Nonsulfur purple (NSP) photosynthetic bacteria are metabolically versatile and can grow under a wide variety of physiological conditions. They assimilate CO₂ via the Calvin-Benson-Bassham (CBB) reductive pentose phosphate pathway with ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) subunit proteins (CbbL and CbbS), as well as the CbbX protein, in cells grown on benzoate without added bicarbonate; such cells used the small amounts of dissolved CO₂ in the medium to support growth. These proteins were not observed in extracts from cells grown in the presence of high levels (10 mM) of added bicarbonate. To confirm the results of the proteomics studies, it was shown that the total RubisCO activity levels were significantly higher (five- to sevenfold higher) in wild-type (CGA010) cells grown on benzoate with a low level (0.5 mM) of added bicarbonate. Immunoblots indicated that the increase in RubisCO activity levels was due to a specific increase in the amount of form I RubisCO (CbbLS) and not in the amount of form II RubisCO (CbbM), which was constitutively expressed. Deletion of the main transcriptional regulator gene, cbbR, resulted in impaired growth on benzoate-containing low-bicarbonate media, and it was established that form I RubisCO synthesis was absolutely and specifically dependent on CbbR. To understand the regulatory role of the CbbRRS two-component system, strains with nonpolar deletions of the cbbRRS genes were grown on benzoate. Distinct from the results obtained with photoautotrophic growth conditions, the results of studies with various CbbRRS mutant strains indicated that this two-component system did not affect the observed enhanced synthesis of form I RubisCO under benzoate growth conditions. These studies indicate that diverse growth conditions differentially affect the ability of the CbbRRS two-component system to influence cbb transcription.

R. palustris is an NSP photosynthetic bacterium that belongs to the alphaproteobacterial group. The extreme metabolic versatility of this organism allows photosynthetic growth by fixation of CO₂ (photoautotrophy) or by assimilation of organic carbon (phototrophery), as well as aerobic chemoheterotrophic and chemolithoautotrophic growth (17, 20, 21). The organization of the cbb genes in R. palustris is unique, with juxtaposition of genes encoding a novel two-component regulatory (CbbRRS) system in the cbb region (20, 21) (Fig. 1). The CbbRRS genes encode an atypical two-component system that is comprised of a large hybrid sensor kinase (CbbSR) and two response regulator proteins (CbbRR1 and CbbRR2), and there are no apparent DNA

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**Differential Accumulation of Form I RubisCO in *Rhodopseudomonas palustris* CGA010 under Phototrophic Growth Conditions with Reduced Carbon Sources**

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binding domains on any of these proteins (21). The cbbRRS genes are found between the major transcription regulator gene (cbbR) and genes encoding the large (cbbL) and small (cbbS) subunits of form I Rubisco. Recently, it was shown that this two-component system modulates the expression of the cbbRII operon under photoautotrophic growth conditions, possibly in response to a redox signal (20–22). The presence of genes encoding this system in the cbbRII region (Fig. 1) and the role of this system in modulating the activity of form I Rubisco and expression of cbbLS in photoautotrophically grown cells (21) are unique to R. palustris, as similar systems have not been described for related NSP bacteria, such as R. sphaeroides and R. capsulatus (6, 20). Inasmuch as this two-component system specifically influences cbbLS transcription in photoautotrophically grown R. palustris cells, the role of the cbbRRS system under photoheterotrophic benzoate growth conditions was analyzed in the current study.

