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Transcriptional Control of Expression of Genes for Photosynthetic Reaction Center and Light-Harvesting Proteins in the Purple Bacterium *Rhodovulum sulfidophilum*

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The purple photosynthetic bacterium *Rhodovulum sulfidophilum* synthesizes photosynthetic apparatus even under highly aerated conditions in the dark. To understand the oxygen-independent expression of photosynthetic genes, the expression of the *puf* operon coding for the light-harvesting 1 and reaction center proteins was analyzed. Northern blot hybridization analysis showed that *puf* mRNA synthesis was not significantly repressed by oxygen in this bacterium. High-resolution 5′ mapping of the *puf* mRNA transcriptional initiation sites and DNA sequence analysis of the *puf* upstream regulatory region indicated that there are three possible promoters for the *puf* operon expression, two of which have a high degree of sequence similarity with those of *Rhodobacter capsulatus*, which shows a high level of oxygen repression of photosystem synthesis. Deletion analysis showed that the third promoter is oxygen independent, but the activity of this promoter was not enough to explain the aerobic level of mRNA. The posttranscriptional *puf* mRNA degradation is not significantly influenced by oxygen in *R. sulfidophilum*. From these results, we conclude that *puf* operon expression in *R. sulfidophilum* is weakly repressed by oxygen, perhaps as a result of the following: (i) there are three promoters for *puf* operon transcription, at least one of which is oxygen independent; (ii) readthrough transcripts which may not be affected by oxygen may be significant in maintaining the *puf* mRNA levels; and (iii) the *puf* mRNA is fairly stable even under aerobic conditions.

The purple photosynthetic bacterium *Rhodovulum sulfidophilum* is a marine bacterium that can grow by either photosynthesis or respiration on a wide range of organic compounds (20). Like those of other purple bacteria, the photosynthetic apparatus of *R. sulfidophilum* is composed of three membrane-spanning pigment protein complexes known as the reaction center (RC) and the light-harvesting 1 and 2 complexes (LH1 and LH2, respectively). The light energy captured by LH1 and LH2 is transferred to the RC, where the primary photochemical reaction takes place. The RC complexes of most purple bacteria are known to consist of at least L, M, and H subunits. The light-harvesting complexes are composed of two membrane-spanning polypeptides, α and β subunits, which bind bacteriochlorophylls (BChls) and carotenoids (13, 15, 48). It is known that these proteins are encoded by three operons: the *puf* operon, which encodes the LH1 α and β polypeptides and the RC-L and -M polypeptides; the *puc* operon, which encodes the LH2 α and β polypeptides; and the *puh* operon, which encodes RC-H polypeptide (9, 26, 44, 46, 47). Some purple bacteria have a *pufC* gene in the *puf* operon that encodes an RC-bound cytochrome subunit with four *c*-type hemes. Recently, we sequenced the whole *puf* operon of *R. sulfidophilum* and showed that this bacterium has a *pufC* gene in the *puf* operon encoding a unique cytochrome subunit that contains only three possible heme-binding sites (30).

In some purple bacteria such as *Rhodobacter* species, the synthesis of the photosynthetic apparatus is regulated by oxygen concentration and light intensity. Many studies using *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* have demonstrated that photosystem synthesis is controlled at a number of different levels in the cells (2–4, 11, 15, 24, 34, 45). Much of the environmental regulation appears to be exerted at the level of photosynthesis gene transcription (2, 3, 34, 45). The importance of the posttranscriptional mRNA degradative processes and posttranslational effects in determining final levels of membrane-bound photosynthetic apparatus has also been reported (15, 24). In contrast to *Rhodobacter* species, *R. sulfidophilum* synthesizes the photosynthetic apparatus even under highly aerated conditions in the dark (14). A study of the *puc* operon of *R. sulfidophilum* showed that *puc* mRNA synthesis is weakly repressed by oxygen but markedly suppressed by high-intensity light (19). This is distinctly different from the results shown for the *Rhodobacter* species, which show a high degree of repression by oxygen and a weak repression by light (2, 3).

