTAATAAGGTAGAAGTTCTGTTCGGGAATTTGGAA  120
                    <--      -->     <--      -->
cbbR
ATGGTGCGGCTCGATGCGATCACCCTGAAGCAGCTGCGCGCCCTCGTGGCGGTGGCGGGC  180
 M  V  R  L  D  A  I  T  L  K  Q  L  R  A  L  V  A  V  A  G

AGCGCCTCGCTGACGGGCGGGGCCACGCGCCTCGGTCTGACGCCTCCGGCAATCCACAGC  240
 S  A  S  L  T  G  G  A  T  R  L  G  L  T  P  P  A  I  H  S

CAGATCAGGAACTTGGAGGAAGCCTTCGGCGTGCCGCTCCTCCATCGGCCGCCCGAGACG  300
 Q  I  R  N  L  E  E  A  F  G  V  P  L  L  H  R  P  P  E  T
                                        XhoI
GGCTCCTTCACGCCGACGCTCGCCGGAATCGCCGTTCTCGAGGCCGCTCAGCGGATCGAG  360
 G  S  F  T  P  T  L  A  G  I  A  V  L  E  A  A  Q  R  I  E

GTCATTCTGTCGCAGTGCTCATACCAAGTGATGGCGGTGAGCGAGGGCCGGGCCGGGCAG  420
 V  I  L  S  Q  C  S  Y  Q  V  M  A  V  S  E  G  R  A  G  Q

GTGACGCTGGGTGTGGTCTCGACCGGGCGCTATTTCGCGCCGCGGCTGGTGAAGATGCTG  480
 V  T  L  G  V  V  S  T  G  R  Y  F  A  P  R  L  V  K  M  L

AGCCTCGCCTGCCCCGAGATCCGAATCGCCCTCCGCGTGGGCAACCGCGAGCAGCTGATC  540
 S  L  A  C  P  E  I  R  I  A  L  R  V  G  N  R  E  Q  L  I

GACGATCTGGCGCGGCACATGGTGGATCTGGCGGTCATGGGACGCCCCCCGCGCCAGCCC  600
 D  D  L  A  R  H  M  V  D  L  A  V  M  G  R  P  P  R  Q  P

GAGGTGGCGTCGGTGGCGCTCGGGCCGCATCCGCACGGGATCGTGGCGCCGCCCGATCAT  660
 E  V  A  S  V  A  L  G  P  H  P  H  G  I  V  A  P  P  D  H

CCGCTGGCGGGTCTGGCCGAGGTGCCGGTGCCGGACCTTCTGTCCCAGACCTTTCTGGCG  720
 P  L  A  G  L  A  E  V  P  V  P  D  L  L  S  Q  T  F  L  A

CGCGCGGAAGGGTCGGGGACACGGGTGCTCATGTCGCGCTATCTCGACCGGCTGGGCGAG  780
 R  A  E  G  S  G  T  R  V  L  M  S  R  Y  L  D  R  L  G  E

GGGCAGGTGGTGGACCTGATCGAGATGGATTCGAACGAGACCATCAAGCAGTCGGTCATC  840
 G  Q  V  V  D  L  I  E  M  D  S  N  E  T  I  K  Q  S  V  I

GCGGGGCTGGGGCTCGCCTTCCTGTCGCTGCATGTGGTGATGGACGAGCTGCGCTTCGGC  900
 A  G  L  G  L  A  F  L  S  L  H  V  V  M  D  E  L  R  F  G

CAGCTCGTGCAGTTGGCGGCCCCCGGCCTGCCCATCGAGCGGCACTGGTTCCTCGTCCAT  960
 Q  L  V  Q  L  A  A  P  G  L  P  I  E  R  H  W  F  L  V  H

CCGGTCGATCGGCCGCTGAATCCGGCCGCGCTCAGGGTGCAGGGCGAGATCGTGAAGCTG 1020
 P  V  D  R  P  L  N  P  A  A  L  R  V  Q  G  E  I  V  K  L

