JOURNAL OF BACTERIOLOGY, May 2000, p. 2831–2837 Vol. 182, No. 10
0021-9193/00/$04.00+0
Copyright © 2000, American Society for Microbiology. All Rights Reserved.

Expression of Uptake Hydrogenase and Molybdenum Nitrogenase in *Rhodobacter capsulatus* Is Coregulated by the RegB-RegA Two-Component Regulatory System

SYLVIE ELSEN,[1]† WANDA DISCHERT,[2] ANNETTE COLBEAU,[2] AND CARL E. BAUER[1]*

Department of Biology, Indiana University, Bloomington, Indiana 47405,[1] and UMR 5092 CEA-CNRS-UJF, Laboratoire de Biochimie et Biophysique des Systèmes Intégrés, Département de Biologie Moléculaire et Structurale, CEA/Grenoble, 38054 Grenoble cedex 9, France[2]

Received 2 December 1999/Accepted 16 February 2000

Purple photosynthetic bacteria are capable of generating cellular energy from several sources, including photosynthesis, respiration, and H_2 oxidation. Under nutrient-limiting conditions, cellular energy can be used to assimilate carbon and nitrogen. This study provides the first evidence of a molecular link for the coregulation of nitrogenase and hydrogenase biosynthesis in an anoxygenic photosynthetic bacterium. We demonstrated that molybdenum nitrogenase biosynthesis is under the control of the RegB-RegA two-component regulatory system in *Rhodobacter capsulatus*. Footprint analyses and in vivo transcription studies showed that RegA indirectly activates nitrogenase synthesis by binding to and activating the expression of *nifA2*, which encodes one of the two functional copies of the *nif*-specific transcriptional activator, NifA. Expression of *nifA2* but not *nifA1* is reduced in the *reg* mutants up to eightfold under derepressing conditions and is also reduced under repressing conditions. Thus, although NtrC is absolutely required for *nifA2* expression, RegA acts as a coactivator of *nifA2*. We also demonstrated that in *reg* mutants, [NiFe]hydrogenase synthesis and activity are increased up to sixfold. RegA binds to the promoter of the hydrogenase gene operon and therefore directly represses its expression. Thus, the RegB-RegA system controls such diverse processes as energy-generating photosynthesis and H_2 oxidation, as well as the energy-demanding processes of N_2 fixation and CO_2 assimilation.

The purple nonsulfur photosynthetic bacterium *Rhodobacter capsulatus* exhibits remarkable metabolic diversity (30). This bacterium is capable of generating energy from light via photosynthesis as well as from dark aerobic and anaerobic respiration. Another feature is the capacity to grow heterotrophically as well as autotrophically. When growing autotrophically, the cells are also capable of generating cellular energy and reducing power by the oxidation of H_2, which occurs at a membrane-bound [NiFe]hydrogenase complex. Several decades ago, Gest and colleagues described the presence of redox-related interrelationships among carbon assimilation, N_2 fixation, and photophosphorylation (26; reviewed in reference 27). However, the nature and even the existence of specific molecular mechanisms for balancing the use of reducing equivalents have remained unclear.

Recent studies have suggested that balancing different metabolic processes could result, at least partially, from the activity of a global two-component regulatory system that regulates the synthesis of the enzymes involved in different energetic processes. Indeed, the three fundamental biological processes catalyzed by photosynthetic bacteria, i.e., photosynthesis, CO_2 fixation, and N_2 assimilation, are affected by the RegB-RegA global two-component transduction signal system in *Rhodobacter sphaeroides* (32). In *R. capsulatus*, the RegB-RegA system functions as a classic two-component system, with RegB being a membrane-spanning histidine kinase capable of autophosphorylating in the presence of ATP (3, 40, 47). The phosphate group is then transferred to its cognate partner, the cytosolic response regulator RegA (3, 31, 47). Phosphorylation of RegA increases its DNA-binding to the *puf* and *puc* photosynthesis promoters where it functions as an activator of transcription (3, 15). Homologous systems have been found in many species of the α proteobacteria, including such photosynthetic species as *R. sphaeroides*, *Rhodovulum sulfidophilum*, and *Roseobacter denitrificans* (21, 22, 37), as well as in nonphotosynthetic species such as *Bradyrhizobium japonicum* and *Rhizobium meliloti* (2, 49). RegA and its homologs exhibit an unprecedented 79% degree of conservation, especially in the C-terminal DNA-binding helix-turn-helix structure, which is 100% conserved (37). This suggests that the RegB-RegA system plays a fundamental role in this group of bacteria.

Originally the RegB-RegA system was discovered for its role in anaerobic activation of the *puf*, *puc*, and *puh* photosynthetic gene operons from *R. capsulatus* (40, 47). In addition to its involvement in photosynthesis, a related RegB-RegA system from *R. sphaeroides* (PrrB-PrrA) has been implicated in the positive regulation of the *cbbI* and *cbbII* operons that encode enzymes of the Calvin cycle CO_2 fixation pathway (45; J. M. Dubbs, T. H. Bird, C. E. Bauer, and F. R. Tabita, submitted for publication). The *R. sphaeroides* system was also shown to be involved in the nitrogen fixation process, since the derepression of nitrogenase synthesis that occurs in the absence of the CO_2 fixation pathway requires a functional *regB* gene (32). It has also been shown that inactivation of a RegA homolog in the nonphotosynthetic bacterium *B. japonicum* (RegR) reduces nitrogen fixation to a level where nodules are ineffective in fixing nitrogen. This effect on nitrogen fixation is caused by

* Corresponding author. Mailing address: Department of Biology, Indiana University, Jordan Hall, Bloomington, IN 47405. Phone: (812) 855-6595. Fax: (812) 855-6705. E-mail: cbauer@bio.indiana.edu.

† Present address: UMR 5092 CEA-CNRS-UJF, Laboratoire de Biochimie et Biophysique des Systèmes Intégrés, Département de Biologie Moléculaire et Structurale, CEA/Grenoble, 38054 Grenoble cedex 9, France.

