Interactions of the \textit{cbb}_{II} \ Promoter-Operator Region with CbbR and RegA (PrrA) Regulators Indicate Distinct Mechanisms to Control Expression of the Two \textit{cbb} Operons of \textit{Rhodobacter sphaeroides}\textsuperscript{*}

James M. Dubbs\textsuperscript{5} and F. Robert Tabita\textsuperscript{5§¶}

\textsuperscript{5}From the Department of Microbiology and the Plant Molecular Biology/Biotechnology Program, The Ohio State University, Columbus, Ohio 43210-1292

Author for Correspondence: F. Robert Tabita

Department of Microbiology

The Ohio State University

484 West 12\textsuperscript{th} Avenue

Columbus, OH 43210-1292

Telephone: (614) 292-4297

Fax: (614) 292-6337

E-mail: tabita.1@osu.edu

Running Title: Regulation of \textit{cbb}_{II} operon gene expression
SUMMARY

In a previous study [Dubbs, J. M., Bird, T. H., Bauer, C. E., and Tabita, F. R. (2000) J. Biol. Chem. 275, 19224-19230], it was demonstrated that the regulators CbbR and RegA (PrrA) interacted with both promoter proximal and promoter distal regions of the form I (cbbI) promoter operon specifying genes of the Calvin-Benson-Bassham (CBB) cycle of *Rhodobacter sphaeroides*. To determine how these regulators interact with the form II (cbbII) promoter, three *cbbFII::lacZ* translational fusion plasmids were constructed containing various lengths of sequence 5' to the *cbbII* operon of *R. sphaeroides* CAC. Expression of β-galactosidase was monitored under a variety of growth conditions in both the parental strain and knockout strains that contain mutations that affect synthesis of CbbR and RegA. The binding sites for both CbbR and RegA were determined by DNase I footprinting. A region of the *cbbII* promoter from +38 bp to –227 bp contained a CbbR binding site and conferred low-level regulated *cbbII* expression. The region from –227 bp to –1025 bp contained six RegA binding sites and conferred enhanced *cbbII* expression under all growth conditions. Unlike the *cbbI* operon, the region between –227 bp and –545 bp, that contains one RegA binding site, was responsible for the majority of the observed enhancement. Both RegA and CbbR were required for maximal *cbbII* expression. Two potentially novel and specific *cbbII* promoter-binding proteins that did not interact with the *cbbI* promoter region were detected in crude extracts of *R. sphaeroides*. These results, combined with the observation that chemoautotrophic expression of the *cbbI* operon is RegA independent, indicated that the mechanisms controlling *cbbI* and *cbbII* operon expression during chemoautotrophic growth are quite different.
INTRODUCTION

The nonsulphur purple bacterium *Rhodobacter sphaeroides* utilizes the Calvin-Benson-Bassham (CBB)\(^1\) reductive pentose cycle as its primary pathway for CO\(_2\) fixation. In this metabolically diverse organism the CBB cycle plays two very different roles. Under autotrophic growth conditions, CO\(_2\) serves as the sole carbon source and the CBB cycle is the primary source for nearly all of the fixed carbon utilized by the cell. This may entail aerobic chemoautotrophic growth in the dark (i.e. in a minimal medium lacking organic carbon under an atmosphere of 5%CO\(_2\)-45%H\(_2\)-50% air) or anaerobic photoautotrophic in the light (i.e. in a minimal medium bubbled with 1.5%CO\(_2\)/98.5%H\(_2\)). Photoheterotrophic growth in the presence of a fixed carbon source causes the role of the CBB cycle to shift, such that CO\(_2\) serves primarily as an electron sink, with excess reducing equivalents generated by the oxidation of fixed carbon compounds funneled to CO\(_2\) (1). When grown under conditions where the CBB cycle is required, *R. sphaeroides* maintains the appropriate level of CBB cycle activity through the coordinate expression of two CBB cycle operons, denoted *cbbI* and *cbbII* (2, 3). In addition to structural genes that encode CBB cycle enzymes, each operon encodes one of two distinct forms of ribulose bisphosphate carboxylase/oxygenase (Rubisco). The *cbbI* operon contains the genes for a form I (L\(_8\)S\(_8\)) Rubisco (*cbbL\(_I\)cbbS\(_I\)*) (4) while the *cbbII* operon encodes the large subunit of a form II type Rubisco (*cbbM\(_II\)*) (5). The regulation of *cbb* gene expression in *R. sphaeroides* is quite complex (2). Expression of the genes in both the *cbbI* and *cbbII* operons is highly induced during anaerobic phototrophic growth and moderately induced during aerobic chemoautotrophic growth (6). During growth under CO\(_2\) fixing conditions, expression of each operon is modulated independently in response
to a number of environmental parameters such as the level of CO$_2$ and the reduction state of organic carbon compounds supplied for growth (7-11). This independent regulation results in shifts in the relative abundance of proteins encoded within each operon. In general, growth under photoheterotrophic conditions, with a fixed (organic) carbon source, results in an excess of $cbb_{II}$ expression over $cbb_I$. Maximal expression from both operons is observed under photoautotrophic and chemoautotrophic conditions; i.e., when CO$_2$ is used as the sole carbon source, with $cbb_I$ operon expression exceeding that for the $cbb_{II}$ operon (11). In addition to the apparent independent regulation of $cbb_I$ and $cbb_{II}$ gene expression, a mechanism for interdependent regulation also exists that results in a compensatory increase in the expression of one operon when the other is inactivated (4, 7, 9, 10). The $cbbR$ gene, which encodes a LysR-type transcriptional regulator, is located immediately upstream and divergently transcribed from $cbbF_I$ (12) and mediates this compensatory effect. CbbR is a positive regulator of the expression of both the $cbb_I$ and $cbb_{II}$ operons (12, 13). The $regA-regB$ ($prrA-prrB$) two component regulatory system, encoding sensor kinase RegB (PrrB) and response regulator RegA (PrrA), also plays a role in $cbb$ regulation. Although originally identified as a regulator of photosystem biosynthesis genes in both $R$. capsulatus (14, 15) and $R$. sphaeroides (16), the $regA-regB$ ($prrA-prrB$) two component regulatory system was implicated in $cbb$ regulation by genetic studies which demonstrated that a $R$. sphaeroides $regB$ insertion mutant exhibited reduced $cbb_I$ and $cbb_{II}$ expression during photoautotrophic growth in a 1.5% CO$_2$-98.5% H$_2$ atmosphere (17). It was subsequently shown that $regA$ is required for $cbb_I$ and $cbb_{II}$ expression during incubation under photoautotrophic growth conditions (18). It has also been demonstrated that RegA binds directly to $cbb$ operon promoters in both $R$. 
capsulatus and R. sphaeroides (18, 19). A growing number of studies have shown that the regA-regB (prrA-prrB) two-component system, and its homologs, regulate the expression of genes involved in a wide variety of metabolic processes such as nitrogen fixation and nitrogen metabolism (20-22), hydrogen utilization and evolution (20, 22), electron transport (23) and the oxidation of formaldehyde (24).

