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Structural and Functional Analyses of Photosynthetic Regulatory Genes *regA* and *regB* from *Rhodovulum sulfidophilum*, *Roseobacter denitrificans*, and *Rhodobacter capsulatus*

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Genes coding for putative RegA, RegB, and SenC homologues were identified and characterized in the purple nonsulfur photosynthetic bacteria *Rhodovulum sulfidophilum* and *Roseobacter denitrificans*, species that demonstrate weak or no oxygen repression of photosystem synthesis. This additional sequence information was then used to perform a comparative analysis with previously sequenced RegA, RegB, and SenC homologues obtained from *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*. These are photosynthetic bacteria that exhibit a high level of oxygen repression of photosystem synthesis controlled by the RegA-RegB two-component regulatory system. The response regulator, RegA, exhibits a remarkable 78.7 to 84.2% overall sequence identity, with total conservation within a putative helix-turn-helix DNA-binding motif. The RegB sensor kinase homologues also exhibit a high level of sequence conservation (55.9 to 61.5%) although these additional species give significantly different responses to oxygen. A *Rhodovulum sulfidophilum* mutant lacking *regA* or *regB* was constructed. These mutants produced smaller amounts of photopigments under aerobic and anaerobic conditions, indicating that the RegA-RegB regulon controls photosynthetic gene expression in this bacterium as it does as in *Rhodobacter* species. *Rhodobacter capsulatus regA*- or *regB*-deficient mutants recovered the synthesis of a photosynthetic apparatus that still retained regulation by oxygen tension when complemented with *reg* genes from *Rhodovulum sulfidophilum* and *Roseobacter denitrificans*. These results suggest that differential expression of photosynthetic genes in response to aerobic and anaerobic growth conditions is not the result of altered redox sensing by the sensor kinase protein, RegB.

Many species of purple nonsulfur photosynthetic bacteria regulate the synthesis of their photosynthetic apparatus in response to alterations in oxygen tension and light intensity. Perhaps the best-characterized species with regard to oxygen control are *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, which are known to repress the synthesis of their photosystem almost completely in response to the presence of high levels of oxygen (13). The *Rhodobacter* response to oxygen contrasts with that of *Rhodovulum sulfidophilum* and *Rhodospirillum centenum*, which are known to repress photosystem synthesis only slightly when grown aerobically (17, 69). A group of obligate aerobic bacteria, represented in this study by *Roseobacter denitrificans*, have been found to synthesize a photosynthetic apparatus aerobically. Interestingly, these "aerobic photosynthetic bacteria" cannot utilize light as the sole energy source for growth. Instead, these organisms rely on respiration (56, 58, 59). *Roseobacter denitrificans* grows slowly as a result of anaerobic respiration in the presence of alternative electron acceptors such as trimethylamine *N*-oxide or nitrate (60). However, this bacterium synthesizes much less of the photosynthetic apparatus under these growth conditions than under aerobic conditions (data not shown) (58, 60). The two *Rhodobacter* species, *Rhodovulum sulfidophilum*, and *Roseobacter denitrificans*, belong to the α-3 subgroup of purple bacteria (proteobacteria) (30). Thus, there is a wide range of responses of closely related members of the subgroup to oxygen.

Although the molecular basis for different responses of photosynthetic bacteria to oxygen is unknown, previous studies with *Rhodobacter capsulatus* and *R. sphaeroides* have indicated that the molecular mechanism of the oxygen inhibition of photosystem synthesis is controlled, in large part, by regulating the transcription of photosynthesis genes. The regulation of photosynthetic gene expression by oxygen is controlled by many transcriptional factors (1–3, 6, 48, 51). One such factor, RegA, was identified in *Rhodobacter capsulatus* as a response regulator of a bacterial two-component system that anaerobically activates the light-harvesting and reaction center structural genes located in the *puf*, *puh*, and *puc* operons (42, 55). RegB is a histidine kinase protein that under anaerobic conditions phosphorylates the cognate response regulator RegA, resulting in the activation of *puf*, *puh*, and *puc* operon expression (31). Mutants lacking *reg* genes are unable to grow under low-intensity light conditions but can grow under high-intensity light conditions (55). In *Rhodobacter sphaeroides*, genes homologous to *regA* and *regB* were found (50) and named *prrA* and *prrB*, respectively (19, 20). In contrast to *regA*-deficient mutant of *Rhodobacter capsulatus*, the *prrA*-deficient mutant of *R. sphaeroides* was unable to grow phototrophically at any light intensity (19). The PrrA-PrrB regulon was shown to regulate *puf*, *puh*, and *puc* operon expression as well as the genes involved in CO_2 reduction and synthesis of cytochrome *c* (19, 52).

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TABLE 1. Bacteria and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source or reference
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* λ^-	Bethesda Research Laboratories
JM109	*endA1 recA1 gyrA96 hsdR17 supE44* λ^- Δ(*lac-proAB*)/F′ [*traD*36 *proAB*$^+$ *lacZ*ΔM15]	68
JM109 λ *pir*	JM109 lysogenized with λ *pir* bacteriophage	47
S17-1	Tp^r Sm^r *hsdR pro recA* RP4-2-Tc::Mu-Km::Tn*7* in chromosome	61
S17-1 λ *pir*	S17-1 lysogenized with λ *pir* bacteriophage	15
XL1-Blue MR.	*recA1 endA1 gyr-96 thi-1 hsdR17 supE44 relA1 lac*/F′ [*proAB*$^+$ *lacZ*ΔM15::Tn*10* (Tc^r)]	11
C600/pDPT51	Mobilizing vector	63
R. capsulatus		
ATCC 11166	Wild type	49
TB2	SB1003 *regA*Δ*Eco*RI-*Pfl*MI::Km^r	7
SD01	SB1003 *regB*Δ640bp N-terminus::Km^r	18
R. sulfidophilum		
W4	Wild type	27
RESA1	*regA*Δ*Pin*AI-*Pin*AI::Km^r	This study
RESB20	*regB*::Km^r	This study
R. denitrificans		
OCh114	Wild type	57
Plasmids		
SuperCos 1	Ap^r; cosmid vector	Stratagene
pUC118	Ap^r; cloning vector	68
pJP5603	Km^r; R6K-based suicide vector	47
pSM3065	Tc^r; pJP5603 derivative	This study
pUC7Tc	Ap^r Tc^r; source of the Tc^r gene	33
pUCKM1	Ap^r Km^r; source of the Km^r gene	53
pCB532Ω	Ap^r Sm^r Sp^r; translational fusion of *pufQ* to *lacZ*	4
pJRD215	Km^r Sm^r; broad-host-range cosmid vector	14
pSLA	Ap^r; 5.0-kb *Eco*RI fragment (*R. sulfidophilum regB-senC-regA*) in pUC118	This study
pROA	Ap^r; 5.5-kb *Eco*RI fragment (*R. denitrificans regB-senC-regA*) in pUC118	This study
pSLKm4	Ap^r Km^r; 6.3-kb fragment (*R. sulfidophilum regA*Δ*Pin*AI-*Pin*AI::Km^r) in pUC118	This study
pSA10	Ap^r Km^r Tc^r; 9.4-kb fragment (pSLKm4 digested with *Kpn*I) in pSM3065	This study
pSB07	Km^r; 0.4-kb *Sac*I-*Sal*I fragment (part of *R. sulfidophilum regB*) in pJP5603	This study
pMWS3.1	Km^r Sm^r; 12-kb fragment (*R. capsulatus senC-regA*) in pJRD215	55
pCSM9e	Km^r Sm^r; 5.1-kb fragment (*R. capsulatus regB-senC*) in pJRD215	42
pMCS003	Km^r Sm^r; 2.8-kb *Eco*RI-*Nru*I fragment (*R. sulfidophilum senC-regA*) in pJRD215	This study
pMCS010	Km^r Sm^r; 2.6-kb *Eco*RI-*Nde*I fragment (*R. sulfidophilum regB*) in pJRD215	This study
pMCR001	Km^r Sm^r; 3.2-kb *Eco*RI-*Sac*I fragment (*R. denitrificans senC-regA*) in pJRD215	This study
pMCR010	Km^r Sm^r; 2.5-kb *Hinc*II fragment (*R. denitrificans regB-senC*) in pJRD215	This study