R. palustris can metabolize a wide array of organic compounds, which serve as electron donors and major carbon sources. These compounds include organic acids, such as malate, succinate, and butyrate, as well as aromatic acids, such as benzoate. Among the NSP bacteria, R. palustris stands out because of its capacity for anaerobic photoheterotrophic growth on reduced aromatic compounds, such as benzoate (12, 17, 29). Photoheterotrophic growth of R. palustris on benzoate requires bicarbonate/CO2 or some other added electron acceptor. R. palustris can degrade benzoate anaerobically through a reductive benzoate pathway in which benzyol-coenzyme A (benzoyl-CoA) is the central intermediate. The final products of benzoate degradation are 3 molecules of acetyl-CoA, 1 molecule of CO2, and 6 reducing equivalents. Acetyl-CoA is used for the synthesis of cell building blocks (12, 13). In this study, we demonstrated that photoheterotrophic growth on benzoate in the presence of low levels of bicarbonate obligately required synthesis of form I Rubisco and that the CbbR regulator protein played an essential role. Unlike its role under photoautotrophic growth conditions, the CbbRRS two-component system did not appear to play a regulatory role under benzoate growth conditions, suggesting that there is differential utilization of this regulatory system in R. palustris.

Materials and Methods

Strains and growth conditions. R. palustris strains used in this study are listed in Table 1. R. palustris CGA010 was kindly provided by F. Rey and C. S. Harwood (University of Washington). It was derived from parent strain CGA009 after a frameshift in the hupL gene was repaired. Strains with nonpolar deletions (with the exception of polar antibiotic cassette insertions in strains CGA2025 and CGA2064) in various genes in the cbbRII region were prepared as previously described (21) and are listed in Table 1. R. palustris was grown under photoheterotrophic conditions in PM medium (15) in the presence of 3 mM sodium benzoate. Freshly prepared bicarbonate (NaHCO3) at concentrations of 10 nM and 0.5 mM was added as the electron acceptor. Photoheterotrophic growth was carried out in crimp-sealed tubes containing 10 ml of PM medium prequilled by an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). R. palustris cells in the late logarithmic to early stationary phase were harvested by centrifugation (4,000 × g for 10 min at 4°C) and washed once with TED buffer (100 mM Tris-Cl, 1 mM EDTA, 1 mM dithiothreitol; pH 7.0), and the cell pellets were stored at −80°C.

Proteomic analysis. Soluble extracts from benzoate-grown R. palustris cells were prepared for shotgun proteomics as previously described for resolution of the baseline proteome of this organism (29). The methodology used for proteome analysis involved protein fractionation by centrifugation, subsequent digestion with trypsin, and analysis of peptides by liquid chromatography coupled with tandem mass spectrometry. These analyses resulted in deep proteome coverage, and marked differences in the proteome were readily revealed and correlated well with different metabolic modes of growth. Analysis of the data was thoroughly described previously (29). The entire data set is available and described at http://compbio.ornl.gov/rpal_proteome. Control growth with 3 mM benzoate plus 10 mM bicarbonate was not done in the original study. To check for the presence of CbbL, CbbS, and CbbX, such a control was prepared, and the results were analyzed by using the shotgun proteomics method that was described previously (29) and compared to the results for cells grown on 3 mM benzoate with no added bicarbonate (Table 2).

Rubisco assay and immunoblot analysis. R. palustris cell pellets were resuspended in 600 μl of TED buffer. Glass beads (diameter, 0.10 to 0.25 mm; 750 mg; Retsch) were then added to the cell suspension; the cells were disrupted in a mixer mill (type MM2; Retsch, Haare, Germany) for 9 min at 30 Hz. The soluble protein fraction obtained after centrifugation at 13,000 × g for 15 min at 4°C was assayed for Rubisco activity as previously described (32). The protein concentration was determined by the Bradford method (2) using bovine serum albumin as a standard and chromogenic reagents (Bio-Rad Laboratories, Hercules, CA). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electro-

![FIG. 1. Gene arrangement in the cbbRII and cbbRIII regions of R. palustris CGA010.](image-url)
phoresis and transferred to a nitrocellulose membrane (Immobilon-P; Millipore, Bedford, MA) using a semidy electroblotting apparatus. Immunoblot analysis was performed according to standard protocols (1). The blots were probed with specific antibodies against the R. palustris form I (CbbLS) holoenzyme or form II (CbbM) at a dilution of 1:3,000. Alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (Bio-Rad Laboratories, Hercules, CA) was used as the secondary antibody.