The overall goal of our ongoing investigation is to understand the mechanism(s) involved in the regulation of cbb gene expression in R. sphaeroides. Prior to this work, the primary model system for our cbb gene regulation studies was the R. sphaeroides cbbI operon. Previous studies, using cbbI::lacZ promoter fusions, showed that the cbbI promoter contains a promoter proximal region (-100 bp to +1 bp) that confers low-level regulated expression of cbbI that is CbbR-dependent (13). DNaseI footprinting studies showed that this region contained a binding site for CbbR (-10 bp to -70 bp) along with two RegA binding sites (-61 to -110) (18). A promoter distal upstream activating region was also identified, between -280 bp and -636 bp that significantly enhanced cbbI expression under all growth conditions tested. This region was found to contain two RegA binding sites (-301 to -415 bp) (18). Although, earlier work determined that the cbbII promoter occurred within 1000 bp of the cbbII transcription start (25), details of the structure of the R. sphaeroides cbbII promoter have not been previously investigated. In this study, cbbII::lacZ translational fusions with different amounts of upstream sequence were constructed to facilitate monitoring of gene expression under a variety of growth conditions. Evidence for upstream activating sequences was obtained within the cbbII promoter region and DNaseI footprint analyses enabled binding sites for both CbbR and RegA to be identified within the cbbII promoter region. An important byproduct of these
studies was the demonstration that two potentially novel and specific $cbb_{II}$ promoter binding proteins were present in cell extracts of $R. \text{sphaeroides}$. The results of this investigation indicated that the structure of the $R. \text{sphaeroides} \ cbb_{II}$ promoter exhibited both similarities and differences to the $R. \text{sphaeroides} \ cbb_{I}$ promoter (18), with consequent effects on differential regulation of the $cbb$ operons.
EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, and culture conditions - *R. sphaeroides* strains CAC (6), and CAC::regAΩ (17) were grown photoautotrophically under a gas atmosphere of 1.5 % CO₂/98.5% H₂ and chemoautotrophically under a gas atmosphere of 5 % CO₂/45 % H₂/50 % Air as previously described (11, 13). *R. sphaeroides* strains CAC (6), CAC::regAΩ (26) and 1312 (12) were also grown photoheterotrophically under a 100% argon atmosphere in Ormerod's medium supplemented with 0.4% malate (27). Aerobic chemoheterotrophic growth was performed using Ormerod's medium supplemented with 0.4% malate while shaking vigorously in the dark at 30 °C. Growth on solid media was performed with the addition of 1.5% agar and anaerobiosis was achieved using a BBL Gas Pak anaerobic system (Becton Dickinson Microbiology Systems, Cockeysville, MD). Triparental matings were performed using helper plasmid pRK2013 according to methods previously described (28). Antibiotics were added to the media, as required, at the following concentrations (in µg/ml): for *E. coli*, ampicillin (100-200), tetracycline (12.5), spectinomycin (20), and kanamycin (50); for *R. sphaeroides*, trimethoprim (50), kanamycin (25), tetracycline (3.5), and spectinomycin (20).

β-galactosidase assays - Cultures of *R. sphaeroides* strains CAC, CAC::regAΩ and 1312 were sampled (10 ml) at an O.D.₆₆₀nm of between 0.8 and 1.1. Sonicated extracts were generated in a buffer containing 10 mM Tris-Cl, pH 8.0, 1 mM EDTA and 5 mM β-mercaptoethanol. Total protein was determined using the Bio-Rad protein assay dye-binding reagent (Bio-Rad Laboratories, Hercules, CA). β-galactosidase assays were performed as previously described (13).
Purification of RegA*, RegB’’ and CbbR - RegA* was purified from *E. coli* strain BL21(DE3) carrying the plasmid pET29CBD::regA* using a method previously described (29).

RegB’’, a truncated form of RegB lacking a membrane spanning region, was purified from *E. coli* strain BL21(DE3) carrying plasmid pET28alt::regB’’ as previously described (30).

Preparations of recombinant *R. sphaeroides* CbbR were obtained from *E. coli* strain BL21(DE3) carrying plasmid pET11R-11 as previously described (13).