It is still not known how RegB senses alterations in oxygen concentration. It was reported that *Rhodobacter capsulatus* and *R. sphaeroides* mutants lacking cytochrome oxidase cbb_3 exhibit elevated photosynthesis gene expression under both anaerobic and aerobic conditions (9, 71). Cytochrome cbb_3 is a dominant terminal cytochrome *c* oxidase under semiaerobic growth conditions in *R. sphaeroides* and a sole terminal cytochrome *c* oxidase in *R. capsulatus* (22, 64). Although no direct evidence was demonstrated, the electron transfer pathway involving cytochrome *c* oxidase was suggested to be an important signal for controlling the RegA-RegB phosphorelay cascade (9, 28, 44, 71).

The regulation of photosynthesis gene expression in species other than *Rhodobacter* has been examined only recently (26, 43). Transcription of the *puf* operon of *Roseobacter denitrificans* was observed in atmospheric oxygen tension (43), and that of the *puc* operon of *Rhodovulum sulfidophilum* was shown to be weakly repressed by oxygen (26), whereas these transcriptions were markedly suppressed by high-intensity light (26, 43). This is distinctly different from the results obtained with the *Rhodobacter* species, which show a high degree of repression by oxygen (>30-fold) and weak repression by light (~2-fold). Thus, the expression of photosynthesis genes in species that can synthesize a photosystem under aerobic growth conditions is somewhat different from that in the *Rhodobacter* species (17, 26, 29, 43).

In this study, we have cloned and sequenced genes corresponding to *regA* and *regB* from the aerobic photosynthetic bacteria *Rhodovulum sulfidophilum* and *Roseobacter denitrificans*. Deletion analysis of these genes in *Rhodovulum sulfidophilum* and complementation analysis of *Rhodobacter capsulatus reg* mutations were carried out by using *reg* genes from *Rhodovulum sulfidophilum* and *Roseobacter denitrificans*. These results suggest that RegB is not the oxygen-sensing component controlling photosynthesis gene expression in these species.

MATERIALS AND METHODS

Bacteria and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. *Rhodobacter capsulatus* was anaerobically grown at 30°C in 30-ml screw-cap bottles filled with RCV medium (67). *Rhodovulum*

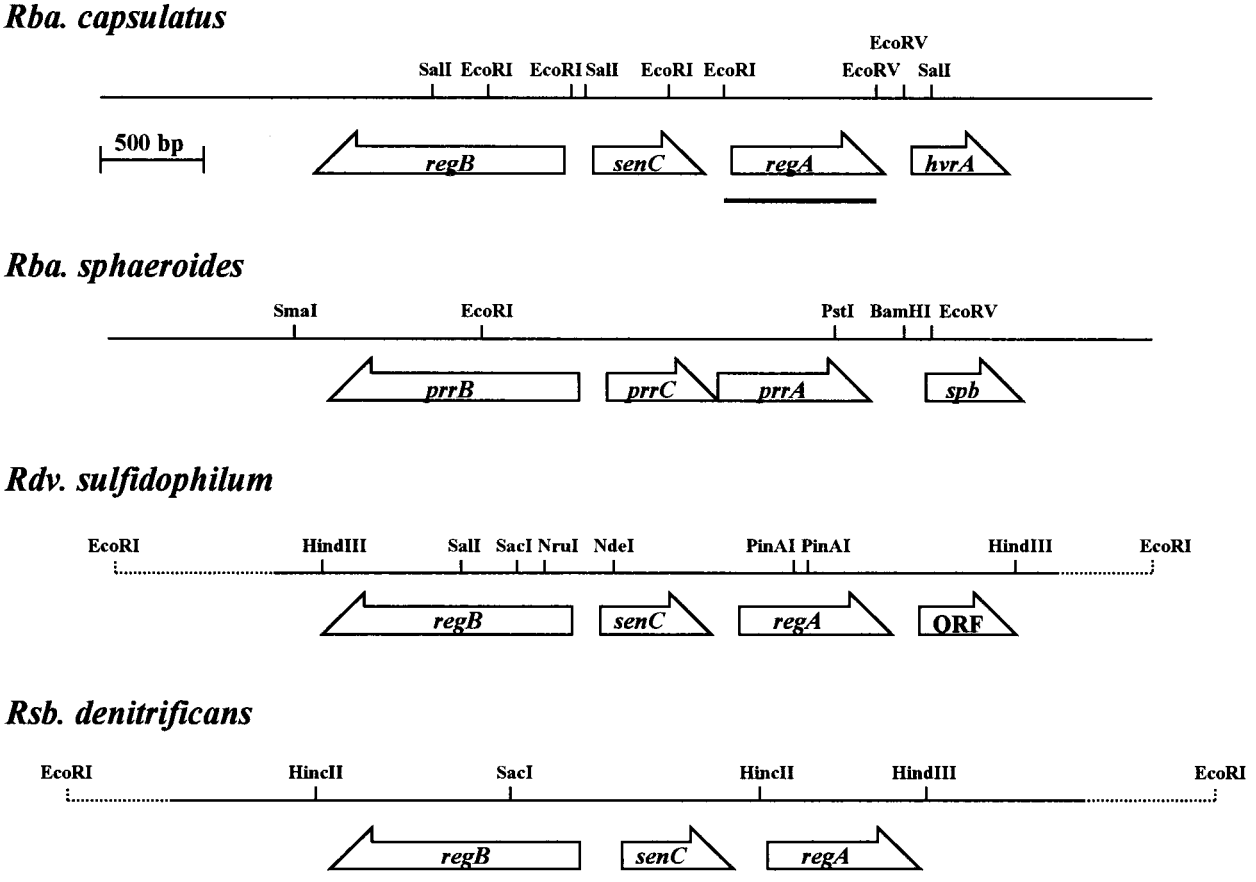

FIG. 1. Physical and genetic maps of the photosynthetic regulatory gene cluster. ORFs and their directions of transcription are represented by open arrows. The broken lines shown in *Rhodovulum* (*Rdv*.) *sulfidophilum* and *Roseobacter* (*Rsb*.) *denitrificans* indicate the unsequenced regions. The DNA template used in the Southern hybridization is indicated by a thick line shown in *Rhodobacter* (*Rba*.) *capsulatus. Rhodobacter* (*Rba*.) *sphaeroides prrB*, *prrC*, *prrA*, and *spb* genes are thought to be equivalent to the homologous *regB*, *senC*, *regA*, and *hvrA* genes, respectively (see the text).