TABLE 1. Nitrogenase and β-galactosidase activities of the wild-type (SB1003), *regA*-disrupted (MS01), and *regB*-disrupted (SD01) strains harboring plasmid pNF:Q-ZΩ (*nifH*::*lacZ*)

Strain	Genotype	MG[a]		MN[a]	
		Nitrogenase activity	β-Galactosidase activity	Nitrogenase activity	β-Galactosidase activity
SB1003	Wild type	63.6	23,868	<0.1	3.3
MS01	*regA*	13.1	6,485	<0.1	1.1
SD01	*regB*	16.3	8,138	<0.1	1.2

[a] The cells were grown under anaerobiosis in MG or MN medium. Nitrogenase activities represent nanomoles of C_2H_4 formed per minute per milligram of protein. β-Galactosidase activities are given in nanomoles of *o*-nitrophenol formed per minute per milligram of protein. These results represent the means of at least three independent measurements.

a dependence of RegR for optimal expression of NifA, which is a *nif*-specific transcriptional activator of nitrogenase structural genes (2). However, to date, direct binding of RegR to the *nifA* promoter has not been established.

In this study, we have demonstrated that the RegB-RegA system from *R. capsulatus* indirectly activates the synthesis of nitrogenase. Indeed, our in vivo and in vitro studies demonstrate that RegA binds to and activates expression of the *nifA2* gene, which encodes one of the two functional copies of the NifA transcriptional activator of the nitrogenase structural genes. We also demonstrate that RegA directly represses [NiFe]hydrogenase structural gene expression by binding to the *hupSLC* promoter.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The *R. capsulatus* strains used in the study are wild-type strain SB1003 (55), the *regA*-disrupted strain MS01 (47), and the *regB*-disrupted strain SD01 (15). *R. capsulatus* strains were grown at 34°C in minimal salts medium (RCV) (54) supplemented with 30 mM DL-malate as a carbon source and either 7 mM L-glutamate (MG medium) or 7 mM ammonium sulfate (MN medium) as a nitrogen source. RCV malate without a nitrogen source was used as a nitrogen-free (NF) medium. For hydrogenase studies, strains were grown either under aerobic dark conditions or under anaerobic photosynthetic conditions in the light (about 2,500 lx) as previously described by Colbeau et al. (10). For the nitrogenase studies, cells were grown anaerobically overnight in MN medium, washed in NF medium, and induced in MG or MN medium aerobically or anaerobically for 12 to 14 h as described previously by Fostner-Hartnett and Kranz (25). *Escherichia coli* strains were grown aerobically in Luria-Bertani medium at 37°C (46). Antibiotics were added at the following concentrations (milligrams per liter) : 100 (ampicillin), 100 (spectinomycin), 50 (trimethoprim), and 10 (tetracycline) for *E. coli*, and 10 (kanamycin), 10 (spectinomycin), and 1 (tetracycline) for *R. capsulatus*.

Plasmids and plasmid mobilization. Mobilization of plasmids pNF:Q-ZΩ (*nifH*::*lacZ*) (43) from *E. coli* to *R. capsulatus* was accomplished with mobilizing strain Tec5 (48) as described by Young et al. (56). Plasmids pDFH100*Bcl* (*nifA1*::*lacZ*) (25), pDFH200*T* (*nifA2*::*lacZ*) (25), and pAC142 (*hupS*::*lacZ*) (12) were mated into *R. capsulatus* recipient strains using the triparental mating system of Ditta et al. (14) using conditions described by Colbeau et al. (9).

Enzyme assays. Hydrogenase activity was assayed in whole cells as previously described with 0.15 mM methylene blue as the electron acceptor (11). Nitrogenase assays were performed as reported by Meyer et al. (39). β-Galactosidase activity was assayed as described by Elsen et al. (20).

DNase I footprint analysis. RegA* was overexpressed and purified as previously described by Du et al. (15). Probes were prepared by PCR amplification as follows. Amplification of the *hupSLC* promoter utilized primers HOse31 (5′-CGACAATTGTCCCTCCCTTGC) and HOse38 (5′-GCGGCGGCAAAATTGGAAAGC), and plasmid pAC142 (12) previously digested with *Bam*HI was used as a template. Primers FOse36 (5′-GGAAGCGCCATTTTTTTCGGC) and FOse37 (5′CATGTCGAGACTTGGTCAAGC) were used for amplification of the *nifA2* promoter, with genomic DNA as the template. For selective labeling of DNA strands, one of the primers of the PCR amplification was 5′-end labeled with ^{32}P prior to amplification and the amplified DNA fragments were purified as described previously (19). A 10-μl binding reaction mixture was first prepared, containing 1 μl of DNA (50 fmol), 7 μl of H_2O, and 2 μl of 5× footprint binding buffer composed of 125 mM HEPES (pH 8.0), 300 mM potassium acetate, 25 mM magnesium actetate, 10 mM calcium chloride, 5 mM dithiothreitol, and 125 μg of bovine serum albumin per ml. The reaction mixture was then added to a 10-μl solution composed of 2 volumes of 1× footprint binding buffer and 1 volume of protein dialysis buffer (15) containing various amounts of RegA*. Digestion with DNase I and subsequent termination of the assays were carried out as previously described by Bird et al. (4). A modified Maxam and Gilbert G+A chemical sequencing reaction was used to determine the location of DNase I protection (38).

RESULTS

The RegB-RegA two-component regulatory system is involved in nitrogenase gene regulation. We assessed whether biosynthesis of the *R. capsulatus* molybdenum nitrogenase was affected by the RegB-RegA signal transduction cascade by assaying nitrogenase enzyme activity in the wild-type parent strain SB1003, the *regA*-disrupted mutant strain MS01, and the *regB*-disrupted mutant strain SD01. As shown in Table 1, nitrogenase activity was high in SB1003 cells that were grown in MG medium. There was no significant nitrogenase activity when these cells were grown in MN medium (Table 1) or when oxygen was present (data not shown). This pattern is similar to that reported by Pollock et al. (43), which reflects the regulation of nitrogenase synthesis in response to fixed nitrogen availability by the *ntr* system and to the oxygen sensitivity of the NifA proteins which function as transcriptional activators of the *nifHDK* operon (reviewed in reference 35). The data in Table 1 also demonstrate that nitrogenase activity was significantly affected by *regA* and *regB* disruptions, as evidenced by five- and fourfold reductions observed in MS01 and SD01 cells, respectively, under derepressing conditions.

We also assayed β-galactosidase activities in strains SB1003, MS01, and SD01 that contained a *nifH*::*lacZ* fusion (pNF:Q-ZΩ) to determine if the observed reduction in nitrogenase activity reflected reduced transcription rates. The β-galactosidase activities shown in Table 1 demonstrate that the nitrogenase activities observed for various strains and growth conditions are also reflected by a similar pattern of *nifH*::*lacZ* expression. Thus, the *R. capsulatus* RegB-RegA two-component system appears to be affecting nitrogenase biosynthesis.

