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Differential Expression of the CO_2 Fixation Operons of *Rhodobacter sphaeroides* by the Prr/Reg Two-Component System during Chemoautotrophic Growth

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In *Rhodobacter sphaeroides*, the two *cbb* operons encoding duplicated Calvin-Benson Bassham (CBB) CO_2 fixation reductive pentose phosphate cycle structural genes are differentially controlled. In attempts to define the molecular basis for the differential regulation, the effects of mutations in genes encoding a subunit of Cbb3 cytochrome oxidase, *ccoP,* and a global response regulator, *prrA* (*regA*), were characterized with respect to CO_2 fixation (*cbb*) gene expression by using translational *lac* fusions to the *R. sphaeroides* cbb_I and cbb_{II} promoters. Inactivation of the *ccoP* gene resulted in derepression of both promoters during chemoheterotophic growth, where *cbb* expression is normally repressed; expression was also enhanced over normal levels during phototrophic growth. The *prrA* mutation effected reduced expression of cbb_I and cbb_{II} promoters during chemoheterotrophic growth, whereas intermediate levels of expression were observed in a double *ccoP prrA* mutant. *PrrA* and *ccoP1 prrA* strains cannot grow phototrophically, so it is impossible to examine *cbb* expression in these backgrounds under this growth mode. In this study, however, we found that PrrA mutants of *R. sphaeroides* were capable of chemoautotrophic growth, allowing, for the first time, an opportunity to directly examine the requirement of PrrA for *cbb* gene expression in vivo under growth conditions where the CBB cycle and CO_2 fixation are required. Expression from the cbb_{II} promoter was severely reduced in the PrrA mutants during chemoautotrophic growth, whereas cbb_I expression was either unaffected or enhanced. Mutations in *ccoQ* had no effect on expression from either promoter. These observations suggest that the Prr signal transduction pathway is not always directly linked to Cbb3 cytochrome oxidase activity, at least with respect to *cbb* gene expression. In addition, *lac* fusions containing various lengths of the cbb_I promoter demonstrated distinct sequences involved in positive regulation during photoautotrophic versus chemoautotrophic growth, suggesting that different regulatory proteins may be involved. In *Rhodobacter capsulatus*, ribulose 1,5-bisphosphate carboxylase-oxygenase (RubisCO) expression was not affected by *cco* mutations during photoheterotrophic growth, suggesting that differences exist in signal transduction pathways regulating *cbb* genes in the related organisms.

Rhodobacter sphaeroides and other nonsulfur purple bacteria exhibit unparalleled metabolic versatility. In part, a functional Calvin-Benson-Bassham (CBB) reductive pentose phosphate cycle is crucial for metabolic versatility. Depending on the growth mode, the CBB pathway plays different roles (28). During photoheterotrophic growth, the CBB pathway functions to enable CO_2 to serve as an electron sink for excess reducing power generated during oxidation of organic carbon substrates. Alternate electron acceptors, such as dimethyl sulfoxide, however, may replace CO_2 (32). In the absence of alternate electron acceptors provided under these growth conditions, the demand for the CBB cycle is directly related to the oxidation state of the carbon source provided (28). Two other growth modes, photoautotrophy and chemoautotrophy, require the CBB cycle to supply fixed carbon to the cell. Under these growth conditions, the demand for CO_2 fixation is much higher than that during photoheterotrophic growth, simply because CO_2 is the sole carbon source. Because of the different roles played by the CBB cycle, a regulatory network that links CO_2 fixation not only to the demand for carbon but also to the oxidation-reduction (or redox) status of the cell has evolved in *R. sphaeroides* (8, 11, 24). In recent years, a variety of genes that appear to be involved in sensing redox have been identified in this organism (4, 5, 21). In particular, a two-component histidine kinase-response regulator pair, encoded by the *prrBA* (*regBA*) genes, has been shown to activate transcription of genes involved in such diverse processes as photosystem biosynthesis, carbon dioxide assimilation, and nitrogen fixation and metabolism (11, 17, 24, 25), and in the related organism *Rhodobacter capsulatus*, the *regBA* system has also been shown to repress genes involved in hydrogen oxidation and dimethyl sulfoxide reduction (3, 13). Although the signal recognized by the *prr* system has remained elusive, recent studies with *R. sphaeroides* suggest the involvement of electron transport through a Cbb3 cytochrome oxidase in transmitting an inhibitory signal to the sensor kinase, PrrB, resulting in decreased levels of phosphorylated PrrA required for activation of photosynthesis genes (Fig. 1) (19). This proposal is based on the observation that photosystem promoters, normally regulated by the response regulator PrrA, become deregulated in cytochrome oxidase mutants. Under aerobic growth conditions,

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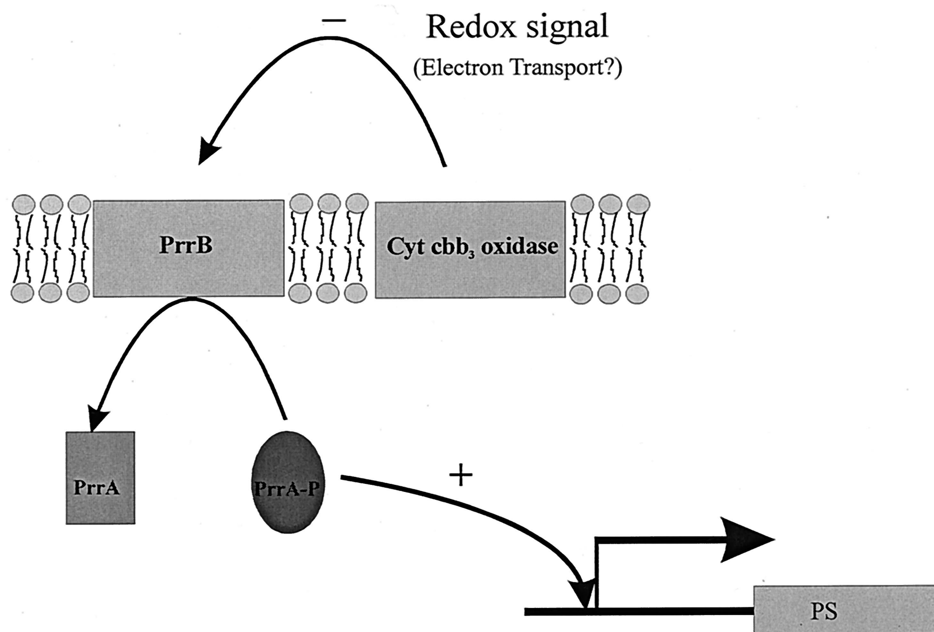

FIG. 1. Model of photosystem gene regulation by PrrA in *R. sphaeroides*. An inhibitory signal generated by Cbb3 cytochrome oxidase in response to the redox status of the cell is suggested to be transmitted to PrrB, thereby inhibiting phosphorylation of PrrA. In the absence of the inhibitory signal, transcription of photosystem (PS) genes is observed. (Data are from reference 19.)

genes that are normally not expressed, or are transcribed at low levels, are turned on; these genes become overexpressed under anaerobic growth conditions. Double mutants impaired in *prrA* and *ccoP* exhibit the nonpigmented phenotype of the single *prrA* mutant (19). This apparent dominance of the *prrA* mutation suggested that both *prrA* and *ccoP* were part of the same signal transduction pathway. Finally, mutations in *ccoQ*, a gene transcribed with cytochrome oxidase structural genes, do not affect cytochrome oxidase activity per se, but *ccoQ* mutants do elicit the same deregulated expression pattern for the pigment genes as observed in *ccoP* mutants, where the Cbb3 cytochrome oxidase is inactivated (20). Recent evidence suggests that the product of the *ccoQ* gene may play a role in stabilizing CcoP (22).