sulfidophilum was grown under the same conditions as *R. capsulatus* in RCV medium supplemented with 2% sodium chloride. *Roseobacter denitrificans* was aerobically grown at 30°C in a medium containing yeast extract, polypeptone, Casamino Acids, and glycerol (59). *Escherichia coli* strains were grown at 37°C in a Luria-Bertani medium. Illumination was provided by 60-W tungsten lamps. Aerobic growth of *R. capsulatus*, *Rhodovulum sulfidophilum*, and *Roseobacter denitrificans* was achieved by shaking a 25-ml culture in a 250-ml conical flask at 200 rpm. Antibiotics, when necessary, were added to the *E. coli* culture to the following final concentrations: ampicillin, 100 μg/ml; kanamycin, 25 μg/ml; tetracycline, 20 μg/ml; spectinomycin, 100 μg/ml; and trimethoprim, 50 μg/ml; they were added to *R. capsulatus* cultures to the following concentrations: kanamycin, 10 μg/ml; streptomycin, 10 μg/ml; and spectinomycin, 10 μg/ml; and they were added to *Rhodovulum sulfidophilum* cultures at the following concentrations: kanamycin, 50 μg/ml; tetracycline, 3 μg/ml.

Preparation of the genomic library. A genomic library of *Rhodovulum sulfidophilum* was constructed with the SuperCos 1 cosmid vector kit (Stratagene). The genomic DNA was partially digested by *Sau*3AI and ligated into the SuperCos 1 cosmid vector at the unique *Bam*HI restriction site. After ligation, the cosmid was packaged into phage particles (Amersham) and then injected into *E. coli* XL1-Blue MR. Approximately 15,000 clones were pooled and amplified. Cells containing the clones were stored in 15% glycerol solution at −80°C. The genomic library of *Roseobacter denitrificans* was prepared by the same methods.

Screening and cloning of the *regA* and *regB* genes. Genomic libraries of *Rhodovulum sulfidophilum* and *Roseobacter denitrificans* DNA were screened by colony hybridization. A 580-bp *Eco*RI-*Eco*RV fragment including the *regA* gene of *Rhodobacter capsulatus* (Fig. 1) was used as a probe after labeling with [α-^{32}P]dATP, using a DNA Megalabel labeling kit (TaKaRa). The labeled probe was hybridized to the DNA on the membrane at 60°C. Colonies showing positive signals were isolated. Inserted DNA fragments (more than 40 kb) in cosmid vectors were digested with *Eco*RI and subcloned into a plasmid pUC118. Clones containing the *regA* gene were then screened by hybridization with the same probe as described in the cosmid screening and named pSLA (derived from *Rhodovulum sulfidophilum* DNA) and pROA (derived from *Roseobacter denitrificans* DNA). Plasmids pSLA and pROA contained 5.0 and 5.5 kb of inserted DNA, respectively (Fig. 1). DNA manipulation and hybridization were carried out by standard methods (37) or as instructed by the enzyme manufacturers.

DNA sequencing. A deletion kit (TaKaRa) was used to obtain the sequential series of overlapping DNA fragments. Nucleotide sequencing was performed with a 373A DNA sequencer with a *Taq* dye primer cycle-sequencing kit (Applied Biosystems). Some of the sequencing data were derived from synthetic oligonucleotide primers and a *Taq* dye terminator cycle-sequencing kit (Applied Biosystems). The data obtained were processed by using a DNASIS (Hitachi) sequence analysis program.

Construction of the *Rhodovulum sulfidophilum regA*-disrupted strain, RESA1. For construction of strain RESA1, a suicide vector, pSM3065, was prepared as follows. Suicide vector pJP5603 (47) was digested with *Bgl*II and *Nco*I to isolate a fragment including *RP4mob* and *R6Kori*. The *Bgl*II-*Nco*I fragment was blunt ended with T4 DNA polymerase and then ligated with an *Hin*cII-*Hin*cII fragment containing a tetracycline resistance gene derived from plasmid pUC7Tc (33) to construct suicide vector pSM3065. The kanamycin cassette in the plasmid pUCKM1 (53) was inserted into the *Pin*AI sites of the *regA* gene of *Rhodovulum sulfidophilum* in plasmid pSLA to create plasmid pSLKm4. The direction of transcription of the kanamycin resistance gene was the same as that of the *regA* gene in this construction. Both pSM3065 and pSLKm4 contain unique *Kpn*I sites in the multiple-cloning sites. These two plasmids were digested with *Kpn*I and ligated to construct a plasmid, pSA10. The plasmid was then transferred into *Rhodovulum sulfidophilum* cells by conjugation with the mobilizing strain S17-1 lysogenized with λ*pir* (47, 61). Km^r Tc^s cells were selected as double-crossover candidates, and the chromosomal insertion was confirmed by Southern hybridization. The mutant strain was designated RESA1.

Construction of the *Rhodovulum sulfidophilum regB*-disrupted strain, RESB20. The *Sac*I-*Sal*I DNA fragment containing the internal region of *regB* of *Rhodovulum sulfidophilum* was cut out from plasmid pSLA and inserted into suicide vector pJP5603 (47) to construct a plasmid, pSB07. The plasmid was then transferred into *R. sulfidophilum* cells by conjugation with the mobilizing strain S17-1 lysogenized with λ*pir* (47, 61). Cells resistant to kanamycin were selected as