**RESULTS**

**Proteome of benzoate-grown R. palustris.** In a prior study, it was noted that proteins involved in phototrophic benzoate metabolism were readily resolved from extracts of benzoate-grown cells (29). Of particular interest was the apparent selective accumulation and high abundance of form I Rubisco peptides (CbbL and CbbS), along with CbbX, from wild-type strain CGA010 cells grown in the absence of added bicarbonate (Table 2). There was no synthesis of these proteins in phototrophically grown cells grown on succinate. Cells grown photoheterotrophically on benzoate with optimum (high) levels (10 mM) of bicarbonate as an electron acceptor did not show any indication of the presence of CbbL, CbbS, or CbbX (Table 2). Although the function of CbbX is not known at this time, it is apparent from the proteomics data that cbbX transcription may be coregulated along with cbbLS transcription in this organism. In R. sphaeroides (10) and Alcaligenes eutrophus (16), cbbX transcription is influenced by and cbbX is cotranscribed with, respectively, the cbbLS genes. Insertional mutagenesis of the cbbX gene in R. sphaeroides resulted in impaired phototrophic growth with 1.5% CO2-98.5% H2; however, photoheterotrophic growth on malate or butyrate remained similar to that of wild-type strain HR. Although no functional role has been assigned to the cbbX gene product, it has been speculated that this protein has a regulatory role in autotrophic CO2 fixation in A. eutrophus (16).

**Control of form I Rubisco synthesis in benzoate-grown cells.** The apparent selective synthesis of form I Rubisco and CbbX in benzoate-grown cells cultured in the absence of added bicarbonate suggested that regulatory control was a function of the level of dissolved CO2 and/or added bicarbonate/CO2 in the growth medium. Variations in growth were observed in media not containing added bicarbonate; i.e., the level of dissolved CO2 used as an electron acceptor was found to vary in media not containing added bicarbonate. In media rigorously purged for dissolved CO2, no growth occurred, indicating that CO2 is required as an external electron acceptor for growth on a reduced substrate, such as benzoate. To create reproducible growth conditions with a defined level of dissolved CO2 and/or added bicarbonate/CO2 in the growth medium. Variations in growth were observed in media not containing added bicarbonate; i.e., the level of dissolved CO2 used as an electron acceptor was found to vary in media not containing added bicarbonate. In media rigorously purged for dissolved CO2, no growth occurred, indicating that CO2 is required as an external electron acceptor for growth on a reduced substrate, such as benzoate. To create reproducible growth conditions with a defined level of dissolved CO2, wild-type R. palustris CGA010 was grown under anaerobic photoheterotrophic conditions on 3 mM benzoate with various concentrations of exogenously added sodium bicarbonate (10 mM, 5 mM, 1 mM, 0.8 mM, and 0.5 mM). Normal growth of wild-type R. palustris was observed with both high (10 mM) and low (0.5 mM) bicarbonate concentrations. (Fig. 2A and B). Late-logarithmic- to early stationary-phase cells were then harvested and extracts were prepared, which was followed by measurement of Rubisco activity and immunoblot analyses using specific antibodies against form I and form II Rubisco. Clearly, the Rubisco activity levels were significantly higher (range, five- to sevenfold) in the wild-type CGA010 cells grown on benzoate with a low level of bicarbonate (0.5 mM) (Table 3). The increased Rubisco activity was due to a specific increase in form I Rubisco protein and not to an increase in form II protein, in agreement with the proteomics studies (Fig. 3 and 4).

**Fig. 2.** Representative growth of R. palustris wild-type (WT) (CGA010) and mutant (CGA2063 [ΔcbbRR2], CGA2091 [ΔcbbRR1 ΔcbbRR2 ΔcbbM], CGA2025 [ΔcbbR], CGA2028 [ΔcbbLS], and CGA2071 [ΔcbbM]) strains on 3 mM benzoate in the presence of (A) 10 mM and (B) 0.5 mM bicarbonate. Turbidity (100 × log10 OD660) is plotted against time.
bicarbonate (the final OD$_{660}$ was no greater than 0.2). The total RubisCO activity and form I RubisCO synthesis did not differ significantly under these conditions. However, much like the results with benzoate, there was good growth on ethanol (50 mM) at low bicarbonate levels ($\leq$5 mM) under microaerobic growth conditions. Under these conditions, like the results with benzoate, accumulation of form I RubisCO was observed with low levels of bicarbonate.