In *R. sphaeroides* duplicated CBB cycle genes are encoded within the cbb_I and cbb_{II} operons, which exhibit distinct growth-dependent gene expression patterns (7). In general, expression of genes within the cbb_{II} operon is more responsive to the redox status of the cell, whereas the cbb_I genes are transcribed in response to the demand for carbon (7, 12, 29). A LysR-type transcriptional regulator, CbbR, and the PrrBA two-component system activate both operons (1, 2, 8, 24). Additional, as-yet-unidentified, regulatory factors may account for differential expression of the *cbb* operons.

Because of the proposed involvement of the Cbb3 oxidase in the signal transduction pathway modulating phosphorylation of PrrA in *R. sphaeroides*, it was of obvious interest to examine the expression of *cbb* genes in *cco* mutant backgrounds. The inability of *prrA* strains of *R. sphaeroides* to grow phototrophically due to the absence of photosynthetic pigments has necessitated the use of alternate conditions of derepression to examine the role of PrrA in gene expression. The effect of PrrA on expression of photosynthesis genes has been examined in cells grown in the dark under lowered oxygen tension, a growth mode in which photosynthesis is not required but that nonetheless causes gratuitous induction of pigment synthesis. For *cbb* expression, CO_2 starvation causes induction of *cbb* genes. In both cases, no induction was observed in the *prrA* mutant (2). *prrB* mutants grow phototrophically, presumably because PrrA is phosphorylated by alternative sensor kinases (10). In addition to decreased pigment production, *cbb* gene expression is down regulated in a *prrB* background (24).

In this investigation, a *prrA* mutant of *R. sphaeroides* was found to be capable of dark chemoautotrophic growth in a H_2-CO_2-O_2 atmosphere (23). Under this growth condition *cbb* gene expression is required, allowing the organism to use CO_2 as the sole carbon source, thus affording the opportunity to study the effects of *prrA* on *cbb* transcription in growing cells. Further, expression of both *cbb* operons was deregulated in a *ccoP* mutant background, similar to the case for genes involved in photosystem biosynthesis under chemoheterotrophic and phototrophic growth conditions. However, drastically different patterns of cbb_I and cbb_{II} promoter activity were found in *prrA* and *prrA ccoP* mutant backgrounds during chemoautotrophic growth, suggesting that the proposed link between *ccoP* and *prrA* in signal transduction may not be absolute.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *R. sphaeroides* and *R. capsulatus* strains and plasmids used in this study are listed in Table 1. For growth experiments, Ormerod's minimal salts medium supplemented with 0.4% DL-malate was used for aerobic chemoheterotrophic growth in flasks at 30°C with rigorous shaking. For anaerobic photoheterotrophic growth, completely filled screw-cap tubes were placed in front of incandescent light bulbs as described previously (12). Photoautotrophic cultures were bubbled continuously with 1.5% CO_2–98.5% H_2, and chemoautotrophically grown cultures were bubbled with 5% CO_2–45% H_2–50% air, in Ormerod's minimal salts medium (9). Adaptation of

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Genotype	Source or reference
R. sphaeroides[a]		
HR	Wild type; Sm^r	33
PrrA	HRCAC, *prrA*::*Sal*I *spc* cartridge of pHP45 inserted into *Xho*I site of *prrA*	Y. Qian and F. R. Tabita, unpublished results
HRΩ	HRCAC *prrB*::ΩSp	24
2.4.1	Wild type	W. Sistrom
CcoP1	2.4.1 *ccoP*::ΩTp	18
CcoP1PrrA	2.4.1 *ccoP*::Ω*TprrA*::ΩSpc,	19
CcoQΔ	2.4.1, in-frame deletion in *ccoQ*	22
R. capsulatus		
MT1131	Wild type, *crtD121* Rif^r	27
GK32	MT1131 *ccoNO*::Km *cox*	14
MG1	MT1131 *ccoP*::Km *cox*	14
M4	MT1131 Δ*ccoN*	27
Plasmids		
pVKC1	pVK102 Tc^r Km^r, cbb_I::*lacZYA*	2
pVKCII	pVK102 Tc^r Km^r, cbb_{II}::*lacZYA*	This study

[a] All *R. sphaeroides* strains except CCOQΔ are CAC.

all *R. sphaeroides* strains, except strain CcoQ, to chemoautotrophic competence (CAC) was accomplished as previously described (23). For genetic exchange experiments, *R. sphaeroides* transconjugants were selected on peptone-yeast extract medium (33) supplemented with the appropriate antibiotic. *Escherichia coli* JM109 (34) and HB101(pRK2013) (6) were grown in Luria broth at 37°C with shaking (26). Conjugal transfer of plasmids pVKC1 and pVKCII from *E. coli* to *R. sphaeroides* strains was accomplished by triparental mating as previously described (33), using helper plasmid pRK2013 (6). Antibiotics were added to media when appropriate at the following concentrations: for *E. coli*, kanamycin at 25 μg/ml and tetracycline at 25 μg/ml; for *R. sphaeroides*, kanamycin at 25 μg/ml, tetracycline at 5 μg/ml, trimethoprim at 20 μg/ml, and spectinomycin at 20 μg/ml.

Preparation of cell extracts and enzyme assays. *Rhodobacter* cultures were grown to mid- to late exponential phase, and 20-ml samples were collected, washed once with TEM (25 mM Tris-Cl, 1 mM EDTA, and 5 mM β-mercaptoethanol), and frozen at −70°C until use. Cell extracts were prepared by sonication followed by centrifugation in an Eppendorf centrifuge at 4°C for 10 min. ribulose 1,5-bisphosphate carboxylase-oxygenase (RubisCO) activity was measured in the supernatant fraction as described previously (7). The protein concentration was determined by a modified Lowry protocol (16) or with the Bio-Rad Laboratories (Hercules, Calif.) protein assay dye-binding reagent. β-Galactosidase assays were based on continuous measurement of o-nitrophenol produced, using the extinction coefficient for o-nitrophenol (1). Absorbance spectra of cell extracts were determined at room temperature by using a Cary 100 spectrophotometer.

Western immunoblot procedure. Proteins resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15) were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, Mass.) according to directions supplied by the manufacturer, using a Bio-Rad Transblot semidry transfer cell. Washes and incubations with antibodies were carried out as described previously (30), using antibodies directed against either the form I or form II RubisCO from *R. sphaeroides*. Immunoblots were developed with the Attophos detection reagent according to the instructions of the manufacturer (Amersham, Buckinghamshire, England) and visualized with a Storm 840 imaging system (Molecular Dynamics, Sunnyvale, Calif.).