**DNaseI footprint analysis** - Probes for DNase I footprint analyses were prepared by PCR amplification of selected regions of the cbbII operon promoter of *R. sphaeroides*, using pJG3 (31) as a template. Selective labeling of DNA strands was performed by 5’ end labeling of one of the oligonucleotide primers with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and [γ-32P] ATP (7000 Ci/nmol, ICN Biochemicals, Costa Mesa, CA)+) prior to amplification. The PCR reactions consisted of 1 µmole of each labeled and unlabeled oligonucleotide primer, 28 ng of template DNA and 2.5 units of Taq polymerase (GIBCO BRL, Gaithersburg, MD). Amplification was performed as follows: denaturation at 95°C for 5 min, then 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. A final elongation step was performed for 7 min at 72°C. PCR fragments were purified on non-denaturing polyacrylamide gels followed by electroelution. The isolated probe DNA was then ethanol precipitated and resuspended in 100 µl of buffer containing 50 mM HEPES (pH 8.0) and 100 mM sodium acetate.

DNase I footprint assays were performed as described previously (18). RegA* was phosphorylated in a reaction containing RegA* (128 µM), RegB’’ (128 µM), ATP (1
mM) in a buffer containing 50 mM HEPES, pH 7.8, 5 mM MgCl₂, 100 mM KCl, 2 mM CaCl₂, 1.5 mM dithiothreitol, 25 µg/ml bovine serum albumin and 25% glycerol. Standard G+A DNA sequence ladders were generated by the chemical cleavage method as described by Ausubel et al. (32). The sequences of the oligonucleotides used to generate the probes used for the CbbR footprinting experiments are as follows: cbbII-1, 5’-CATGGCTCCTCCTGCTCTG-3’; cbbII-2, 5’-TCCTCGAGAGCGGGGTGAGCG-3’. The sequences of the oligonucleotides used to generate the probes used in the RegA* footprinting experiments are as follows: cbbII-5, 5’-CAGGGCCCGGGGTGACC-3’; cbbII-11, 5’-GCCCTGCCCAAGCCTGACGC-3’; cbbII-13, 5’-GACGCCCTGCGGACACGGACC-3’; cbbII-14, GCCCGGCGAGACGCTCGCTC-3’; cbbII-15B, 5’-CCCTGCTCCTGGGTCGCTGC-3’; cbbII-16, 5’-GCAAGGCAGACTCGAGGAG-3’; cbbII-19, 5’-CGCCGCCTGTGCCACCATGC-3’; cbbII-20, 5’-GCATGGTGGCAGACCCGCG-3’.

Preparation of extracts for column chromatography and gel mobility shift assays – Extracts of R. sphaeroides strains grown to late exponential phase (i.e. O.D._{660 nm} ~ 1.0) were sonicated in a buffer containing 10 mM KCl, 20 mM HEPES, pH 8.5, 1 mM EDTA and 5 mM β-mercaptoethanol (buffer A). The cleared supernatant was precipitated with ammonium sulfate at 70% saturation and the precipitated proteins were resuspended and dialyzed against buffer A. The sample was then bound to a 3 ml Uno-Q (Bio-Rad) column equilibrated with buffer A and eluted with a 10 mM to 1.5 M KCl gradient in buffer A. Column chromatography was performed using a Biologic Duo-Flow FPLC (BioRad). Eluted fractions were assayed for cbbII promoter binding activity by the gel mobility shift assay. Gel mobility shift DNA binding reactions were performed in a 50 µl
total volume containing 50% eluted fraction, 36 % buffer A, 1.8 µg poly(dI-dC) · poly(dI-dC), 10% glycerol and 20,000 cpm of $^{32}$P-radiolabeled probe DNA. Gel electrophoresis was performed as previously described (13). Gel mobility shift probes were generated by either filling in restriction fragment overhanging ends with DNA polymerase I Klenow fragment in the presence of [$\alpha$-$^{32}$P] dCTP and dNTPs or PCR amplification using [$\gamma$-$^{32}$P] end labeled oligonucleotides as described earlier for DNaseI footprint analysis. Gel mobility shift probes 1, 2 and 3 in Figure 5 were generated using the cbbII promoter restriction fragments XhoI/BamHI, XhoI/XcmI, and XcmI/BamHI, respectively. The oligonucleotide pairs used to generate probes 4, 5 and 6 in Figure 5 were cbbII-16:cbbII-3, cbbII-15B:cbbII-3 and cbbII-1:cbbII-2, respectively. The sequence of oligonucleotide cbbII-3 is 5’-CGCGTCGAACAGTGGGGCGCC-3’. The sequences of the other oligonucleotides used are listed earlier in Experimental Procedures under DNaseI footprint analysis.