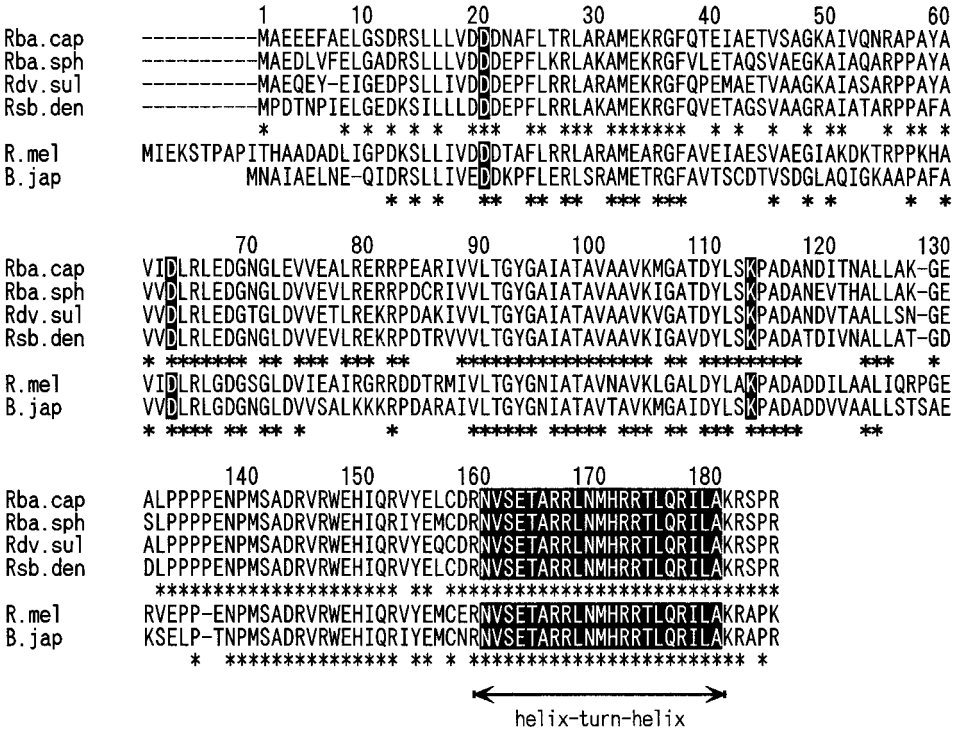

FIG. 2. Alignment of amino acid sequences of RegA of *Rhodobacter capsulatus* (Rba. cap) (55), *Rhodovulum sulfidophilum* (Rdv. sul), and *Roseobacter denitrificans* (Rsb. den), PrrA of *Rhodobacter sphaeroides* (Rba. sph) (19), ActR of *Rhizobium meliloti* (R. mel) (65), and RegR of *Bradyrhizobium japonicum* (B. jap) (5). Asterisks indicate identical amino acids. Residues considered functional and a predicted helix-turn-helix DNA-binding motif are boxed.

single-crossover candidates. The insertion of the plasmid into the chromosome was confirmed by Southern hybridization. The mutant strain was designated RESB20. The single-crossover event in RESB20 took place in the 5′ region of the *Sac*I-*Sal*I *regB* gene segment (Fig. 1). The direction of transcription of the kanamycin resistance gene was the same as that of the *regB* gene in the RESB20 chromosome.

Genetic manipulations. For mobilizing the reporter plasmid pCB532Ω (4) containing a ColE1 origin of replication into *Rhodobacter capsulatus*, *E. coli* C600/pDPT51 (63) was used as a mobilizing strain. Plasmid derivatives of pJRD215 (14) were mobilized into *R. capsulatus* by conjugation with the mobilizing strain S17-1 (61).

Spectral and protein analysis. Membranes for absorption spectrum measurements were obtained by sonicating cells grown to the mid-logarithmic phase and measuring them with a Shimazu UV 160 spectrophotometer. The bacteriochlorophyll content in the cell suspension was determined with acetone-methanol (7:2) extract as described previously (12). Protein content determination was performed with two assay kits from Bio-Rad (kits 500-0001 and 500-0111) as specified by the manufacturer. The β-galactosidase activity of *Rhodobacter capsulatus* cells containing a reporter plasmid for gene expression was determined as described by Young et al. (70).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper are available in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AB010722 (*Rhodovulum sulfidophilum*) and AB010723 (*Roseobacter denitrificans*).

RESULTS

Cloning and sequence analysis of the photosynthesis regulatory gene cluster of *Rhodovulum sulfidophilum* and *Roseobacter denitrificans*. A cosmid DNA library derived from *Rhodovulum sulfidophilum* and *Roseobacter denitrificans* chromosomal DNA was probed with a *Rhodobacter capsulatus* DNA fragment containing *regA*. DNA fragments in several cosmid clones that gave positive hybridization signals were then subcloned into pUC118. Cloned DNA fragments derived from *Rhodovulum sulfidophilum* (pSLA) and *Roseobacter denitrificans* (pROA) were subsequently sequenced (DDBJ, EMBL, and GenBank accession no. AB010722 and AB010723, respectively). Analysis of the primary structures of these nucleotide sequences indicated that these DNA fragments contained at least three open reading frames (ORFs). One ORF showed high sequence identity to the *regA* gene of *Rhodobacter capsulatus* (84.2% in *Rhodovulum sulfidophilum* and 78.7% in *Roseobacter denitrificans*), while the other two ORFs showed significant sequence identity to *regB* (56.6% in *Rhodovulum sulfidophilum* and 55.9% in *Roseobacter denitrificans*) and to the *senC* genes of *R. capsulatus* (51.0% in *Rhodovulum sulfidophilum* and 47.8% in *Roseobacter denitrificans*), respectively. The initiation codon for *regB* of *Roseobacter denitrificans* is assumed to be GTG based on a comparison of the amino-terminal portion of the sequence with *prrB* of *Rhodobacter sphaeroides* and on codon usage after the GTG similar to that observed with the reaction center-core protein genes of *Roseobacter denitrificans* (36). Although the GTG is an irregular initiation codon, translation initiating from GTG has been found for other proteins (35).

The relative order and directions of the putative *regA*, *regB*, and *senC* were the same in *Rhodovulum sulfidophilum* and *Roseobacter denitrificans* as in *Rhodobacter capsulatus* and *R. sphaeroides* (Fig. 1) (10, 20, 42). The ORF found downstream of *regA* in *Rhodovulum sulfidophilum* has no significant homology to any proteins that have been reported, although *Rhodobacter capsulatus* and *R. sphaeroides* have *hvrA* and *spb* at that position, respectively, which function as light-responding *trans*-acting factors for photosynthetic gene expression (10, 40). This result suggests that the counterpart of *hvrA* or *spb* has been lost or is located at a distance from the *regA-regB* gene cluster in *Rhodovulum sulfidophilum*.

Analysis of RegA sequences. Figure 2 shows the similarity of RegA homologues to those of ActR of *Rhizobium meliloti* (65) and RegR of *Bradyrhizobium japonicum* (5). These are re-

sponse regulator proteins thought to be involved in sensing low pH and controlling nitrogen fixation-associated genes, respectively, to which RegA has a high homology score (5, 65). As demonstrated by the alignment, RegA homologues from *Rhodobacter capsulatus*, *Roseobacter denitrificans*, and *Rhodobacter sphaeroides* exhibit an identical sequence length of 184 amino acid residues whereas RegA from *Rhodovulum sulfidophilum* lacks only one residue in the amino-terminal region. All of the RegA homologues from photosynthetic bacteria exhibit a high degree of identity throughout their length (78.7 to 84.2%), including the characteristic two Asp residues (positions 20 and 63) and a Lys residue (position 113). These residues are thought to play central roles in phosphorylation, which affects protein activity (19, 24, 55, 65). The RegA homologues also exhibit a conserved series of four prolines (positions 133 to 136), two of which are also conserved in ActR from *R. meliloti*. This region is immediately followed by a carboxyl-terminal region (positions 137 to 185) exhibiting 93% identity among the RegA homologues. In this region, amino acids 160 to 180 have considerable sequence similarity to known helix-turn-helix DNA-binding motifs (Fig. 2) (5, 18, 38). The putative DNA-binding motif contains an unprecedented 100% sequence identity among RegA homologues in each of these species. Conserved amino acids, which have been suggested to be important in the DNA-binding motifs (16), include Glu at position 163, Ala at position 165, Leu at positions 168, Thr at position 174, and Arg at position 177. As calculated by an amino-acid-versus-position scoring matrix for the evaluation of the helix-turn-helix motif (16), the SD score of the RegA homologues is 5.4, which is well within the range observed for known DNA-binding proteins (2.5 to 7.1) (16).