**Analysis of CbbRRS gene mutants.** The presence of the CbbRRS system genes in the $cbb_y$ region (Fig. 1) and the role of this system in modulating the activity of form I RubisCO and expression of $cbbLS$ in photoautotrophically grown cells (21) are unique to *R. palustris* as similar systems have not been described for related NSP bacteria, such as *R. sphaeroides* and *R. capsulatus* (6, 20). Therefore, the role of the CbbRRS system under photoheterotrophic benzene growth conditions was also analyzed. Various *R. palustris* strains, most of which contained in-frame nonpolar knockouts of key genes, were used for these studies (Table 1) (21). These strains include strains with deletions of the RubisCO genes (e.g., $cbbLS$ [strain CGA2028] or $cbbM$ [strain CGA2071]) or with deletion of the transcriptional regulator gene $cbbR$ (strain CGA2025). The CbbRRS gene mutants include a strain with the entire CbbRRS system deleted by itself (strain CGA2024) or in a $cbbR$ deletion background (strain CGA2064). The strains also include strain CGA2034 with deletion of the $cbbSR$ gene, strain CGA2063 with deletion of both response regulator genes ($cbbRR1$ and $cbbRR2$), and strain CGA2083 with deletion of only the $cbbRR2$ gene. Other strains were constructed in a form II RubisCO ($cbbM$) deletion background, including strain CGA2091 ($cbbRR1$ $cbbRR2$ $cbbM$), strain CGA2098 ($cbbSR$ $cbbM$), and strain CGA2067 ($cbbRS$ $cbbM$). The absence of a form I RubisCO ($cbbLS$ strain CGA2028) or form II RubisCO ($cbbM$ strain CGA2071) gene resulted in a specific phenotypic response during photoheterotrophic growth on benzoate with a low level of bicarbonate (CO$_2$). While growth in the presence of 10 mM bicarbonate was not affected, when $cbbLS$ deletion strain CGA2028 was cultured on low-bicarbonate medium, a significant growth lag was noted (7 days). On the other hand, the absence of form II RubisCO in strain CGA2071 allowed normal growth with both high and low bicarbonate (CO$_2$) levels (Fig. 2A and B). Interestingly, the CbbRRS gene deletions, such as those in strains that have deletions in one or both response regulator genes, a deletion of the sensor kinase gene, or deletions of the entire CbbRRS system, allowed normal growth on benzoate with either high or low levels of added bicarbonate, unlike growth under photoautotrophic conditions (21). The lack of any effect on growth was also observed even in a $cbbM$ background (Fig. 2A and B).