RESULTS

RubisCO activity in CcoP and PrrA/RegA strains. Mutations in *ccoP* that inactivate the high-affinity Cbb3 cytochrome oxidase of *R. sphaeroides* show significant levels of pigment production during aerobic chemoheterotrophic growth. Enhanced pigment synthesis is also observed in *ccoP* strains during anaerobic phototrophic growth, whereas mutants in which *prrA* is impaired are completely unable to synthesize pigments and grow phototrophically (35). The observed derepression of pigment gene expression in the *ccoP* strains appears to be mediated through PrrA, because double *ccoP prrA* mutants exhibit the nonpigmented phenotype of strain PrrA (19). Since expression of the *cbb* genes, which encode enzymes of the CBB CO_2 fixation pathway, have also been shown to be under control of PrrA/RegA, it was of interest to determine the role of the Cbb3 cytochrome oxidase in repression of these genes during growth in the presence of oxygen. Accordingly, *R. sphaeroides* HR and 2.4.1 (wild type), CcoP1 (*ccoP*) (18), PrrA (*prrA*) (Y. Qian and F. R. Tabita, unpublished results), and CcoP1PrrA (a double mutant containing mutations in both *ccoP* and *prrA*) (19) were cultured under different growth conditions; extracts were prepared and assayed for RubisCO activity as a measure of overall *cbb* gene expression. During chemoheterotrophic growth in air in the presence of a fixed carbon source such as malate, RubisCO activity was, as expected, found at very low levels in wild-type strains (Table 2). However, the *ccoP* mutant strain exhibited a 15-fold increase in RubisCO activity over the wild type (Table 2). The *prrA* strain exhibited low RubisCO activity, comparable to that observed in the wild-type strain, whereas the *ccoP prrA* double mutant exhibited activity intermediate between those measured in the wild type and the *ccoP* mutant. In view of the enhanced levels of RubisCO activity in the *ccoP* strain during aerobic chemoheterotrophic growth, it was of interest to examine the effect of this mutation on *cbb*

TABLE 2. RubisCO activities in *R. sphaeroides* strains

Strain	RubisCO activity[a] under the following condition:			
	Chemoheterotrophy (malate-air)	Photoheterotrophy (malate-argon)	Photoautotrophy (1.5% CO_2–98% H_2)	Chemoautotrophy (5% CO_2–45% H_2–50% air)
2.4.1 (wild type)	2 ± 1	46 ± 5	288 ± 10	32 ± 2
HR (wild type)	2 ± 1	55 ± 15	278 ± 6	33 ± 2
CACΩ	ND[b]	17 ± 3	65 ± 38	ND
PrrA	3 ± 1	ND	ND	32 ± 11
CcoP1	31 ± 5	261 ± 17	339 ± 13	142 ± 16
CcoP1PrrA	11 ± 2	ND	ND	138 ± 55
CcoQ	1 ± 0.3	35 ± 8	289 ± 67	ND

[a] Activities are expressed as nanomoles of CO_2 fixed per minute per milligram of protein. Numbers represent the averages and standard deviations from multiple assays of two or three independent cultures.
[b] ND, not determined.

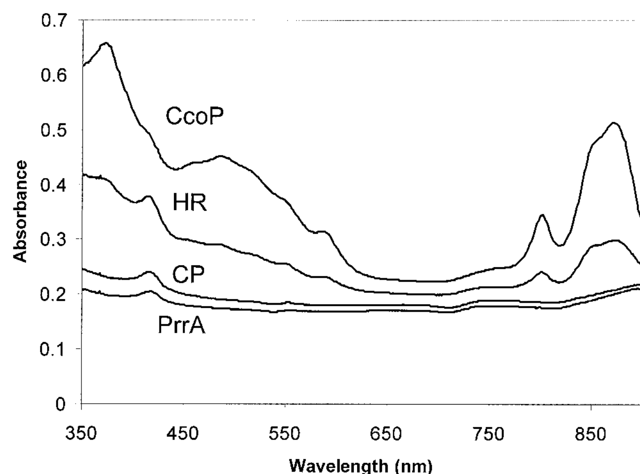

FIG. 2. Absorption spectra of wild-type (HR) and mutant (CcoP1 [CcoP], PrrA, and CcoPPrrA [CP]) strains of *R. sphaeroides*. Spectra were obtained from cell extracts of cultures that were grown chemoautotrophically. Extracts were normalized to a protein concentration of 500 μg/ml.

expression under aerobic chemoautotrophic growth conditions, that is, growth in the presence of O_2, where CO_2 serves as the sole carbon source in the absence of organic carbon. Under aerobic chemoautotrophic growth conditions the *cbb* genes are much more highly expressed than during aerobic chemoheterotrophic growth (23). *R. sphaeroides* does not normally grow under chemoautotrophic conditions unless it is adapted via a gain-of-function mutation (23). We have found that all *R. sphaeroides* strains may be adapted to these growth conditions (G. C. Paoli, J. L. Gibson, and F. R. Tabita, unpublished observations). Such CAC strains are capable of using CO_2 as the sole carbon source and O_2 as the terminal electron acceptor. A previously prepared *prrA* mutant of strain HRCAC (Qian and Tabita, unpublished results) was found to be capable of chemoautotrophic growth with 5% CO_2, albeit with a prolonged lag and a generation time that was greater than that of the wild-type CAC strain. Because a mutation in the *prrA*/*regA* gene does not affect chemoautotrophic growth like it does phototrophic growth, this *prrA* strain afforded, for the first time, the ability to directly assess the effect of the *prrA* mutation on *cbb* gene expression in growing cells. In addition, *R. sphaeroides* strain HRΩ, which contains a mutation in the *prrB* gene (24), was also found to grow chemoautotrophically, albeit after about a 7-day lag period, which was not noted previously. Wild-type strains HR and 2.4.1 grew with a distinct pink coloration during chemoautotrophic growth; this coloration was markedly enhanced in the *ccoP* mutant, whereas the *prrA* and *ccoP prrA* strains were completely nonpigmented. These pigmentation differences for chemoautotrophically grown wild-type and mutant strains were reflected by the absorption spectra obtained (Fig. 2). Similar to phototrophically grown cells, the *ccoP* mutant expressed higher levels of pigments than the wild-type strain, while no pigment production was noted for the *prrA* and *prrA ccoP* strains (Fig. 2).

In conjunction with the photosynthetic pigment content, RubisCO activity levels for the mutant and wild-type CAC strains were assessed when cells were cultured under chemoautotrophic growth conditions (Table 2). As noted for chemoheterotrophic cultures, both wild-type strains and the *prrA* strain exhibited similar levels of RubisCO during chemoautotrophic growth, suggesting that PrrA does not activate or repress *cbb* expression in the presence of oxygen. By contrast, in the *ccoP* and *ccoP prrA* mutant strains, RubisCO activity levels were fourfold greater than that in the wild-type strain. Based on RubisCO activity in chemoheterotrophic and chemoautotrophic cultures, activation of *cbb* genes in the presence of oxygen in the *ccoP* mutant appeared to be independent of PrrA. The effect of the mutation in *ccoP* on anaerobic expression of the *cbb* genes was also examined. RubisCO activity was measured in cells grown photoheterotrophically on malate and photoautotrophically in a 1.5% CO_2–98.5% H_2 atmosphere, growth conditions that in the wild-type strain elicit intermediate and high levels of RubisCO, respectively. The strains that contain a mutation in *prrA* were not included, since they do not grow phototrophically due to the inability to synthesize the photosynthetic apparatus. Therefore, in these experiments, strain CACΩ (containing a mutation in *prrB*/*regB*) was substituted, since this strain is able to grow despite reduced synthesis of pigments (25). In photoheterotrophically grown cells, the *ccoP* strain exhibited nearly a sevenfold increase in RubisCO activity over that of the wild-type strain, comparable to the highly induced levels of RubisCO observed during photoautotrophic growth of the wild-type strain (Table 2). By contrast, RubisCO activity levels were nearly identical in the wild-type strain and the *ccoP* mutant when these strains were grown photoautotrophically. As reported previously (24), RubisCO activity in the *prrB* mutant strain was severely diminished under photoautotrophic conditions, to levels comparable to that observed during photoheterotrophic growth (Table 2).

