JOURNAL OF BACTERIOLOGY, Jan. 1996, p. 12–18
0021-9193/96/$04.00+0
Copyright © 1996, American Society for Microbiology
Vol. 178, No. 1

# A Global Signal Transduction System Regulates Aerobic and Anaerobic $CO_2$ Fixation in *Rhodobacter sphaeroides*

YILEI QIAN[1] AND F. ROBERT TABITA[1,2,3]*

*Ohio State Biochemistry[1] and Plant Molecular Biology/Biotechnology Programs[2] and the Department of Microbiology,[3] The Ohio State University, Columbus, Ohio 43210-1292*

Received 13 July 1995/Accepted 24 October 1995

**Complementation of a mutant of *Rhodobacter sphaeroides* defective in photosynthetic $CO_2$ reduction led to the identification of a gene which encodes a protein that is related to a class of sensor kinases involved in bacterial signal transduction. The nucleotide sequence and deduced amino acid sequence led to the finding that the gene which complemented the mutant is the *regB* (*prrB*) gene, previously isolated from both *R. sphaeroides* and *Rhodobacter capsulatus* and shown to regulate the anaerobic expression of structural genes required for the synthesis of the reaction center and light-harvesting systems of these organisms. The current investigation indicates that in addition to its role in the regulation of photosystem biosynthesis, *regB* (*prrB*) of *R. sphaeroides* is intimately involved in the positive regulation of the $cbb_I$ and $cbb_{II}$ Calvin cycle $CO_2$ fixation operons. In addition to regulating the expression of structural genes encoding enzymes of the primary pathway for $CO_2$ fixation in *R. sphaeroides*, *regB* was also found to be required for the expression of a gene(s) important for the putative alternative $CO_2$ fixation pathway(s) of this organism. A mutation in *regB* also blocked expression of structural genes of the *cbb* regulon in a strain of *R. sphaeroides* capable of aerobic $CO_2$-dependent growth in the dark. It is thus apparent that *regB* is part of a two-component system and encodes a sensor kinase involved in the global regulation of both anoxygenic light-dependent- and oxygenic light-independent $CO_2$ fixation as well as anoxygenic photosystem biosynthesis.**

*Rhodobacter sphaeroides* is a nonsulfur purple bacterium capable of both anoxygenic photosynthetic growth and vigorous aerobic growth in the dark. Under photolithoautotrophic growth conditions, this organism may be cultured with $H_2$ being used as an electron donor and $CO_2$ being used as the electron acceptor and sole carbon source. *R. sphaeroides* also employs various organic compounds as electron donors and major carbon sources (photoheterotrophic growth), and several organic acids, such as malate, succinate, and butyrate, are especially well suited for this growth mode. Although the role of $CO_2$ fixation is diminished under photoheterotrophic conditions, $CO_2$ is still assimilated primarily through the Calvin cycle (33). Thus, ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco), the key enzyme of the primary $CO_2$ fixation pathway (14), is essential for photosynthetic growth when $CO_2$ is the major carbon source or prime electron acceptor. Interestingly, there are two distinct forms of Rubisco in *R. sphaeroides*, each of which, by itself, is capable of supporting photolithoautotrophic and photoheterotrophic growth (7). When the form I and form II Rubisco genes are both deleted, as in the case of *R. sphaeroides* 16 (a Rubisco double deletion mutant, *cbbL cbbS cbbM*), the organism is no longer capable of photolithoautotrophic growth in an $H_2$-$CO_2$ atmosphere; under photoheterotrophic growth conditions in the presence of malate, growth is also impossible unless an alternative electron acceptor, such as dimethyl sulfoxide (DMSO), is provided (8). Interestingly, strain 16 shows a time-dependent tendency to gain the ability to grow photoheterotrophically in a malate medium in the absence of DMSO, presumably using $CO_2$ derived from the metabolism of malate as the electron acceptor. Strain 16PHC has been isolated from strain 16 several times (35), and genetic studies have indicated that strain 16PHC most likely arises as the result of a point mutation of strain 16, so that strain 16PHC exhibits a much greater ability to fix $CO_2$ in vivo than its parent, strain 16 (36). It was therefore proposed that there is an alternative $CO_2$ fixation pathway(s) in *R. sphaeroides* that does not require the presence of Rubisco. In support of this, both Rubisco deletion strains, 16 and 16PHC, were found to grow photolithoautotrophically with reduced sulfur compounds being used as electron donors and $CO_2$ being used as the sole carbon source (36). In addition, the fact that the Rubisco deletion strain of *Rhodospirillum rubrum*, strain I-19, is capable of malate-dependent photoheterotrophic growth with $CO_2$ being used as an electron acceptor (9) further indicates that alternative $CO_2$ fixation routes may be important to nonsulfur purple bacteria. Thus far, several alternative $CO_2$ fixation pathways have been shown to contribute to net carbon fixation in various species (33); whether *R. sphaeroides* employs any of these routes has not been established at this time. In order to ascertain how $CO_2$ may be assimilated and to characterize the mechanism by which this metabolic pathway may be controlled in *R. sphaeroides* (10), we obtained mutants of strain 16PHC in this study that are incapable of malate-dependent photoheterotrophic growth with $CO_2$ as the electron acceptor. A transposon (Tn*5*) mutant of 16PHC, strain A25, was isolated, and it was found to be capable of normal growth under malate-dependent photoheterotrophic conditions, with DMSO being used as the electron acceptor, but grew poorly with $CO_2$ as the electron acceptor. The interrupted gene of strain A25 was cloned, sequenced, and found to exhibit homology to several sensor kinases involved in prokaryotic signal transduction. Surprisingly, the wild-type gene which complemented strain A25 and allowed the organism to grow in malate medium with $CO_2$ being used as the electron acceptor was identified as *regB* (*prrB*) of *R. sphaeroides* (5, 25) and *Rhodobacter capsulatus* (22), a gene that had originally been shown to be part of a regulatory cascade controlling the anaer-

* Corresponding author. Mailing address: Department of Microbiology, The Ohio State University, 484 West 12th Ave., Columbus, OH 43210-1292. Phone: (614) 292-4297. Fax: (614) 292-6337. Electronic mail address: Tabita.1@osu.edu.

TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Phenotype or genotype | Source(s) |
|---|---|---|
| *R. sphaeroides* | | |
| HR | Wild type, Str$^r$, CA−[a] | 37 |
| CAC | Derivative of strain HR, Str$^r$, CA+[b] | 23 |
| 16 | *cbbL cbbS cbbM* Str$^r$ Km$^r$ Tp$^r$, PH−[c] | 8 |
| 16PHC | Derivative of strain 16, *cbbL cbbS cbbM* Str$^r$ Km$^r$ Tp$^r$, PH+[d] | 35 |
| A25 | Tn*5* mutant of strain 16PHC, Gm$^r$ *regB* | This study |
| HRΩ | *regB*-deficient mutant of strain HR, Spc$^r$ | This study |
| CACΩ | *regB*-deficient mutant of strain CAC, Spc$^r$ | This study |
| *E. coli* | | |
| JM109λ*pir* | Derivative of JM109, containing λ*pir* on the chromosome | 21, 24 |
| S17-1λ*pir* | Str$^r$ Tp$^r$ Spc$^r$ λ*pir* and mobilizing factors on chromosome | 24 |
| Plasmids | | |
| pRK415 | Broad-host-range vector, Tc$^r$ | 18 |
| pRK12F2 | pRK415 derivative, containing a 26-kb *Eco*RI insert | This study |
| pRKBgB3 | pRK415 derivative, containing a 7.8-kb *Bgl*II insert | This study |
| pRKXH2.0 | pRK415 derivative, containing a 2-kb *Sal*I insert | This study |
| pHP45Ω | Contains 2-kb Ω fragment (Str$^r$ Spc$^r$ gene) | 26 |
| pJP5603 | Mobilizable suicide vector, Km$^r$ | 24 |
| pJP21 | pJP5603 derivative, containing a 2-kb *Sal*I insert | This study |
| pJP21Ω | pJP21 derivative, containing a Str$^r$-Spc$^r$ Ω cartridge | This study |
| pRK2013 | Helper plasmid for triparental conjugation, Km$^r$ | 3 |
| pGP5Gm | Mobilizable suicide vector, containing transposon Tn*5*::Gm | T. Donohue |
| pVKC1-13 | Translational fusion of $cbb_I$ promoter and *lacZ* | 4 |
| pHX300W21 | Transcriptional fusion of $cbb_{II}$ promoter and *xylE* | 39 |

[a] CA−, cannot grow chemolithoautotrophically in the presence of oxygen.
[b] CA+, can grow chemolithoautotrophically in the presence of oxygen.
[c] PH−, cannot grow photoheterotrophically without the addition of DMSO.
[d] PH+, can grow photoheterotrophically without the addition of DMSO.

obic expression of structural genes involved in the biosynthesis of the photosynthetic reaction center (RC) and the light-harvesting (LH) systems in *R. capsulatus* (15, 22) and now *R. sphaeroides* (6). In this study, we further show that *regB* is involved in the control of $CO_2$ fixation through the Calvin cycle, both anaerobically in the light and aerobically in the dark; *regB* also appears to control the alternative $CO_2$ fixation pathway of *R. sphaeroides*. Thus, *regB* and probably its cognate gene, *regA* (28), are part of a global system that regulates both anoxygenic phototrophic and aerobic chemoautotrophic $CO_2$ metabolism while also controlling the expression of genes important for the biosynthesis of the photosystem (5, 19, 22, 25, 28).

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *R. sphaeroides* and *Escherichia coli* strains employed in this study are listed in Table 1. *R. sphaeroides* strains were grown in defined medium under anoxic photoheterotrophic growth conditions, with malate being used as the electron donor, in standing screw-cap-test-tube cultures or in bottles bubbled with argon. In some cases, cultures were grown in minimal medium in the total absence of organic carbon and in an atmosphere of 1.5% $CO_2$–98.5% $H_2$, as described previously (7). Aerobic chemolithoautotrophic growth of *R. sphaeroides* CAC was achieved by bubbling minimal medium with a mixture of 5% $CO_2$–45% $H_2$–50% air in the dark. Aerobic chemoheterotrophic-grown cells were cultured in malate medium in baffled Erlenmeyer flasks shaken at high speed in the dark. Complex medium (peptone-yeast extract [PYE] [37]) was used for all conjugation experiments and for the routine inoculation of starter cultures obtained from frozen stocks. *E. coli* JM109λ*pir* or S17-1λ*pir* was used to propagate plasmids pGP5Gm and pJP5603 or their derivatives. The concentrations of the antibiotic agents streptomycin, kanamycin, trimethoprim, tetracycline, gentamicin, and spectinomycin for all *R. sphaeroides* cultures were 50, 25, 200, 5, 5, and 25 μg/ml, respectively. *E. coli* strains were grown with the same concentrations of antibiotic agents being used, except that tetracycline and gentamicin were raised to 12 and 10 μg/ml, respectively.

**Transposon mutagenesis of *R. sphaeroides* 16PHC.** Conjugation of *E. coli* donor strain S17-1λ*pir*, harboring Tn*5* suicide vector pGP5Gm, and *R. sphaeroides* recipient strain 16PHC was performed as previously described (7). Transposon mutants that could grow on PYE plates containing gentamicin and kanamycin were picked and grown in the light on malate-containing plates in the presence or absence of DMSO in GasPak jars (7). Those colonies that grew poorly on malate-$CO_2$ plates and yet grew normally on malate-DMSO plates were saved. A genomic library from strain 16PHC was prepared in plasmid pRK415 as previously described (38).

**Sequence analysis.** DNA sequences (from both strands) were obtained from clones containing a series of nested deletions of the target DNA. Sequences were aligned with the AssemblyLign program, and open reading frames (ORFs) were found with the MacVector program. Homology searches in GenBank were carried out with a FastA program.

**Site-directed interposon mutagenesis of *regB*.** Plasmid pJP21Ω was used to disrupt the *regB* gene in various strains of *R. sphaeroides*. It was constructed by first inserting the 2-kb fragment containing the *regB* gene into the *Sal*I site of suicide vector pJP5603 to generate plasmid pJP21. Then, with *Sma*I being used, the Ω Str$^r$-Spc$^r$ resistance cartridge from plasmid pHP45Ω was inserted by blunt-end ligation into the *Not*I site of the 2-kb *Sal*I *regB*-containing fragment. The resultant plasmid, pJP21Ω, was subsequently introduced into wild-type strain HR and strain CAC via triparental mating with *E. coli* JM109λ*pir* and helper plasmid pRK2013 being used. Those *R. sphaeroides* clones that were resistant to spectinomycin and sensitive to kanamycin and were shown by Southern hybridization, with *regB* being used as a probe, to contain the expected mobility shift were deemed to contain a specific *regB* knockout phenotype as a result of homologous recombination of the disrupted gene in place of the wild-type gene on the chromosome.