**TABLE 3.** RubisCO activity in crude extracts of *R. palustris* cells grown on benzoate with 10 mM and 0.5 mM NaHCO$_3$.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Sp act (nmol/min/mg) with:</th>
<th>Difference in sp act (fold)</th>
<th>Immunoblot analysis of form I RubisCO with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 mM HCO$_3^-$</td>
<td>0.5 mM HCO$_3^-$</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td></td>
<td>25.9 ± 6.3</td>
<td>141.2 ± 15.1</td>
<td>5.5</td>
</tr>
<tr>
<td>CGA2025</td>
<td>$\Delta cbbR$</td>
<td>12.8 ± 5.3</td>
<td>80.7 ± 13.8</td>
<td>4.7</td>
</tr>
<tr>
<td>CGA2028</td>
<td>$\Delta cbbLS$</td>
<td>21.4 ± 1.8</td>
<td>100.7 ± 13.8</td>
<td>4.7</td>
</tr>
<tr>
<td>CGA2071</td>
<td>$\Delta cbbM$</td>
<td>43.9 ± 8.7</td>
<td>150.5 ± 48.4</td>
<td>3.4</td>
</tr>
<tr>
<td>CGA2083</td>
<td>$\Delta cbbRR2$</td>
<td>25.9 ± 2.7</td>
<td>191.1 ± 43.1</td>
<td>7.3</td>
</tr>
<tr>
<td>CGA2034</td>
<td>$\Delta cbbSR$</td>
<td>20 ± 5.3</td>
<td>109.4 ± 2.7</td>
<td>5.5</td>
</tr>
<tr>
<td>CGA2098</td>
<td>$\Delta cbbRS$</td>
<td>39.5 ± 20.8</td>
<td>203.9 ± 69.2</td>
<td>5.2</td>
</tr>
<tr>
<td>CGA2024</td>
<td>$\Delta cbbRRS$</td>
<td>23.6 ± 2.6</td>
<td>114.9 ± 8.2</td>
<td>4.8</td>
</tr>
<tr>
<td>CGA2067</td>
<td>$\Delta cbbM$ $\Delta cbbRRS$</td>
<td>49.9 ± 13.6</td>
<td>202.1 ± 34.4</td>
<td>4</td>
</tr>
<tr>
<td>CGA2063</td>
<td>$\Delta cbbRR1$ $\Delta cbbRR2$</td>
<td>25.4 ± 3.1</td>
<td>180.1 ± 53</td>
<td>7.2</td>
</tr>
<tr>
<td>CGA2091</td>
<td>$\Delta cbbRR1$ $\Delta cbbRR2$ $\Delta cbbM$</td>
<td>45.7 ± 1.3</td>
<td>313.6 ± 76.6</td>
<td>6.9</td>
</tr>
<tr>
<td>CGA2064</td>
<td>$\Delta cbbR$ $\Delta cbbRRS$</td>
<td>16.8 ± 1.9</td>
<td>69.2 ± 5.2</td>
<td>4.2</td>
</tr>
</tbody>
</table>

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* +, basal expression of form I RubisCO; ++, compensatory synthesis of form I RubisCO; +++ and ++++, induced expression of form I RubisCO; --, no expression.

1 NG, no growth.

**FIG. 3.** Immunoblot analysis of cell extracts of *R. palustris* wild type (WT) (CGA010) and cbbRRS gene mutants (CGA2063 [$\Delta cbbRR1$ $\Delta cbbRR2$], CGA2091 [$\Delta cbbRR1$ $\Delta cbbRR2$ $\Delta cbbM$], CGA2034 [$\Delta cbbR$], CGA2098 [$\Delta cbbSR$ $\Delta cbbM$], CGA2083 [$\Delta cbbRR2$ $\Delta cbbM$], CGA2025 [$\Delta cbbR$], CGA2028 [$\Delta cbbLS$], CGA2071 [$\Delta cbbM$], CGA2024 [$\Delta cbbRRS$], CGA2067 [$\Delta cbbRS$ $\Delta cbbM$], CGA2064 [$\Delta cbbR$ $\Delta cbbRRS$]) grown on benzoate in the presence of (A) 10 mM and (B) 0.5 mM bicarbonate and probed with antibodies specific for form I RubisCO. Each lane contained 10 μg total soluble protein. A compensatory increase in synthesis of form I RubisCO was observed in form II RubisCO deletion strains CGA2091, CGA2098, CGA2071, and CGA2067 even with 10 mM bicarbonate.
Inactivation of the transcriptional regulator gene cbbR. Functional inactivation of the divergently transcribed regulator gene cbbR in strains CGA2025 (cbbR) and CGA2064 (cbbR cbbRRS) resulted in normal growth with 10 mM bicarbonate (Fig. 2A); however, the mutation was lethal with 0.5 mM bicarbonate. In these two strains, however, the cbbR gene was disrupted by simple insertion of a kanamycin cartridge in the coding sequence. It was subsequently found that repeated passage of strains CGA2025 (cbbR) and CGA2064 (cbbR cbbRRS) resulted in eventual loss of the antibiotic resistance marker, repair of the cbbR gene, and an altered phenotype whereby the strains could grow in the benzoate media with 0.5 mM bicarbonate. Such repaired strains were found to specifically synthesize form I RubisCO, much like the wild-type strain. Since CbbR is specifically involved in regulating cbbLS expression (21), these results support the importance of form I RubisCO synthesis in benzoate-grown cells under conditions when the dissolved CO₂ level is low.