Western immunoblot analysis of form I and form II RubisCO synthesis. *R. sphaeroides* synthesizes two distinct RubisCO enzymes, encoded by genes within separate *cbb* operons. RubisCO activity measurements do not distinguish between the two enzymes; however, advantage may be taken of the fact that the form I and form II RubisCO enzymes from *R. sphaeroides* are immunologically distinct. Therefore, Western immunoblot analysis was used to qualitatively assess the extent to which each operon was affected by the mutations. In chemoautotrophically grown cells (Fig. 3, lanes 1 to 5), the *ccoP* mutation resulted in increased accumulation of both forms of RubisCO (Fig. 3, lanes 2), in accordance with the higher level of RubisCO measured in this strain. In spite of unchanged RubisCO activity, an obvious decrease in the form II RubisCO was observed in the *prrA* mutant (Fig. 3B, lane 3); form I RubisCO accumulation was only slightly affected by the *prrA* mutation (Fig. 3A, lane 3). Under chemoautotrophic growth conditions, the pattern of form I and form II RubisCO accumulation exhibited by the *ccoP prrA* strain was distinct from that exhibited by the single mutants. The level of form I RubisCO protein in the *ccoP prrA* double mutant was similar to that observed in the *ccoP* strain (Fig. 3A, lane 4), whereas the amount of form II RubisCO protein observed in all *prrA* strains was clearly diminished compared to that in the *ccoP* strain (Fig. 3B, lanes 3 and 4). The amount of both form I and form II RubisCO was found to increase as a result of the *ccoP* mutation in cells grown photoheterotrophically (Fig. 3, lanes 6 and 7). In extracts derived from photoautotrophically grown

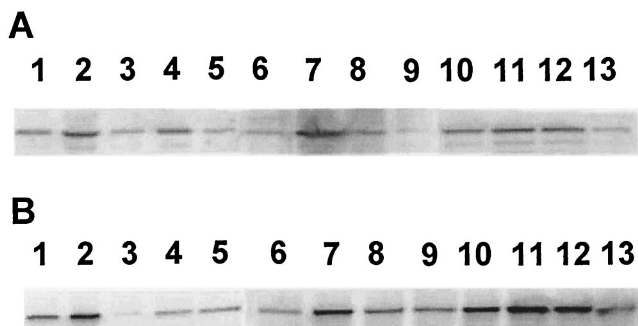

FIG. 3. Western immunoblot analysis of form I and form II RubisCO in *R. sphaeroides cco* and *prr* mutants grown under different conditions. Antisera to form I (A) and form II (B) RubisCO were reacted against immunoblots containing cell extracts of *R. sphaeroides* strain HR (lanes 1, 6, and 10), CcoP1 (lanes 2, 7, and 11), CcoQΔ (lanes 8 and 12), HRΩ (lanes 5, 9, and 13), PrrA (lanes 3), and CcoP1PrrA (lanes 4). Cells were cultured under three growth conditions: lanes 1 to 5, chemoautotrophic growth; lanes 6 to 9, photoheterotrophic growth; and lanes 10 to 13, photoautotrophic growth. For photoheterotrophic and photoautotrophic growth samples, 35 μg of protein was loaded onto sodium dodecyl sulfate gels, and for chemoautotrophic growth samples, 50 μg of protein was applied to gels.

cells, the level of form I and form II RubisCO protein increased only slightly in the *ccoP* strain compared to the wild-type strain (Fig. 3, lanes 11). The mutation in *prrB*, as reported previously (24) and shown here for comparison, resulted in decreased accumulation of both form I and form II RubisCO in cells cultured photoheterotrophically and photoautotrophically (Fig. 3, lanes 9 and 13).

RubisCO activity in the CcoQ mutant. It was previously shown that inactivation of the *ccoN*, *ccoO*, or *ccoP* gene results in complete loss of Cbb3 cytochrome oxidase activity and a concomitant derepression of the expression of the photosystem biosynthesis genes in *R. sphaeroides* (18, 20). By contrast, although deregulation of pigment gene transcription in the presence of oxygen is observed in a *ccoQ* mutant, cytochrome oxidase activity is unchanged (20). Interestingly, inactivation of *ccoQ* did not affect the level of RubisCO activity in cultures of *R. sphaeroides* grown chemoheterotrophically on malate (Table 2), suggesting that cytochrome oxidase activity is required for the repressive effect under aerobic conditions. In addition, RubisCO activity was unaltered in the *ccoQ* mutant in cells grown phototrophically (Table 2). Analysis of cell extracts by Western immunoblot analysis also revealed little difference in form I and form II RubisCO synthesis between phototrophically grown *ccoQ* and wild-type strains (Fig. 3, lanes 8 and 12).

R. sphaeroides ***cbb_I*** **and** ***cbb_{II}*** **reporter-promoter assays.** To determine whether or not the *ccoP* mutation was affecting transcription and to quantitate the effect, pVKC1 and pVKCII, broad-host-range plasmids containing the cbb_I and cbb_{II} promoters, respectively, translationally fused to *lacZ* were transferred to the various strains. β-Galactosidase activity was measured under the different growth conditions to quantitate the level of transcription. It was apparent that the effect of the *ccoP* mutation was exerted on both promoters at the level of transcription (Fig. 4 and 5). In the *prrA* mutant, cbb_I and cbb_{II} promoter activity was very low or undetectable for wild-type and *prrA* strains cultured chemoheterotrophically, whereas significant derepression was observed in the *ccoP* and *ccoP prrA* strains under these growth conditions (Fig. 4A and 5A). β-Galactosidase specific activities for strains harboring pVKC1, the cbb_I promoter fusion (391 and 133 nmol/min/mg for the *ccoP* and *ccoP prrA* mutants, respectively), were somewhat higher than the activities obtained when these strains harbored cbb_{II} promoter fusion pVKCII (131 and 100 nmol/min/mg, respectively). Although the trend is the same, during aerobic chemoheterotrophic growth, the negative effect of the *prrA* mutation on the *ccoP* strain was considerably less pronounced for expression directed by the cbb_{II} promoter than for that directed by the cbb_I promoter. Under chemoautotrophic growth conditions, strains containing the cbb_I promoter fusion (pVKC1) exhibited increased levels of β-galactosidase activity (i.e., 1.8- to 1.9-fold-higher levels) in the *ccoP* and *ccoP prrA* strains compared to the wild type. This pattern of increased activity is reminiscent of the enhanced levels of total RubisCO activity previously observed for these strains (Table 2). Like RubisCO activity levels, the *prrA* strain exhibited cbb_I promoter activity similar to that of the wild-type strain (Fig. 4B). By contrast, the activity profile of β-galactosidase with the cbb_{II} promoter plasmid pVKCII in these strains was very different from that of RubisCO under chemoautotrophic growth conditions. Both *prrA* strains exhibited reduced β-galactosidase activity compared to the wild-type strain (Fig. 5B), with activity in the *ccoP prrA* and *prrA* mutants only 58 and 23% of that of the wild-type strain, respectively. In the *ccoP* strain containing the cbb_{II} fusion, β-galactosidase activity was threefold higher than that measured in the wild-type strain. Based on the fusion activities and the Western immunoblot analyses, the *prrA* mutation appeared to negatively affect cbb_{II} expression during chemoautotrophic growth. In addition, although enhanced expression from both promoters was observed in the *ccoP* mutant, the secondary *prrA* mutation affected expression from the two promoters differently. For the cbb_{II} promoter, the positive effect of the *ccoP* mutation was almost completely negated in a *prrA* background, whereas exactly the opposite was observed for the cbb_I promoter (Fig. 4B and 5B).