Analysis of the RegB sequence. The RegB homologues from all four photosynthetic bacteria are less highly conserved (55.9 to 61.5%) than are the RegA homologues (78.7 to 84.2%). However, sequence conservation is still quite high relative to that observed among other sensor kinases (covered in more detail in Discussion). Figure 3 shows the amino acid sequence alignment of RegB of *Rhodobacter capsulatus*, *Rhodovulum sulfidophilum*, and *Roseobacter denitrificans* and of PrrB of *Rhodobacter sphaeroides* and ActS and RegS, which are thought to function as sensor kinase proteins responsible for phosphorylating ActR in *R. meliloti* and RegR in *B. japonicum*, respectively (5, 65). The region designated the H block, which contains His-224 and has been suggested to be an autophosphorylation site, is highly conserved throughout the RegB homologues and in ActS and RegS (20, 42, 45, 46). Blocks G1, G2, F, and N, known to be conserved in other sensor kinases (45, 46), are also highly conserved. Blocks G1 and G2 are considered the ATP-binding domain because they resemble glycine-rich portions of nucleotide-binding domains (46). Since mutations in block N, G1, or G2 eliminate the autokinase activity of the osmolarity sensor EnvZ in *E. coli* (32), these blocks are likely to be necessary for the autokinase activity of RegB homologues from the four photosynthetic bacteria as well as of ActS and RegS. Besides the well-characterized G1, G2, F, and N blocks, the cytosolic portion of the protein (C-terminal half) showed extensive sequence similarity among RegB homologues. Some of these homologous portions presumably represent areas involved in docking with RegA, which, as discussed below, is highly conserved.

Analysis of SenC sequences. Additional genes, i.e., *senC* in *Rhodobacter capsulatus* (9) and *prrC* in *R. sphaeroides* (20), are also known to be located between *regB* and *regA* in these species. As indicated in Fig. 1, *senC* is also present in a similar position between *regB* and *regA* in *Rhodovulum sulfidophilum* and in *Roseobacter denitrificans*. The SenC homologues from the four photosynthetic bacteria exhibit 37.4 to 51.0% sequence identity, which is significantly lower than that observed with RegA or RegB homologues. The *senC* gene product was also previously observed to have high sequence identity to a yeast nucleus-encoded protein, SCO1 (9, 20), which is thought to be an element of the assembly of cytochrome *c* oxidase in yeast (8, 54). The amino acid sequence alignment of SenC homologues from the four photosynthetic bacteria to SCO1 is shown in Fig. 4. Notable areas of conservation include a very hydrophobic patch among the 38 amino acid residues at the amino-terminal end, as well as a putative iron-binding domain at positions 83 to 89 (9).

Effects of *regA* and *regB* disruptions in *Rhodovulum sulfidophilum*. *Rhodovulum sulfidophilum regA*- and *regB*-defective mutants were constructed and named RESA1 and RESB20, respectively (see Materials and Methods). The profiles of the photosynthetic growth of these mutants and wild-type *Rhodovulum sulfidophilum* are shown in Fig. 5. Under high-intensity light conditions (100 W/m^2 [Fig. 5A]), RESA1 grew more slowly, with a doubling time of 6.7 h, than wild-type cells, with a doubling time of 4.5 h. The phenotypes were more pronounced when assayed under low-intensity light conditions (3 W/m^2 [Fig. 5B]), when the doubling times of RESB20 and RESA1 were three and four times as long, respectively, as that of wild-type cells. These observations are similar to those for *Rhodobacter capsulatus* mutants in terms of the ability of photosynthetic growth of the *regA* defective mutant and are different from that of the *R. sphaeroides regA* (*prrA*) mutant, which was unable to grow photosynthetically at any light intensity (19, 55).

Figure 6 shows the absorption spectra of membranes of *Rhodovulum sulfidophilum* wild-type, RESA1, and RESB20 cells grown under anaerobic low-intensity light (3 W/m^2) (Fig. 6A), anaerobic high-intensity light (100 W/m^2) (Fig. 6B), aerobic-dark (Fig. 6C), and aerobic high-intensity light (100 W/m^2) (Fig. 6D) conditions. The RESA1 mutant showed reduced synthesis of photopigments compared to that of the wild type under all of these growth conditions. In the RESB20 mutant, the synthesis of photopigments was also reduced to a lesser extent than compared in RESA1. The bacteriochlorophyll (BChl) content per membrane protein of RESA1 and RESB20 cells grown under anaerobic high-intensity light conditions was 40 and 72%, respectively, of that of the wild-type cells. Under aerobic-dark conditions, these mutants synthesized 78 and 91% of the wild-type levels, respectively. These differences between two mutants support the idea that RegA may be phosphorylated via cross talk by a protein kinase other than RegB (23, 42). The effects of the mutations on the expression of light-harvesting complex 2 (LH2), which peaked at 800 and 850 nm, were more pronounced than on the expression of light-harvesting complex 1 (LH1), which appeared as a shoulder at 870 nm (Fig. 6). This observation is similar to that for the *Rhodobacter* species (19, 55).

Complementation analysis of *reg* genes among different species. We carried out a complementation analysis of *Rhodobacter capsulatus reg* mutations by using *reg* genes from *Rhodovulum sulfidophilum* and *Roseobacter denitrificans* to examine whether these genes are responsible for the different aerobic and anaerobic expression patterns of photosynthesis genes between *Rhodobacter capsulatus*, *Rhodovulum sulfidophilum*, and *Roseobacter denitrificans*. DNA fragments containing *regA* or *regB* from *Rhodobacter capsulatus*, *Rhodovulum sulfidophilum*, and *Roseobacter denitrificans* were cloned into wide-host-range vectors (Table 1) and introduced into the *Rhodobacter capsulatus regA* or *regB* disruption mutants TB2 and SD01, respectively. As shown in Table 2, TB2 transconju-