Role of the CbbRRS system. To further understand the physiological role of the CbbRRS system, total RubisCO activity (the activities of both form I and form II RubisCO) was measured at pH 8.0 in crude extracts of cells grown on benzoate with 10 mM and 0.5 mM bicarbonate. Significantly higher total RubisCO activities (five to sevenfold higher) and form I RubisCO protein levels were observed for the cbbRRS gene mutants with low bicarbonate levels (Table 3 and Fig. 3). Previous studies with the cbbRRS gene mutants showed that there was reduced total RubisCO activity when cells were grown photolithoautotrophically (Fig. 5) (21). The total RubisCO activity levels in the cbbRRS mutants increased further in a form II RubisCO (cbbM) background, which was not observed previously for photolithoautotrophically grown cells (20, 21). This was especially evident for strain CGA2091, in which both of the response regulator genes were deleted in a background lacking form II RubisCO. Enhanced RubisCO activity that was about twofold higher than that of the wild type was observed in this strain with both 10 mM and 0.5 mM bicarbonate (Table 3).

RubisCO protein accumulation in mutant strains. Disruption of the cbbR transcriptional regulator gene in strains CGA2025 (cbbR) and CGA2064 (cbbR cbbRRS) resulted in impaired growth with 0.5 mM bicarbonate and no form I RubisCO synthesis with 10 mM bicarbonate (Fig. 3). However, cbbR disruption did not affect form II RubisCO (CbbM) accumulation when cells were grown on benzoate with 10 mM bicarbonate, confirming the previous finding that cbbR selectively regulates form I RubisCO synthesis in photolithoautotrophically grown cells (Fig. 4). Deletion of the structural genes encoding form I RubisCO (CGA2028, cbbLS) did not result in a compensatory increase in the level of form II RubisCO with either 10 or 0.5 mM bicarbonate, as revealed by immunoblot analyses (Fig. 4). It is noteworthy that strain CGA2025 (cbbLS) is capable of growth on benzoate with a low bicarbonate level, while CGA2025 (cbbR) is unable to grow under these conditions, considering the fact that both strains lack form I RubisCO. This suggests that the presence of CbbR is important for growth with a low level of bicarbonate, with CbbR probably having an as-yet-unknown more global regulatory role within the cell.

The significantly high level of total RubisCO activity observed in strain CGA2091 (cbbRR1 cbbRR2 cbbM) was explained after analysis of the immunoblots, which revealed an increase in form I accumulation with both high (10 mM) and low (0.5 mM) bicarbonate levels (Fig. 3A and B).

Increased total RubisCO activity levels in all the cbbRRS gene mutants (CGA2063, CGA2091, CGA2083, CGA2024, CGA2067, CGA2034, and CGA2098) can be attributed to a specific increase in form I RubisCO levels with 0.5 mM bicarbonate (Fig. 3). In these mutants, however, the form II RubisCO levels remained unchanged with both 10 and 0.5 mM bicarbonate (Fig. 4). Interestingly, slightly less form I RubisCO accumulation was observed with 0.5 mM bicarbonate in strains...
CGA2024 (cbbRRS) and CGA2034 (cbbSR) than in the other cbbR mutants. However, both strain CGA2024 (cbbRRS) and strain CGA2034 (cbbSR) show the same general pattern of increased form I RubisCO synthesis with 0.5 mM bicarbonate that was observed for the other cbbR mutants. This was not observed in a form II deletion background for strains CGA2067 (cbbR cbbM) and CGA2098 (cbbSR cbbM) due to a compensatory increase in expression of form I RubisCO with both 10 mM and 0.5 mM bicarbonate (Fig. 3 and 4). In general, it was observed that the cbbR genes deletions did not have an adverse effect on phototrophotrophic growth on benzoate or form I RubisCO accumulation, and in fact, a compensatory increase in form I RubisCO synthesis was observed in a form II deletion background; this result differs from the results obtained with photolithoauotrophically grown cells (21).