**Cell extracts and enzyme assays.** Cells were harvested, washed in buffer, and sonicated, and the resultant crude extract was separated from cell debris as previously described (36). Rubisco and catechol 2,3-dioxygenase (product of the *xylE* reporter gene) were assayed by established procedures (8, 39). β-Galactosidase (product of the *lacZ* reporter gene) was measured (27), with some modification of the usual assay (as established by T. Wahlund of this laboratory); color development of *o*-nitrophenyl was continuously monitored over a period of 10 to 15 min, and the change in steady-state $A_{405}$ per minute was used to calculate the rate of the reaction.

**Nucleotide sequence accession number.** The sequence for *regA*, *regB*, and *regC* of strain 16PHC has been submitted to the GenBank-EMBL data bank under accession number U22925.

## RESULTS

**Isolation of a transposon mutant of strain 16PHC.** About 1,000 gentamicin-resistant *R. sphaeroides* colonies were screened for photoheterotrophic growth on malate-supplemented minimal medium plates in the presence or absence of

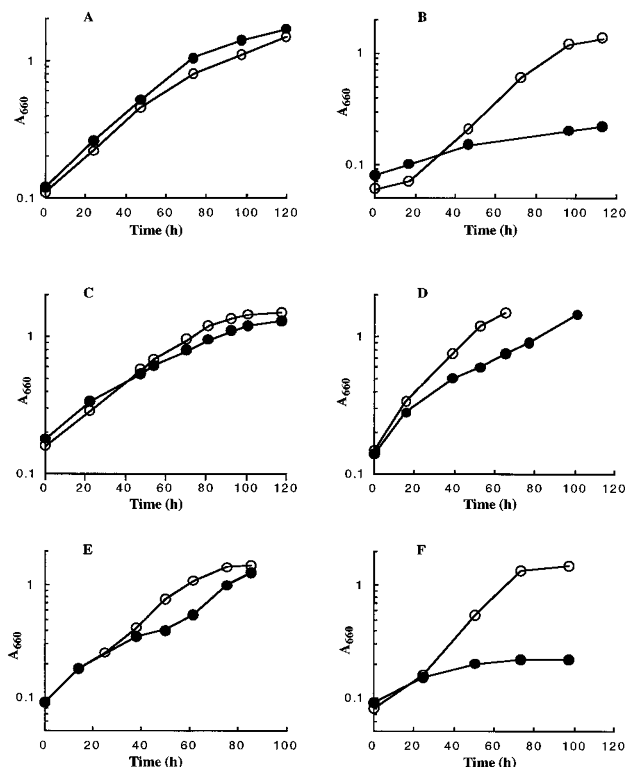


FIG. 1. Growth of *R. sphaeroides* strains. Strains 16PHC (A) and A25 (B) were cultured in malate-supplemented minimal medium. Cells were grown in the light in the presence (○) or absence (●) of DMSO. Strain A25 was also grown in the presence of plasmids pRK12F2 (C), pRKBgB3 (D), pRKXH2.0 (E), and vector pRK415 only (F).

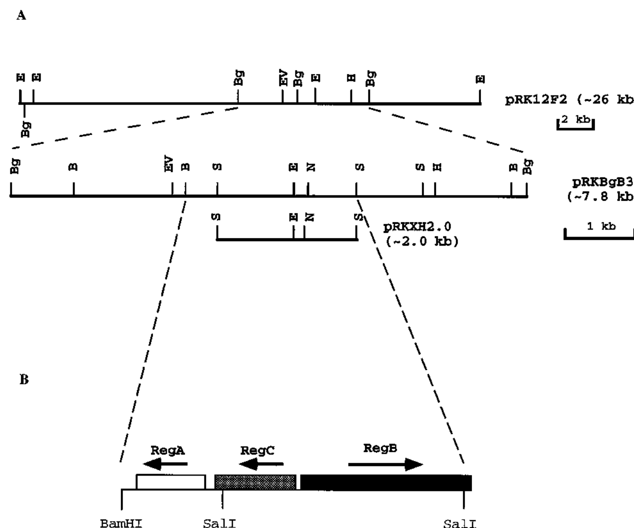


FIG. 2. (A) Cloning of complementing plasmids pRK12F2, pRKBgB3, and pRKXH2.0. (B) Gene organization of *regA*, *regB*, and *regC* in *R. sphaeroides*. E, *Eco*RI; Bg, *Bgl*II; H, *Hin*dIII; EV, *Eco*RV; S, *Sal*I; N, *Not*I.

DMSO under anaerobic conditions. Several potential transposon mutants that showed poor growth on malate plates lacking DMSO but that grew normally in the presence of DMSO were isolated. One of these, strain A25, was further examined. In standing liquid test-tube cultures, strain A25 grew poorly in malate medium with $CO_2$ as the electron acceptor and yet grew well in malate medium containing DMSO as the electron acceptor, in contrast to its parent (strain 16PHC), which grew well with both electron acceptors (Fig. 1A and B).

**Cloning and identification of the site of Tn*5* transposition in strain A25.** A 10.5-kb *Sal*I fragment from the chromosome of strain A25 conferring gentamicin resistance was cloned into pUC19. By using the genomic portion of that fragment as probe, a clone containing a 26-kb *Eco*RI insert was isolated from a genomic library of strain 16PHC prepared in broad-host-range vector pRK415. The 2-kb *Sal*I fragment, the site of transposition of Tn*5*::Gm in strain A25, was subcloned into pUC19, as were the adjacent fragments. These fragments were sequenced, and three ORFs were found in this region (Fig. 2B). A GenBank search revealed that one of the ORFs (*regA*) showed 100% identity with the *prrA* gene (5) and 99.5% identity with a gene previously identified as *regA* (an only 5-bp difference) (25), both of which are from *R. sphaeroides*. Another ORF was identical to *regB* (*prrB*) of *R. sphaeroides* (6) and shared 57% amino acid sequence identity with the deduced sequence of *regB* of the related organism *R. capsulatus* (22). This gene encodes a sensor kinase that is part of a two-component system which regulates the expression of structural genes required for the biosynthesis of the LH system and photosynthetic RCs of *R. sphaeroides* and *R. capsulatus* (5, 22).

