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MINIREVIEW

# CbbR, the Master Regulator for Microbial Carbon Dioxide Fixation

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**Biological carbon dioxide fixation is an essential and crucial process catalyzed by both prokaryotic and eukaryotic organisms to allow ubiquitous atmospheric $CO_2$ to be reduced to usable forms of organic carbon. This process, especially the Calvin-Bassham-Benson (CBB) pathway of $CO_2$ fixation, provides the bulk of organic carbon found on earth. The enzyme ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase (RubisCO) performs the key and rate-limiting step whereby $CO_2$ is reduced and incorporated into a precursor organic metabolite. This is a highly regulated process in diverse organisms, with the expression of genes that comprise the CBB pathway (the *cbb* genes), including RubisCO, specifically controlled by the master transcriptional regulator protein CbbR. Many organisms have two or more *cbb* operons that either are regulated by a single CbbR or employ a specific CbbR for each *cbb* operon. CbbR family members are versatile and accommodate and bind many different effector metabolites that influence CbbR's ability to control *cbb* transcription. Moreover, two members of the CbbR family are further post-translationally modified via interactions with other transcriptional regulator proteins from two-component regulatory systems, thus augmenting CbbR-dependent control and optimizing expression of specific *cbb* operons. In addition to interactions with small effector metabolites and other regulator proteins, CbbR proteins may be selected that are constitutively active and, in some instances, elevate the level of *cbb* expression relative to wild-type CbbR. Optimizing CbbR-dependent control is an important consideration for potentially using microbes to convert $CO_2$ to useful bioproducts.**

The CbbR protein is a LysR-type transcriptional regulator (LTTR) that functions to control expression of genes of the $CO_2$ fixation (*cbb*) operons that specify enzymes of the Calvin-Bassham-Benson (CBB) pathway. CbbR-dependent regulation occurs in diverse organisms, including nonsulfur and sulfur purple bacteria, marine and freshwater chemoautotrophic bacteria, cyanobacteria, methylotrophic bacteria, several varieties of hydrogen-oxidizing bacteria, and different *Pseudomonas*, *Mycobacterium*, and *Clostridium* strains (1–14). In addition, CbbR regulates carbon fixation gene expression in chloroplasts of eukaryotic red algae (15).

For many prokaryotic and eukaryotic organisms, $CO_2$ is often the sole source of carbon, with the CBB pathway acting as the paramount metabolic pathway that enables such organisms to synthesize all the building blocks and macromolecules required for life. The net goal of the enzymes of the CBB cycle is to provide one triose phosphate molecule as the fundamental reduced form of useable carbon from an intake of three $CO_2$ molecules. Although several enzymes are dedicated to the CBB pathway, ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase (RubisCO) is the enzyme that catalyzes the actual "fixation" of $CO_2$ onto the ene-diol form of RuBP, resulting in the production of two phosphorylated three-carbon molecules of 3-phosphoglyceric acid (3-PGA). Because this enzyme is a relatively poor catalyst and must contend with $CO_2$ and $O_2$, competing for the same active site under aerobic conditions, cells very often compensate by synthesizing huge amounts of RubisCO (e.g., up to 50% of the soluble protein under appropriate growth conditions [16]). Clearly, this is a highly regulated system, and under some physiological conditions, especially in bacteria, it is necessary for the organism to either upregulate or downregulate expression of genes of the CBB pathway. Transcriptional control of the *cbb* genes is thus vital because of the heavy energy demands and the burden of additional protein synthesis placed on the cell by $CO_2$ assimilation. In bacteria, the master regulator protein in all cases is CbbR.

Like all LTTR family members, CbbR binds as a tetramer to the promoter region of the *cbb* operon. The generalized consensus DNA binding sequence for LTTRs is T-$N_{11}$-A, and all CbbR proteins interact with this DNA binding motif (17). Typically, each of two DNA binding sites (within approximately 6 to 20 bp of each other) is bound by a CbbR dimer, creating a dimer of dimers (tetramer). Like all LTTRs, the CbbR protein structure is about 300 to 330 amino acids in length and is composed of three functional domains. There is a DNA binding domain (DBD) at the N terminus that binds to the *cbb* promoter region. The LTTR DBD is classified as a winged helix-turn-helix (HTH) motif (18, 19). All four HTH motifs within a CbbR tetramer interact with DNA when bound to the *cbb* promoter. A linker helix domain functions to connect the DBD and the recognition domains (RD) of the protein (RD-I and RD-II; also referred to as effector domains). The linker domain is a 30-amino-acid α-helix that operates as a rigid linker helix to prevent interaction between the DBD and the RD. The linker helix also contributes to dimer formation through coiled-coil interactions (20–26). Figure 1 illustrates a generalized structure for all CbbR proteins. Regions of conservation that distinguish CbbR proteins from other LTTRs are found in the recognition domains and are discussed below with respect to effector interactions.

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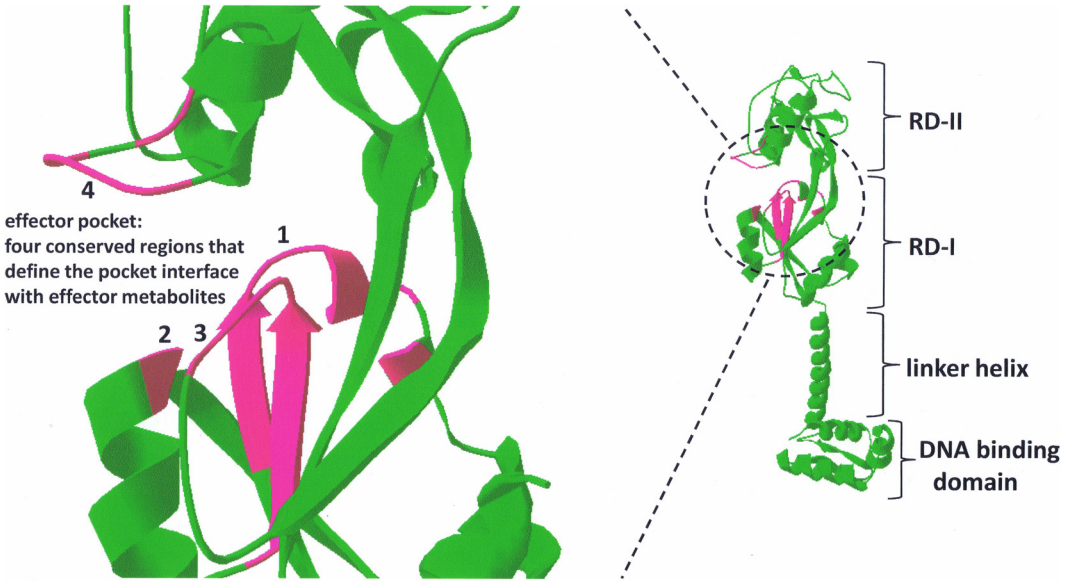