RegA indirectly activates nitrogenase gene expression. We next addressed whether RegA directly controls nitrogenase expression by binding to the *nifHDK* promoter region, by performing DNase I protection assays on both DNA strands of a 321-bp fragment that contains the *nifHDK* promoter region (from bp −264 to +57). Using the RegA* protein, which exhibits constitutive activity in vivo (15) and high DNA-binding affinity in vitro (3), we observed no RegA*-mediated protection of either strand of the *nifHDK* promoter region (data not shown). This suggests that RegA may indirectly regulate nitrogenase gene expression.

In species where it has been tested, the direct activator of *nifHDK* expression under conditions of fixed nitrogen and oxygen limitation is the NifA protein (reviewed in references 23 and 35). Two identical copies of *nifA*, termed *nifA1* and *nifA2*, are present in *R. capsulatus*, and the product of either gene is sufficient for diazotrophic growth (36). To determine whether

TABLE 2. β-Galactosidase activity measurements of *nifA1*::*lacZ* and *nifA2*::*lacZ* reporter gene fusions in wild-type strain SB1003, *regA*-disrupted strain MS01, and *regB*-disrupted strain SD01

Plasmid	Strain	Genotype	β-Galactosidase activity[a] under:			
			Anaerobiosis		Aerobiosis	
			MG	MN	MG	MN
nifA1::*lacZ*	SB1003	Wild type	1,950	199	1,226	125
	MS01	*regA*	1,825	115	1,157	149
	SD01	*regB*	1,949	255	ND[b]	ND
nifA2::*lacZ*	SB1003	Wild type	11,023	902	6,626	454
	MS01	*regA*	1,329	53	1,214	66
	SD01	*regB*	1,466	85	ND	ND

[a] β-Galactosidase activities are given in nanomoles of *o*-nitrophenol produced per minute per milligram of protein. Results are means of at least three independent measurements.
[b] ND, not determined.

RegB-RegA indirectly controls *nifHDK* expression by affecting the transcription of *nifA1* and/or *nifA2*, we introduced *nifA1*::*lacZ* (pDFH100Bcl) and *nifA2*::*lacZ* (pDFH200T) fusion plasmids into SB1003, MS01, and SD01 cells and subsequently measured β-galactosidase activities under different growth conditions. As shown in Table 2, *nifA1* and *nifA2* expression in the wild-type strain SB1003 had a similar pattern of high expression in MG medium and an approximate 10-fold reduction in activity when the cells were grown in presence of ammonium (MN medium). The reduction of activity observed in MN medium-grown cells is in agreement with a role of the transcriptional activator NtrC, which activates *nifA* expression only in ammonium-free medium (25, 29).

When *nifA1*::*lacZ* expression was measured in the *regA*- and *regB*-disrupted strains MS01 and SD01, respectively, no effect on β-galactosidase activities in cells grown in all of the tested media was observed (Table 2). There was also no evidence of RegA* binding to the *nifA1* promoter region as measured by DNase I protection assays (data not shown). On the other hand, expression of *nifA2* is significantly reduced in both MS01 and SD01 cells relative to the level observed in wild-type cells under all of the tested growth conditions (Table 2). Specifically, *nifA2* expression in MS01 was reduced 8.3-fold in MG medium and 17-fold in MN medium under anaerobiosis. Similarly, strain SD01 exhibited a 7.5-fold reduction in MG medium and a 10.6-fold reduction in MN medium. The RegB-RegA system also appears to control *nifA2* expression in the presence of oxygen, as illustrated by a 5.5- and 7-fold reduction of expression in MG and MN media, respectively, in the *regA*-disrupted strain under aerobic growth conditions (Table 2). Furthermore, expression of *nifA2* is still under the control of nitrogen availability, as evidenced by higher β-galactosidase activities in MG than MN medium. The strong effect of *regB* and *regA* inactivation on *nifA2* expression could explain the reduction in *nifHDK* gene expression in the mutants described above.

***nifA2* expression is directly controlled by RegA.** DNase I protection analyses of RegA* binding to the *nifA2* promoter were undertaken to determine if RegA directly regulates *nifA2* expression. As illustrated by a protected region from bp −43 to −61 on the top strand (Fig. 1A) and from bp −43 to −72 on the bottom strand (Fig. 1B), RegA does indeed bind to the *nifA2* promoter. A hypersensitive site was also observed on each strand, which corresponds to position −61. As shown in Fig. 2A, RegA binds to the *nifA2* promoter between the transcription start sites and the previously mapped NtrC DNA-binding sites (24, 25). These results indicate that RegA indirectly activates *nifHDK* gene expression by participating in the activation of *nifA2* gene expression.

RegB-RegA controls hydrogenase biosynthesis. Nitrogenase is capable of generating hydrogen as a by-product of N_2 fixation. Thus, there appears to be a metabolic link between nitrogenase and hydrogenase synthesis, since the presence of hydrogen stimulates synthesis of the H_2 uptake hydrogenase (reviewed in reference 53). Consequently, we also addressed whether disruption of *regB* or *regA* could directly or indirectly affect the expression of the uptake hydrogenase. For this analysis, we assayed hydrogenase activity and *hup* expression patterns in wild-type and *regB*- and *regA*-disrupted strains that contained the *hupS*::*lacZ* reporter plasmid, pAC142 (12). The data in Table 3 show that hydrogenase activity values and β-galactosidase measurements of *hupS*::*lacZ* expression varied in parallel for each of the various strains and growth conditions tested. In the wild-type strain, SB1003, activity was high when H_2 was evolved from nitrogenase, which occurs under anaerobic growth conditions in MG medium. In contrast, activity was low when the hydrogen concentration was low or absent, which occurs when cells are grown in MN medium under aerobic or anaerobic conditions, when nitrogenase does not evolve hydrogen. However, activity was restored to high levels when 10% hydrogen was exogenously added to MN medium. This demonstrates that there is a dependence of *hup* expression on hydrogen, and not simply on synthesis of nitrogenase. The pattern of H_2 dependence of *hup* expression in SB1003 is very similar to that previously reported by Colbeau and Vignais (12) with wild-type strain B10 of *R. capsulatus*. An involvement of RegA and RegB in the control of hydrogenase synthesis is evidenced by a strong increase in β-galactosidase and hydrogenase activities in the *reg* mutants under all growth conditions tested (Table 3). Indeed, disruption of *regA* or *regB* both led to the same three- to sixfold increase in *hupS*::*lacZ* expression and hydrogenase enzyme activity under the various tested growth conditions. Interestingly, hydrogenase synthesis is still regulated by H_2 in the *regA*- and *regB*-disrupted strains, as evidenced by the stimulation of *hup* expression in the presence of endogenously produced (MG medium) or exogenously added H_2 in strains MS01 and SD01 (Table 3). Thus, the RegB-RegA signal transduction system represses hydrogenase synthesis by a mechanism that is independent of the HupT-HupR system, which activates *hupSLC* synthesis in response to the presence of H_2 (13, 51). Interestingly, the RegB-RegA system appears to be modulating *hup* expression under both aerobic and anaerobic growth conditions even though RegB kinase activity is thought to be affected by the oxygen status of the cell.