AAGGGAGCCTATCTGCCCGGCGCCCCCGCCTGAGCCGTCAGACGCCGACGTAGCGGGCGA 1080
 K  G  A  Y  L  P  G  A  P  A  *

ACTGCTGTGCGGCGGGGGAGAGATCGGCCGCGGGCAGGGTTTCGGCCACGCGGCCCGTCT 1140
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FIG. 1. Nucleotide and deduced amino acid sequence of *R. sphaeroides cbbR*. The amino-terminal portion of the *cbbF*$_I$ coding sequence (7) is shown to illustrate its position relative to the *cbbR* gene. Putative ribosome binding sites are overlined, and the stop codon is indicated with an asterisk. The *Xho*I site at position 337 is shown. The small arrows beneath the sequence in the intergenic region between *cbbF*$_I$ and *cbbR* denote small inverted repeats of potential *lysR*-type binding sites (11).

bank searches, significant similarity was found to sequences belonging to the LysR family of transcriptional regulatory proteins. Interestingly, four proteins that have been assigned to the LysR family on the basis of sequence comparisons are divergently oriented with respect to RubisCO coding sequences in other bacteria. These sequences include *cfxR* from *A. eutrophus* (35), *rbcR* from *Chromatium vinosum* (31), *rbcR* from *Thiobacillus ferrooxidans* (21), and a partial sequence, *orfD*, from *Xanthobacter flavus* (23). The complete deduced amino acid sequences of the proteins from *A. eutrophus*, *C. vinosum*, and *T. ferrooxidans* share approximately 35% identity with the CbbR protein of *R. sphaeroides*. For the sequence available for the *orfD* protein of *X. flavus*, the same degree of identity as the two other CbbR sequences is noted. Other members of the LysR family, such

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CBBRS      MVRLDAITLKQLRALVAVAGSASLTGGATRLGLTPPAIHSQIRNLEE  47
CBBRA    MSSF.R.L..R..QIF.T..RH..FVRA.EE.H..Q..VSM.VKQ..S
CBBRC        MHVS.R...VFE...RHN.Y.RA.EE.H.SQ..VSM.V.Q..D
CBBRT      MSIRHA..H..KIFA...RHM.FARA.EE.H.....LSI.V.Q.A.
CBBRX      MAPHW..R...LVALA.A.G.YAKA.QDM..S...VTA.MKA...

CBBRS  AFGVPLLHRPPRDGLLHADARRNRRSRGAQRIEVILSQCSYQVMAVSEGR  97
CBBRA  VV.MA.FE.VKGQLT.TEPG--D.LLHH.S.ILGEVKDAEEGLQ..KDCE
CBBRC  EI.LS.FE.LGKQVV.TEAG--REVFHYSRA.GQS.REMEEVLESLKGVS
CBBRT  .V.Q..FDQIGKKIY.T.AG--EALTSACHD.LDR.EYFTQEIA.LQGLE
CBBRX  DI...MFE.VDGRLRPT.AG--QELL.AQE..ARA..EAERAIA.LKSPE

CBBRS  AGQVTLGVVSTGRYFAPRLVKMLSLACPEIRIALRVGNREQLIDDLARHM 147
CBBRA  Q.SI.I.LI..SK....K.LAGFTALH.GVDLRIAE....T.LRL.QDNA
CBBRC  R.SLRIA.A..VN......MAIFQQRHSG.GLR.D.T...S.VQM.DSNS
CBBRT  K.SLKVATL..TK..I..MLGGFCTEH.GVATV.FI....V.LER...NQ
CBBRX  R.S.VV...A.AK....MALAAFRRRR...ELR.II....DI.RGIVSLD

CBBRS  VDLAVMGRPPRQPEVASVALGPHPHGIVAPPDHPLAGLAEVPVPDLLSQT 197
CBBRA  I...L......ELDAV.EPIAA...VL..S.R...HDAKGFDLQE.RHE.
CBBRC  ...VL..V...NV..EAE.FMDN.LVVI.........ERAISLAR.AEE.
CBBRT  D..YIL.Q..EHMN.VAE.FADN.LVV..RL....TQEKDIEPSR.RDVP
CBBRX  F.V.I..