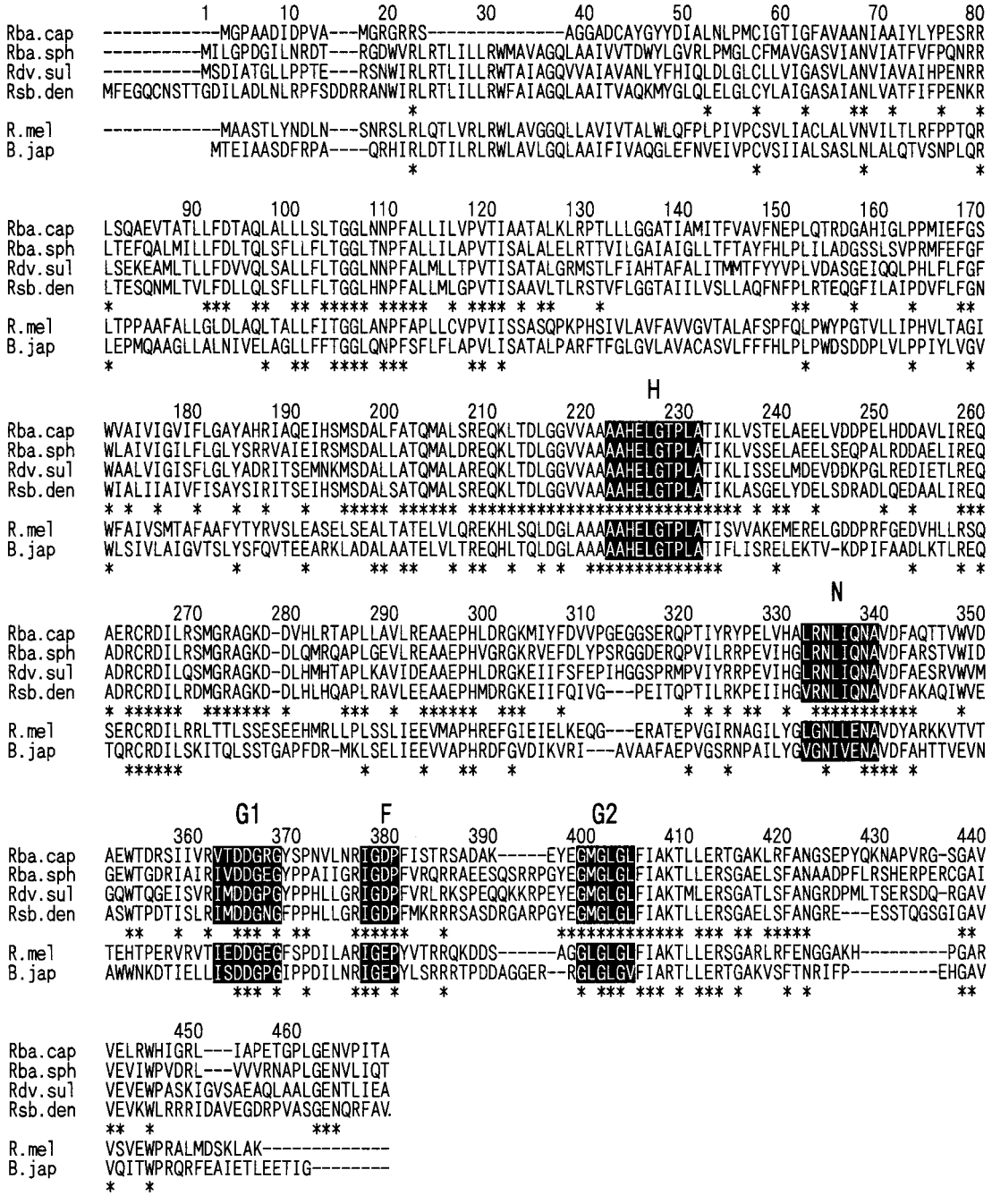

FIG. 3. Alignment of amino acid sequences of RegB of *Rhodobacter capsulatus* (Rba. cap) (42), *Rhodovulum sulfidophilum* (Rdv. sul), and *Roseobacter denitrificans* (Rsb. den), PrrB of *Rhodobacter sphaeroides* (Rba. sph) (20), ActS of *Rhizobium meliloti* (R. mel) (65), and RegS of *Bradyrhizobium japonicum* (B. jap) (5). Asterisks indicate identical amino acids. The histidine block (H), an asparagine-rich block (N), glycine-rich domains (G1 and G2), and a variable-length spacer (F) which are roughly conserved in the histidine kinases (45, 46) are boxed.

gants containing the plasmid carrying *regA* and *senC* from *Rhodobacter capsulatus* (pMWS3.1), *Rhodovulum sulfidophilum* (pMCS003), and *Roseobacter denitrificans* (pMCR001) all complemented the chromosomal disruption of *regA*. Similar results were obtained by complementing the *regB* defect in SD01 with plasmids containing *regB* and *senC* from *Rhodobacter capsulatus* (pCSM9e) and *Roseobacter denitrificans* (pMCR010) and *regB* from *Rhodovulum sulfidophilum* (pMCS010) (Table 2). Furthermore, all of the *Rhodobacter capsulatus* transconjugants retained the aerobic repression of photosynthesis (*puf*) gene expression regardless of the species origin of the complementing *regA* or *regB* gene (Table 2). The notable difference observed with all of the transconjugants was an increased aerobic level of *puf* expression in comparison to wild-type cells. This may be due to an increased copy number of *regA* or *regB* genes in the cells as a result of being plasmid located, as reported previously (21). Another possible explanation of this difference is that the presence of genes other

FIG. 4. Alignment of amino acid sequences of SenC of *Rhodobacter capsulatus* (Rba. cap) (9), *Rhodovulum sulfidophilum* (Rdv. sul), and *Roseobacter denitrificans* (Rsb. den), PrrC of *Rhodobacter sphaeroides* (Rba. sph) (20), and yeast nucleus-encoded protein SCO1 (8, 54). Asterisks indicate identical amino acids. The residues hypothesized to contribute to a predicted iron-binding domain are boxed (9).

than *regA* and *regB*, such as *senC*, affects *puf* operon expression. The increase of *puf* expression in comparison to wild-type cells was especially prominent in TB2 and SD01 transconjugants containing *Rhodovulum sulfidophilum regA-senC* and *Rhodobacter capsulatus regB-senC*, respectively. The *puf* expression in these transconjugants was, however, still highly repressed by oxygen.

DISCUSSION

The results of this study demonstrate that the *regA-senC-regB* regulatory gene cluster is present in photosynthetic bacteria that aerobically synthesize the photosynthetic apparatus. As shown in Fig. 2, RegA homologues are highly conserved, with over 78% identity among the four photosynthetic bacteria and over 60% identity to ActR from *R. meliloti* and RegR from *B. japonicum*. This is in contrast to the observation that the average identity between two response regulators is 23% based on pairwise alignments of 79 response regulators that have different functions (for example, PhoB and OmpR) (66). The identity score improves only slightly in comparisons of response regulators from different species that have the same function. For example, the identity score is 26% for NtrC homologues, 24% for FixJ homologues, and 37% for OmpR homologues. Thus, the RegA homologues exhibit an unprecedented level of conservation, which suggests that there are significant constraints on the ability of RegA to undergo mutational changes in these species. This could be a function of the diverse roles of RegA (PrrA), which is known to be involved in controlling the expression of the photosynthetic apparatus as well as that of the cytochrome c_2 gene and carbon fixation (2, 19, 52).

Comparison of the amino acid sequences of RegA homologues with that of CheY, a well-known response regulator of bacterial chemotaxis (66), shows that at the precise location where CheY ends, the RegA homologues from photosynthetic bacteria contain a stretch of four prolines, presumably providing a flexible region. The stretch of prolines is followed by a highly conserved carboxyl-terminal region (amino acids 137 to 185) exhibiting 93.6% sequence identity among the photosynthetic homologues and 89.8% when including ActR and RegR. An alignment of the conserved carboxyl-terminal region with known DNA-binding sequences (16) reveals a region (NVSETARRLNMHRRTLQRILA) suggestive of a helix-turn-helix DNA-binding motif. This sequence is 100% conserved among the various RegA homologues that have been cloned. Assuming that this region does indeed contain a DNA-binding motif, the remarkable 100% level of sequence identity in this region would indicate that the target DNA sequences are highly conserved in these different species.