RegA directly represses hydrogenase gene expression. We also tested whether RegA affects *hup* expression by direct binding to the *hupSLC* promoter region, by performing a

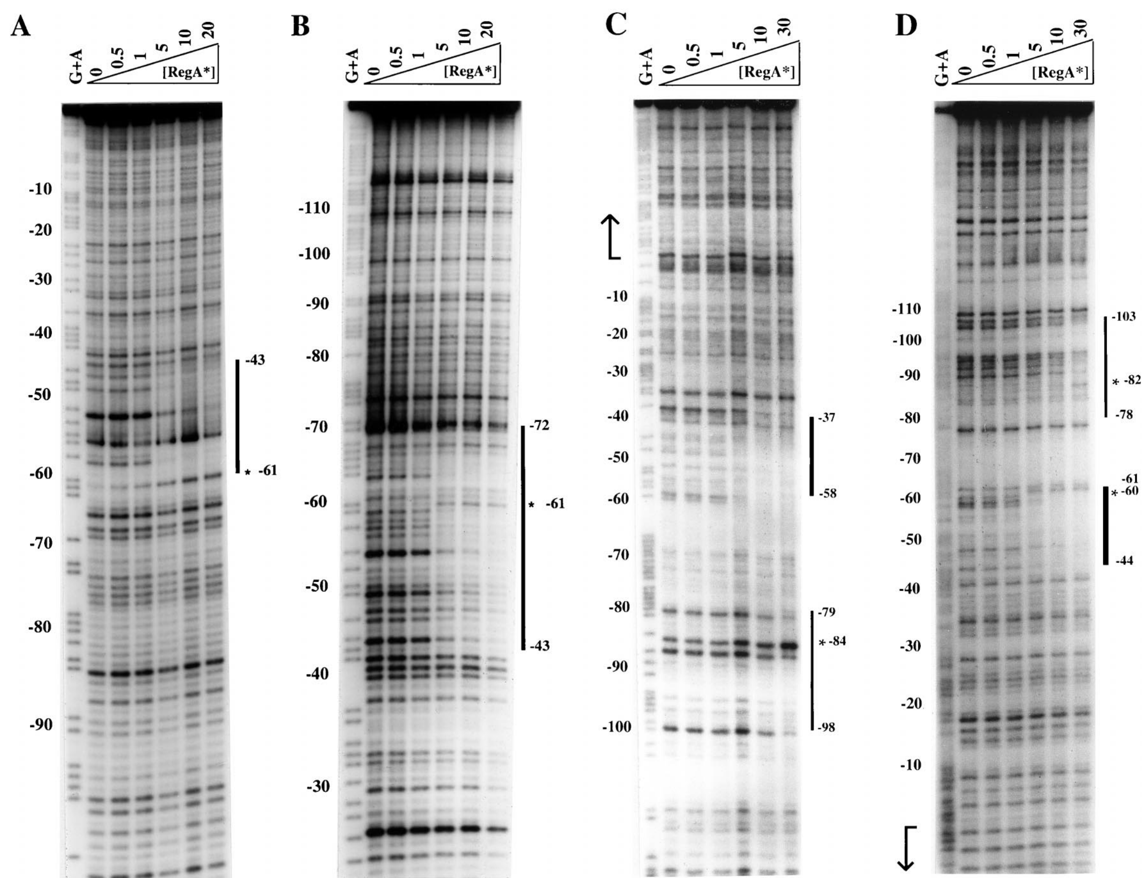

FIG. 1. DNase I footprint analysis of RegA* binding to the *nifA2* and *hupSLC* promoters. RegA*-mediated DNase I protection patterns to the top (A) and bottom (B) strands of the *nifA2* promoter and to the top (C) and bottom (D) strands of the *hupSLC* promoter are presented. G+A indicates a Maxam-Gilbert sequencing ladder. Each of the subsequent lanes are protection patterns generated in the presence of increasing micromolar concentrations of purified RegA*. The thick and thin vertical bars represent the major and minor RegA* DNA-binding sites, respectively. The arrows show the start and direction of transcription of the genes. DNase I-hypersensitive sites are indicated by asterisks.

DNase I protection analysis with RegA*. A RegA* DNA-binding site, which extends from bp −37 to −58 on the top strand (Fig. 1C) and from bp −44 to −61 on the bottom strand (Fig. 1D), was observed with as little as 5 μM RegA*. As also indicated in Fig. 1, when the RegA* concentration was increased from 10 to 30 μM, a second protected region was detected from bp −79 to −98 on the top strand and from bp −78 to −103 on the bottom strand. This second RegA*-protected region overlaps with a previously described integration host factor (IHF) DNA-binding site (50) that is located from bp −93 to −81 (Fig. 2B). Expression of *hupSLC* is known to be strongly activated in the presence of IHF (50), indicating that RegA may function as a repressor of hydrogenase gene expression in *R. capsulatus* by competing with IHF for binding to this region. Hydrogenase expression is also highly dependent on the presence of H_2, which is mediated by the two-component transcriptional regulator HupR, which binds upstream from the IHF-binding site, as indicated in Fig. 2B (13, 51).