The sequence of the third ORF, which we have called *regC*, is basically identical to that of the *prrC* gene previously isolated from *R. sphaeroides* (6), with some notable exceptions (see accession number U22925). The deduced amino acid sequence of PrrC was shown to exhibit homology to sequences of genes that encode mitochondrial membrane proteins required for assembly of cytochrome oxidase (6). RegB exhibited homology with several other sensor kinases that have been shown to be associated with prokaryotic signal transduction pathways, e.g., FixL from *Azorhizobium caulinodans* (17), HydH from *E. coli* (29), NtrB from *R. capsulatus* (16), RcsC from *E. coli* (3), and SpoIIJ from *Bacillus subtilis* (1). In all of these proteins and several others, a histidine residue (His-221 in RegB of *R. sphaeroides*) is conserved, as are several other residues in the region thought to be essential for autophosphorylation of sensor kinases and the transfer of the phosphate group to their cognate response regulators of bacterial two-component regulatory systems (Fig. 3). A hydropathy plot shows that the N terminus of RegB contains a potential membrane-spanning portion (data not shown), much like the *R. capsulatus* protein (22), suggesting that RegB might be a membrane-associated sensor kinase.

**Complementation studies with strain A25.** Since the original fragment from the genomic library of strain 16PHC was on plasmid pRK415, a broad-host-range and mobilizable vector, it was feasible to study its function in *R. sphaeroides* directly. A 7.8-kb *Bgl*II fragment containing the 2-kb *Sal*I portion and the 2-kb *Sal*I fragment itself were subcloned into pRK415 (Fig. 2A). Each of the three fragments, when mated into strain A25, was found to restore the ability of strain A25 to grow photosynthetically in a malate minimal medium with $CO_2$ being used as the electron acceptor (Fig. 1C, D, and E), while the vector alone did not (Fig. 1F). It should be noted that the 2-kb *Sal*I fragment does not include 15 amino acids at the C terminus of RegB, which is apparently not important for the function of RegB. From these results, it would appear that a mutation in the wild-type copy of *regB* on the chromosome contributes to the phenotypic switch from strain 16PHC to strain A25.

**The function of *regB*.** The $Str^r$-$Spc^r$ $\Omega$ cartridge was inserted into the *regB* genes of wild-type strain HR and strain CAC in

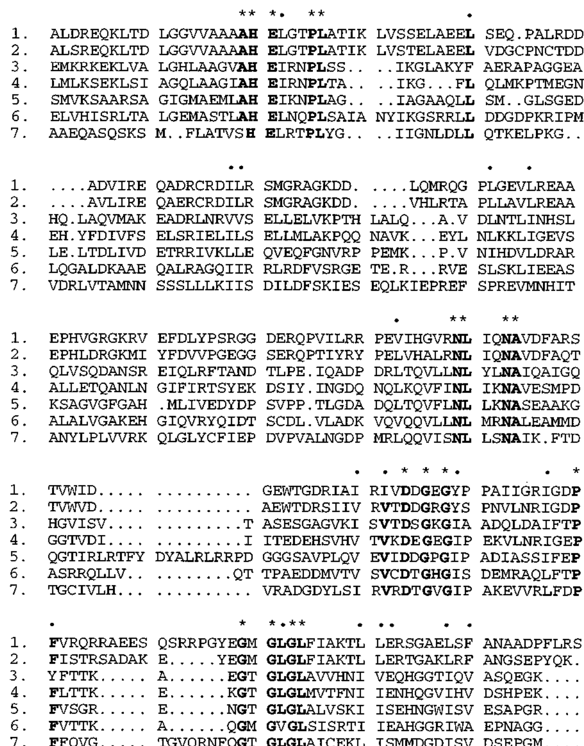


FIG. 3. C-terminal sequence homology among seven sensor kinases, including (1.) RegB from *R. sphaeroides* (6), (2.) RegB from *R. capsulatus* (22), (3.) HydH from *E. coli* (29), (4.) SpoIIJ from *B. subtilis* (1), (5.), NtrB from *R. capsulatus* (16), (6.) FixL from *A. caulinodans* (17), and (7.) RcsC from *E. coli* (31). Asterisks indicate identical residues, and solid dots indicate similar residues.

order to construct specific knockout strains. Colonies that were chosen for study were Spc$^r$ Km$^s$ and were subsequently verified to contain a specific disruption of the *regB* gene (see Materials and Methods). HRΩ, the *regB* mutant of strain HR, was examined under different growth conditions. It was found that HRΩ could grow normally under aerobic chemoheterotrophic conditions but that it had a characteristic long lag (3 to 4 days) during photoheterotrophic growth in malate with $CO_2$ being used as the electron acceptor, compared with wild-type strain HR. After this initial lag, there were no differences in, e.g., generation times, final cell densities, pigmentation, etc. Under photolithoautotrophic growth conditions, however, strain HRΩ grew poorly in a 1.5% $CO_2$–$H_2$ atmosphere but normally, with a lag, in a 5% $CO_2$–$H_2$ atmosphere (results not shown). Under carbon starvation conditions, when strains HR and HRΩ were both bubbled with argon, the characteristic derepression of Rubisco was observed for strain HR (37) but not strain HRΩ (Fig. 4). Since these results suggested that *regB* affected the expression of Calvin cycle $CO_2$ fixation genes (*cbb*), experiments were undertaken to examine the impact of the *regB* mutation on both the $cbb_I$ and $cbb_{II}$ operons. A construct (pVKC1-13) containing a translational fusion of the $cbb_I$ promoter and *lacZ* (4) was introduced into strains HR and HRΩ. It was shown that in strain HR, the Rubisco and β-galactosidase activities were both substantially enhanced under photosynthetic growth conditions, and this effect was much more pronounced under photolithoautotrophic growth conditions compared with photoheterotrophic growth conditions (Fig. 5A and C). In strain HRΩ, low levels of Rubisco activity and β-galactosidase activity were found under both photohet-

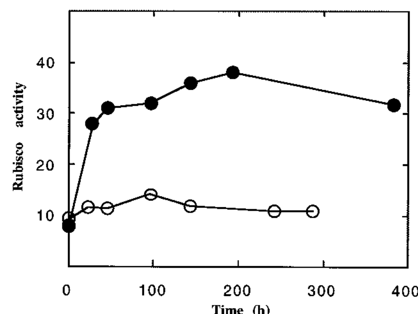


FIG. 4. Induction of Rubisco activity in strain HR (●) and strain HRΩ (○) under carbon starvation conditions (37). Cells were first grown to the late exponential phase in minimal medium bubbled with argon in the light. The cells were then washed, incubated in organic carbon-free minimal medium, and bubbled with argon, and samples were taken at the indicated times for Rubisco activity measurements (37), which are expressed as nanomoles per minute per milligram of product formed.