CBBRS  FLARAEGSGTRVLMSRYLDRLGEGQVVDLIEMDSNETIKQSVIAGLGLAF 247
CBBRA  ..L.EP.....TVAEYMFRDHLFT-PAKV.TLG.......A.M..M.ISL
CBBRC  .VM.E......QA.E.FFSER.QT-IRHGMQ.TR..AV..A.RS....SV
CBBRT  .IL.EP.....LAAEKFFEQH.VI-LKTRM.FG...AV....AG...ITV

CBBRS  LSLHVVMDELRFGQLVQLAAPGLPIERHWFLVHPVDRPLNPAALRVQGEI 297
CBBRA  ....TLGL...T.EIGL.DVA.T....I.HVAHMSSKR.S..SESCRAYL
CBBRC  V...TIEL..ETRR..T.DVE.F.DR.Q.Y..YRRGKR.S...GAFREFV
CBBRT  ..ASTIRA..AS.K.AI.DVR.F.L..K.YA.Y.AGKRIS.RTKAFMEYL

CBBRS  VKLKGAYLPGAPA 310
CBBRA  LEHTAEF.GREYGGLMPGRRVA
CBBRC  LSEAARMHCRLG
CBBRT  FAASADGEN.PSLPKP
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FIG. 2. Amino acid alignments of known CbbR sequences. Gaps are indicated by dashes; identical residues are indicated by dots. The alignments are of CbbR sequences from the following organisms: CBBRS, *R. sphaeroides*; CBBRA, *A. eutrophus* (35); CBBRC, *C. vinosum* (31); CBBRT, *T. ferrooxidans* (21); and CBBRX, *X. flavus* (23).

as the LysR protein itself (27), typically share less than 25% identity with CbbR. Identical residues common to all the CbbR sequences are distributed throughout the molecule, with striking similarity evident between residues corresponding to amino acids 100 to 240 of the CbbR sequence from *R. sphaeroides* (Fig. 2). The strong homology in this region is in contrast to that noted between other members of the LysR family, in which the amino-terminal portion of the coding sequence is usually conserved. The latter region contains the helix-turn-helix motif, which constitutes the potential DNA binding domain (14). It is tempting to speculate that the conserved residues situated in the middle of the CbbR coding regions may reflect sequences involved in binding to a common inducer molecule.

One motif that has been found common to all LysR DNA binding sites is a T-N_{11}-A box situated upstream from the regulated genes (11). This box is characterized by 3-bp inverted repeats at either end and is usually repeated two or three times. Two such boxes are located in the intergenic region between *cbbR* and *cbbF*$_I$ (Fig. 1).

Inactivation of *cbbR*. Because the predicted sequence of CbbR was found to be similar to those of bacterial regulatory proteins and it was located adjacent to the form I Calvin cycle gene cluster, it was of interest to determine whether the CbbR gene product was involved in the regulation of CO_2 fixation in *R. sphaeroides*. To test this possibility, an antibiotic resistance cartridge was inserted into the *cbbR* coding sequence, thereby inactivating the gene. The disrupted *cbbR* gene was introduced into *R. sphaeroides* as described in Materials and Methods, and the resultant *cbbR*-negative strain (strain 1312) was tested for the ability to grow photosynthetically. The *cbbR* strain exhibited severely reduced growth on agar plates when malate or butyrate was supplied as carbon source under photosynthetic conditions, compared with wild-type strain HR. During photosynthetic growth on malate in liquid cultures, the mutant strain exhibited a generation time of 16 h compared with 8 h for the wild type. No growth of the *cbbR* strain was observed when cells were incubated under photolithoautotrophic conditions, in which CO_2 is the sole source of carbon.

TABLE 2. RubisCO levels in *R. sphaeroides* HR, 1312, and 1312 complemented with various plasmid constructs

Strain	Plasmid	Growth condition	RubisCO protein[a]		RubisCO sp act (nmol/min/mg of protein)[b]
			I	II	
HR		Mal[c]	1.8	1.5	26.6
		Aut[d]	9.2	2.9	363.7
1312		Mal	—[e]	0.5	8.0
		Aut	ND[f]	ND	ND
1312	pJG336	Mal	1.7	0.7	39.5
		Aut	7.5	1.3	290.3
1312	pJG106	Mal	—	2.2	32.1
		Aut	—	2.0	16.1
1312	pVK12	Mal	—	4.3	43.0
		Aut	0.9	28.5	271.4
1312	pRPS429	Mal	2.3	0.3	64.0
		Aut	ND	ND	ND

[a] RubisCO protein as determined by rocket immunoelectrophoresis expressed as percent total soluble protein; the average of duplicate determinations.
[b] Values represent the average of duplicate or triplicate determinations.
[c] Photoheterotrophic growth on 0.4% malate.
[d] Photolithoautotrophic growth on 1.5% CO_2 in H_2.
[e] —, not present or found in the strain indicated.
[f] ND, not determined; strain does not grow under these conditions.