Construction of cbbII::lacZ promoter fusion plasmids - A 1056 bp fragment containing the cbbII promoter was amplified from pJG3 (31) via PCR. The primers used for the amplification were designed to create an EcoRI site 1020 bp upstream of the cbbII transcription start and a BglII site 47 bp downstream of the cbbII transcription start, within cbbFII. This fragment was blunt-end cloned into the HincII site of pK18 to generate plasmid pKcbbII and sequenced. The 1056 bp EcoRI/BglII fragment of pKcbbII was ligated into EcoRI/BamHI digested pMC1403 to yield plasmid pMCCII. The BamHI/BglII ligation in pMCCII resulted in an in frame fusion of cbbFII to lacZ that contains the first three codons of cbbFII. EcoRI digested pMCCII was then ligated into the EcoRI site of the conjugative plasmid pVK101 (33) to yield plasmid pVKCII.
To construct plasmid pVKCIIXho, the sequence upstream of the XhoI site at -638 bp was deleted by digestion of pKcbbII with Smal/XhoI. The overhanging ends were filled using Klenow polymerase and dNTPs followed by religation. Ligation products that had not deleted the Smal/XhoI fragment were selected against by digestion with KpnI before transformation into E. coli JM109. The plasmid (pKIIΔXho) selected was found to have deleted an additional 93 bp downstream of the XhoI site to -545 bp. A 606 bp EcoRI/BglII fragment of pKIIΔXho was ligated into EcoRI/BamHI digested pMC1403 to generate pMCCIIXho. EcoRI digested pMCCIIXho was then ligated into the EcoRI site of the conjugative plasmid pVK101 to yield pVKCIIXho. The method used for the construction of pVKCIIXcm was the same as that for pVKCIIXho except that XcmI was used in place of XhoI to generate plasmids pKIIΔXcm and pMCCIIXcm. This resulted in a lacZ fusion containing 227 bp upstream of the cbbII transcription start.
RESULTS

Regulation of the cbbII operon in R. sphaeroides CAC- In order to identify the DNA sequences required for transcriptional regulation of the cbbII operon a number of lacZ translational fusion plasmids containing increasing amounts of DNA upstream of the cbbFII translation start were constructed. Previous studies, using transcriptional fusions to xylE (25), determined that the cbbII promoter was located within 991 bp of the cbbFII translation start. It was with this in mind that we constructed cbbFII::lacZ translational fusion plasmids containing 1025 bp (pVKCII), 545 bp (pVKCIIXho) and 227 bp (pVKCIIXcm) of sequence upstream of the cbbFII transcription start (Fig. 1). These plasmids were transferred into R. sphaeroides CAC, a spontaneous gain of function mutant of R. sphaeroides HR that has the ability to grow chemoautotrophically in the presence of 10% O2 concentrations (i.e., in minimal medium lacking organic carbon bubbled with 5%CO2-45% H2-50% air) (6). The plasmid-containing strains were then assayed for β-galactosidase activity under chemoheterotrophic, photoheterotrophic, photoautotrophic, and chemoautotrophic growth conditions (Table II). As expected, aerobic chemoheterotrophic growth in malate media yielded the lowest LacZ activity for each plasmid-containing strain. There was a dramatic increase in LacZ activity in cells grown under photoautotrophic conditions (i.e. CO2 as the sole carbon source), while photoheterotrophic growth with malate as the carbon source resulted in significantly lower activity. During aerobic chemoautotrophic growth, fusion-plasmids pVKCIIXcm, pVKCIIXho and pVKCII mediated levels of LacZ activity that were 77%, 44% and 74% lower, respectively, than levels obtained under photoautotrophic growth conditions.
It is known that form II Rubisco synthesis and \textit{cbbM} transcription is enhanced under photoautotrophic growth conditions (4, 11). The enhanced level of LacZ activity during photoautotrophic growth using plasmid pVKCIIXcm is consistent with these prior studies and suggested that the \textit{cbbII} promoter contained a proximal regulatory region within 227 bp upstream of the \textit{cbbF}_{II} transcription start (pVKCIIXcm) that conferred the proper regulatory pattern.

Two regions upstream of –227 bp were found to affect \textit{cbbII} transcription. The first upstream regulatory region (region 1) strongly activated \textit{cbbII} expression under all growth conditions and is situated between -227 bp and -545 bp (Fig. 1). This region activated \textit{cbbII} expression 10-fold, 16-fold and 39-fold under photoheterotrophic, photoautotrophic and chemoautotrophic growth conditions, respectively (Table II, compare pVKCIIXcm to pVKCIIXho). Even during aerobic chemoheterotrophic growth conditions, region 1 conferred a 2-fold enhancement of \textit{cbbII} expression. The second upstream regulatory region (region 2) spans the sequence between -545 and -1025 bp. This region enhanced \textit{cbbII} expression only during chemoheterotrophic and photoautotrophic growth conditions, where it conferred 6-fold and 1.5-fold increases in \textit{cbbII} expression, respectively (Table II, compare pVKCIIXcm to pVKCIIXho). During chemoautotrophic growth region 2 had a slightly negative effect, reducing \textit{cbbII} expression by approximately 29%.

\textit{Regulation of cbbII promoter activity in R. sphaeroides cbbR insertion strain 1312}- In order to investigate the role that the trans-acting transcriptional activator CbbR plays in \textit{cbbII} operon regulation, fusion plasmids pVKCIIXcm, pVKCIIXho and pVKCII were introduced into \textit{cbbR} insertion strain 1312 and lacZ expression was monitored under
chemoheterotrophic and photoheterotrophic growth conditions (Table II). Strain 1312 harbors an inactivated CbbR due to the insertion of a trimethoprim resistance cassette within the *cbbR* gene (12). Consequently, strain 1312 does not synthesize form I Rubisco and accumulates only low levels of form II Rubisco compared to wild-type *R. sphaeroides*. As a result, strain 1312 is capable of growth under chemoheterotrophic and photoheterotrophic conditions but not under photoautotrophic or chemoautotrophic conditions (12). It was apparent that normal regulated expression exhibited by each of the *cbbFII-lacZ* fusion plasmids upon switching from chemoheterotrophic to photoheterotrophic growth was abolished in this strain (Table II) indicating that a functional *cbbR* gene was necessary in order for the upstream activating sequence contained in plasmids pVKCIIIXho and pVKCII to influence *lacZ* expression.