**FIG 1** Proposed structure of CbbR. On the right is the generalized ribbon structure of the CbbR monomer based on the structure of the LTTR family member CbnR (20), illustrating the four major domains of the LTTR. On the left is the enlarged structure of the CbbR effector pocket. Four regions of the effector pocket, denoted 1 to 4, are highlighted in magenta, with residues of regions 1 to 4 conserved among CbbR family members. These four conserved regions define the effector pocket and are positioned at the interface between the effector metabolite(s) and CbbR. The conserved amino acid sequences (magenta) for each region are as follows: for region 1, GVVSTAKYFXP; for region 2, NR; for region 3, DLAIMGRPP; and for region 4, REXGSGTR ("X" represents a residue position that is not conserved). The effector pocket conservation also applies to CmpR, CcmR, and QscR, which are CbbR subfamily members. Conservation is high within the four regions of the effector pocket for all CbbRs examined: 81.5% for region 1, 95% for region 2, 86% for region 3, and 97% for region 4. DeepView/Swiss-Pdb Viewer software was used to generate the structural model.

## *cbbR* AND *cbb* GENE ORGANIZATION

The *cbbR* gene, like most LTTR family members, is usually located directly upstream of the *cbb* operon that it regulates but in the opposite orientation. There are some notable exceptions to this general rule of gene organization. For example, *cbbR* of *Rhodospirillum rubrum* is in the same orientation as and adjacent to *cbbM* (encoding form II RubisCO); however, the orientation of *cbbR* is opposite that of the remainder of the *cbb* operon (4, 27). Another interesting exception is provided by *Hydrogenophilus thermoluteolus*; there, *cbbR* is located within a split *cbb* operon in an orientation opposite that of all the *cbb* genes. *Rhodobacter capsulatus* also presents an interesting situation where this organism contains two *cbbR* genes encoding two distinct CbbR proteins ($CbbR_I$ and $CbbR_{II}$) that regulate two separate *cbb* operons, one of which, along with its cognate *cbbR* gene, was apparently derived from a chemoautotrophic ancestor (7, 28). In the chemoautotroph *Hydrogenovibrio marinus*, there also are two *cbbR* genes (*cbbR1* and *cbbRm*) and three *cbb* operons (*cbbLS-1*, *cbbLS-2*, and *cbbM*), with *cbbR1* located upstream and in an orientation opposite that of *cbbLS-1*, while the *cbbRm* gene is located upstream of but in the same orientation as *cbbM* (10, 29–31). The *cbbLS-2* operon contains genes encoding carboxysomes and is expressed under conditions of low $CO_2$ concentrations, independently of CbbR1 or CbbRm regulation (30, 31). On the other hand, CbbR1 and CbbRm of *H. marinus* may be involved in repressing expression of carboxysome genes contained within the *CbbLS-2* operon at high levels of $CO_2$ (31). Finally, CbbR has also been shown to regulate the expression of carboxysome genes in *Acidithiobacillus ferrooxidans*, a chemoautotrophic gammaproteobacterium that characteristically grows in acidic environments (3, 13). Notably, the single CbbR from *A. ferrooxidans* regulates four distinct *cbb* operons (3, 13).

While the foregoing represent some very interesting situations where CbbR plays an important physiological role, in addition to regulating *cbb* gene expression, the usual situation is that a single *cbbR* gene is used to exclusively regulate the two major *cbb* operons that are found in most prokaryotic organisms. Many phototrophic and chemoautotrophic organisms contain multiple RubisCO genes, usually encoding distinct form I (CbbLS) and form II (CbbM) enzymes that function to fix $CO_2$ at low and high $CO_2$ levels, respectively (32–35). The most thoroughly studied examples where a single CbbR regulates *cbb* operons containing distinct form I and form II RubisCO genes are *Rhodobacter sphaeroides* (5, 36, 37) and *Rhodopseudomonas palustris* (11). Several additional autotrophic bacterial species, including *Ralstonia eutropha* strain H16, contain two *cbb* operons regulated by the product of a single *cbbR* gene. *R. eutropha* has a well-characterized *cbb* regulon where a single *cbbR* gene controls both chromosomal and megaplasmid-borne *cbb* genes (2); however, the RubisCO enzymes encoded by these separate operons are virtually identical (38). *Mycobacterium* sp. strain JC1 DSM 3803 also has two *cbb* operons regulated by one CbbR, but the *cbbR* gene is directly downstream of and in the same orientation as *cbbLS-1* (12, 39).

## PHYLOGENETIC RELATEDNESS OF CbbRS AND CLOSELY RELATED LTTRS

Amino acid identities accurately reflect the general relatedness of CbbR proteins from different organisms. Yet there is a striking drift of amino acid homologies among the CbbR family similar to the general lack of amino acid identity within the LTTR family as

a whole. Despite these differences in primary sequence, there is a high degree of structural and conformational similarity of the monomeric, dimeric, and tetrameric states of all LTTR proteins. Regions of residue similarity and identity within the LTTR and CbbR families include the DBD (HTH motif), regions defining the effector pocket, and areas of the protein important for the formation of dimers and tetramers. As determined on the basis of amino acid identities, the CbbR subfamily also includes QscR, CmpR, and CcmR, three closely related LTTRs that are more similar to some CbbRs than some CbbRs are to each other. QscR regulates the expression of two operons involved in the one-carbon serine assimilatory pathway of some methylotrophic bacteria (40, 41). CmpR regulates transcription of operons involved with bicarbonate transport in cyanobacteria (42) and specifically regulates expression of certain genes involved in the $CO_2$-concentrating mechanism (CCM) (43–46). The CCM allows cyanobacteria to actively transport $HCO_3^-$ into the cytoplasm and then into the carboxysome. Subsequently, carboxysomal carbonic anhydrase catalyzes the conversion of $HCO_3^-$ to $CO_2$ such that $CO_2$ becomes highly concentrated in this microcompartment and is readily made available and saturates the active site of RubisCO (47). In *Synechocystis* sp. strain PCC 6803, CmpR activates transcription of the *cmpABCD* operon (high-affinity bicarbonate transporter), but another CbbR-like protein, CcmR, represses expression of the *ndhD3*, *ndhF3*, and *chpY* genes which are required for expression of the inducible high-affinity $CO_2$ transporter, NDH-$1_3$ (48, 49).