In order to determine whether or not the growth phenotype was actually related to alterations in the pattern of Calvin cycle enzyme synthesis, it was necessary to measure expression. As mentioned previously, several Calvin cycle genes in *R. sphaeroides*, including those encoding PRK, FBP, and RubisCO, are duplicated and organized within two physically distinct operons (6, 7). Because in previous studies it was shown that these three enzymes followed a similar induction pattern (7), the level of RubisCO can conveniently be used as a general indicator of the relative expression of all gene products encoded by the form I and form II Calvin cycle operons. Furthermore, because the form I and form II RubisCO enzymes are immunologically distinct, immunoelectrophoresis can be used to distinguish between and at the same time quantitate expression from either the form I or form II operon.

Extracts prepared from cells grown photoheterotrophically on malate were initially assayed for RubisCO. The RubisCO activity measured in malate-grown cells of the CbbR mutant was only 30% of the level measured in the wild-type strain HR (Table 2). Furthermore, no form I RubisCO was detected when extracts from the mutant strain were analyzed by rocket immunoelectrophoresis. In addition, quantitation of the levels of form II RubisCO protein showed that there was only 33% of the amount found in the wild-type strain HR (Table 2).

Complementation studies. Given the likelihood that the

cbbR gene product acts in *trans* as a transcriptional activator for the expression of CO_2 fixation genes, various plasmid constructs were introduced into the *cbbR* strain by conjugation. Transconjugants were initially screened for the ability to grow photolithoautotrophically. Three plasmids complemented the *cbbR* strain for wild-type growth characteristics: pVK12, a pVK102 derivative carrying an intact *cbbR* gene; pJG336, a cosmid carrying the form I Calvin cycle gene cluster including *cbbR*; and pJG106, a cosmid carrying the form II Calvin cycle gene cluster. An additional plasmid, pRPS429, which carries the form I RubisCO and FBA genes under the control of the *Rhodospirillum rubrum* RubisCO promoter (4), failed to restore the capacity for photolithoautotrophic growth to the *cbbR* strain.

To examine the basis for complementation, extracts were prepared and assayed for RubisCO activity from plasmid-containing cells grown either photoheterotrophically on malate or photolithoautotrophically with 1.5% CO_2 as the sole source of carbon. The results show that in malate-grown cells, all of the plasmids restored the level of RubisCO activity in the *cbbR* strain to the approximate levels observed in the wild-type strain HR. However, when these extracts were examined by immunoelectrophoresis in order to assess the contribution of form I and form II RubisCO to overall enzyme activity, form I RubisCO was detected only in strains carrying pJG336 or pRPS429 (Table 2). Therefore, all of the RubisCO activity measured in the *cbbR* strains carrying pVK12 and pJG106 can be attributed to expression of the form II RubisCO.

Also shown in Table 2 are the levels of RubisCO activity measured in the complemented strains grown photolithoautotrophically, with the exception of the strain carrying pRPS429, which cannot grow under these conditions. Most striking is the extremely low RubisCO activity observed in the strain carrying plasmid pJG106, which contains all the known form II Calvin cycle genes; the RubisCO levels were only 6% of that observed for photolithoautotrophically grown wild-type strain HR and were similar to levels observed in malate-grown cells. It should be noted that although pJG106 enabled the mutant to grow photolithoautotrophically, the growth rate was noticeably slower than that of the wild-type strain HR (data not shown). As observed in photoheterotrophically grown cells, only form II RubisCO could be detected by rocket immunoelectrophoresis. In contrast to the uninduced levels of RubisCO in the mutant complemented with the form II cosmid clone, the *cbbR* strain carrying either pVK12 or pJG336 expressed wild-type levels of RubisCO activity following growth under photolithoautotrophic conditions. However, immunoelectrophoresis revealed that only the strain carrying plasmid pJG336 was able to induce expression of form I RubisCO protein to levels that approximated that for the wild type. Although the RubisCO activity on the basis of enzyme assays was similar to that observed for the wild-type strain, the strain complemented by pVK12 produced only about 10% of the normal level of form I carboxylase protein. Overexpression of the form II RubisCO accounted for the wild-type levels of enzyme activity measured in crude extracts of this strain.