*Regulation of cbbII promoter activity in R. sphaeroides regA insertion mutant strain CAC::regAΩ* - Previous studies had demonstrated that the RegA-RegB (PrrA-PrrB) two-component regulatory system is also required, along with *cbbR*, for maximum *cbb* expression in *R. sphaeroides* (17, 18). The *regA*-insertion mutant strain *R. sphaeroides* CAC::regAΩ is unable to grow under phototrophic growth conditions, however, it does have the ability to grow under aerobic chemoheterotrophic and aerobic chemoautotrophic conditions. Consequently, the *cbbFII-lacZ* fusions were introduced into *R. sphaeroides* CAC::regAΩ; *lacZ* expression was monitored during growth under aerobic chemoheterotrophic and chemoautotrophic conditions and compared to strain CAC (Table II). In chemoautotrophically grown *R. sphaeroides* CAC::regAΩ, the promoter proximal regulatory region mediated a dramatic 7-fold increase in *cbbII* expression relative to that in the parental strain (Table II, pVKCIIIXcm). These results
indicated that the Reg/Prr system may have an indirect negative regulatory affect on this region under this growth condition. Under aerobic chemoautotrophic growth conditions, the positive regulatory effect of region 1 and the somewhat lessened affect of region 2 in the wild-type were completely negated in the regA background. In fact, region 2 (Table II, pVKCII) conferred a moderate enhancement of cbbII expression during aerobic chemoautotrophic growth suggesting that the negative effect of this region is regA dependent in the wild-type. Clearly, regA was necessary for high-level regulated expression of the cbbII operon during aerobic chemoautotrophic growth. Interestingly, regA appeared also to be necessary for maximal aerobic chemoheterotrophic expression of the cbbII operon as well, since cbbII activation in the wild-type strain via region 1, and especially region 2, was no longer observed in the regA strain (Table II, pVKCIIXho and pVKCII).

**Binding of R. sphaeroides CbbR to the cbbII promoter** - Previous gel mobility shift studies had indicated that CbbR binds to the cbbII promoter within 200 bp of the cbbII transcription start (data not shown). In order to define the site of CbbR binding, DNase I protection assays, using [32P]-labeled probes spanning the region from +41 bp to -149 bp relative to the cbbII transcription start were performed. CbbR protected two closely spaced regions, as seen by DNaseI digestion (Figs. 2). The first site (site A) was located from -1 bp to -31 bp and the second site (site B), stretched from -35 bp to -61 bp. A DNaseI hypersensitive site was found between sites A and B at -32 bp.

**Binding of R. capsulatus RegA* to the R. sphaeroides cbbII promoter** - Previous studies had shown that the RegA-RegB (PrrA-PrrB) system is involved in cbb regulation in *R. sphaeroides* (17) and a constitutively active RegA protein (RegA*) from the related
nonsulphur purple bacterium *R. capsulatus*, was shown to bind specific regions of the *cbb*₇ operon promoter-operator of *R. sphaeroides* (18). To define the RegA binding sites, we used *R. capsulatus* RegA* in DNase I footprinting experiments of the *R. sphaeroides* *cbb*₁₁₁ promoter-operator region. [³²P]-labeled probes that covered a region from +41 bp to -1038 bp were employed. The results indicated that RegA* bound to the *R. sphaeroides* *cbb*₁₁₁ promoter at six distinct sites (Fig. 3A, B, C, D). Protection at the first site (site 1) was found from -282 bp to -308 bp with DNase I hypersensitive sites occurring at -280 bp and -302 bp (Figs. 3A). The second site (site 2) was widely separated from site 1 and consisted of protection from -583 bp to -601 bp with a hypersensitive site located at -597 bp (Fig. 3B). The third RegA* binding site (site 3) protected the region from -733 bp to -749 bp with a hypersensitive site at -744 bp (Fig. 3C). The fourth site (site 4) was comprised of an area of protection from -761 bp to -784 bp (Fig. 3C ). Protection at site 5 spanned the region from -836 bp to -851 bp with a DNase I hypersensitive site at -839 bp (Fig. 3D). The final RegA* binding site (site 6) showed protection from -857 bp to -883 bp with DNaseI hypersensitive sites at -867 bp and -882 bp (Fig. 3D).