DISCUSSION

Hydrogenase and nitrogenase syntheses are coregulated by the RegB-RegA system. In the present study, we have identified two important new elements of the *reg* regulon controlled by

TABLE 3. Hydrogenase and β-galactosidase activities of the wild-type (SB1003), *regA*-disrupted (MS01), and *regB*-disrupted (SD01) strains harboring plasmid pAC142 (*hupS*::*lacZ*)

Growth condition[a]	SB1003		MS01		SD01	
	Hydrogenase activity[b]	β-Galactosidase activity[b]	Hydrogenase activity	β-Galactosidase activity	Hydrogenase activity	β-Galactosidase activity
MG, anaerobiosis	43.4	4,360	122.2	10,790	75.5	9,680
MN, anaerobiosis	6.6	730	37.3	3,230	30.0	3,750
MN, aerobiosis	7.3	750	37.4	4,100	31.9	4,770
MN, aerobiosis, H_2	33.3	4,700	60.0	10,800	55.4	10,810

[a] The cells were grown under anaerobiosis or aerobiosis in MG or MN medium, in the presence or absence of exogenous 10% hydrogen.

[b] Hydrogenase activities are given in micromoles of methylene blue reduced per hour per milligram of protein, and β-galactosidase activities are given in nanomoles of *o*-nitrophenol formed per minute per milligram of protein. These results represent the means of at least three independent measurements.

A

NtrC NtrC

GAAGCGCCATTTTTTTCGGCGCTCCGGCCATTCCCGCCTCAAAGTTGATCTTAACGAAGCCTCAATCACCCCCAA

*

GTTGCGACATATTGGCACCTGAGACTGTTTGCGCCGTGCATCCGGGCGCAAGCTTGACCAAGTCTCGACATGTCC

nifA2

B

HupR

GCCCTTGTCGCGGCGGCAAAATTGGAAAGCAACTGACCGAAGTGGAAATGGAATCGGTCAAAATACTTTCGGTTT

IHF * * *

TTTTACGTGACAAAATCACACACCATTGAAAAACAAGGAAAAATTTTTCTGCGACAATTTGCCGTTTCTCATTGG

CGGAAACCGGCCCAACCATGAGAATTCCACTCTACCCATG

hupSLC

FIG. 2. Features of the *nifA2* (A) and *hupSLC* (B) promoters. Horizontal arrows in *nifA2* (25) and *hupSLC* (51) indicate previously published transcription start sites. Putative −35 and −10 promoter sequences are underlined. Black boxes indicate regions of top- and bottom-strand protection from DNase I digestion by RegA*, and asterisks correspond to DNase I-hypersensitive sites. Also indicated are the DNA-binding sites of NtrC (gray boxes) (24), HupR (white box) (13), and IHF (bracket) (50).

the RegB-RegA two-component regulatory system in *R. capsulatus* (Fig. 3). Using in vitro footprint analysis and in vivo *lacZ* fusion studies, we have demonstrated that the response regulator RegA indirectly activates the *nifHDK* nitrogenase genes and directly represses the *hupSLC* hydrogenase structural genes. Uptake hydrogenase and nitrogenase synthesis are coregulated in *Rhizobium leguminosarum* bv. viciae by the nitrogen fixation regulator NifA (7) and two FnrN proteins (28). However, no genetic relationship had yet been established between nitrogenase and hydrogenase in *R. capsulatus*. This study is the first demonstration of coregulation between hydrogenase and nitrogenase synthesis in *R. capsulatus* which occurs by the RegB-RegA two-component regulatory system.

The *nif* genes required for biosynthesis and assembly of the molybdenum nitrogenase are activated under conditions of both fixed nitrogen and oxygen limitation (reviewed in reference 35). Under nitrogen limitation, the *ntr* system activates the expression of the two functional copies of *nifA* in *R. capsulatus*. It has been demonstrated that NtrC binds to two tandem sites centered more than 100 bp upstream of the *nifA1* and *nifA2* transcriptional start sites and that NtrC~P activates the σ^{70}-containing RNA polymerase holoenzyme (6, 24). The two NifA proteins then activate the expression of all the other *nif* genes in the absence of oxygen (35). Our results indicate that RegA enhances *nifA2* expression under all growth conditions tested. Since it has previously been shown that NtrC is absolutely required for *nifA2* expression (25), RegA appears to function as a coactivator with NtrC to ensure optimal *nifA2* expression.

The membrane-bound [NiFe]hydrogenase, encoded by the *hupSLC* operon, catalyzes hydrogen oxidation in *R. capsulatus*. This enzyme allows cells to grow autotrophically, with hydrogen as an electron source. Under photoheterotrophic growth conditions, *hupSLC* expression is activated by hydrogen gas evolved by nitrogenase as a by-product of nitrogen assimilation (reviewed in reference 53). Hydrogenase synthesis is known to be activated in the presence of hydrogen by the two-component system HupT-HupR. The HupU and HupV proteins have been proposed to sense the hydrogen stimulus and to transfer information to the histidine protein kinase HupT (17, 18, 52). HupT is then thought to control the phosphorylated state of the response regulator HupR, which activates *hupSLC* transcription by directly binding to the promoter (13, 51). Another regulatory factor required for *hupSLC* expression is the histone-like IHF protein that binds to a region between RNA polymerase and HupR DNA-binding sites (13, 50). Our results indicate that the RegB-RegA two-component regulatory system is involved in repression of hydrogenase gene expression under heterotrophic growth conditions. We have also observed that RegA* binds to a region that overlaps the IHF DNA-binding site. Presumably, competition between IHF and RegA for binding to this region is the reason for the repressing effect of RegA.

In earlier studies, RegB and RegA were demonstrated to be required for direct activation of transcription of the photosynthesis genes (reviewed in reference 1). It has also recently been demonstrated that RegA directly activates the *cbb* operons that code for enzymes of the Calvin CO_2 fixation cycle in *R. capsulatus* (P. Vichivanives, C. E. Bauer, and R. Tabita, submitted). Furthermore a functional *regB* gene is required for the derepression of nitrogenase synthesis that occurs in *R. sphaeroides* when the Calvin cycle is mutationally disrupted, even in the presence of ammonium (32). Recent studies have shown that the system also regulates aerobic respiration, by controlling the biosynthesis of the two identified terminal oxidases in *R. capsulatus*, as well as the electron transfer system shared by respiration and photosynthesis processes (L. Swem, S. Elsen, T. H. Bird, H. Myllykallio, F. Daldal, and C. E. Bauer, unpublished data). Therefore, the RegB-RegA system appears to control energy-generating and energy-consuming metabolisms involved in the consumption and production of reduced equivalents. Specifically, this two-component system represses hydrogenase synthesis that catalyzes H_2 oxidation, which is a net generator of reducing equivalents, and activates CO_2 assimilation and N_2 fixation, which are processes that utilize reducing equivalents. RegB-RegA appears to function in a manner that would lower the reducing-equivalent level in the cell. The system appears to be a master cellular redox regulator that ensures that cells do not become overreduced.