Finally, cbb_I and cbb_{II} promoter activities were measured under phototrophic growth conditions in the wild-type and *ccoP* mutant strains in the presence or absence of organic carbon. In cultures grown photoheterotrophically on malate, the levels of β-galactosidase activity directed by the cbb_I promoter in the *ccoP* mutant were 9.5-fold higher than those measured in the wild-type strain (Fig. 4C). The difference between cbb_I::*lacZ* expression in the *ccoP* mutant and the wild-type strain was less pronounced (about 3.7-fold) during photoautotrophic growth (Fig. 4D), where the highest level of cbb_I expression is normally observed. Expression from the cbb_{II} promoter was approximately 1.7- to 1.9-fold higher in the *ccoP* mutant than in the wild type under both photoheterotrophic and photoautotrophic growth conditions (Fig. 5C and 5D).

cbb_I **promoter elements required for chemoautotrophic gene expression.** The *prrA*-independent expression of the cbb_I::*lacZ* translational fusion (pVKC1) during chemoautotrophic growth indicated that additional regulators might be involved in cbb_I activation under this growth condition. Regions of the cbb_I promoter important for chemoautotrophic expression were mapped and compared to those regions known to be required for phototrophic expression (1, 2). cbb_I::*lacZ* fusion plas-

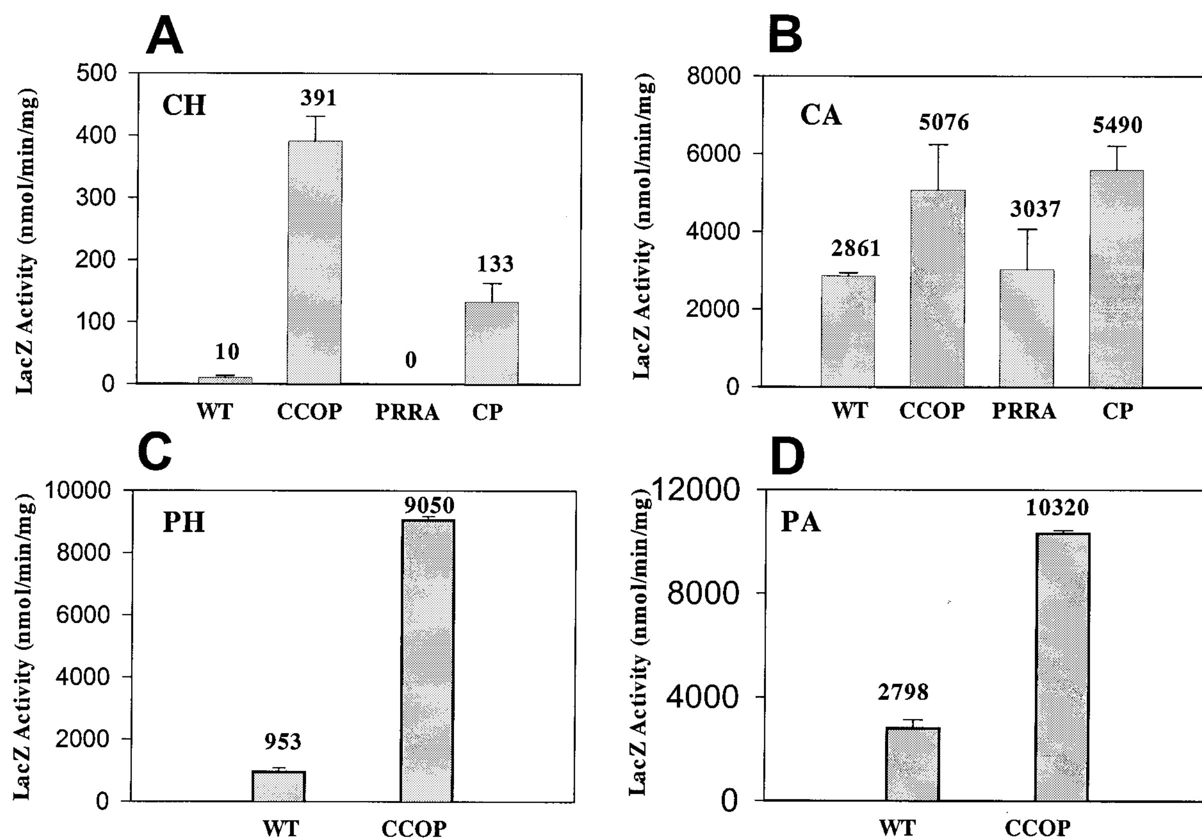

FIG. 4. cbb_I::*lacZ* fusion quantitation in *R. sphaeroides ccoP* and *prrA* mutant strains grown under different culture conditions. β-Galactosidase activity was measured in cell extracts of *R. sphaeroides* strains harboring plasmid pVKC1 grown to mid-exponential phase under the following growth conditions: chemoheterotrophic (CH) (A), chemoautotrophic (CA) (B), photoheterotrophic (PH) (C), or photoautotrophic (PA) (D). WT, wild-type strain 2.4.1; CCOP, strain CcoP1; PRRA, strain PrrA; CP, strain CcoPPrrA. Standard deviations are indicated as error bars. Numbers above bars are the means. All values are based on multiple assays of at least three independent cultures.

mids (1) containing 103 bp (pVKH1), 280 bp (pVKB1), 330 bp (pVKG1), 501 bp (pVKF1), and 636 bp (pVKC1) of upstream sequence (Fig. 6) were introduced into *R. sphaeroides* strains HR and PrrA, and β-galactosidase was measured after chemoautotrophic growth. Under these growth conditions, all of the fusion plasmids, with the exception of pVKG1 and pVKF1, yielded higher expression levels in the *prrA* mutant strain. The levels ranged from slightly over twofold for pVKB1 to over eightfold for pVKH1, suggesting that *prrA* may exert a negative effect on cbb_I expression during chemoautotrophic growth. Examination of β-galactosidase expression patterns with successively longer cbb_I promoter fusions revealed three regions primarily responsible for induction during chemoautotrophic growth. The first region, situated between bp −103 and −280 relative to the mapped cbb_I transcription start site (1), was responsible for 12-and 3.5-fold increases in β-galactosidase expression in the wild-type and *prrA* backgrounds, respectively. The second region, between bp −501 and −636, resulted in increases in β-galactosidase expression of over 3-fold in the wild-type strain and 3.9-fold in the *prrA* mutant. Similar analysis of photoautotrophically grown cells using these fusions revealed a different pattern in the wild-type background. While the region between bp −103 (pVKH1) and −280 (pVKB1) conferred a sevenfold induction of β-galactosidase under photoautotrophic growth conditions, addition of the 50-bp region between bp −280 (pVKB1) and −330 (pVKG1) contributed an additional eightfold induction. This is in stark contrast to the pattern observed under chemoautotrophic growth conditions, in which the region between pVKB1 and pVKG1 is responsible for only a 1.6-fold increase in β-galactosidase activity in the wild-type strain and a 1.8-fold decrease in the *prrA* mutant. These results indicate that the regions upstream of the cbb_I promoter that are important for activation under photoautotrophic growth conditions (1, 2) are distinct from those that are important for activation under chemoautotrophic growth conditions.

Effect of mutations in the *ccoNOQP* operon on *cbb* promoter activity in *Rhodobacter capsulatus*. PrrA (RegA) has been shown to bind to *cbb* promoters and activate transcription of *cbb* genes in *R. capsulatus* (31). Therefore, it was of interest to assess the effect of mutations in *cco* genes on *cbb* expression in this background. Several mutants of *R. capsulatus*, in which cytochrome oxidase was disrupted (14), were grown chemoheterotrophically and photoheterotrophically on malate, and extracts were assayed for RubisCO activity. The *cco* mutations had no apparent effect on RubisCO activity levels under chemoheterotrophic (data not shown) or photoheterotrophic (Table 3) growth conditions, growth regimens that effected the most pronounced change in RubisCO gene expression and protein accumulation in *R. sphaeroides*.