*Detection of cbb*₁₁₁ *promoter binding activities in extracts of R. sphaeroides strain CAC*-The results of the *cbb*₁₁₁::*lacZ* fusion studies suggested that other proteins or other factors might be involved in *cbb*₁₁₁ regulation in addition to CbbR and RegA under aerobic chemoautotrophic growth conditions. This was particularly evident since a *cbb*₁₁₁::*lacZ* fusion plasmid containing 227 bp of sequence upstream of the *cbb*₁₁₁ transcription start (pVKCIIXcm) showed a higher level of *lacZ* expression under chemoautotrophic growth conditions in a *regA* mutant background, compared to the parental strain. In addition, the involvement of potential alternative regulators was consistent with the observation that
*cbb* II expression during aerobic chemoautotrophic growth was significantly lower than during anaerobic photoautotrophic growth. In order to determine if additional *cbb* II regulatory proteins were present in *R. sphaeroides*, a crude extract of chemoautotrophically grown *R. sphaeroides* CAC::regAΩ was subjected to ammonium sulfate precipitation to 70% saturation. The 70% ammonium sulfate fraction was separated by anionic exchange chromatography (Uno-Q, Bio-Rad) and eluted with a 50 mM to 1.5 M KCl gradient. The resulting fractions were tested for *cbb* II promoter binding activity in a gel mobility shift assay. The results of the gel mobility shift assay indicated that two DNA binding activities were present that bound to a probe spanning a region from -4 bp to -637 bp (*Bam* HI/*Xho* I fragment) upstream of the *cbb* II transcription start (Fig. 4). The first *cbb* II promoter binding activity (activity X) eluted in several fractions between 0.23 M and 0.44 M KCl. The second binding activity (activity Y) eluted in a single fraction at approximately 0.62 M KCl. Both of the *cbb* II promoter binding activities could also be separated using heparin sepharose affinity chromatography (data not shown). In order to more closely define the region of the *cbb* II promoter to which activity X binds, additional gel mobility shifts were performed using a heparin agarose fraction derived from extracts of *R. sphaeroides* grown under photoautotrophic conditions using DNA probes spanning various regions between -4 bp to -637 bp upstream of the *cbb* II transcription start (Fig. 5 A, B). Activity X bound to probe 4 (spanning -91 bp to -650 bp) resulting in one “high intensity” shifted band and an additional less abundant more slowly migrating band. A gel mobility shift assay using the shorter probe 5, spanning -91 to -357 bp, produced a single “high intensity” shifted band. Use of probe 6 (spanning +41 bp to -149 bp) detected a single “faint” shifted signal. The
gel mobility shift experiments also showed that cleavage of probe 1, spanning –4 bp to –637 bp, by digestion with XcmI, resulted in no binding to either of the resulting fragments (probes 2 and 3 in Fig. 5 B). The results suggest that activity X binds to the \textit{cbb}II promoter region at a high affinity site between positions -91 bp and -357 bp, probably in the vicinity of the \textit{XcmI} site at -221 bp. Additional lower affinity binding sites may also occur between +41 bp and -149 bp and between -357 bp and -650 bp.
DISCUSSION

This investigation and previous studies (13, 18) indicate that the *R. sphaeroides cbb*<sub>I</sub> and *cbb*<sub>II</sub> promoters have similar structural features. Both promoters are composed of a promoter proximal regulatory region, containing a CbbR binding site sufficient to confer low level regulated *cbb* expression; in addition, a more distal upstream activating region, containing RegA binding sites, enhances expression. While each upstream activating region contains multiple RegA binding sites, a single site in each operon (located at –301 bp in *cbb*<sub>I</sub> and –282 bp in *cbb*<sub>II</sub>) is responsible for the majority of this activation suggesting that both of these sites function similarly during *cbb* activation.

The placement of the CbbR and RegA binding sites and the involvement of upstream sequences in regulated expression of the *cbb*<sub>II</sub> operon is summarized (Fig. 6). Not surprisingly the regulation of the *cbb*<sub>II</sub> operon mirrored that of the *cbb*<sub>I</sub> operon with low expression during aerobic chemoheterotrophic growth and high expression during phototrophic growth. Maximal *cbb* expression during phototrophic growth has been shown to be dependent on *cbbR* as well as *reg* (12, 13, 18). Thus far, the involvement of upstream activating sequences appears to be unique to *R. sphaeroides* as such sequences are not involved with the regulation of the *cbb*<sub>I</sub> and *cbb*<sub>II</sub> operons of the related organism *R. capsulatus* (19).

The discovery that maximal aerobic chemoautotrophic expression of *cbb*<sub>II</sub> required *regA* was unexpected, given that chemoautotrophic expression of *cbb*<sub>I</sub> is *regA* independent (34). This indicates that molecular mechanisms involved with regulating the two operons are quite distinct under this growth condition. While the nature of the
different chemoautotrophic regulatory mechanisms is not known, the need for different control mechanisms may stem from the fact that O₂ serves as a terminal electron acceptor during chemoautotrophic growth. Previously, it was shown that the action of the Reg/Prr two-component system of *R. sphaeroides* may be mediated by electron flow through a cbb₃-type terminal cytochrome oxidase since inactivation of the operon (*ccoNOPQ*) that encodes this oxidase resulted in aberrant *regA* dependent activation of photopigment gene expression under aerobic growth conditions (35). It is possible that during chemoautotrophic growth electron flow to O₂ via the cbb₃-oxidase may dampen the Reg/Prr mediated activation of *cbb* gene expression to the extent that RegA activation alone would produce an insufficient level of CBB cycle enzymes to support optimal growth. The fact that the *cbbII* promoter is expressed at reduced levels during chemoautotrophic growth relative to photoautotrophic growth is consistent with this idea. An inability to support high level *cbb* expression might necessitate the recruitment of an additional positive regulatory system(s). The most probable target for chemoautotrophic up-regulation would be the *cbbI* operon since it encodes the form I (L₈S₈) Rubisco, used as the major autotrophic enzyme in the CBB pathway (1). However, form II Rubisco and enzymes encoded by the *cbbII* operon, allow the CBB pathway to play a somewhat more specialized role such that CO₂ may be employed as a terminal electron acceptor (11). Thus, retaining Reg/Prr control over *cbbII* gene expression during chemoautotrophic growth may give *R. sphaeroides* an enhanced ability to regulate redox poise when growing at the expense of highly reduced electron donors (i.e., molecular H₂).

Additional regulators may also affect *cbbII* expression during aerobic chemoautotrophic growth. In a *regA* background, the level of chemoautotrophic *cbbII*
expression from the promoter proximal regulatory region (pVKCIIXcm) was significantly higher than that in parental strain CAC. This increase in cbbII expression in the regA mutant could be due to either activation by CbbR or additional cbbII-specific regulatory proteins whose expression may or may not be affected by regA. The detection of two cbbII promoter-binding proteins, X and Y, in extracts of R. sphaeroides grown both chemoautotrophically and photoautotrophically, provided direct evidence for additional cbbII-specific proteins that bind physiologically significant regulatory sequences. Although proteins X and Y have not yet been identified, binding cannot be attributed to RegA due to the fact that these proteins were present in extracts of a R. sphaeroides regA strain. Moreover, protein X was detected in extracts of photoheterotrophically-grown R. sphaeroides cbbR mutant strain 1312.