Total amino acid identities for individual CbbRs range from 22% to 56%, with the majority of identities falling between 35% and 45%. The CbbRs with the highest identities include CbbR of *Bradyrhizobium japonicum* and CbbR of *R. palustris* at 56%, CbbR of *Allochromatium vinosum* and CbbR of *Methylococcus capsulatus* at 55%, CbbR of *R. sphaeroides* and $CbbR_{II}$ of *R. capsulatus* at 54%, and CmpR of *Synechococcus elongatus* PCC 7942 and CcmR of *Synechocystis* PCC 6803 at 54%. The phylogenetic analysis of CbbR proteins is in good agreement with the overall phylogenetic relationship of microorganisms that possess *cbbR* genes.

## INTERACTIONS WITH CbbR: THE CASE OF DUELING TRANSCRIPTION FACTORS

There are several studies that show LTTR interactions with RNA polymerase or sigma factors (50–55), but there are few examples of interactions of LTTR proteins (not including CbbRs) with other transcriptional regulators (56, 57). By and large, this is a testament to the ability of LTTRs to independently and adequately regulate their operons in the prokaryotic kingdom. However, in the case of some phototrophic bacteria, regulation of *cbb* expression is much more complex, imposing additional layers of regulation on the energetically costly process of $CO_2$ assimilation. There are two well-studied systems that illustrate this regulatory complexity: interaction of CbbR with additional (and different) transcription regulators in *R. sphaeroides* and *R. palustris* (58–61).

In *R. sphaeroides*, CbbR interacts with the RegA response regulator, which is part of a global two-component system (RegA/RegB) that controls expression of both the $cbb_I$ and $cbb_{II}$ operons of this organism (58, 62). In addition to the *cbb* regulon, RegA and its cognate sensor kinase, RegB, maintain control over several operons involved with energy-related (redox) metabolism in phototrophic nonsulfur purple bacteria (63, 64). Thus, the response regulator of this two-component system, RegA, binds to multiple sites within the promoter regions of both *cbb* operons of *R. sphaeroides* (37, 65). This scenario is similar to that seen with the *hemA* gene in *R. sphaeroides*, where RegA and FnrL bind the *hemA* promoter at positions where FnrL takes the place of CbbR in the *hemA* promoter and phosphorylation of RegA changes the affinity of RegA for the *hemA* promoter (66). For the $cbb_I$ operon of *R. sphaeroides*, it has been demonstrated that RegA has a higher affinity for the promoter when RegA binding site 3 is present, and site 3 is also necessary for optimal expression of the $cbb_I$ operon *in vivo* (58, 65). It was further shown that RegA greatly enhances the stability of the CbbR/promoter DNA complex and that CbbR must be bound to the *cbb* promoter in order to interact with RegA but that it is not necessary for RegA to be bound to DNA to interact with CbbR (58). This scenario presumably prevents interactions between the two regulator proteins unless CbbR is bound to the *cbb* promoter, pointing to a finely tuned attenuation strategy limiting potential nonspecific interactions between the two proteins. It may be surmised that a deleterious situation would be avoided if the proteins did not interact unless bound to the *cbb* operon promoter. Adding to the complexity, some studies have shown that phosphorylation of RegA greatly increases its DNA binding stability, while other studies have illustrated that phosphorylation of RegA enhances activation of transcription (64). Cross-linking experiments, using a bifunctional binding compound, indicated that RegA and CbbR form a stoichiometric complex, results that were buttressed by gel mobility shift assays that also showed specific interactions between RegA and CbbR-bound DNA (58). In addition, extensive mutational analyses provided evidence that CbbR and RegA interact with each other through their DBDs (59). A model for this rather complex regulatory scenario, which is based on several lines of evidence and provides a likely explanation for how CbbR and RegA interact to regulate *cbb* gene expression and, subsequently, $CO_2$ fixation in *R. sphaeroides*, is presented in Fig. 2. Also considered in this model is the involvement of various small-molecule effectors that influence CbbR function (Fig. 2) (discussed below).

*R. palustris* represents an interesting and even more complex regulatory system involving CbbR and several additional protein regulators. While *R. palustris* also contains a Reg-type two-component system (67), it is not clear what this system regulates in this organism, as it apparently does not control photosystem biosynthesis as in *R. sphaeroides* and *R. capsulatus* (J. T. Beatty and F. R. Tabita, unpublished observations), nor is there any evidence to date to indicate that the Reg system controls *cbb* gene expression in *R. palustris* (J. L. Gibson and F. R. Tabita, unpublished observations). However, the regulation of the $cbb_I$ operon in *R. palustris* has proven to be extremely complex, involving a novel three-protein two-component system (11, 68). The regulatory proteins involved, CbbR, CbbRR1, CbbRR2, and CbbSR, are all encoded by genes that are closely juxtaposed within the $cbb_I$ operon region, with *cbbR* divergently transcribed from *cbbRR1*, *cbbRR2*, and *cbbSR*, which are immediately upstream from the *cbbL* and *cbbS* genes encoding the large and small subunits, respectively, of form I RubisCO (11). The CbbSR protein is a large transmembrane sensor kinase which, like many sensor kinases, is capable of autophosphorylation. In addition, CbbSR contains a consensus phosphate acceptor site, with a conserved aspartate-containing motif typical of many response regulators. Studies have shown that, upon autophosphorylation at a specific histidine residue, the phosphate may be transferred to the acceptor site of CbbSR (11, 68). Thus, this large protein basically acts as its own two-compo-