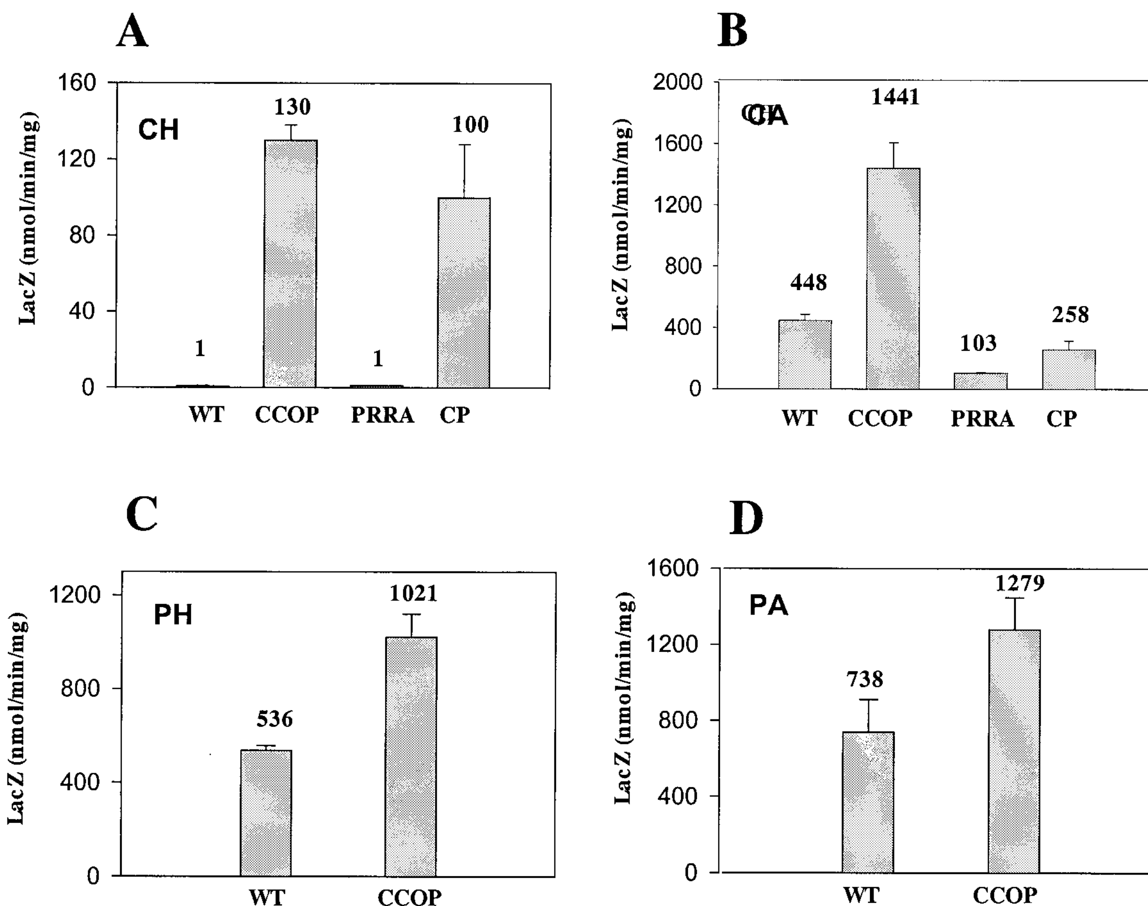

FIG. 5. cbb_{II}::*lacZ* fusion quantitation in *R. sphaeroides ccoP* and *prrA* mutant strains grown under different culture conditions. β-Galactosidase activity was measured in cell extracts of *R. sphaeroides* strains harboring plasmid pVKCII grown to mid-exponential phase under the following growth conditions: chemoheterotrophic (CH) (A), chemoautotrophic (CA) (B), photoheterotrophic (PH) (C), or photoautotrophic (PA) (D). WT, wild-type strain 2.4.1HR; CCOP, strain CcoP1; PRRA, strain PrrA; CP, strain CcoPPrrA. Standard deviations are indicated as error bars. Numbers above bars are the means. All values are based on multiple assays of at least three independent cultures.

DISCUSSION

In *R. sphaeroides* the *prrA*/*regA* gene product has been shown to activate a number of promoters involved in various aspects of metabolism, including photosystem biosynthesis (4), nitrogen fixation and metabolism (11, 25), and CO_2 assimilation (24). Inactivation of any of the genes within the *ccoNOQP* operon results in aerobic derepression as well as enhanced anaerobic expression of photosystem biosynthesis genes that are regulated by *prrA* (4). Curiously, inactivation of *ccoQ* does not seem to affect cytochrome oxidase activity but does affect expression of photosystem biosynthesis genes in the same manner as in mutants in which cytochrome oxidase is inactive. Based on these observations, it was proposed that electron flow through the high-affinity Cbb3 cytochrome oxidase within the membrane of *R. sphaeroides* transmits a signal to sensor kinase PrrB in the presence of oxygen; this signal somehow inhibits phosphorylation of PrrB, with the *ccoQ* gene product mediating signal transduction from cytochrome oxidase to PrrB (20). More recent evidence suggests that in *R. sphaeroides*, the *ccoQ* gene product plays a role in protecting CcoP from proteolytic degradation under aerobic conditions and inactivation of *ccoQ* does indeed actually effect a decrease in cytochrome oxidase activity which is most pronounced under high aeration, explaining the deregulation of photopigment genes in the presence of O_2 (22). Among the genes in *R. sphaeroides* that are regulated by the PrrAB (RegAB) global two-component system are the *cbb* operons, which encode enzymes of the CBB reductive pentose phosphate CO_2 fixation pathway. Expression of both cbb_I and cbb_{II} promoters is down regulated in a *prrB* mutant grown photosynthetically, suggesting that the PrrA-PrrB signal transduction system positively regulates *cbb* gene expression (24). In the *prrB* mutant, pigments are also expressed at lower levels than in the wild type (10). PrrA from *R. capsulatus* (RegA) has been shown to bind to the cbb_I and cbb_{II} promoters of *R. sphaeroides*; however, direct evidence for PrrA involvement in *cbb* expression in vivo has been lacking, because the *prrA* mutant of *R. sphaeroides* is incapable of phototrophic growth (2), although the ability to express both form I and form II RubisCO under anaerobic carbon starvation conditions was restored in a *R. sphaeroides prrA* (*regA*) mutant complemented with plasmid-borne *R. capsulatus regA* (2).