In conclusion, despite the involvement of similar upstream activation sequences, it is clear that distinct molecular mechanisms serve to regulate gene expression of the two major cbb operons of R. sphaeroides. Moreover, it is apparent that the global two-component Reg/Prr system is only selectively involved with cbb control, with Reg/Prr required to activate transcription of only the cbbII operon, and not the cbbI operon under aerobic chemoautotrophic growth conditions. Future studies will focus on further elucidating regulatory mechanism(s) involved in cbb activation that are both distinct and common to both operons, as well as identifying and determining the role of recently discovered proteins that bind specifically to the cbbII promoter.

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REFERENCES


**FOOTNOTES**

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¶To whom correspondence should be addressed: Department of Microbiology, The Ohio State University, 484 West 12th Ave., Columbus, OH 43210-1292. Tel: (614) 292-4297; Fax: (614) 292-6337; E-mail: tabita.1@osu.edu

¹The abbreviations used are: CBB, Calvin-Benson-Bassham; Rubisco, ribulose bisphosphate carboxylase/oxygenase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HEPES, hydroxyethylpiperazineethane sulfonic acid; bp, base pairs; PCR, polymerase chain reaction.
FIGURE LEGENDS

FIG. 1. Map of cbbII::lacZ fusion plasmids. The restriction fragments corresponding to each fusion construct are shown and the amount of sequence upstream of the cbbII transcription start site is indicated. The positions of three cbbII upstream ORFs are indicated. Sections of the cbbII promoter designated as upstream regulatory regions 1 and 2 are also shown. The function of ORFs U1, U2 and V have not been determined. It should be noted that the distal end of the insert in plasmid (pVKCIIXho) occurs at –545 bp, 93 bp downstream of the XhoI site (see “Experimental Procedures”).

FIG. 2. DNase I footprint analysis of the binding of CbbR to the R. sphaeroides cbbII promoter-operator region. The oligonucleotides cbbII-1 (bottom strand) and cbbII-2 (top strand) were used to PCR amplify the probes used in the experiment. The labeled oligonucleotide (see “Experimental Procedures”) used to generate each probe is indicated above each panel. The amount of partially purified CbbR (µg) added to each reaction is indicated at the top of the panel. Standard (Std) lanes contain a Maxam and Gilbert A+G cleavage ladder of the probe used in the experiment. Bars indicate regions of protection and asterisks (*) denote DNase I hypersensitive sites. Sequence reference positions are indicated.

FIG. 3. DNase I footprint analysis of the binding of phosphorylated RegA* to the R. sphaeroides cbbII promoter-operator region. The oligonucleotide pairs (see Experimental Procedures) used to generate the probes used in each experiment are: cbbII-
15B (top strand) and cbbII-5 (bottom strand) (Panel A); cbbII-16 (top strand) and cbbII-11 (bottom strand) (Panel B); cbbII-20 (top strand) and cbbII-13 (bottom strand) (Panel C); cbbII-14 (top strand) and cbbII-19 (bottom strand) (Panel C). The labeled oligonucleotide used to generate each probe is indicated at the top of each panel along with the concentration of RegA* (µM) in each reaction mixture. Standard (Std) lanes contain a Maxam and Gilbert A+G cleavage ladder of the probe used in the experiment. Bars indicate regions of protection and asterisks (*) denote DNase I hypersensitive sites. Sequence reference positions are indicated. Arrows (>) indicate the position of the cbbII transcription start.

**FIG. 4. Detection of cbbII promoter binding proteins in extracts of a R. sphaeroides regA mutant grown chemoautotrophically.** A phosphorimage of a gel shift assay performed on fractions eluted from an anionic exchange column (Lanes 1-14, 15-28) of an extract of chemoautotrophically grown *R. sphaeroides* CAC::regAΩ. Positive control reactions using a heparin agarose fraction containing protein X were included (lanes C). Fractions containing DNA binding activities X and Y are indicated.

**FIG. 5. Binding of protein X to the cbbII promoter region.** A) Restriction sites used to generate probes 1, 2 and 3 are shown. The start and end positions of each probe are indicated in parentheses. Probes 4, 5 and 6 are described in Experimental Procedures. B) Shown is a compilation of phosphorimages of several gel mobility shift assays using probes of varying sizes that span different regions of the cbbII promoter-operator (A) and 15 µl of a heparin sepharose fraction containing protein X. Lane 1, probe 4, no protein
added; lane 2, probe 4 plus protein X; lane 3, probe 5, no protein added; lane 4, probe 5 plus protein X; lane 5, probe 6, no protein added; lane 6, probe 6 plus protein X; lane 7, probe 1, no protein added; lane 8, probe 1 plus protein X; lane 9, probe 2, no protein added; lane 10, probe 2 plus protein X; lane 11, probe 3, no protein added; lane 12, probe 3 plus protein X. All reactions contained 1.8 µg poly(dI-dC) · poly(dI-dC).

**FIG. 6. Summary of DNase I footprinting results of CbbR and RegA* binding to the *R. sphaeroides* cbb II promoter-operator region.** The sequence is numbered relative to the cbb II transcription start at +1. Brackets indicate regions of protection on the top (above) and bottom (below) strands with DNase I hypersensitive sites indicated by asterisks. The translation start site for cbbF II is indicated along with the cbb II transcription start sites (>). The relative increase in LacZ activity (i.e. 1X, 16X and 1.5X) conferred by the three promoter fusions used in this study during photoautotrophic growth is indicated above the sequence.
Table I.