erotrophic and photolithoautotrophic conditions in a 1.5% $CO_2$–$H_2$ atmosphere. Immunological studies (11, 12) confirmed that the synthesis of form I and form II Rubisco was drastically affected by the mutation in the *regB* gene (results not shown). However, the Rubisco and β-galactosidase activities both in-

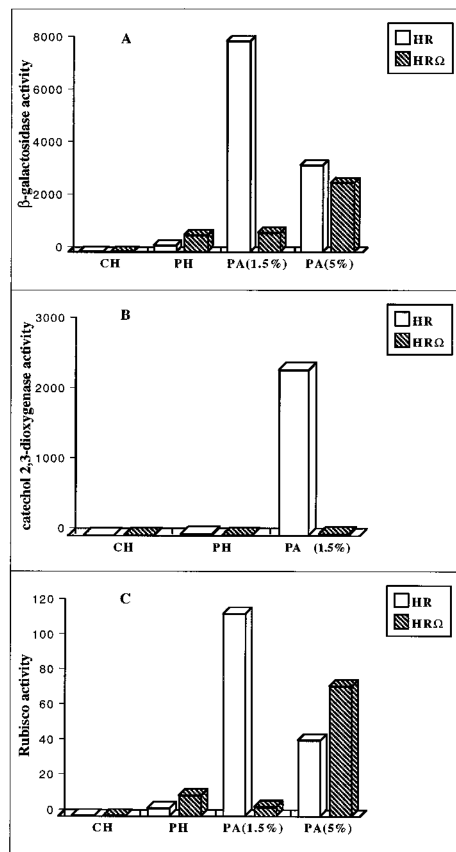


FIG. 5. Levels of β-galactosidase activity (A) in strains HR(pVKC1-13) and HRΩ(pVKC1-13), catechol 2,3-dioxygenase activity (B) in strains HR (pHX300W21) and HRΩ(pHX300W21), and Rubisco activity (C) in strains HR and HRΩ under anoxic photoheterotrophic (PH), photolithoautotrophic (1.5% $CO_2$–98.5% $H_2$ or 5% $CO_2$–95% $H_2$) (PA), and aerobic chemoheterotrophic (CH) growth conditions. Units of enzyme activity are expressed as nanomoles per minute per milligram of product formed.

creased to similar levels or even higher levels in strains HR and HRΩ in a 5% $CO_2$–$H_2$ atmosphere. Therefore, the levels of expression of β-galactosidase, a measure of the activity of the $cbb_I$ operon promoter, and Rubisco activity, a measure of the level of the product of the last gene of each *cbb* operon (11, 12), indicated that transcription of the *cbb* regulon was affected by a mutation in *regB* under photolithoautotrophic conditions in an atmosphere of 1.5% $CO_2$–$H_2$. To determine if transcription of the $cbb_{II}$ operon was also affected, an *xylE*-$cbb_{II}$ transcriptional promoter fusion (39), plasmid pHX300W21, was introduced into strains HR and HRΩ. Similar results were obtained (Fig. 5B), namely, a dramatic increase in promoter activity under photolithoautotrophic conditions in a 1.5% $CO_2$–$H_2$ atmosphere for strain HR but not strain HRΩ.

Strain CAC is a spontaneous gain of function mutant which was isolated from strain HR and which grows chemolithoautotrophically in the dark in a $CO_2$-$H_2$-$O_2$ atmosphere, resulting in a large increase in Rubisco synthesis (23). To test if *regB* is involved in dark aerobic $CO_2$ fixation, procedures similar to those described above were employed to isolate a specific *regB*-deficient mutant in this strain. Southern blotting experiments (results not shown) established that homologous recombination occurred to yield a specific disruption of the *regB* gene in strain CACΩ. Strain CACΩ was found to be incapable of aerobic chemolithoautotrophic growth and exhibited impaired anoxygenic photolithoautotrophic growth; no significant Rubisco derepression was observed when chemoheterotrophically grown cells were incubated in the absence of organic carbon in an atmosphere of 5% $CO_2$–45% $H_2$–50% air (Fig. 6C). However, Rubisco activity and synthesis (verified immunologically) substantially increased in parent strain CAC (23). The promoter activities of the $cbb_I$ and $cbb_{II}$ operons were also examined under chemoheterotrophic and chemolithoautotrophic conditions in both strain CAC and strain CACΩ. Both the β-galactosidase activity (*lacZ* fusion to the $cbb_I$ promoter [4]) and the catechol 2,3-dioxygenase activity (*xylE* fusion to the $cbb_{II}$ promoter [39]) were greatly increased in strain CAC when growth was switched from chemoheterotrophic to chemolithoautotrophic conditions. However, this increase was not observed for strain CACΩ (Fig. 6A and B). Therefore, it is apparent that the mutation in RegB influences transcription of the *cbb* regulon in the presence of oxygen in the dark as well as in the absence of oxygen in the light. Finally, the specificity of *regB* in regulating the function of the *cbb* regulon of *R. sphaeroides* was further underlined by the ability of plasmid pRKXH2.0, as well as plasmids pRKBgB3 and pRK12F2, to complement strain CACΩ for photolithoautotrophic growth in a $CO_2$-$H_2$ atmosphere. Given that active transcription of Rubisco and *cbb* is absolutely required under these growth conditions (8), the complementation studies confirm that *regB* is involved in the control of autotrophic $CO_2$ fixation.

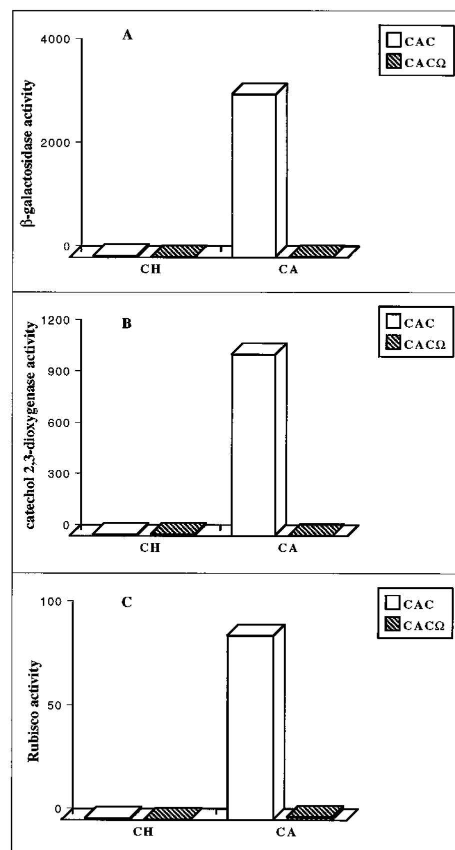


FIG. 6. Levels of β-galactosidase activity (A) in strains CAC(pVKC1-13) and CACΩ(pVKC1-13), catechol 2,3-dioxygenase activity (B) in strains CAC (pHX300W21) and CACΩ(pHX300W21), and Rubisco activity (C) in strains CAC and CACΩ under aerobic chemoheterotrophic (CH) and chemolithoautotrophic (CA) growth conditions. Units of enzyme activity are expressed as nanomoles per minute per milligram of product formed.