The RegA-RegB two-component regulatory system was identified as a transcriptional factor that anaerobically activates the expression of the *puf*, *puc*, and *puh* operons in *R. capsulatus* (31, 37). Genes homologous to *regA* and *regB* were also found in *R. sphaeroides* and named *prrA* and *prrB*, respectively (17, 36). Recently, we have found and characterized the RegA-RegB regulatory system in *R. sulfidophilum* and showed that it controls the photosynthetic gene expression in this bacterium (29), as it does in *R. capsulatus* (37). We also demonstrated that the species-dependent difference in the repression of photosynthetic gene expression under aerobic conditions is not the result of altered redox sensing by the sensor kinase protein, RegB (29). These observations suggested the presence of another redox-responding protein that affects the RegB activity.

To date, high-resolution mRNA mapping for the identification of transcription initiation sites in combination with detailed promoter deletion studies for the *puf* operon has been undertaken only with *Rhodobacter* species, although the location of the 5′ ends of *puf* mRNA has been reported for several purple bacteria (1, 5, 6, 12, 22, 27, 32, 33, 41). Genetic analyses

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TABLE 1. Bacteria and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source or reference
E. coli		
DH5α	F^- *recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44 gyr96 relA1* Δ(*lacZYA-argF*)*U169* ϕ80d*lacZ*ΔM15 *deoR thi*	Bethesda Research Laboratories
S17-1	Tp^r Sm^r *hsdR pro recA* RP4-2-Tc::Mu-Km::Tn*7* in chromosome	38
R. sulfidophilum		
W4	Wild type	20
RESA1	*regA*Δ*Pin*AI-*Pin*AI::Km^r	29
R. capsulatus ATCC 11166	Wild type	35
Plasmids		
pUC119	Ap^r, multiple cloning sites in *lacZ′*	42
pUFS101	pUC118 with 10-kb *Eco*RI insert encoding *R. sulfidophilum puf* operon	30
pUFS001	2,580-bp *Eco*RI-*Bam*HI fragment containing *pufQ*, *pufB*, *pufA*, and part of *pufL* from pUFS101 cloned into *Eco*RI-*Bam*HI-cut pUC119	This study
pCF1010	Transcriptional fusion vector containing unique *Pst*I, *Not*I, *Nsi*I, *Avr*II, *Stu*I, *Bsp*MII, and *Xba*I restriction sites between the 2.0-kb Ω Sm^r/Sp^r and the 5.1-kb *lacZYA′*, IncQ/IncP4	28
pPS001	2.4-kb *Hin*cII-*Xba*I fragment from pUFS001 cloned into *Stu*I-*Xba*I-cut pCF1010	This study
pPS002	2.1-kb *Nru*I-*Xba*I fragment from pUFS001 cloned into *Stu*I-*Xba*I-cut pCF1010	This study
pPS003	1.9-kb *Sma*I-*Xba*I fragment from pUFS001 cloned into *Stu*I-*Xba*I-cut pCF1010	This study
pPS004	1.5-kb *Pst*I-*Xba*I fragment from pUFS001 cloned into *Pst*I-*Xba*I-cut pCF1010	This study
pPS005	1.4-kb *Bal*I-*Xba*I fragment from pUFS001 cloned into *Stu*I-*Xba*I-cut pCF1010	This study
pPS100	1.0-kb *Hin*cII-*Bal*I fragment from pUFS001 cloned into *Stu*I-cut pCF1010	This study
pPS200	656-bp PCR product from pUFS001 template cloned into *Stu*I-*Xba*I-cut pCF1010	This study
pPS300	374-bp PCR product from pUFS001 template cloned into *Stu*I-*Xba*I-cut pCF1010	This study

of the *puf* operon promoter from *R. capsulatus* have indicated that transcription initiation sites and a *cis*-acting regulatory site involved in oxygen and light control of promoter activity are located far upstream from the 5′ end of the stable *puf* mRNA (1, 5). Our previous study showed that *R. sulfidophilum* has a *pufQ* gene as the first gene of the operon, as in *R. capsulatus*, and that the DNA sequences upstream of the operons of *R. sulfidophilum* and *R. capsulatus* showed high similarity (30). Thus, comparative studies of the *R. sulfidophilum puf* operon promoter would be useful for understanding the molecular basis of the regulation of *puf* operon expression with respect to the oxygen-independent synthesis of the photosynthetic apparatus in this bacterium.