*Strains and Plasmids*

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJG3</td>
<td>Ap⁺; pUC8 containing a 3.8 kb <em>EcoRI</em> fragment containing <em>cbbFII</em>, <em>cbbPII</em> and 2.1 kb of upstream sequence</td>
<td>31</td>
</tr>
<tr>
<td>pK18</td>
<td>Kn⁺; derivative of pUC18</td>
<td>36</td>
</tr>
<tr>
<td>pKcbbII</td>
<td>Kn⁺; pK18 containing a 1046 bp PCR fragment of the <em>cbbII</em> promoter region flanked by engineered <em>EcoRI</em> and <em>BglII</em> sites.</td>
<td>This study</td>
</tr>
<tr>
<td>pKIIXho</td>
<td>Kn⁺; pK18 carrying a 595 bp <em>EcoRI/BglII</em> fragment that contains 545 bp of sequence upstream of the <em>cbbII</em> transcription start.</td>
<td>This study</td>
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<tr>
<td>pKIIXcm</td>
<td>Kn⁺; pK18 carrying a 277 bp <em>XcmI/BglII</em> fragment of pKcbbII</td>
<td>This study</td>
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<tr>
<td>pMC1403</td>
<td>Ap⁺; LacZ translational fusion vector</td>
<td>This study</td>
</tr>
<tr>
<td>pMCcII</td>
<td>Ap⁺; pMC1403 carrying a 1046 bp <em>EcoRI/BglII</em> fragment of pKcbbII</td>
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<tr>
<td>pMCcIIIXho</td>
<td>Ap⁺; pMC1403 carrying a 595 bp <em>EcoRI/BglII</em> fragment of pKIIXho</td>
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<td>Kn⁺; conjugative helper plasmid</td>
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<tr>
<td>pVK101</td>
<td>Kn⁺ Te⁺; broad host range vector</td>
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<tr>
<td>pVKcII</td>
<td>Kn⁺ Te⁺ Ap⁺; pVK101 carrying pMCcII inserted in the <em>EcoRI</em> site</td>
<td>This study</td>
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<td>pVKcIIIXho</td>
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*R. sphaeroides* strains

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<tr>
<th>Strain</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>CAC</td>
<td>Chemoautotrophic competent variant of <em>R. sphaeroides</em> HR</td>
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<tr>
<td>1312</td>
<td><em>cbbR::Tp¹</em></td>
<td>12</td>
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<tr>
<td>CAC::<em>regAΩ</em></td>
<td><em>regA::Spe²</em></td>
<td>26</td>
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</table>
$E. \text{coli}$ strains

| JM109 | 37 |
Table II

Expression of cbbR-lacZ translational fusions in *R. sphaeroides* strains CAC, 1312 and CAC::regAΩ under varying growth conditions

All values are the average of values determined in three independent experiments. The standard deviation for each value appears in parentheses. Dashes (---) indicate conditions under which a particular strain is incapable of growth.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Chemoheterotrophic Growth (Malate/Air)</th>
<th>Photoheterotrophic Growth (Malate/Argon)</th>
<th>Photoautotrophic growth (1.5%CO₂/98.5%H₂)</th>
<th>Chemoautotrophic Growth (5%CO₂/45%H₂/50%Air)</th>
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<td></td>
<td>LacZ Activity (nmol/min/mg)</td>
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<tr>
<td>pVKCIIXcm</td>
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<td>23</td>
<td>137</td>
<td>32</td>
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<td></td>
<td>(0.1)</td>
<td>(2)</td>
<td>(4)</td>
<td>(1)</td>
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<tr>
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<td>2208</td>
<td>1235</td>
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<td></td>
<td>(0.6)</td>
<td>(37)</td>
<td>(334)</td>
<td>(189)</td>
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<td>pVKCII</td>
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<td>250</td>
<td>3366</td>
<td>871</td>
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<td>(1)</td>
<td>(12)</td>
<td>(157)</td>
<td>(45)</td>
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<tr>
<td>pVKCIIXho</td>
<td>6.3</td>
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<td>(1.0)</td>
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<td>(91)</td>
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<tr>
<td>pVKCIIXho</td>
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<td>---</td>
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<td>(154)</td>
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<tr>
<td>pVKCII</td>
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<td>---</td>
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<td></td>
<td>(0.2)</td>
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<td>(66)</td>
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</table>
Fig. 1

The diagram illustrates the genetic structure of a viral genome fragment. The restriction sites EcoRI, XhoI, and XcmI are indicated. The ORFs (ORFV, ORFU1, ORFU2) are represented as boxes, with the flanking regions labeled as Upstream Regulatory region 1 and Upstream Regulatory region 2.

- **pVKCIIXcm**: Contains the cbbF and lacZ genes.
- **pVKCIIXho**: Contains the ORFU2 gene.
- **pVKCII**: Contains the ORFU1 gene.

The distances between these regions are specified: -227 bp, -545 bp, and -1025 bp, respectively.
Fig. 5

A

Xho I (-637 bp)  Xcm I (-221 bp)  BamHI(-4 bp)

probe 1

probe 2

probe 3

(-650 bp)  probe 4  (-91 bp)

(-357 bp)  probe 5

(-149 bp)  probe 6  (+41 bp)

B

1  2  3  4  5  6  7  8  9  10  11  12

<--

<--

<--
Interactions of the cbbII promoter-operator region with CbbR and RegA (PrrA) regulators indicate distinct mechanisms to control expression of the two cbb operons of rhodobacter sphaeroides

James M. Dubbs

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