The RegB-RegA system functions as a global regulator. A common occurrence among many RegB-RegA-controlled genes is the presence of additional activators and/or repressors that regulate their expression. Indeed, RegA activity appears to involve competition or synergy at its target promoters, with

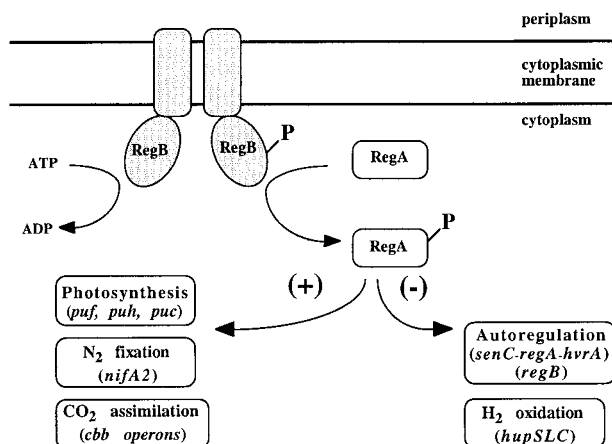

FIG. 3. The *reg* regulon of *R. capsulatus*. The RegB-RegA signal transduction system activates photosynthesis (*puf*, *puh*, and *puc*), nitrogen fixation (*nifA2*), and carbon assimilation (*cbb*) genes and represses hydrogenase structural genes (*hupSLC*) and its own expression. References are given in the text.

a broad range of transcription factors such as histone-like proteins (IHF), LysR family proteins (CbbR), and response regulators (HupR, NtrC). For example, expression of the light-harvesting photosystem II apoproteins encoded by the *puc* operon is anaerobically activated by RegA as well as aerobically repressed by CrtJ (15, 19, 44). For H_2 oxidation, *hup* expression is activated by the presence of H_2 via the response regulator HupR (13, 51) and repressed by RegA. In N_2 fixation, *nifA2* expression is clearly dependent on limitation of fixed nitrogen via activation by NtrC (24; reviewed in reference 35), as well as activation by RegA. In carbon fixation, regulation of the *cbb* operons that code for Calvin cycle enzymes involves activation by CbbR in response to fixed carbon levels as well as activated by RegA (16; J. M. Dubbs, T. H. Bird, C. E. Bauer, and F. R. Tabita, submitted for publication). Hence, the RegB-RegA two-component system appears to function as a secondary regulator that provides an overlying layer of control on these otherwise specifically regulated processes. In many respects, the global nature of the *reg* regulon is similar to what has been observed for the ArcB-ArcA two-component system in *E. coli*. The ArcB-ArcA global system provides redox-responsive regulation of a variety of metabolic genes, many of which are also regulated by additional transcription factors (reviewed in reference 33).

A question that is currently being addressed involves the mechanisms that allow RegA to interact with such diverse transcription factors to control target gene expression. Recently, Bowman et al. (5) demonstrated that RegA and CrtJ proteins compete for overlapping DNA-binding sites on the *puc* promoter. As suggested above, one could envision that repression exerted by RegA on *hupS* expression may also result from competition with IHF for binding to the *hup* promoter, since IHF and RegA DNA-binding sites overlap. Bowman et al. (5) also demonstrated that RegA recruits RNA polymerase-σ^{70} to the *puf* and *puc* promoters by establishing protein-protein interactions. It is interesting that the *nifA1* and *nifA2* promoters in *R. capsulatus* are atypical in that, although they do require NtrC for activation, they are recognized by the housekeeping RNA polymerase-σ^{70} rather than RNA polymerase-σ^{54} (6). As observed for *puc* and *puf*, RegA could play a role in recruiting RNA polymerase-σ^{70} to the *nifA2* promoter, with NtrC then playing a role in promoting activation. In such a case, RegA would not directly activate transcription but instead would increase expression by providing more RNA polymerase bound to the promoter region that would then be activated by NtrC. Clearly, continued studies of the mechanism of RegA activities on these target promoters are warranted, given the diversity of promoter types that RegA regulates.

Oxygen is not a direct inhibitor of the histidine protein kinase, RegB. The RegB-RegA system was first identified as being responsible for anaerobic activation of photosynthesis gene expression (40, 47). Oxygen was originally thought to directly inhibit RegB kinase activity, but this has never been directly demonstrated. The results of this study indicate that RegB and RegA are capable of repressing *hup* gene expression even under conditions where oxygen is present (chemoheterotrophic conditions), suggesting that RegB may be phosphorylating RegA in the presence of oxygen (Table 3). This conclusion is supported by a previous study by Madigan and Gest (34), which demonstrated that *R. capsulatus* cells exhibited full pigment production under chemoautotrophic conditions where O_2, H_2, and CO_2 are present. The current model is that the RegB-RegA system responds to the overall redox state of the cell rather than to oxygen directly. This model is supported by studies which demonstrate that *R. capsulatus* and *R. sphaeroides* mutants lacking cbb_3-type cytochrome *c* oxidase exhibit elevated photosynthesis gene expression under both aerobic and anaerobic conditions (8, 41). Presumably electron flow through a functional cbb_3-type cytochrome *c* oxidase is required for a normal regulation of photosystem synthesis by transmitting a redox signal to RegB. The redox pathway in *R. sphaeroides* appears to involve the protein CcoQ, one of the cytochrome *c* oxidase components, and the protein RdxB (41, 42). However, it is not yet clear if these proteins are also involved in RegB sensing in *R. capsulatus*. A better understanding of the redox-sensing pathway is needed to elucidate how RegB-RegA is able to control the expression of such different metabolic processes in *R. capsulatus*.

ACKNOWLEDGMENTS

We thank Howard Gest, Fevzi Daldal, and Terry Bird for stimulating discussions and Robert Kranz for the *nifA1* and *nifA2* reporter plasmids.

This work is supported by NIH grant GM53940 (to C.E.B.) and CEA-CNRS-UJF grant (UMR 5092) (to A.C.).