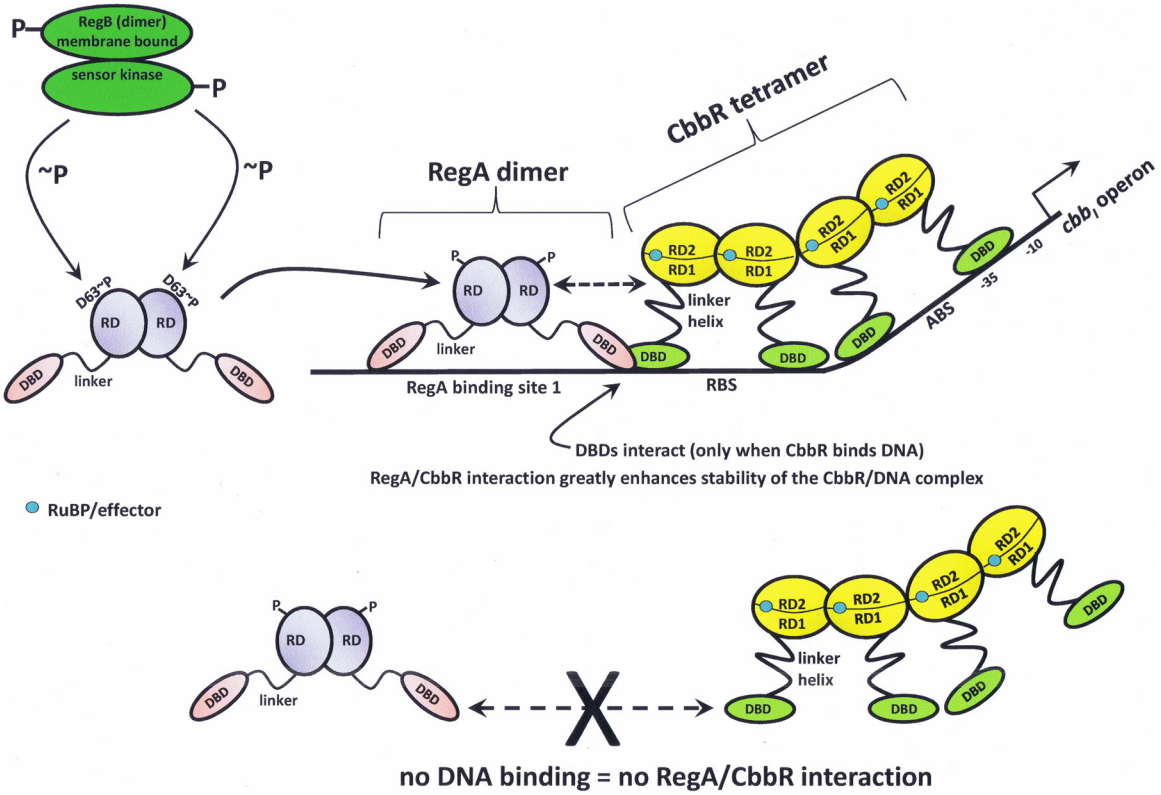


**FIG 2** Transcriptional regulation of the *cbb* operons of *Rhodobacter sphaeroides*. CbbR and RegA interact on the $cbb_I$ and $cbb_{II}$ promoters. There are four RegA binding sites upstream of the $cbb_I$ operon and six RegA binding sites upstream of the $cbb_{II}$ operon (37, 65). RegA DNA binding site 1 and the RegA binding site (RBS) overlap upstream of the $cbb_I$ transcriptional start site. The DBDs of CbbR and RegA interact and generate a CbbR/RegA/DNA complex at the $cbb_I$ promoter (59). The interaction of RegA with CbbR greatly increases the stability of the CbbR/DNA complex (58). CbbR does not interact with RegA if CbbR is not bound to the *cbb* promoter (58). RuBP (positive effector) is shown within the effector pocket of a CbbR monomer; the pocket is a small cleft formed between RD-1 and RD-2. Dashed arrows represent interactions with CbbR. ABS, activation binding site. RD-1 and RD-2, recognition domain 1 and recognition domain 2, respectively. DBD, DNA binding domain. RD (RegA), receiver domain. ~P, phosphorylation at residue D63 of RegA. The −10 and −35 regions of the $cbb_I$ promoter are indicated.

nent system. However, CbbSR also catalyzes phosphorylation of both response regulators, CbbRR1 and CbbRR2 (11), the specificity for which is influenced by specific PAS domains on CbbSR (68). Both physiological/genetic and *in vitro* studies indicate that CbbRR1 and CbbRR2 bind to CbbR and influence $cbb_I$ gene transcription (60). In addition, various effector molecules influence these interactions in a concentration-dependent fashion and stabilize CbbR binding to the *cbbLS* promoter. CbbR/CbbRR1 interactions enhance the binding affinity of CbbR to the promoter, and CbbRR1, in concert with effectors ATP, RuBP, and fructose-1,6-bisphosphate, stabilizes the CbbR/promoter complex (69). A model for CbbR/CbbRR1/CbbRR2 interaction proposes that CbbRR2 acts as an antiactivator in the absence of effectors and that CbbRR1, by binding to CbbR and altering the conformation of CbbR, thus prevents CbbR from binding the *cbbLS* promoter (61). The presence of CbbRR1 and effectors negates the effect that CbbRR2 has on CbbR and allows binding of CbbR to the promoter and subsequent expression of the $cbb_I$ operon (61). A model summarizing the information relative to CbbR involvement with all these additional factors in *R. palustris* is presented (Fig. 3). Surface plasmon resonance (SPR) experiments were crucial to providing quantitative results concerning the effects of effectors on protein interactions as well as forming the basis for interpreting the interplay between CbbR, CbbRR1, CbbRR2, and effectors and how these factors all influence the regulation of the *cbbLS* promoter in *R. palustris* (60, 61, 69). Indeed, it is interesting that such a very complex system has been adopted by *R. palustris* to ensure control of the expression of form I RubisCO (*cbbL* and *cbbS*) and the genes of the $cbb_I$ operon. Of note, the $cbb_{II}$ operon, including the gene (*cbbM*) encoding form II RubisCO, does not appear to be controlled by CbbR or by CbbRR1, CbbRR2, or CbbSR (11).