DISCUSSION

Differential synthesis of form I RubisCO. The current physiological study was undertaken to probe the control of form I RubisCO synthesis during growth of R. palustris on a reduced electron donor, benzoyl. The expression of the cbb operons during photosynthetic growth is dependent on the CO₂ level and the redox state of the fixed carbon supplied (11, 14, 27). In general, the overall levels of gene expression for each cbb operon are low for growth on oxidized fixed organic carbon sources, with slightly greater (about twofold) expression of the cbb₁ operon than of cbb₂. Both cbb operons are highly and maximally expressed under photolithoauotrophic growth conditions, with cbb₂ expression exceeding that of cbb₁ by a factor of two (14). When organisms are grown photolithoauotrophically with more reduced carbon sources (electron donors), much higher levels of cbb gene expression are obtained. Thus, when NSP bacteria such as R. sphaeroides, R. capsulatus, and Rhodospirillum rubrum are cultured with reduced electron donors like hydrogen, propionate, or butyrate, RubisCO synthesis is greatly enhanced, especially synthesis of form I RubisCO (23, 27). The differential expression of both forms of RubisCO under various growth conditions emphasizes their functional diversity. Thus, form I RubisCO becomes responsible for providing cellular carbon and predominates under CO₂ limiting conditions, while form II RubisCO balances the intracellular redox potential under conditions when there is carbon and electron abundance (14). In the current study with R. palustris we observed that bicarbonate (CO₂) levels influenced cbbLS transcription when R. palustris was cultured with a reduced electron donor, such as benzoyl. During anaerobic photolithoauotrophic growth on benzoate at a low CO₂ concentration (0.5 mM bicarbonate), increased levels of form I RubisCO activity and expression were observed. The predominant synthesis of form I RubisCO over form II under CO₂ limiting conditions is consistent with the idea that form I RubisCO has a much higher affinity for CO₂ than form II RubisCO (28). These results suggested that this growth condition created an intracellular milieu that favored form I RubisCO synthesis. Excess reducing equivalents (reduced pyridine nucleotides) and other CoA ester intermediates generated during growth on benzoate are all possible indicators of the carbon and energy status of the cell, thus influencing the activity and expression of form I RubisCO. The enhanced level of form I RubisCO is thus presumably a response to carbon limitation and may be important for scavenging the low levels of dissolved CO₂ to maintain growth and CO₂ fixation, as previously reported for R. rubrum cells grown on butyrate (23). Given the constitutive expression of form II RubisCO under these conditions, form I RubisCO specifically becomes responsible for allowing CO₂ to function as an electron acceptor. Measurements of total RubisCO activity and immunoblot studies confirm proteomics findings that indicated that there was specific accumulation of form I RubisCO subunits proteins (CbbL and CbbS) in cells grown on benzoyl in the presence of the low endogenous levels of CO₂ normally dissolved in the media.

In the related NSP bacteria, R. sphaeroides, R. capsulatus, and R. rubrum cbb gene expression is regulated specifically by CbbR, a LysR family regulator. R. sphaeroides contains a single cbbR gene, the protein product of which regulates both cbb₁ and cbb₂ operon expression (9, 20). In R. palustris, a different mechanism of CbbR-mediated regulation of cbb operon expression seems to be functional during photolithoauotrophic growth on benzoyl. Deletion of the main transcriptional regulator gene, cbbR, in strain CGA2025 completely abolished the synthesis of form I RubisCO, while form II RubisCO accumulation remained unaffected (Fig. 3 and 4). This suggested that CbbR selectively regulated form I RubisCO (cbbLS) expression, while form II RubisCO (cbbM) was constitutively expressed, corroborating previous observations for photosynthotrophically grown cells.