As expression of the form I genes was not completely restored when *cbbR* was supplied in *trans*, the possibility that the trimethoprim resistance cassette was inserted into a sequence required for expression of the form I operon was examined with the plasmid pRK411. This construct contains $cbbF_I$ $cbbP_I$ in addition to sequences upstream of these genes up to the *Xho*I site where the *cbbR* gene was disrupted. This plasmid was conjugally transferred into *R. sphaeroides* 1844, in which expression of the form I genes is blocked by an insertion in $cbbF_I$ (7). Since the form I gene products are not produced in this strain, expression of the plasmid-encoded form I PRK can be monitored by Western immunoblot analysis. Form I PRK was detected in photoheterotrophically grown cells of strain 1844 that contained the plasmid (data not shown). Although the results are not quantitative, expression was shown to be regulated as no form I PRK was detected in aerobically grown cells. These results suggest that incomplete complementation of the $Cbbr^-$ mutant harboring the *cbbR* gene is not due to the lack of sufficient sequence upstream of the form I operon, but to other, as yet unexplained, effects of the insertion.

TABLE 3. RubisCO activity in strains grown aerobically on malate minimal medium

Strain	Plasmid	RubisCO sp act (nmol/min/mg of protein)[a]
HR		1.9
1312		2.1
1312	pVK12	0.5
1312	pJG336	1.1
1312	pJG106	11.3
16		—[b]
16	pJG106	18.0
16	pJG336	—
$cbbM_{II}$ mutant		—

[a] Values represent the average of duplicate or triplicate determinations.
[b] —, not detected in this strain.

Aerobic expression of RubisCO. In order to determine the effect of the *cbbR* mutation on aerobic expression of Calvin cycle genes in *R. sphaeroides*, RubisCO levels were determined in extracts of aerobically grown cells. The level of RubisCO activity observed in the *cbbR* strain (strain 1312) was not significantly different from that measured in the wild-type strain. Similarly, the *cbbR* strain, harboring either pJG336 or the plasmid carrying the wild-type *cbbR* gene, exhibited extremely low levels of RubisCO activity, typical of that usually observed in extracts of aerobically grown cells (Table 3). Interestingly, however, 5- to 10-fold greater levels of RubisCO were routinely observed in the *cbbR* strain carrying plasmid pJG106. The relatively high RubisCO activity was apparently unrelated to the *cbbR* background because similarly high activities were observed in strain 16, a RubisCO deletion mutant harboring plasmid pJG106 (Table 3), whereas no activity could be detected in either strain 16 alone or the form II RubisCO ($cbbM_{II}$) deletion mutant.

DISCUSSION

In the *cbbR* strain, synthesis of form I gene products was completely abolished, while the level of form II enzyme synthesis was found to be only about 33% of that observed in the wild-type strain HR. Attempts to complement the *cbbR* mutant with the intact *cbbR* gene supplied in *trans* completely restored expression of genes within the form II operon, but expression of the form I genes was only partially regained. These results suggest that synthesis of the form II enzymes is not entirely dependent on the *cbbR* gene product, but that they may be expressed at a low, possibly constitutive level in its absence. The *cbbR* gene product (CbbR) is apparently needed for increased synthesis of the form II gene products, typically seen during photolithoautotrophic growth, since the *cbbR* strain harboring the form II

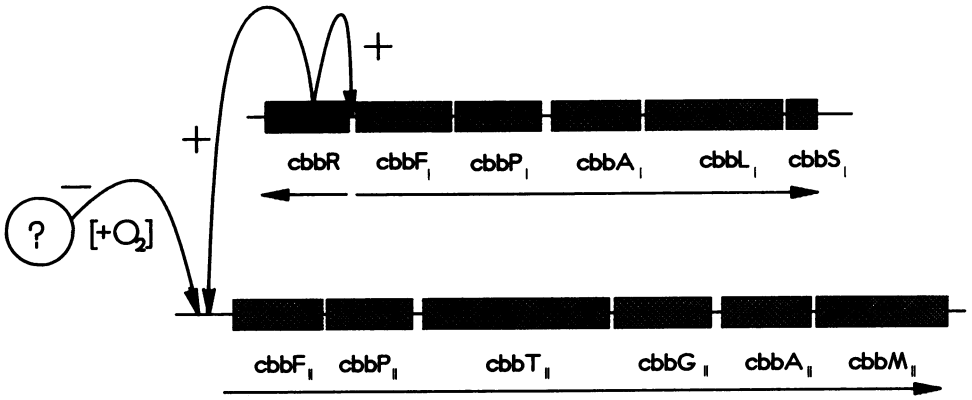

FIG. 3. Model of Calvin cycle structural gene regulation in *R. sphaeroides*. Positive transcriptional regulation of the form I and form II operons by *cbbR* is indicated by a plus sign. Also shown is the potential negative transcriptional regulation of the form II operon that is influenced by O_2, shown as a minus sign.