## POSTRANSLATIONAL REGULATION OF CbbR FUNCTION: THE ROLE OF EFFECTOR METABOLITES

As prototypical LTTRs, CbbRs require a bound coinducer molecule or effector to activate transcription from the *cbb* promoter (17, 19). Common to most of the members of the LTTR family, the effector usually is an intermediate metabolite of the pathway that is regulated (17, 19). Effector binding occurs in a small cleft formed between RD-I and RD-II (Fig. 1) and is a hallmark of the LTTR family (22, 70–80). A recent study using nondenaturing mass spectrometry has illustrated for the first time that an LTTR tetramer binds four molecules of its effector in a stepwise pattern while bound to DNA (81). Binding of the effector produces a change in the angle at which an LTTR bends the promoter DNA to

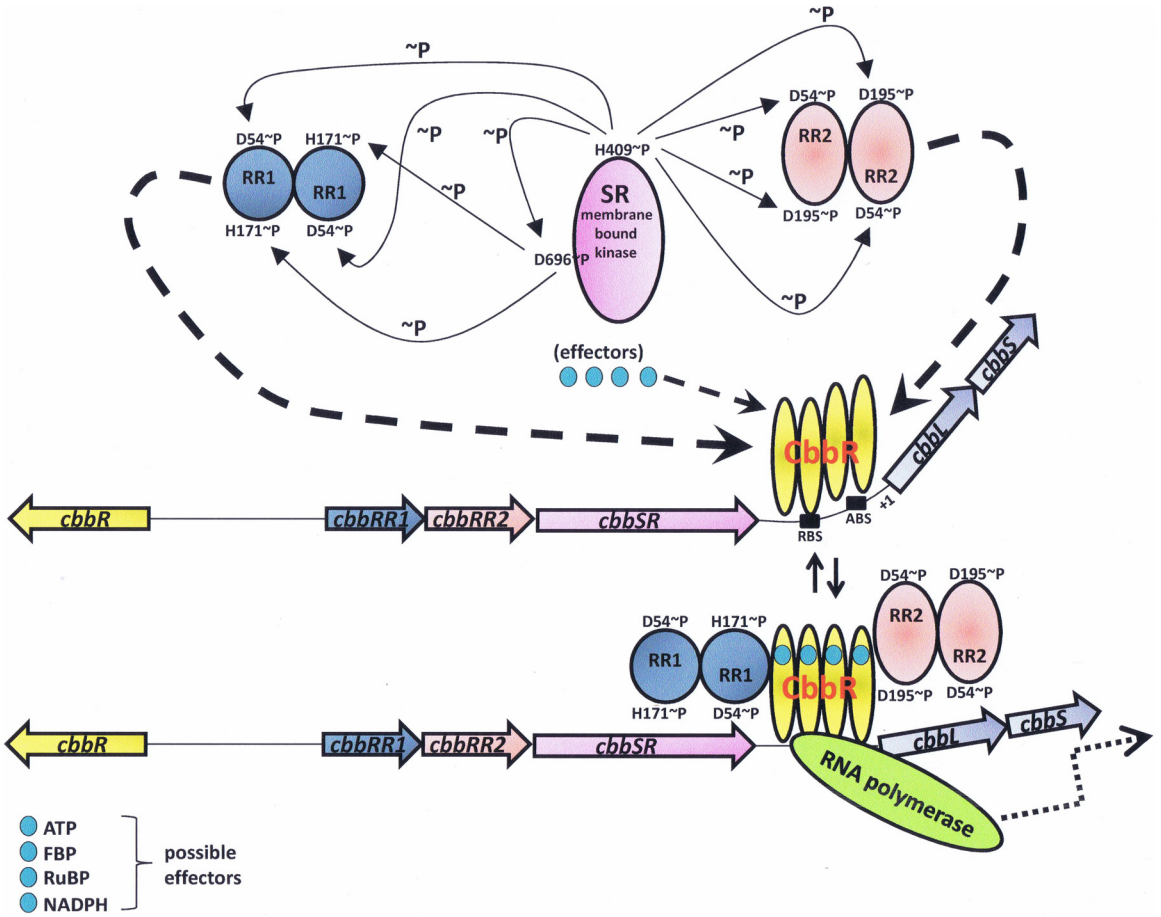


**FIG 3** Transcriptional regulation of the $cbb_I$ operon of *Rhodopseudomonas palustris* and the role of the four regulatory factors in the expression of $cbb_I$ under autotrophic conditions. SR is the membrane-bound sensor kinase (CbbSR) that autophosphorylates and catalyzes phosphorylation of the two response regulators, RR1 (CbbRR1) and RR2 (CbbRR2). RR1 and RR2 subsequently interact with CbbR. CbbR binds the $cbb_I$ promoter at the recognition binding site (RBS) and the activation binding site (ABS). Potential positive effectors ATP, FBP, RuBP, and NADPH (68) are shown. Dashed arrows represent interactions with CbbR (60, 61, 69). Oppositely pointing solid arrows represent reversible interactions with CbbR. Dotted arrows represent transcriptional activation. Relaxation of the bend angle that CbbR imposes on the $cbb_I$ promoter is brought about by effector binding and precedes transcription. ~P, phosphorylation at specific residues of CbbSR, CbbRR1, and CbbRR2 (68). The transcriptional start site is denoted +1.

which it is bound (17, 19). For most scenarios, this change in DNA bend angle initiates contact or alters contact with the RNA polymerase, activating transcription. Effector binding to the LTTR can also inhibit transcription (19, 82). Studies illustrating DNA bend angle modification or initiation of transcription brought about by effector binding have been reported for some CbbRs (8, 76, 82, 83). The effector molecules may be different for each CbbR, depending on the organism. Figure 4 illustrates the relaxation of the *cbb* promoter bend angle imposed by CbbR subsequent to conformational changes elicited by effector (RuBP) binding after the switch to autotrophic growth for *R. sphaeroides*, *R. capsulatus*, *Xanthobacter flavus*, *and H. thermoluteolus* (8, 76, 82, 83).

In *R. sphaeroides*, RuBP, which of course is a unique metabolite of the CBB pathway, was suggested to be the effector for CbbR. The suggestion was based on a study employing a strain deleted for form I and form II RubisCO, leading to an accumulation of RuBP and a subsequent increase in transcription for both the $cbb_I$ and $cbb_{II}$ operons (84). Additionally, *in vitro*, the CbbR from *R. sphaeroides* was shown to alter the angle at which it bends the $cbb_I$ promoter DNA in the presence of RuBP, as illustrated by a change in the mobility of the CbbR/DNA complex in gel mobility shift assays (76). DNase I footprint analysis also demonstrates that RuBP improves protection of the $cbb_I$ promoter by CbbR (76).