REFERENCES

1. **Bauer, C. E., and T. H. Bird.** 1996. Regulatory circuits controlling photosynthesis gene expression. Cell **85:**5–8.
2. **Bauer, E., T. Kaspar, H. M. Fischer, and H. Hennecke.** 1998. Expression of the *fixR-nifA* operon in *Bradyrhizobium japonicum* depends on a new response regulator, RegR. J. Bacteriol. **180:**3853–3863.
3. **Bird, T. H., S. Du, and C. E. Bauer.** 1999. Autophosphorylation, phosphotransfer and DNA-binding properties of the RegB/RegA two-component regulatory system in *Rhodobacter capsulatus*. J. Biol. Chem. **274:** 16343–16348.
4. **Bird, T. H., J. K. Grimsley, J. A. Hoch, and G. B. Spiegelman.** 1996. The *Bacillus subtilis* response regulator Spo0A stimulates transcription of the *spoIIG* operon through modification of RNA polymerase promoter complexes. J. Mol. Biol. **256:**436–448.
5. **Bowman, W. C., S. Du, C. E. Bauer, and R. G. Kranz.** 1999. *In vitro* activation and repression of photosynthesis gene transcription in *Rhodobacter capsulatus*. Mol. Microbiol. **33:**429–437.
6. **Bowman, W. C., and R. G. Kranz.** 1998. A bacterial ATP-dependent, enhancer binding protein that activates the housekeeping RNA polymerase. Genes Dev. **12:**1884–1893.
7. **Brito, B., M. Martinez, D. Fernandez, L. Rey, E. Cabrera, J. M. Palacios, J. Imperial, and T. Ruiz-Argüezo.** 1997. Hydrogenase genes from *Rhizobium leguminosarum* bv. viciae are controlled by the nitrogen fixation regulatory protein NifA. Proc. Natl. Acad. Sci. USA **94:**6019–6024.
8. **Buggy, J., and C. E. Bauer.** 1995. Cloning and characterization of *senC*, a gene involved in both aerobic respiration and photosynthesis gene expression in *Rhodobacter capsulatus*. J. Bacteriol. **177:**6958–6965.
9. **Colbeau, A., A. Godfroy, and P. M. Vignais.** 1986. Cloning of DNA fragments

carrying hydrogenase genes of *Rhodopseudomonas capsulata*. Biochimie **68:** 147–155.