In this study, we identified and characterized the *R. sulfidophilum puf* operon promoters. The results suggest that the *R. sulfidophilum puf* operon has three possible transcription initiation sites, one of which is oxygen and *regA* independent. The promoter region was compared with that of the *R. capsulatus puf* promoter.

MATERIALS AND METHODS

Bacterial strains and cultures. The bacterial strains and plasmids used in this study are listed in Table 1. *R. capsulatus* was anaerobically grown at 30°C in 30-ml screw-cap bottles filled with RCV medium (39). Wild-type *R. sulfidophilum* W4 and the *regA*-disrupted strain RESA1 were grown under the same conditions as *R. capsulatus* in RCV medium supplemented with 0.35 M sodium chloride. Aerobic growth of *R. capsulatus* and *R. sulfidophilum* was achieved by shaking a 10-ml culture in a 100-ml conical flask at 200 rpm. Illumination was provided by 60-W tungsten lamps. *Escherichia coli* strains DH5α and S17-1 were grown at 37°C in Luria-Bertani medium. Antibiotics were added at the final concentrations given: to *E. coli* cultures, ampicillin (100 μg/ml) or tetracycline (20 μg/ml), and to *R. sulfidophilum* cultures, kanamycin (50 μg/ml) or streptomycin (20 μg/ml), where necessary.

Conjugation techniques. The pCF1010-derived plasmids were mobilized into *R. sulfidophilum* cells by conjugation with the mobilizing strain *E. coli* S17-1 (38).

Plasmid construction. The plasmid pCF1010 (28) was used to make transcriptional fusions of the *R. sulfidophilum puf* operon promoter region to a promoterless *lacZ* gene including a ribosome-binding site. First, a 2,580-bp *Eco*RI-*Bam*HI fragment containing *pufQ*, *pufB*, *pufA*, and part of *pufL* as well as 1,073 bp upstream of *pufQ* was cut out from the plasmid pUFS101 (30) and cloned into *Eco*RI/*Bam*HI-cut pUC119, resulting in pUFS001. This plasmid has a unique *Xba*I site just downstream of the *Bam*HI site (in the polycloning site). DNA fragments digested by *Hin*cII-*Xba*I, *Nru*I-*Xba*I, *Sma*I-*Xba*I, and *Bal*I-*Xba*I from pUFS001 (see Fig. 3) were cloned into *Stu*I/*Xba*I-cut pCF1010 to construct the *puf-lacZ* fusions named pPS001, pPS002, pPS003, and pPS005, respectively. A *Pst*I-*Xba*I fragment from pUFS001 was cloned into *Pst*I/*Xba*I-cut pCF1010 to construct pPS004. A *Hin*cII-*Bal*I fragment from pUFS001 was cloned into *Stu*I-cut pCF1010 and named pPS100. For the construction of pPS200 and pPS300, two DNA fragments were amplified by PCR using pUFS001 as a template DNA. The fragments were amplified by the combination of forward primer M13-F (5′-CGACGTTGTAAAACGACGGCCAGT-3′) and reverse primer Z1200RXbaI (5′-TTTCTAGACCGAGCGGCAGGATATGAG-3′) and forward primer M13-F and reverse primer Z940RXbaI (5′-TTTCTAGAGCAAA GGCGCAGGGCAGCC-3′). Both Z940RXbaI and Z1200RXbaI primers have additional polynucleotides at the 5′ ends to give the *Xba*I sites in the fragments (underlined). The DNA fragments amplified by M13-F and Z1200RXbaI primers and M13-F and Z940RXbaI primers were digested with *Hin*cII and *Xba*I and then ligated into the *Stu*I/*Xba*I-cut pCF1010, resulting in pPS200 and pPS300, respectively. The sequence accuracy of the amplified fragments was confirmed by sequencing using a 310A Genetic Analyzer (Applied Biosystems).

β-Galactosidase assays. Cell extracts were prepared from 10-ml cultures showing an optical density of 0.5 to 0.6 at 660 nm, and the β-galactosidase activity of the extracts was determined as described by Young et al. (43). Protein content determination was performed with a Bradford assay kit (Bio-Rad Laboratories). Final results were obtained as the amount of *o*-nitrophenyl-β-galactoside (ONPG) hydrolyzed per minute per milligram of total protein. Results are the averages based on at least three independent assays.