For *R. capsulatus*, the metabolites that may influence CbbR-mediated expression present a more complex situation. Since *R. capsulatus* has two CbbRs ($CbbR_I$ and $CbbR_{II}$) regulating two *cbb* operons ($cbb_I$ and $cbb_{II}$, respectively) (7), each CbbR has its own set of effector molecules. In gel mobility shift assays, expression of $CbbR_I$ was shown to result in a significant increase in binding to the $cbb_I$ promoter DNA in the presence of 3-phosphoglycerate, 2-phosphoglycolate, ATP, $KH_2PO_4$, and RuBP and a small increase in binding to the $cbb_I$ promoter in the presence of NADPH, fructose-6-phosphate, fructose-1,6-bisphosphate (FBP), and ribose-5-phosphate (85). DNase I footprint analyses illustrated modified protection of the $cbb_I$ promoter DNA by $CbbR_I$ in the presence of RuBP, indicating a change in conformation of $CbbR_I$ and suggesting an altered bend angle of the $cbb_I$ promoter DNA (85). For $CbbR_{II}$, gel mobility shifts demonstrated enhanced binding to the $cbb_{II}$ promoter in the presence of RuBP, 2-phosphoglycolate, 3-phosphoglycerate, phosphoenolpyruvate, and FBP (85).

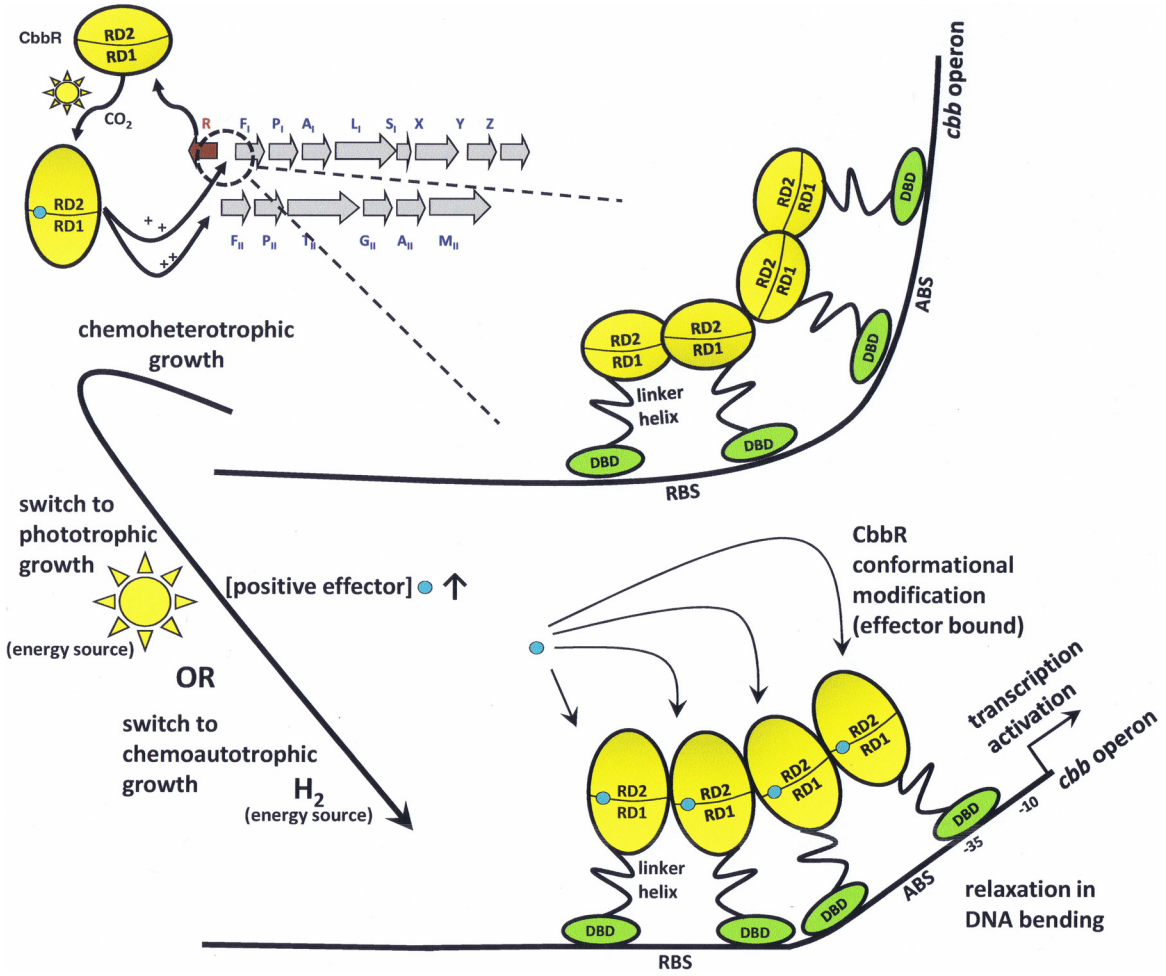


**FIG 4** Consequences of relaxation of the promoter DNA bend angle upon effector binding (blue circle) to CbbR. For four CbbR-containing bacteria, a change in the promoter DNA bend angle has been shown to occur upon effector binding to the effector pocket of the protein. In *R. sphaeroides*, as illustrated here, CbbR undergoes a conformational change upon binding of a positive effector such as RuBP as a result of switching from heterotrophic to photo- or chemoautotrophic $CO_2$-dependent growth conditions, thus allowing a relaxation of DNA bending and subsequent transcription of the *cbb* genes. Under appropriate physiological conditions, RuBP concentrations greatly increase and RuBP binds to CbbR of *R. sphaeroides* (76) and $CbbR_I$ of *R. capsulatus* (84), while phosphoenolpyruvate (PEP), FBP, and 3-PGA concentrations greatly increase and each binds to $CbbR_{II}$ of *R. capsulatus* during photoautotrophic growth (85). NADPH concentrations rapidly increase in *X. flavus* (83, 87) and *H. thermoluteolus* (8) during chemoautotrophic growth. This relaxation of the DNA bend angle leads to appropriate contact with RNA polymerase and activates transcription of the *cbb* operon. RBS, recognition binding site; ABS, activation binding site. RD-1 and RD-2, recognition domains 1 and 2, respectively.