Most sensor kinases exhibit significant homology only in the conserved G1, G2, F, N, and H domains located in the cytosolic C-terminal region (Fig. 3). Not surprisingly, the alignment of RegB in Fig. 3 indicates that the six RegB homologues also exhibit extensive homology in these domains. However, further inspection of the alignment indicates that there also exist several additional regions with significant conservation, including extensive sequence conservation (93% identity) in the Q-linker region (positions 196 to 221), which is an area located immediately upstream of the H domain. There is also an additional area of extensive sequence conservation located between the H and N domains (positions 258 to 303). Given that RegA has a

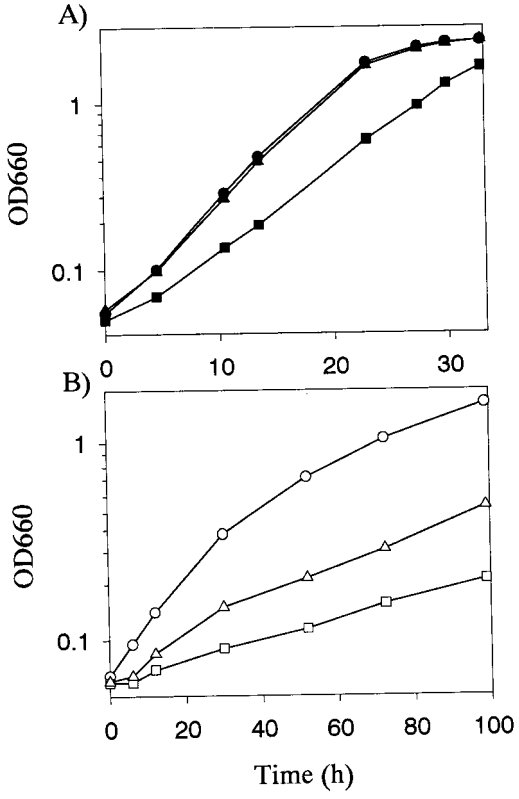

FIG. 5. Growth profiles of *Rhodovulum sulfidophilum* wild-type, *regA*-disrupted (RESA1), and *regB*-disrupted (RESB20) strains. Cells were grown under anaerobic high-intensity light conditions (100 W/m^2) (solid circles, wild-type cells; solid squares, RESA1 cells; solid triangles, RESB20 cells) (A) and anaerobic low-intensity light conditions (3 W/m^2) (open circles, wild-type cells; open squares, RESA1 cells; open triangles, RESB20) (B).

very high degree of homology, these additional areas of RegB conservation could represent RegA-docking domains. Indeed, the area between the H and N domains has previously been implicated in the docking of the response regulator CheY to the sensor kinase CheA (62).

There is also a well-conserved (73% identity) region in the amino-terminal membrane-spanning region of RegB (amino acids 91 to 113). This region could be involved in redox signal transduction or in the formation of stable dimers (45, 46). The amino acid sequences of the membrane-spanning region of RegB homologues showed no significant similarities to those of the oxygen-related sensor kinase FixL or the redox-sensitive sensor kinase ArcB (25, 41). The S-boxes that are present in a large family of proteins thought to be involved in sensing the oxygen-redox potential (72) are not present in RegB, whereas they are reported to be present in FixL and ArcB. The heme-binding site located between the membrane-spanning region and the kinase domain in FixL is not located in any of the RegB homologues either.

The occurrence of *senC* (*prrC*) between *regA* and *regB* in each of the four photosynthetic bacteria suggests an important role for *senC* in the cascade of the regulation mechanisms of photosynthetic gene expression via the RegA-RegB phosphorelay circuit. Horne et al. (28) suggested that the *senC* homologue from *Rhodobacter sphaeroides* senses the redox state of cytochrome *c*, and this would indirectly influence the PrrB (RegB) activity. Buggy and Bauer (9) demonstrated that strains disrupted in SenC had reduced levels of cytochrome *c*

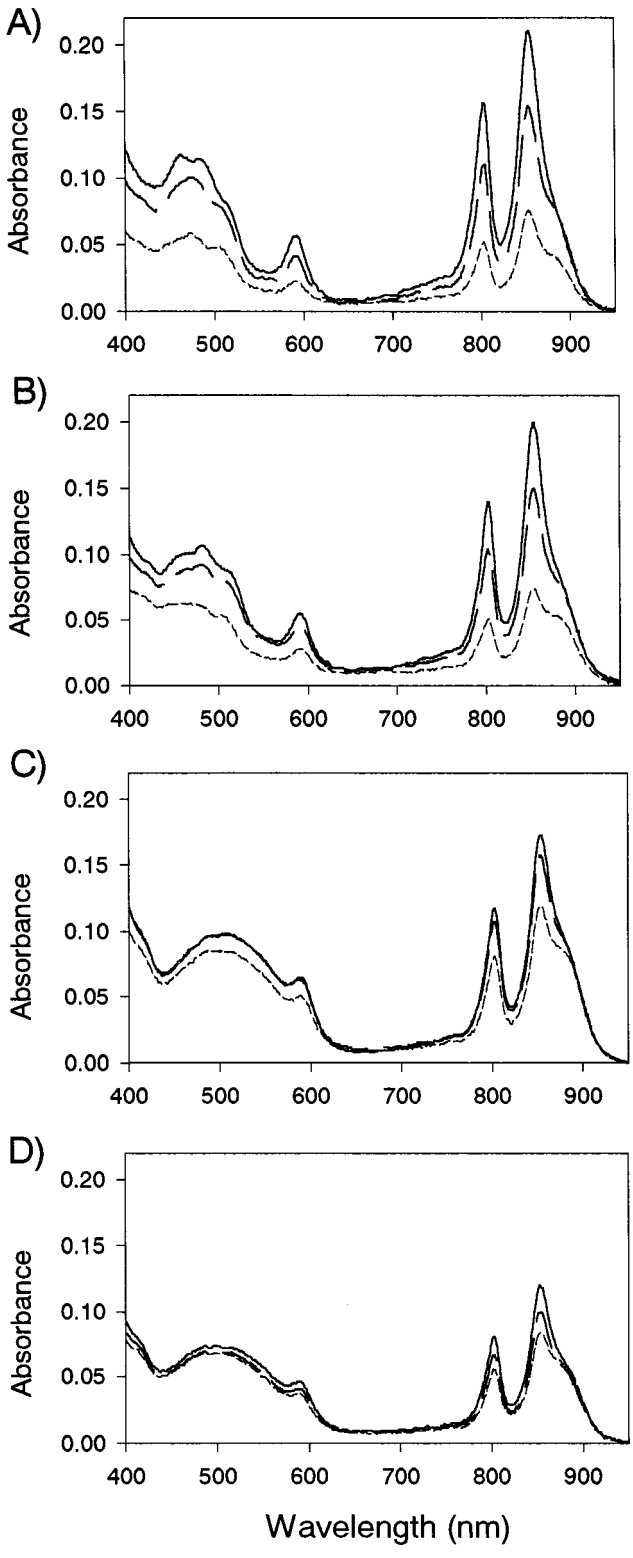

FIG. 6. Absorption spectra of *Rhodovulum sulfidophilum* wild type (solid lines), RESA1 (short dashed lines) and RESB20 (long dashed lines) grown under anaerobic low-intensity light conditions (3 W/m^2) (A), anaerobic high-intensity light conditions (100 W/m^2) (B), aerobic-dark conditions (C), and aerobic high-intensity light conditions (100 W/m^2) (D). Cells in the mid-logarithmic growth phase were harvested and sonicated, and spectra were measured in membrane preparations. All samples contained 75 μg of protein per ml.