Northern hybridization analysis. Total RNA of *R. sulfidophilum* and *R. capsulatus* cells grown till the logarithmic growth phase, showing an optical density of 0.5 to 0.6 at 660 nm, was extracted using an RNeasy kit (Qiagen). The total amounts of RNA obtained from the same amounts of the cells estimated from the optical density varied by less than 15% among the various conditions. Electrophoresis of the total RNA was performed in 1.0% agarose gels containing formaldehyde (40 mM MOPS [morpholinepropanesulfonic acid], 10 mM sodium acetate, 1 mM EDTA, and 2.2 M formaldehyde, pH 7.0). After electrophoresis, the RNA was transferred to positively charged nylon membranes (Boehringer Mannheim) or a Hybond-N+ positively charged nylon membrane (Amersham). Four probes were used (probes A, B, C, and D, shown in Fig. 1a). Probe A is a PCR product amplified with forward primer ZENDF (5′-AGAGGGAGCTCG CATGT-3′) and reverse primer L350R (5′-CCGGGTTTGTAGTGGAA-3′) from cell lysates of *R. sulfidophilum* or *R. capsulatus* as described previously (30). Probe B is a 1.2-kb DNA fragment excised from pUFS101 by *Apa*I endonuclease. Probe C is a PCR product amplified with forward primer Z0F (5′-CGGAGTT

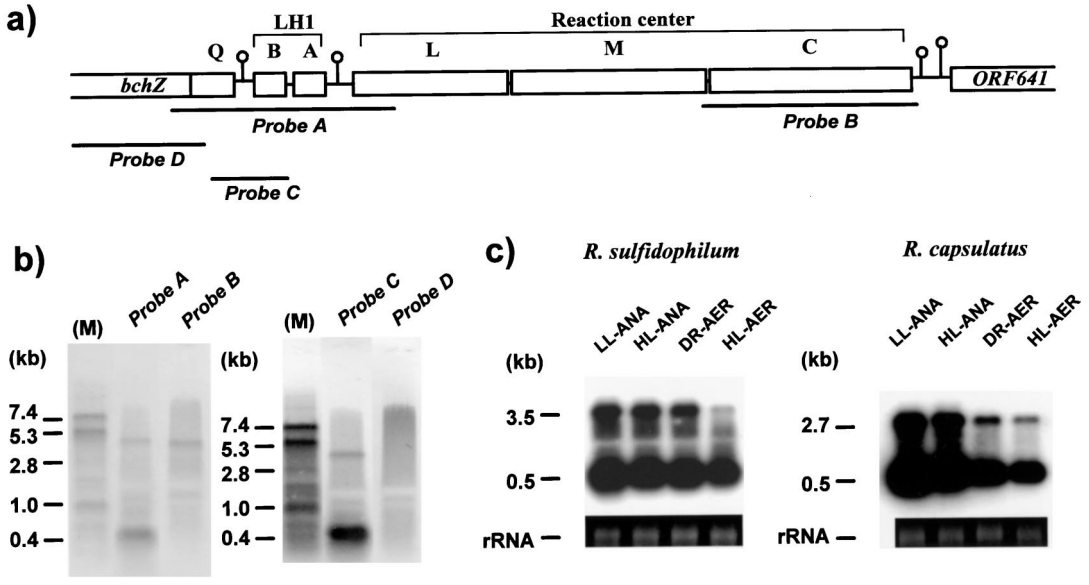


FIG. 1. Physical map and expression of the *puf* operon of *R. sulfidophilum*. (a) Gene arrangement of the *R. sulfidophilum puf* operon. The genes are indicated by open boxes: Q, *pufQ*; B, *pufB*; A, *pufA*; L, *pufL*; M, *pufM*; C, *pufC*. The positions of putative hairpin structures are indicated by open circles. DNA probes used in Northern hybridization experiments are indicated by thick lines. (b) Northern hybridization analysis of mRNA transcripts with four different specific probes for the *R. sulfidophilum puf* operon. Total RNA was extracted from *R. sulfidophilum* grown under anaerobic light conditions. The digoxigenin-dUTP-labeled probe A, probe B, probe C, and probe D were used. The lengths of the standard RNAs are indicated on the left. (c) Effects of oxygen and light on *puf* operon transcription in *R. sulfidophilum* and *R. capsulatus*. Three micrograms of total RNA isolated from cells grown under four different conditions (LL-ANA, anaerobic low-intensity light [3 W/m^2]; HL-ANA, anaerobic high-intensity light [100 W/m^2]; DR-AER, aerobic dark; and HL-AER, aerobic high-intensity light [100 W/m^2]) was loaded on each lane. Northern hybridization was performed with the ^{32}P-labeled probe A of *R. sulfidophilum* or *R. capsulatus*. Ethidium bromide staining of 16S rRNA (lower panel, rRNA) was used to confirm equivalent loading of total RNA.