In the present study, a mutation in *prrA*, in *R. sphaeroides* strains that have the capacity for aerobic chemoautotrophic growth, did not eliminate or diminish the aerobic chemoau-

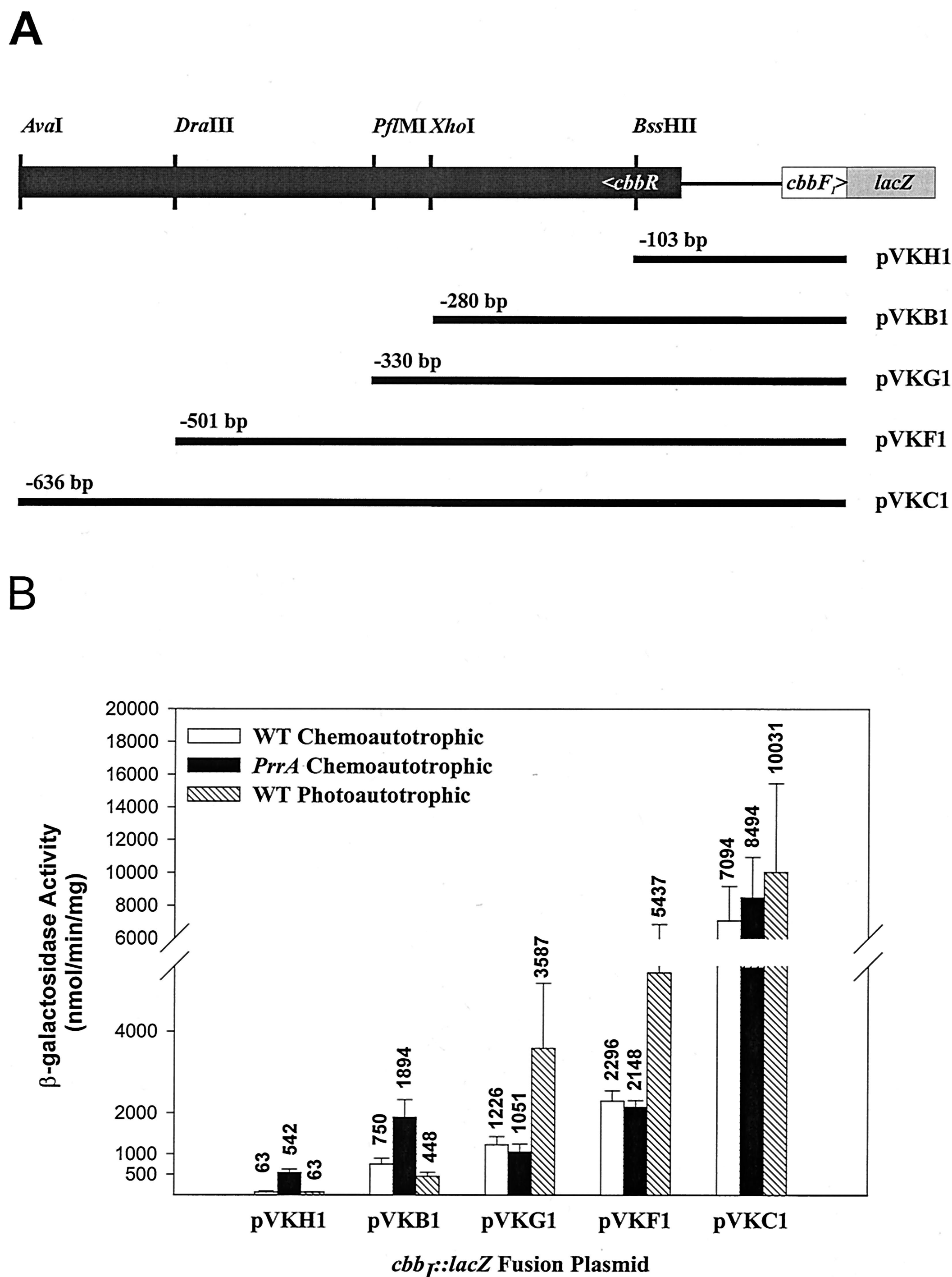

FIG. 6. cbb_I::*lacZ* fusions in *R. sphaeroides* wild-type and *prrA* mutant strains grown chemoautotrophically. (A) Restriction map of the cbb_I promoter region, illustrating restriction sites used to make cbb_I::*lacZ* fusions. Numbers above restriction fragments indicate the number of nucleotides from the transcriptional start site. (B) β-Galactosidase activity in cell extracts of *R. sphaeroides* that contained cbb_I::*lacZ* fusions in wild-type (WT) and *prrA* mutant strains. Error bars indicate standard deviations.

totrophic growth potential. Chemoautotrophic growth favors enhanced *cbb* gene transcription, as this growth condition requires that CO_2 be used as the sole source of carbon. This growth condition thus allows one the opportunity to directly test the effect of the *prrA* mutation on *cbb* expression in vivo. Using cbb_I and cbb_{II} promoter-*lacZ* fusions, it was shown that cbb_{II} expression was significantly decreased in the *prrA* mutant grown chemoautotrophically, whereas cbb_I expression remained unchanged. Expression from both the cbb_I and cbb_{II} promoters increased in the *ccoP* mutant during chemoautotrophic growth, but expression of the two *cbb* promoters in the *ccoP prrA* double mutant was completely different. Expression

TABLE 3. RubisCO activity in *Rhodobacter capsulatus cco* mutants

Strain (genotype)	RubisCO activity[a] during photoheterotrophic growth
MT1131 (wild type)	44 ± 1
GK32 (MT1131 *ccoNO*)	43 ± 3
MG1 (MT1131 *ccoP*)	46 ± 3
M4 (*ccoN*)	56 ± 2

[a] Activities are expressed as nanomoles of CO_2 fixed per minute per milligram of protein. Numbers represent the averages and standard deviations from multiple assays of two independent cultures.

from the cbb_I promoter in the double mutant was slightly elevated compared to that measured in the *ccoP* mutant, whereas cbb_{II} expression was fivefold lower in the double *ccoP prrA* mutant compared to the *ccoP* strain. Further, cbb_{II} promoter activity in the double mutant was twofold the activity measured in the *prrA* mutant, suggesting that the *prrA* mutation was not completely dominant over the *ccoP* mutation under these growth conditions.

Derepression of both *cbb* promoters in the mutant backgrounds during aerobic chemoheterotrophic growth was very similar. In each case, activity was very low or not detectable in the wild-type strain and the *prrA* mutant. Activity from both promoters was highest in the *ccoP* strain and was reduced to 34 and 76% in the *ccoP prrA* double mutant for the cbb_I and cbb_{II}-*lac* fusions, respectively. For the cbb_{II} promoter, this mirrors the chemoautotrophic growth results exactly, in which the *ccoP* mutation caused a reproducible increase in activity in the *prrA* background.

During phototrophic growth, expression from the *cbb* promoters in the *ccoP* background was elevated. For the cbb_I promoter the enhancement was most pronounced during photoheterotrophic growth. However, neither RubisCO enzyme activity measurements nor Western immunoblot analyses showed any evidence that the cbb_I or cbb_{II} promoter was activated in a *ccoQ* background under chemoheterotrophic or phototrophic growth conditions, suggesting that *ccoQ* is a specific conduit for signal transmission from cytochrome oxidase to genes required for photosystem biosynthesis or that photosystem gene expression is more sensitive to slight changes in cytochrome oxidase activity than is *cbb* gene expression.

The finding that expression of the cbb_I promoter is either unaffected or, in some cases, enhanced by the *prrA* mutation, whereas expression from the cbb_{II} promoter is severely reduced during chemoautotrophic growth is both puzzling and intriguing. There are several possible explanations that might clarify these results. An alternate transcriptional activator that may also respond to a signal derived from the Cbb3 cytochrome oxidase competing for PrrA binding sites during chemoautotrophic growth might explain the unchanged cbb_I transcription in the *prrA* strain, an idea that is supported by the results of expression studies using the cbb_I::*lacZ* promoter fusions that possess different amounts of upstream sequence. With the exception of plasmids pVKG1 and pVKF1, all of the cbb_I::*lacZ* fusion plasmids showed a higher expression level in the *prrA* mutant background relative to the wild-type background during chemoautotrophic growth (Fig. 5). This observation indicated that PrrA might actually function as a negative regulator of cbb_I expression during chemoautotrophic growth. It is possible that PrrA could interfere with the action of other cbb_I transcriptional activators by competing for binding sites. A similar mechanism has been proposed for PrrA-mediated negative regulation of the *hup* operon in the related organism *R. capsulatus* (3). PrrA binding sites within the *hup* operon promoter were found to overlap those of the transcriptional activator integration host factor. It was proposed that PrrA exerts its negative effect by competing with integration host factor for binding. It is also possible that the negative regulatory effect is indirectly due to a disruption in other systems controlled by PrrA.