TABLE 2. Photopigment synthesis and photosynthetic gene expression in *reg* mutants of *Rhodobacter capsulatus* complemented with *reg* genes from various species

Strain	BChl content (nmol/mg)[c]		Expression of *puf* operon[d]	
	$-O_2$	$+O_2$	$-O_2$	$+O_2$
Wild type (*R. capsulatus*)	34.5	0.5	544 ± 30	15 ± 5
TB2(pJRD215)[a] vector only	13.9	0.3	40 ± 10	9 ± 5
TB2(pMWS3.1) +*Rhodobacter regA-senC*	37.2	0.7	382 ± 50	62 ± 20
TB2(pMCS003) +*Rhodovulum regA-senC*	51.5	0.6	1,971 ± 150	125 ± 25
TB2(pMCR001) +*Roseobacter regA-senC*	42.5	0.6	315 ± 30	60 ± 15
SD01(pJRD215)[b] vector only	21.6	0.5	135 ± 70	18 ± 10
SD01(pCSM9e) +*Rhodobacter regB-senC*	42.8	0.5	1,050 ± 145	108 ± 40
SD01(pMCS010) +*Rhodovulum regB*	45.7	0.5	855 ± 150	60 ± 20
SD01(pMCR010) +*Roseobacter regB-senC*	40.1	0.4	499 ± 125	63 ± 25

[a] TB2; *R. capsulatus* (Δ*regA*).
[b] SD01; *R. capsulatus* (Δ*regB*).
[c] BChl content in membranes (nanomoles per milligram of membrane protein). Data are based on the average of three independent assays. Uncertainty limits in this assay are within 5% in all transconjugants and the wild type. $-O_2$, anaerobic photosynthetic growth; $+O_2$, aerobic-dark growth.
[d] Values are β-galactosidase activity (micromoles of *o*-nitrophenol-β-D-galactoside hydrolyzed per minute per milligram of protein) of strains with the *puf*::*lacZ* translational fusion in pCB532Ω (4).

oxidase as well as of *puf*, *puc*, and *puh* expression. They suggested that SenC may be involved in controlling RegB phosphorylation activity in response to alterations in cytochrome *c* oxidase activity (9). Clearly, the conservation of *senC* in each of these photosynthetic species indicates that it plays an important role deserving of further study.

The disruption of *regA* and *regB* causes the reduction of photopigment accumulation in *Rhodovulum sulfidophilum*, indicating that the RegA-RegB regulon plays important roles in photosystem synthesis in this species, as it does as in *Rhodobacter* species (Fig. 6). Recently, RegA was shown to bind to the promoter regions for the *puf* (−22 to −80) and *puc* (−52 to −80) operons in *Rhodobacter capsulatus* (18). The nucleotide sequence of the *Rhodovulum sulfidophilum puc* operon was determined and shown to have a similar promoter sequence (−77 to −60) to that of *Rhodobacter capsulatus puc* operon (26). We sequenced the whole *puf* operon of *Rhodovulum sulfidophilum* and found that it contains a similar sequence to the RegA-binding site of the *Rhodobacter capsulatus puf* operon (39). These findings suggest that the RegA homologue from *Rhodovulum sulfidophilum* also binds to the *puf* and *puc* operon promoters.

Because no consensus sequence was found around the 5′ ends of *puf* mRNA, the regulation of the transcription of the *puf* operon in *Roseobacter denitrificans* has been suggested to be different from that in the *Rhodobacter* species (43). However, because the transcription initiation sites of the *Rhodobacter capsulatus puf* operon were far upstream from the stable 5′ end of the *puf* mRNA (4), the transcription starting point of *puf* operon in *Roseobacter denitrificans* may also exist far upstream of the 5′ ends of the stable *puf* mRNA transcripts. If so, a similar sequence to the RegA-binding site of *Rhodobacter capsulatus* may be present in its regulatory region. Recently, it was reported that the *Roseobacter denitrificans puf* operon could be expressed in *Rhodobacter capsulatus* under the control of its promoter (34). This supports the idea that the *puf* operon promoter of *Roseobacter denitrificans* is similar to that of *Rhodobacter capsulatus*.

Less pigment-protein complex is formed in the RESA1 mutant grown under anaerobic-light conditions than in that grown under aerobic-dark conditions, while the wild type produces more pigment-protein complex under anaerobic-light conditions than under aerobic-dark conditions (Fig. 6A to C). The mutants exhibit a 1.8-fold-higher BChl content per membrane protein under aerobic-dark conditions than under anaerobic high-intensity light conditions. These findings indicate that activation of photosynthetic gene expression caused by the RegA-RegB regulon is more apparent under anaerobic than aerobic conditions. In addition, both the wild type and the RESA1 mutants produce smaller amounts of photopigments under aerobic-light than aerobic-dark conditions (Fig. 6C and D), indicating that the aerobic regulatory system which controls the photosystem construction responding to light intensity seems to be present in *Rhodovulum sulfidophilum*.

When *Rhodobacter capsulatus regA* or *regB* mutants were complemented with *regA* or *regB* genes from *Rhodovulum sulfidophilum* and *Roseobacter denitrificans*, they produced photosynthetic pigments under anaerobic conditions but not under aerobic conditions, which is the pattern observed with wild-type *Rhodobacter capsulatus* cells (Table 2). In all transconjugants, expression of the *puf* operon under aerobic conditions increased compared to that of the wild type, although the BChl contents in the transconjugants under aerobic conditions were almost minimal and did not reflect the increased aerobic *puf* operon expression. This result indicates that BChl levels continue to be limiting in all transconjugants under aerobic conditions. This may be due to low levels of expression of the genes involved in tetrapyrrole and BChl biosynthesis, which require other regulatory proteins for activation (1). Indeed, all of the transconjugants exhibited a similar 5- to 10-fold-higher level of *puf* operon expression under anaerobic than aerobic growth conditions (Table 2). Not only does this result indicate functional complementation but also it indicates that RegB from bacteria that aerobically synthesize photopigments also responds to oxygen tension when expressed in *Rhodobacter capsulatus*. There are several interpretations for this finding. One possibility is that *Rhodovulum sulfidophilum* and *Roseobacter denitrificans* cells retain similar reduced states under aerobic and anaerobic growth conditions via a metabolic quirk, such as a high level of respiration, which could effectively scrub out oxygen from these cells. Another possibility is that RegB from *Rhodovulum sulfidophilum* and *Roseobacter denitrificans* are incapable of a redox response in their native species but are capable of a redox response in *Rhodobacter capsulatus*. Perhaps the most intriguing possibility is that RegB is not itself a redox-responding sensor kinase. Instead, its activity may be affected by interacting with another redox-responding protein that is present only in *Rhodobacter capsulatus*. Alternatively,

Rhodobacter capsulatus (but not *Rhodovulum sulfidophilum* or *Roseobacter denitrificans*) may have a phosphatase that removes phosphate from RegA in a redox-responsive manner. Clearly, additional in vivo and in vitro studies of RegB activity from these species must be undertaken. Such comparative studies should be useful in clarifying the details of the control mechanisms of anaerobic gene expression in purple bacteria.

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