DNase I footprint analyses illustrated modified protection of the $cbb_{II}$ promoter DNA by $CbbR_{II}$ in the presence of FBP, phosphoenolpyruvate, and 3-phosphoglycerate, indicating a change in conformation of $CbbR_{II}$ and suggesting an altered bend angle of the $cbb_{II}$ promoter DNA (85).

Several compounds act as effectors of CbbR from *R. palustris*. Gel mobility shift assays demonstrated that ATP, FBP, RuBP, and NADPH all enhance binding of CbbR to the *cbbLS* promoter and that phosphoenolpyruvate inhibits binding of CbbR to the promoter (69). Quantitative SPR studies provided rate constant information and verified that *R. palustris* CbbR exhibits greater affinity for the *cbbLS* promoter in the presence of RuBP, FBP, ATP, and NADPH (61, 69).

Based on *in vitro* transcription experiments, the presence of phosphoenolpyruvate was shown to severely inhibit transcription of the *cbb* promoter by CbbR in *R. eutropha* strain H16 (82). This makes the effector metabolite, phosphoenolpyruvate, a corepressor for CbbR in *R. eutropha*, binding CbbR in the effector pocket but with the opposite effect on transcription. Gel mobility shift studies indicated that phosphoenolpyruvate enhances binding of CbbR to the *cbb* promoter (82, 86). Recent results also indicate that RuBP, ATP, and NADPH increase binding of wild-type CbbR to the *cbb* promoter of *R. eutropha* (86). Several mutant CbbRs with single-amino-acid substitutions near the effector pocket have reduced binding affinities in the presence of phophoenolpyruvate, RuBP, and ATP (86). Two other organisms, *Xanthobacter flavus* and *Hydrogenophilus thermoluteolus*, have CbbRs that show altered promoter DNA bending or increased promoter affinity in the presence of NADPH (8, 83, 87).

The CbbR from *Cyanidioschyzon merolae* (referred to as plastid-encoded transcription factor Ycf30) displays increased binding affinity for its promoter in the presence of NADPH and RuBP, as reported using gel mobility assays (15). *In vivo* experiments in permeabilized chloroplasts also indicated that RubisCO gene

transcription is activated by 3-phosphoglyceric acid, RuBP, and NADPH (15). Ycf30 controls expression of the nucleus-independent RubisCO operon in chloroplasts in this red alga (15).

Studies have demonstrated that CmpR and CcmR can use the same effector molecules that are utilized by many cyanobacterial CbbRs. CmpR from *S. elongatus* PCC 7942 has high affinity for 2-phosphoglycolate and low affinity for RuBP, as illustrated in gel mobility shift studies that demonstrated enhanced binding of CmpR to the *cmp* operon regulatory region (45). CcmR from *Synechocystis* PCC 6803 regulates the promoter regions of a variety of genes involved in the CCM through the use of $NADP^+$ and α-ketoglutarate as effectors (49). SPR studies illustrated that both $NADP^+$ and α-ketoglutarate enhanced binding of CcmR to the *ndhF3* promoter, the regulatory region for several genes involved in high-affinity $CO_2$ uptake (49). Similarly to the case with *R. eutropha*, where CbbR utilizes phosphoenolpyruvate as a corepressor (82), CcmR binds $NADP^+$ and α-ketoglutarate as corepressors to repress expression of the genes involved in the inducible high-affinity CCM of *Synechocystis* sp. strain PCC 6803 (49).

Finally, in the methylotrophic bacterium *Methylobacterium extorquens* AM1, QscR regulates two serine-cycle pathway operons, *qsc1* and *qsc2*, and also regulates the expression of a third gene, *glyA* (40, 41). Intermediate metabolites of the serine-cycle and traditional-energy metabolites (effectors of CbbRs) were found not to be effectors of QscR (40). Formyl-tetrahydrofolate, an intermediate for formaldehyde assimilation which is linked to the serine cycle, was shown to be a candidate effector for QscR (41). Gel mobility shift assays demonstrated that formyl-tetrahydrofolate enhances binding of QscR to the promoters of both the *qsc1* and *qsc2* serine-cycle operons (41).

To better understand how effector molecules interact with CbbR, it is instructive to consider an enlargement of the effector pocket structure of the CbbR subfamily as a distinguishing feature to separate CbbRs from other LTTRs (Fig. 1). The four conserved regions that define the effector pocket contain positively charged residues, usually arginine (sometimes lysine), and polar residues that attract and accommodate negatively charged effectors. The conserved amino acid sequences (highlighted in Fig. 1 in magenta) for each region are as follows: for region 1, GVVSTAKY FXP; for region 2, NR; for region 3, DLAIMGRPP; and for region 4, REXGSGTR ("X" represents a residue position that is not conserved) (Fig. 1). All analyzed bacterial CbbRs utilize similar effector metabolites that have negatively charged phosphate moieties, usually two phosphate moieties, or that may be organic acids that contain two negatively charged acid groups (Table 1). Many of the CbbR effectors, such as RuBP, 3-PGA, FBP, 2-phosphoglycolate, and 2-phosphoglycerate, are metabolites of the CBB pathway and would be expected to be present at higher concentrations in the cell during active biosynthetic $CO_2$ assimilation.

**TABLE 1** CbbRs and subfamily members from various organisms and their effectors

| Source and protein | Effector metabolite(s) (reference[s]) |
|---|---|
| *R. sphaeroides* CbbR | RuBP[a,b] (76) |
| *R. capsulatus* | |
| $CbbR_I$ | RuBP,[c,b] PEP,[b] 3-PGA,[b] 2-phosphoglycolate,[b] 2-phosphoglycerate,[b] ATP,[b] $KH_2PO_4$[b] (85) |
| $CbbR_{II}$ | FBP,[c] 3-PGA,[c,b] PEP,[c,b] RuBP,[b] 2-phosphoglycolate[b] (85) |
| *R. eutropha* CbbR | PEP[b,d] (corepressor), RuBP,[b] ATP,[b] NADPH[b] (82, 86) |
| *R. palustris* CbbR | RuBP,[b,e] ATP,[b,e] FBP,[b,e] NADPH,[b] PEP (inhibits DNA binding) (61, 69) |
| *X. flavus* CbbR | NADPH[a,b] (83, 87) |
| *H. thermoluteolus* CbbR | NADPH[a] (8) |
| *C. merolae* CbbR | RuBP,[b,d] NADPH,[b,d] 3-PGA[d] (15) |
| *S. elongatus* CmpR | RuBP,[b] 2-phosphoglycolate[b] (45) |
| *Synechocystis* PCC 6803 CcmR | $NADP^+$,[e] α-ketoglutarate[e] (49) |
| *M. extorquens* QscR | Formyl-tetrahydrofolate[b] (41) |