10. **Colbeau, A., B. C. Kelley, and P. M. Vignais.** 1980. Hydrogenase activity in *Rhodopseudomonas capsulata*: relationship with nitrogenase activity. J. Bacteriol. **144:**141–148.
11. **Colbeau, A., and P. M. Vignais.** 1983. The membrane-bound hydrogenase of *Rhodopseudomonas capsulata* is inducible and contains nickel. Biochim. Biophys. Acta **748:**128–138.
12. **Colbeau, A., and P. M. Vignais.** 1992. Use of *hupS*::*lacZ* gene fusion to study regulation of hydrogenase expression in *Rhodobacter capsulatus*: stimulation by H_2. J. Bacteriol. **174:**4258–4264.
13. **Dischert, W., P. M. Vignais, and A. Colbeau.** 1999. The synthesis of *Rhodobacter capsulatus* HupSL hydrogenase is regulated by the two-component HupT/HupR system. Mol. Microbiol. **34:**995–1006.
14. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system from Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
15. **Du, S., T. H. Bird, and C. E. Bauer.** 1998. DNA binding characteristics of RegA*. A constitutively active anaerobic activator of photosynthesis gene expression in *Rhodobacter capsulatus*. J. Biol. Chem. **273:**18509–18513.
16. **Dubbs, J. M., and F. R. Tabita.** 1998. Two functionally distinct regions upstream of the *cbbI* operon of *Rhodobacter sphaeroides* regulate gene expression. J. Bacteriol. **180:**4903–4911.
17. **Elsen, S., A. Colbeau, J. Chabert, and P. M. Vignais.** 1996. The *hupTUV* operon is involved in negative control of hydrogenase synthesis in *Rhodobacter capsulatus*. J. Bacteriol. **178:**5174–5181.
18. **Elsen, S., A. Colbeau, and P. M. Vignais.** 1997. Purification and in vitro phosphorylation of HupT, a regulatory protein controlling hydrogenase gene expression in *Rhodobacter capsulatus*. J. Bacteriol. **179:**968–971.
19. **Elsen, S., S. N. Ponnampalam, and C. E. Bauer.** 1998. CrtJ bound to distant binding sites interacts cooperatively to aerobically repress photopigment biosynthesis and light harvesting II gene expression in *Rhodobacter capsulatus*. J. Biol. Chem. **273:**30762–30769.
20. **Elsen, S., P. Richaud, A. Colbeau, and P. M. Vignais.** 1993. Sequence analysis and interposon mutagenesis of the *hupT* gene, which encodes a sensor protein involved in repression of hydrogenase synthesis in *Rhodobacter capsulatus*. J. Bacteriol. **175:**7404–7412.
21. **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.
22. **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.
23. **Fisher, H. M.** 1994. Genetic regulation of nitrogen fixation in rhizobia. Microbiol. Rev. **58:**352–386.
24. **Fostner-Hartnett, D., P. J. Cullen, E. M. Monika, and R. G. Kranz.** 1994. A new type of NtrC transcriptional activator. J. Bacteriol. **176:**6175–6187.
25. **Fostner-Hartnett, D., and R. G. Kranz.** 1992. Analysis of the promoters and upstream sequences of *nifA1* and *nifA2* in *Rhodobacter capsulatus*: activation requires *ntrC* but not *rpoN*. Mol. Microbiol. **6:**1049–1060.
26. **Gest, H.** 1972. Energy conversion and generation of reducing power in bacterial photosynthesis. Adv. Microb. Physiol. **7:**243–282.
27. **Gest, H.** 1999. Bioenergetic and metabolic process patterns in anoxyphototrophs, p. 11–19. *In* G. A. Pescheck, W. Löffelhardt, and G. Schmetterer (ed.), The phototrophic prokaryotes. Kluwer Academic/Plenum Publishers, New York, N.Y.
28. **Gutiérrez, D., Y. Hernando, J. M. Palacios, J. Imperial, and T. Ruiz-Argüeso.** 1997. FnrN controls symbiotic nitrogen fixation and hydrogenase activities in *Rhizobium leguminosarum* biovar viciae UPM791. J. Bacteriol. **179:**5264–5270.
29. **Hübner, P., J. C. Willison, P. M. Vignais, and T. A. Bickle.** 1991. Expression of regulatory *nif* genes in *Rhodobacter capsulatus*. J. Bacteriol. **173:**2993–2999.
30. **Imhoff, J. F., and H. G. Truper.** 1992. The genus *Rhodospirillum* and related genera, vol. 3, p. 2141–2155. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes: a handbook on the biology of bacteria, 2nd edition. Springer-Verlag, New York, N.Y.
31. **Inoue, K., J. L. K. 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.
32. **Joshi, H. M., and F. R. Tabita.** 1996. A global two-component signal transduction system that integrates the control of photosynthesis, carbon dioxide assimilation, and nitrogen fixation. Proc. Natl. Acad. Sci. USA **93:**14515–14520.
33. **Lynch, A. S., and E. C. C. Linn.** 1996. Responses to molecular oxygen, p. 1526–1538. *In* F. C. Neidhardt et *al*. (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
34. **Madigan, M. T., and H. Gest.** 1979. Growth of the photosynthetic bacterium *Rhodopseudomonas capsulata* chemoautotrophically in darkness with H_2 as energy source. J. Bacteriol. **137:**524–530.
35. **Masepohl, B., and W. Klipp.** 1996. Organization and regulation of genes encoding the molybdenum nitrogenase and the alternative nitrogenase in *Rhodobacter capsulatus*. Arch. Microbiol. **165:**80–90.
36. **Masepohl, B., W. Klipp, and A. Pühler.** 1988. Genetic characterization and sequence analysis of the duplicated *nifA*/*nifB* gene region of *Rhodobacter capsulatus*. Mol. Gen. Genet. **212:**27–37.
37. **Masuda, S., Y. Matsumoto, K. V. P. Nagashima, K. Shimada, K. Inoue, C. E. Bauer, and K. Matsuura.** 1999. Structural and functional analyses of photosynthetic regulatory genes *regA* and *regB* from *Rhodovulum sulfidophilum*, *Roseobacter denitrificans*, and *Rhodobacter capsulatus*. J. Bacteriol. **181:**4205–4215.
38. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
39. **Meyer, J., B. C. Kelley, and P. M. Vignais.** 1978. Effect of light on nitrogenase function and synthesis in *Rhodopseudomonas capsulata*. J. Bacteriol. **136:**201–208.
40. **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.
41. **O'Gara, J. P., J. M. Eraso, and S. Kaplan.** 1998. A redox-responsive pathway for aerobic regulation of photosynthesis gene expression in *Rhodobacter sphaeroides* 2.4.1. J. Bacteriol. **180:**4044–4050.
42. **Oh, J. I., and S. Kaplan.** 1999. The cbb_3 terminal oxidase of *Rhodobacter sphaeroides* 2.4.1: structural and functional implications for the regulation of spectral complex formation. Biochemistry **38:**2688–2696.
43. **Pollock, D., C. E. Bauer, and P. A. Scolnik.** 1988. Transcription of the *Rhodobacter capsulatus nifHDK* operon is modulated by the nitrogen source. Construction of plasmid expression vectors based on the *nifHDK* promoter. Gene **65:**269–275.
44. **Ponnampalam, S. N., J. J. Buggy, and C. E. Bauer.** 1995. Characterization of an aerobic repressor that coordinately regulates bacteriochlorophyll, carotenoid, and light-harvesting II expression in *Rhodobacter capsulatus*. J. Bacteriol. **177:**2990–2997.
45. **Qian, Y., and F. R. Tabita.** 1996. A global signal transduction system regulates aerobic and anaerobic CO_2 fixation in *Rhodobacter sphaeroides*. J. Bacteriol. **178:**12–18.
46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
47. **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.
48. **Taylor, D. P., S. N. Cohen, W. G. Clark, and B. L. Marrs.** 1983. Alignment of genetic and restriction maps of the photosynthesis region of the *Rhodopseudomonas capsulata* chromosome by a conjugation-mediated marker rescue technique. J. Bacteriol. **154:**580–590.
49. **Tiwari, R. P., W. G. Reeve, M. J. Dilworth, and A. R. Glenn.** 1996. Acid tolerance in *Rhizobium meliloti* strain WSM419 involves a two-component sensor-regulator system. Microbiology **142:**1693–1704.
50. **Toussaint, B., I. Delic-Attree, R. de Sury d'Aspremont, L. David, M. Vincon, and P. M. Vignais.** 1993. Purification of the integration host factor homolog of *Rhodobacter capsulatus*: cloning and sequencing of the *hipA* gene, which encodes the β-subunit. J. Bacteriol. **175:**6499–6504.
51. **Toussaint, B., R. de Sury d'Aspremont, I. Delic-Attree, V. Berchet, S. Elsen, A. Colbeau, W. Dischert, Y. Lazzaroni, and P. M. Vignais.** 1997. The *Rhodobacter capsulatus hupSLC* promoter: identification of *cis*-acting regulatory elements and of *trans*-activating factors involved in H_2 activation of *hupSLC* transcription. Mol. Microbiol. **26:**927–937.
52. **Vignais, P. M., B. Dimon, N. A. Zorin, A. Colbeau, and S. Elsen.** 1997. HupUV proteins of *Rhodobacter capsulatus* can bind H_2: evidence from the H-D exchange reaction. J. Bacteriol. **179:**290–292.
53. **Vignais, P. M., B. Toussaint, and A. Colbeau.** 1995. Regulation of hydrogenase gene expression, p. 1175–1190. *In* R. E. Blankenship, M. T. Madigan, and C. E. Bauer (ed.), Anoxygenic photosynthetic bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
54. **Weaver, P. F., J. D. Wall, and H. Gest.** 1975. Characterization of *Rhodopseudomonas capsulata*. Arch. Microbiol. **105:**207–216.
55. **Yen, H. C., and B. L. Marrs.** 1976. Map of genes for carotenoid and bacteriochlorophyll biosynthesis in *Rhodopseudomonas capsulata*. J. Bacteriol. **126:**619–629.
56. **Young, D. A., C. E. Bauer, J. C. Williams, and B. L. Marrs.** 1989. Genetic evidence for superoperonal organization of genes for photosynthetic pigments and pigment-binding proteins in *Rhodobacter capsulatus*. Mol. Gen. Genet. **218:**1–12.