## DISCUSSION

Transposon mutagenesis of *R. sphaeroides* 16PHC and subsequent selection for strains with a deficiency in the ability to grow photoheterotrophically in the presence of malate, with $CO_2$ being used as the electron acceptor, led to the discovery that the *regB* (*prrB*) gene is somehow involved in a putative alternative $CO_2$ reduction pathway. The *regB* gene encodes a protein which had previously been found to function as a sensor kinase important for the anaerobic induction of structural genes of the photosynthetic LH and RC apparatus of *R. capsulatus* and *R. sphaeroides* (6, 22). The *regA* and *regB* genes of both organisms are organized similarly, and the *regB* (*prrB*)-*regA* (*prrA*) system exhibits similar functions in regulating light-dependent and oxygen-sensitive photosynthesis gene expression (5, 6, 22, 28). However, there appear to be differences relative to the severity of a mutation in *regA* (*prrA*) in the two organisms (5, 19, 28). Studies with *R. capsulatus* indicated that like RegA, RegB also influences the expression of LH and RC genes and yet a *regB* mutant still maintains a low level of anaerobic induction of the LH and RC genes (22). The results of the current investigation with *R. sphaeroides*, however, indicate that both anoxygenic $CO_2$ fixation and aerobic autotrophic $CO_2$ fixation are also regulated by the *reg* signal transduction system. In this organism, it was found that the levels of Rubisco and $cbb_I$ and $cbb_{II}$ promoter activity were significantly lower in a *regB* mutant of wild-type strain HR under photolithoautotrophic growth conditions (1.5% $CO_2$–$H_2$). Yet, Rubisco activity and $cbb_I$ promoter activity were markedly enhanced relative to photoheterotrophic growth for both the mutant (strain HRΩ) and the wild type (strain HR) when these strains were supplied with 5% $CO_2$–$H_2$. In a result similar to previous results (7), the levels of Rubisco were not as high at 5% $CO_2$–$H_2$; this is also reflected by the $cbb_I$ promoter activity in these cultures. There was no significant effect of the *regB* mutation when these strains were grown under photoheterotrophic or aerobic chemoheterotrophic growth conditions, since the *cbb* regulon is already repressed under these conditions (32), suggesting that there are additional regulatory signals besides that elicited by *regB*. These results indicate, how-

ever, that the function of RegB is influenced by the carbon source and the exogenous $CO_2$ concentration in addition to the light intensity and oxygen tension provided for photosynthetically grown cells. Indeed, oxygen did not negatively regulate the system when the CAC cells were grown chemolithoautotrophically, suggesting that this strain may possess a mutation in some gene(s) that interacts with the *reg* sensory transduction system. Strain A25, shown to be the result of a mutation in *regB*, is incapable of coupling malate-dependent photoheterotrophic growth with the use of $CO_2$ as an electron acceptor. Yet, this strain grows normally when an alternative acceptor (DMSO) is provided. Since strain 16PHC is a Rubisco double deletion strain (35) with absolutely no ability to use the Calvin cycle to fix $CO_2$, it is apparent that *regB* influences the putative alternative $CO_2$ fixation pathway(s) (36) of strain 16PHC. Likewise, a mutation in *regB* appeared to have a drastic effect on the ability of strain CAC to derepress aerobic *cbb* expression and Rubisco synthesis in the dark in a $CO_2$–$H_2$-$O_2$ atmosphere. From these results, it is apparent that the *regA-regB* system might play a role in the regulation of both anoxygenic light-dependent and aerobic light-independent autotrophic and heterotrophic metabolism in *R. sphaeroides*. The global nature of this system is underlined by its ability to sense redox status (when DMSO substitutes for $CO_2$ as the electron acceptor [35]) as well as the concentration and specificity of the carbon source, oxygen tension, and light intensity (5, 28).

The fact that a *regB* mutation has a less severe effect than a mutation in *regA* in both *R. sphaeroides* (5) and *R. capsulatus* (22) suggests that other sensor kinases exist or other kinases can interact with RegA when RegB is absent. In *R. capsulatus*, the *senC regB* double mutant showed the same severe phenotype as a *regA* mutant (22, 28). It is not known, however, if SenC can interact with RegA in a nonspecific fashion or if it is one of the branches in the signal transduction circuit. Recently, a multisensory activation or relay pathway to regulate the initiation of sporulation of *B. subtilis* was proposed (2). KinA (or SpoIIJ) is the first identified kinase involved in this process and is one of the sensor kinases with which RegB from *R. sphaeroides* has homology. KinA and the subsequently identified KinB sense different signals, and both activate the same secondary messenger by enhancing its level of phosphorylation, thus influencing other cell components to cause the activation and repression of different sets of genes (34). Both KinA and KinB mutants are still able to sporulate, although there is a lag. This lag is reminiscent of the phenotype of the *regB* mutant of *R. sphaeroides*, which exhibits a long lag under photoheterotrophic growth conditions when $CO_2$ is the electron acceptor. Whether there is another sensor kinase working in concert with RegB in this organism remains to be established.