cluster on a plasmid exhibited virtually the same RubisCO activity under both photoheterotrophic and photolithoautotrophic growth conditions. The lack of induction, along with preliminary sequence data of the region upstream of $cbbF_{II}$, and the results of hybridization studies (data not shown) suggest that the form II cluster does not contain a *cbbR* counterpart. The ability of plasmid pJG106 to complement the mutant strain is most probably due to a copy number effect in which constitutive expression of the form II enzymes reaches a critical level required for growth.

The reason for the inability of *cbbR* supplied in *trans* on plasmid pVK12 to restore expression of the form I genes to wild-type levels is not clear, but the trimethoprim resistance cartridge is inserted near the 5′ end of the *cbbR* gene and may be interfering with promoter elements required in *cis* for transcription of the form I operon. On the other hand, the insertion could be interfering with transcription of a gene located downstream from *cbbR* that is also required for expression of the form I genes. This possibility is considered unlikely because wild-type expression of form I RubisCO is observed in the *cbbR* strain harboring plasmid pJG336. This plasmid contains the same amount of sequence downstream from *cbbR* as does plasmid pVK12. Presumably, form I RubisCO expressed in the pJG336-complemented strain is derived predominantly from the plasmid-borne rather than chromosomal copies of the form I genes, in which any essential *cis* function(s) is not disrupted. Although the *cbbR* mutant complemented with plasmid pVK12 did not exhibit normal expression of form I gene products, overproduction of the form II gene products was observed, indicating that CbbR mediates the ability of the organism to compensate for the loss of one set of genes, presumably by providing communication between the two sets of Calvin cycle genes through a common signal or inducer. Finally, the lack of complementation by plasmid pRPS429 suggests that RubisCO may not be the limiting factor for photolithoautotrophic growth, since the level of RubisCO was higher in malate-grown cells than in the mutant complemented for photolithoautotrophic growth by plasmid pJG106. The CbbR protein does not appear to affect expression of either set of Calvin cycle genes during aerobic growth. Therefore, another level of regulation must be realized in the presence of oxygen, since constitutive expression in the form II genes observed in the absence of CbbR is apparently repressed by oxygen. The apparent loss of repression of RubisCO synthesis observed in strains harboring the plasmid carrying the form II genes is reminiscent of that observed for plasmid-encoded *puf* genes in *R. sphaeroides* (15) and may result from the titration of a low-copy-number negative regulatory factor due to multiple copies of the plasmid. If this is the case, these results offer preliminary evidence suggesting that the form II operon may be regulated both positively and negatively at the transcriptional level. The simple model illustrated in Fig. 3 summarizes these results. The form I and form II gene clusters can thus be considered to be part of a regulon, since expression of the two operons is apparently regulated by the same regulatory gene; hence, the common gene designation, *cbb*, has been substituted for the previous symbols (30). A similar situation has previously been reported in *A. eutrophus*, a facultative chemoautotroph that also possesses duplicate sets of Calvin cycle genes (16). As in *R. sphaeroides*, the two gene clusters are located on distinct genetic elements: one set is plasmid encoded, and the second is located on the chromosome (16). Unlike *R. sphaeroides*, in which the form I and form II operons not only differ considerably in homology between the isofunctional genes but also in the genes that actually compose each cluster, the two operons in *A. eutrophus* are virtually identical (16). In addition, the gene arrangement in the two organisms differs in that the RubisCO genes in *A. eutrophus* are situated at the 5′ end of the operons, opposite to the 3′ location of the RubisCO genes in *R. sphaeroides* (16). Inactivation of the single functional *lysR*-type regulatory gene, divergently transcribed from the chromosomal operon in *A. eutrophus*, was shown to abolish expression from both chromosomal and plasmid operons (35). Complementation of the mutant strain with a plasmid containing the regulatory gene restored wild-type levels of Calvin cycle enzymes, although no distinction was made as to which enzymes were synthesized, plasmid or chromosomal or both. In any case, synthesis of isofunctional Calvin cycle enzymes in both organisms appears to be coordinated by a common regulatory protein.

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