[a] Metabolite that allows a change in the bend angle CbbR imposes on promoter DNA via gel mobility shift assay.
[b] Metabolite that increases CbbR binding affinity for promoter DNA (i.e., enhances stability of CbbR on promoter DNA) via gel mobility shift assay.
[c] Metabolite that alters the region of promoter DNA protected by CbbR via DNase I footprinting/protection assay.
[d] Metabolite that allows CbbR to activate (or inhibit) transcription from the *cbb* promoter via *in vitro* transcription assay.
[e] Metabolite that increases CbbR binding affinity for promoter DNA (i.e., enhances stability of CbbR on promoter DNA) via SPR.

## CONSTITUTIVELY ACTIVE CbbR PROTEINS

LTTR constitutive activity may be defined as activation of gene expression under conditions that normally repress gene transcription, typically in the absence of the LTTR's effector. When certain residues were altered, various LTTR proteins were found to constitutively activate gene expression; each LTTR appears to be unique with respect to which amino acid substitutions confer constitutive activity. This is probably a logical adaptation, as one might assume that the residues that are important for effector binding or for specific interactions with target DNA might be specific for each LTTR. ("LTTR*" denotes an LTTR variant with constitutive activity.) Many of these amino acid substitutions are centered at the effector pocket, but substitutions in other areas of the LTTR, such as at residues within the linker helix or hinge region or throughout RD-I and RD-II, can generate constitutive activity (70, 72, 76, 86, 88–94). Typically, single-amino-acid substitutions encompass the vast majority of the reported changes identified for constitutive proteins, and most constitutive proteins bind their effectors but may behave differently from the wild-type LTTR in gel mobility shift assays, DNase I footprinting assays, or *in vitro* transcription assays (76, 88, 89, 92–94). Amino acid substitutions that confer constitutive activity are thought to change the conformation of the LTTR tetramer to mimic the conformation seen when it is bound with the effector or to change the conformation of the LTTR/promoter complex to produce a favorable interaction with RNA polymerase to activate transcription. Studies of LTTR* proteins from several LTTR family members, including NodD, AmpR, OccR, CysB, OxyR, NahR, GtlR, XapR, and Cbl, have been previously published (70–72, 88–96).

A large set of CbbR* variants from both *R. sphaeroides* and *R. eutropha* have been isolated (76, 86). Constitutive proteins were generated by specific biological selection strategies involving the use of a reporter construct containing the *cbb* promoter fused to the *lacZ* open reading frame (ORF) integrated into the genome of a *cbbR* deletion strain for both organisms. The mutated *cbbR* proteins contain mutations that encode CbbR* proteins. For *R. sphaeroides*, several of the amino acid substitutions that confer constitutive activity clustered around the effector pocket which proved to be critical in defining the effector pocket for LTTRs (76). Sev-

eral of the CbbR* proteins interact differently with promoter DNA in the presence of RuBP (effector) compared to wild-type CbbR. Interestingly, several of the CbbR* proteins activate expression of the $cbb_I$ promoter to a much greater extent than wild-type CbbR under conditions of *cbb* activation (76). Under conditions repressive for *cbb* activation (chemoheterotrophic growth), the amounts of *cbb* expression produced by the CbbR* proteins differed greatly; that is to say, some CbbR* proteins were better than others at activating gene expression.

CbbR* proteins from *R. eutropha* with amino acid substitutions located in all regions of the protein except the DBD were isolated. Substitutions were localized in the effector pocket, throughout RD-I and RD-II, and in the C terminus, and several residue changes were located in the linker helix. All of these were previously characterized (86). One particular CbbR* of interest is a truncation, leaving only the DBD and the linker helix to act as a transcriptional regulator. Nonetheless, this truncated CbbR* was able to support growth under chemoautotrophic conditions and to activate the *cbb* operons under repressive conditions in *R. eutropha* (86). This truncated protein illustrates that the DBD/linker helix region of CbbR is sufficient to activate expression from the *cbb* promoter, demonstrating that either the DBD or the linker helix or both make contact with the RNA polymerase (86). Similarly to some CbbR* proteins from *R. sphaeroides*, some of the CbbR* proteins from *R. eutropha* activated expression at levels severalfold greater than those seen with wild-type CbbR under autotrophic growth conditions (76, 86). The CbbRs of *R. sphaeroides* and *R. eutropha* exhibit only 35.6% identity, and each species appears to have a specific suite of residue changes that lead to CbbR* activity. Indeed, conserved residues whose presence is known to result in constitutive activity in *R. sphaeroides* CbbR did not confer constitutive activity when similar residues were changed in *R. eutropha* CbbR (86).

## CONCLUDING REMARKS

CbbR controls the assimilation of carbon in autotrophic bacteria. It is the master regulator of the CBB $CO_2$ assimilation pathway, playing an essential role to ensure that the *cbb* genes are actively transcribed. It is clear that CbbR must be posttranslationally modified, and there are various ways in which this is accomplished, including the binding of small-molecule effectors as well as interactions with other transcription factors. Studies that investigate the interaction of other proteins with CbbR will advance understanding of how $CO_2$ fixation is regulated and of how LTTRs regulate transcription in general. CbbR also plays an important role in ensuring that $CO_2$-assimilatory organisms generate essential carbon metabolic intermediates that can be subsequently diverted into the synthesis of economically and globally meaningful biological molecules, such as biofuels. Constitutive CbbR variants have been proven to greatly increase the level of expression from the *cbb* promoter; thus, additional modification of the CbbR protein will further enhance the power of the *cbb* promoter as a tool for the production of biological compounds (76, 86). Other LTTRs are also amenable to constitutive modification, which may be important for the enhanced expression of other pathways in bacteria, since LTTRs are the most common transcriptional regulators in prokaryotes.

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