The relationship among strains 16, 16PHC, and A25 is not resolved, since the same fragment that complements strain A25 to the 16PHC phenotype does not enable strain 16 to couple malate-dependent growth to $CO_2$ reduction. The fact that a mutation in *regB* results in impaired photoheterotrophic growth of strain A25 under conditions in which $CO_2$ is the electron acceptor suggests that RegB has a positive effect on the alternative $CO_2$ fixation system. The positive effect of RegB might be inhibited or repressed in strain 16, which cannot grow photoheterotrophically on malate unless DMSO is used as the electron acceptor. Thus, strain 16PHC may be the result of a dominant mutation occurring either in *trans* (the loss of function of a strong repressor that usually inhibits the alternative $CO_2$ fixation pathway) or in *cis* (a point mutation in the repressor binding site in the upstream regulatory region of genes involved in this pathway). If this putative repressor is downstream and under the control of the RegB-RegA system, the loss of RegB will have no effect on strains that do not show repression (such as strains 16PHC and A25). However, photoheterotrophic growth of strain A25 is obviously drastically affected. It is also hard to conceive that this putative repressor is upstream and controls the RegB-RegA system. If true, this repressor and the RegB-RegA system might jointly control genes of the alternative $CO_2$ fixation pathway; they would then have antagonizing functions so that inhibition by the repressor might be stronger and play the major role in wild-type strain 16, the parent to strain 16PHC (35). When somehow this repressor loses its function, as in strain 16PHC, the genes of the alternative $CO_2$ fixation pathway are turned on by the activation of the RegB-RegA system under photoheterotrophic growth conditions; when the function of *regB* is lost, as in strain A25, gene expression is not activated or is activated poorly by other sensor kinases. It is also apparent that the RegB-RegA system may also control the functions of other activities in the cell. At this time, it is not known whether DMSO reduction plays any role in controlling gene expression of the alternative $CO_2$ fixation pathway; however, the presence of DMSO and its use as an alternative $CO_2$ acceptor drastically affect the ability of strain 16PHC to assimilate $CO_2$ in whole-cell $CO_2$ fixation assays (35). The possible role of RegA as a transcriptional factor is still uncertain, although there is evidence that *regA* (*prrA*) might function through downstream regulatory sequences in the *puc* operon of *R. sphaeroides* (5).

The mechanism involved in the switch from strain HR to CAC is also not clear, since derepression of Rubisco synthesis historically requires the proper light intensity, low oxygen tension, and the appropriate level of $CO_2$ and/or organic carbon source supplied to the cells (32, 33). Obviously, when strain CAC is grown under chemolithoautotrophic conditions, high levels of Rubisco are synthesized in the absence of light and in the presence of $O_2$ concentrations that normally result in diminished synthesis in wild-type strain HR (23). Although wild-type *R. capsulatus* naturally grows under chemolithoautotrophic growth conditions similar to those just discussed (20), the amount of $CO_2$ fixed is half as much as that fixed under photolithoautotrophic conditions (30) and Rubisco levels increase dramatically (23). What is apparent from the results of this study is that RegB seems to be involved in the ability of *R. sphaeroides* CAC to grow chemolithoautotrophically, because the *regB* mutant of strain CAC was no longer capable of chemolithoautotrophic growth. This lack of growth is due to the inability of strain CACΩ to derepress Rubisco synthesis and transcription of the $cbb_I$ and $cbb_{II}$ operons in the absence of a functional *regB* gene.

In summary, this work establishes that the RegB-RegA system not only plays a role in anoxygenic induction or light regulation of LH and RC photosynthesis genes, but this two-component regulatory system has a larger global effect on both photosynthetic and nonphotosynthetic aerobic chemolithoautotrophic carbon metabolism. The identification of the target genes and the genes that interact with this system will be helpful for the absolute understanding of this global regulatory system. In this connection, previous results from our laboratory had indicated that the product of the divergently transcribed *cbbR* gene, located immediately upstream from the $cbb_I$ promoter, positively regulates transcription of both the $cbb_I$ operon and the $cbb_{II}$ operon of *R. sphaeroides* (13). Inasmuch as the phenotype of a *cbbR* mutant of strain HR closely resembles that of the *regB* mutant prepared in the current investigation, it may be fruitful to probe the relationship of the one-component and two-component signal transduction systems and determine how they may interact to control $CO_2$ fixation in this and related organisms.

## ACKNOWLEDGMENTS

The comments and suggestions of Janet Gibson relative to the manuscript are gratefully acknowledged. We also thank Tim Donohue for providing transposon vector pGP5Gm.

This work was supported by Public Health Services grant GM 24497 from the National Institutes of Health and by Department of Energy grant DE-FG02-94ER20033.