Role of the CbbRRS system. The location of the genes encoding the CbbRRS two-component system, between the transcriptional regulator gene (cbbR) and the structural genes encoding form I RubisCO (cbbLS) (Fig. 1), raised further questions concerning the functional significance of this system under photolithoauotrophic conditions with benzoyl as an electron donor. It is known that ATP-dependent autophosphorylation of the sensor kinase protein occurs, followed by subsequent phosphotransfer of the terminal phosphate to the response regulators of this system (21, 22). From the results of this study and the prior study (21), it is apparent that the CbbRRS two-component system differentially affects the accumulation of form I RubisCO, depending on whether the cells are grown photolithoauotrophically or photolithoauotrophically on benzoate. The total RubisCO activity levels were significantly higher (four to five times higher) in the wild type grown photolithoauotrophically under low-bicarbonate (CO₂) conditions, although the levels of form I and II proteins were not significantly different. Interestingly, total RubisCO activity was reduced (50 to 60%) when both response regulators were deleted (CGA2063) and was further reduced in a form II deletion background (CGA2091), a significant difference compared with the results obtained for photolithoauotrophic conditions (Fig. 5). Thus, it can be speculated that the differential utilization of the CbbRRS system in modulation of cbbLS expression is probably the result of the oxidation state of the electron donor (benzoyl or hydrogen), as well as an alteration (depletion) in the intracellular concentration of a metabolite(s) of the central carbon and energy pathways occurring as a result of its metabolism. It is also conceivable that the CbbRRS system provides a regulatory mechanism in addition to that of CbbR for fine-tuning the expression of form I RubisCO under
conditions where CO₂ becomes important for providing the cell with fixed carbon during autotrophic growth.

In the more extensively studied NSP bacteria *Rhodobacter sphaeroides* and *R. capsulatus*, it was found that inactivating one RubisCO gene results in a compensatory and CbbR-dependent increase in expression of the second RubisCO (7–9, 11, 19). This type of compensatory RubisCO accumulation was not observed previously for *R. palustris* grown photolithoautotrophically. However, with benzoate-grown cells we observed a compensatory increase in form I RubisCO in strains CGA2091 (*cbbRR1*) and CGA2098 (*cbbSS cbbM*), and CGA2071 (*cbbM*), in which form II RubisCO was not observed (Fig. 3). A CbbR-dependent increase in form II RubisCO was not observed in *R. palustris* strain CGA2028 (*cbbLS*) with a deletion of the structural genes encoding form I RubisCO (Fig. 4). This finding correlated well with the growth pattern; CGA2028 exhibited a long lag of about 7 days with benzoate and a low bicarbonate concentration before growing to levels similar to the wild-type levels (Fig. 2B). This observation strongly suggested that form I RubisCO (*cbb*) alone is the regulatory target for CbbR and that form II RubisCO is constitutively expressed.

Finally, based on studies with photolithoautotrophically grown cells, we previously speculated that the CbbRRS system influences *cbbLS* transcription, most likely by somehow interacting with the master transcriptional regulator CbbR. The components of the CbbRRS system, especially the response regulators, contain no apparent DNA binding domain, further fueling speculation that this system somehow interacts with CbbR in order to influence *cbbLS* transcription. Preliminary studies using a bacterial two-hybrid system (BacterioMatch II two-hybrid system vector kit; Stratagene) have recently enabled identification of a protein-protein interaction between the transcriptional regulator CbbR and CbbRR1 (response regulator 1 of the CbbRRS system) (G. Joshi and F. R. Tabita, unpublished data). Further work identifying specific regions of the proteins critical for this interaction is in progress in order to understand the physiological role of the CbbRRS two-component system in vivo. In addition, these studies should also provide a framework for understanding how and why this two-component system appears to show a growth substrate-dependent influence on *cbbLS* transcription.

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