The results of the cbb_I-*lacZ* promoter fusion expression studies with chemoautotrophic competent wild-type and *prrA R. sphaeroides* strains also suggest the existence of an additional transcriptional activator(s). Different regions of the cbb_I promoter are important for activation during chemoautotrophic versus photoautotrophic growth (Fig. 6). During photoautotrophic growth, the portion of the cbb_I promoter between bp −103 and −330 imparts the largest positive effect (57-fold) on cbb_I expression. In contrast, two regions of the cbb_I promoter are responsible for the majority of the observed enhancement of expression during chemoautotrophic growth. The first of these spans the sequence from bp −103 to −280 and confers increases in expression of approximately 12-and 3.5-fold in the wild-type and *prrA* strains, respectively. The second region occurs between bp −501 and −636 and enhances chemoautotrophic cbb_I expression 3- and 3.9-fold in wild-type and *prrA* strains, respectively. The fact that during chemoautotrophic growth both of these regions function similarly in the wild-type and *prrA* mutant backgrounds suggests that they may contain binding sites for additional transcriptional activators. The presence of an additional response regulator in the PrrB pathway specific for the cbb_I promoter could also explain the observed pattern of reduced cbb_I and cbb_{II} expression during phototrophic growth in the *prrB* mutant yet unaffected expression of the cbb_I promoter during chemoautotrophic growth in the *prrA* mutant. Arguments against this include the lack of cbb_I induction during carbon starvation in the *prrA* mutant and the fact that in the related organism *R. capsulatus*, PrrA alone is responsible for activation of both *cbb* operons. Finally, in the absence of PrrA, enhanced expression of cbb_I genes may be influenced by the other known and specific transcriptional regulator, CbbR, to compensate for the loss of cbb_{II} transcription. Indeed, CbbR has been shown to promote compensatory *cbb* transcription of one operon when the other is inactivated (9). A model illustrating *cbb* regulation during chemoautotrophic growth is shown in Fig. 7.

Whatever the reason for the lack of a negative effect of the *prrA* mutation on cbb_I transcription under chemoautotrophic growth conditions, it is difficult to reconcile the observed *cbb* expression pattern and the proposed pathway for redox signal transduction via PrrA in the *ccoP prrA* double mutant. If PrrA is an obligatory component in the signal transduction pathway leading from CcoP (19), then cbb_I and cbb_{II} promoter activity in the double *ccoP prrA* mutant would be expected to be identical to that observed in the single *prrA* mutant unless PrrA was acting to repress activity. Although evidence obtained with the cbb_I promoter-*lac* fusions suggests this possibility during chemoautotrophic growth, it cannot explain the pattern of chemoheterotrophic *cbb* expression. Finally,

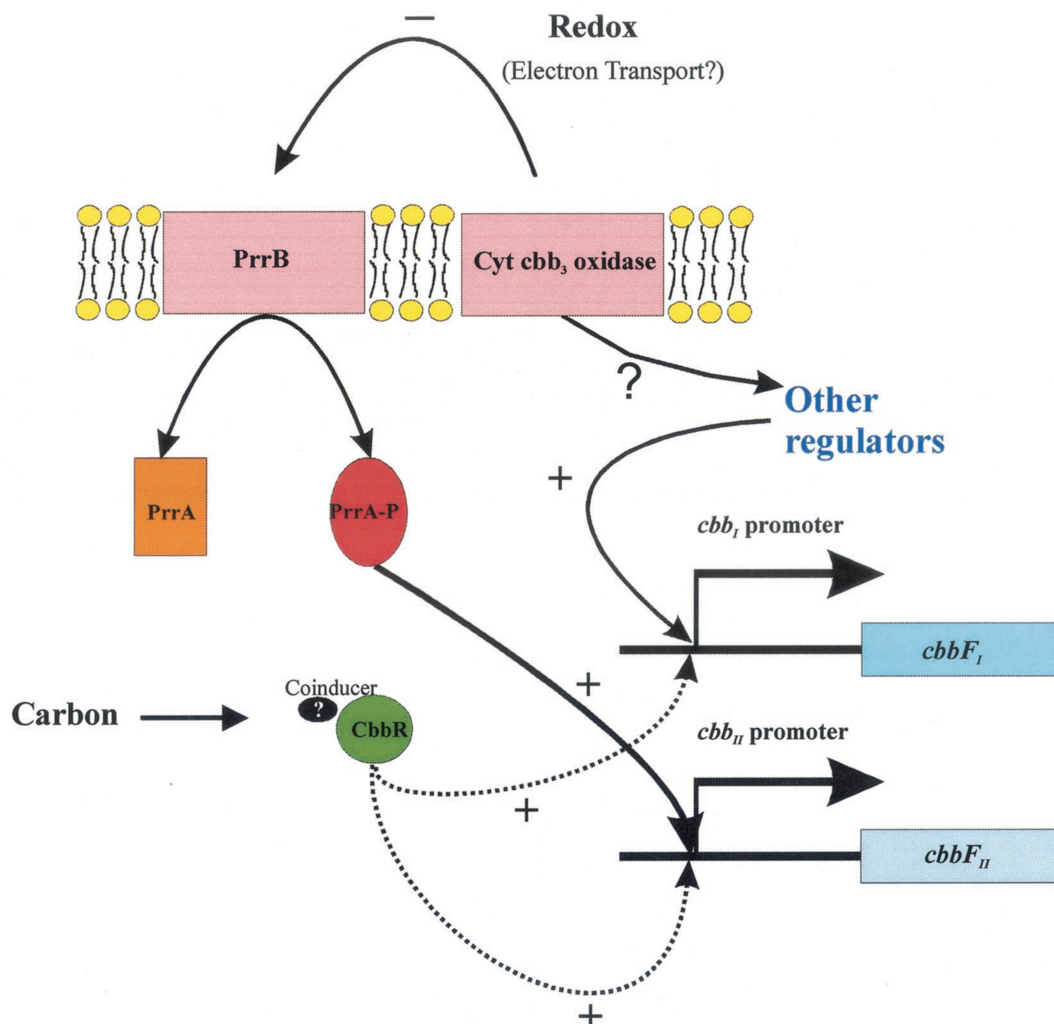

FIG. 7. Proposed model of *cbb* gene regulation in *R. sphaeroides* during chemoautotrophic growth. The Cbb3 cytochrome (Cyt) oxidase transmits an inhibitory signal to PrrB, resulting in a shift in the equilibrium towards unphosphorylated PrrA and subsequent lack of activation of the cbb_{II} promoter. Also shown is a signal derived from Cbb3 cytochrome oxidase activity going to an alternate transcriptional regulator that in turn activates the cbb_I promoter. The model depicts transcriptional activation of both *cbb* operons by CbbR in response to a signal that presumably reflects the carbon status of the cell.

the fact that RubisCO activity was unchanged in various *cco* mutants of *R. capsulatus* during photoheterotrophic growth on malate suggests that signal transduction pathways from Cbb3 cytochrome oxidase are different in the two organisms.

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