## REFERENCES


1. **Antoniewski, C., B. Savelli, and P. Stragier.** 1990. The *spoIIJ* gene, which regulates early developmental steps in *Bacillus subtilis*, belongs to a class of environmentally responsive genes. J. Bacteriol. **172:**86–93.
2. **Burbulys, D., K. A. Trach, and J. A. Hoch.** 1991. Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. Cell **64:**545–552.
3. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
4. **Dubbs, J. M., and F. R. Tabita.** 1994. Analysis of expression of the form I *cbb* operon of *Rhodobacter sphaeroides* using translational fusions to *lacZ*, abstr. H-16, p. 202. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology. American Society for Microbiology, Washington, D.C.
5. **Eraso, J. M., and S. Kaplan.** 1994. *prrA*, a putative response regulator involved in oxygen regulation of photosynthesis gene expression in *Rhodobacter sphaeroides*. J. Bacteriol. **176:**32–43.
6. **Eraso, J. M., and S. Kaplan.** 1995. Oxygen-insensitive synthesis of the photosynthetic membranes of *Rhodobacter sphaeroides*: a mutant histidine kinase. J. Bacteriol. **177:**2695–2706.
7. **Falcone, D. L., R. G. Quivey, Jr., and F. R. Tabita.** 1988. Transposon mutagenesis and physiological analysis of strains containing inactivated form I and form II ribulose bisphosphate carboxylase/oxygenase genes in *Rhodobacter sphaeroides*. J. Bacteriol. **170:**5–11.
8. **Falcone, D. L., and F. R. Tabita.** 1991. Expression of endogenous and foreign ribulose 1,5-bisphosphate carboxylase-oxygenase (RubisCO) genes in a RubisCO deletion mutant of *Rhodobacter sphaeroides*. J. Bacteriol. **173:** 2099–2108.
9. **Falcone, D. L., and F. R. Tabita.** 1993. Complementation analysis and regulation of $CO_2$ fixation gene expression in a ribulose 1,5-bisphosphate carboxylase-oxygenase deletion strain of *Rhodospirillum rubrum*. J. Bacteriol. **175:**5066–5077.
10. **Gibson, J. L.** 1995. Genetic regulation of $CO_2$ fixation, p. 1107–1124. *In* R. E. Blankenship, M. T. Madigan, and C. E. Bauer (ed.), Anoxygenic photosynthetic bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
11. **Gibson, J. L., J.-H. Chen, P. A. Tower, and F. R. Tabita.** 1990. The form II fructose 1,6-bisphosphatase and phosphoribulokinase genes form part of a large operon in *Rhodobacter sphaeroides*: primary structure and insertional mutagenesis analysis. Biochemistry **29:**8085–8093.
12. **Gibson, J. L., D. L. Falcone, and F. R. Tabita.** 1991. Nucleotide sequence, transcriptional analysis, and expression of genes encoded within the form I $CO_2$ fixation operon of *Rhodobacter sphaeroides*. J. Biol. Chem. **266:**14646–14653.
13. **Gibson, J. L., and F. R. Tabita.** 1993. Nucleotide sequence and functional analysis of CbbR, a positive regulator of the Calvin cycle operons of *Rhodobacter sphaeroides*. J. Bacteriol. **175:**5778–5784.
14. **Hartman, F. C., and M. R. Harpel.** 1993. Chemical and genetic probes of the active site of D-ribulose-5,bisphosphate carboxylase/oxygenase; a retrospective based on the three dimensional structure. Adv. Enzymol. Relat. Areas Mol. Biol. **67:**1–75.
15. **Inoue, K., J.-L. Kouadio, C. S. Mosley, and C. E. Bauer.** 1995. Isolation and *in vitro* phosphorylation of sensory transduction components controlling anaerobic induction of light harvesting and reaction center gene expression in *Rhodobacter capsulatus*. Biochemistry **34:**391–396.
16. **Jones, R., and R. Haselkorn.** 1989. The DNA sequence of *Rhodobacter capsulatus ntrA*, *ntrB* and *ntrC* gene analogues required for nitrogen fixation. Mol. Gen. Genet. **215:**507–516.
17. **Kaminski, P. A., K. Mandon, F. Arigoni, N. Desnoues, and C. Elmerich.** 1991. Regulation of nitrogen fixation in *Azorhizobium caulinodans*: identification of a *fixK*-like gene, a positive regulator of *nifA*. Mol. Microbiol. **5:**1983–1991.
18. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene **70:**191–197.
19. **Lee, J. K., and C. Kaplan.** 1992. Isolation and characterization of *trans*-acting mutations involved in oxygen regulation of *puc* operon transcription in *Rhodobacter sphaeroides*. J. Bacteriol. **174:**1158–1171.
20. **Madigan, M. T., and H. Gest.** 1979. Growth of the photosynthetic bacterium *Rhodopseudomonas capsulata* chemoautotrophically in darkness with $H_2$ as the energy source. J. Bacteriol. **137:**524–530.
21. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
22. **Mosley, C. S., J. Y. Suzuki, and C. E. Bauer.** 1994. Identification and molecular genetic characterization of a sensor kinase responsible for coordinately regulating light harvesting and reaction center gene expression in response to anaerobiosis. J. Bacteriol. **176:**7566–7573.
23. **Paoli, G. C., and F. R. Tabita.** 1994. Aerobic chemoautotrophic growth of a spontaneous mutant of *Rhodobacter sphaeroides* strain HR, abstr. H-62, p. 210. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology. American Society for Microbiology, Washington, D.C.
24. **Penfold, R. J., and J. M. Pemberton.** 1992. An improved suicide vector for construction of chromosomal insertion mutations in bacteria. Gene **118:**145–146.
25. **Phillips-Jones, M. K., and C. N. Hunter.** 1994. Cloning and nucleotide sequence of *regA*, a putative response regulator gene of *Rhodobacter sphaeroides*. FEMS Microbiol. Lett. **116:**269–276.
26. **Prentki, P., and H. M. Krisch.** 1994. *In vitro* insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Sganga, M. W., and C. E. Bauer.** 1992. Regulatory factors controlling photosynthetic reaction center and light-harvesting gene expression in *Rhodobacter capsulatus*. Cell **68:**945–954.
29. **Stoker, J., W. N. M. Reijnders, L. F. Oltmann, and A. H. Stouthamer.** 1989. Initial cloning and sequencing of *hydHG*, an operon homologous to *ntrBC* and regulating the labile hydrogenase activity in *Escherichia coli* K-12. J. Bacteriol. **171:**4448–4456.
30. **Stoppani, A. O. M., R. C. Fuller, and M. Calvin.** 1955. Carbon dioxide fixation by *Rhodopseudomonas capsulata*. J. Bacteriol. **69:**491–501.
31. **Stout, V., and S. Gottesman.** 1990. RcsB and RcsC: a two-component regulator of capsule synthesis in *Escherichia coli*. J. Bacteriol. **172:**659–669.
32. **Tabita, F. R.** 1988. Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. Microbiol. Rev. **52:**155–189.
33. **Tabita, F. R.** 1995. The biochemistry and metabolic regulation of carbon metabolism and $CO_2$ fixation in purple bacteria, p. 885–914. *In* R. E. Blankenship, M. T. Madigan, and C. E. Bauer (ed.), Anoxygenic photosynthetic bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
34. **Trach, K. A., and J. A. Hoch.** 1993. Multisensory activation of the phosphorelay initiating sporulation in *Bacillus subtilis*: identification and sequence of the protein kinase of the alternate pathway. Mol. Microbiol. **8:**69–79.
35. **Wang, X., D. L. Falcone, and F. R. Tabita.** 1993. Reductive pentose phosphate-independent $CO_2$ fixation in *Rhodobacter sphaeroides* and evidence that ribulose bisphosphate carboxylase/oxygenase activity serves to maintain the redox balance of the cell. J. Bacteriol. **175:**3372–3379.
36. **Wang, X., H. V. Modak, and F. R. Tabita.** 1993. Photolithoautotrophic growth and control of $CO_2$ fixation in *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* in the absence of ribulose bisphosphate carboxylase-oxygenase. J. Bacteriol. **175:**7109–7114.
37. **Weaver, K. E., and F. R. Tabita.** 1983. Isolation and partial characterization of *Rhodopseudomonas sphaeroides* mutants defective in the regulation of ribulose bisphosphate carboxylase/oxygenase. J. Bacteriol. **156:**507–515.
38. **Weaver, K. E., and F. R. Tabita.** 1985. Complementation of a *Rhodopseudomonas sphaeroides* ribulose bisphosphate carboxylase-oxygenase regulatory mutant from a genomic library. J. Bacteriol. **164:**147–154.
39. **Xu, H. H., and F. R. Tabita.** 1994. Positive and negative regulation of sequences upstream of the form II *cbb* $CO_2$ fixation operon of *Rhodobacter sphaeroides*. J. Bacteriol. **176:**7299–7308.