CATGGTCTATT-3′) and reverse primer B140R (5′-CCACGGGCGCCACTG CCA-3′) using pUFS001 as a template DNA. Probe D is a PCR product amplified with forward primer Z590F (5′-CCATGTCGCCGAGATGCG-3′) and reverse primer Z0R (5′-AATAGACCATGAACTCCG-3′) using pUFS001 as a template DNA. These DNA fragments were labeled with digoxigenin-dUTP as instructed by the manufacturer (Boehringer Mannheim). Probe A of *R. sulfidophilum* and *R. capsulatus* was also labeled with [α-^{32}P]dCTP using a Random Primer DNA labeling kit (Takara). RNA Molecular Weight Marker I (Boehringer Mannheim) was used as a molecular weight standard in some cases. Hybridization was carried out with DIG Easy Hyb Granules (Boehringer Mannheim) at 50°C for over 12 h. After hybridization, the membrane was washed twice with 2× SSC (20× SSC is 3 M sodium chloride and 0.3 M sodium citrate, pH 7.0) and 0.1% sodium dodecyl sulfate (SDS) for 5 min at room temperature and twice with 0.1× SSC and 0.1% SDS for 15 min at 65°C. For quantification of the radiolabeled bands, a BAS 2000 photoimaging system (Fuji film) was used.

Half-life measurement of *puf* mRNA by Northern blot analysis. Cells of *R. sulfidophilum* were grown till the logarithmic growth phase, showing an optical density of 0.5 to 0.6 at 660 nm. Then, rifampin was added to the cultures (500 μg/ml), and cells were collected at various time points. Total RNA was isolated with an RNeasy kit. Total RNA (3 μg per lane) was electrophoresed on 1.0% formaldehyde agarose gels and transferred to a Hybond-N+ positively charged nylon membrane. Probe C was used as a *puf*-specific DNA probe (see "Northern hybridization analysis" and Fig. 1a). The probe was labeled with [α-^{32}P]dCTP using a Random Primer DNA labeling kit. DNA-RNA hybridization was carried out with DIG Easy Hyb at 50°C as instructed by the manufacturer. After hybridization, the membrane was washed twice with 2× SSC and 0.1% SDS for 5 min at room temperature and twice with 0.1× SSC and 0.1% SDS for 15 min at 65°C. For half-life measurement, the radiolabeled bands were quantified using a BAS 2000 photoimaging system.

Primer extension experiment. Total RNA of *R. sulfidophilum* grown till the logarithmic growth phase, showing an optical density of 0.5 to 0.6 at 660 nm, was extracted with an RNeasy kit from aerobically and photosynthetically grown cells. Primers used in this experiment were Z1100R (5′-GTCGAGCAGTG CCTGGGCGTCCTC-3′), Z0R (5′-AATAGACCATGAACTCCG-3′), and AENDR (5′-TCATGGGCGTGATGATCC-3′) (nucleotide numbers of the 5′ ends of the primers in the sequence with GenBank accession no. AB020784 are 975, 1218, and 1353, respectively). These primers were labeled at the 5′ end with [γ-^{32}P]ATP using a DNA MEGALABEL labeling kit (Takara). To determine the 5′ end of the *puf* operon mRNA, a mixture containing 20 μg of RNA, 50 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, and 0.5 pmol of primer in a final volume of 50 μl was incubated at 80°C for 2 min and then at 60°C for 45 min for DNA-RNA hybridization. Then, four deoxynucleoside triphosphates (final concentration, 0.5 mM each) and 20 U of reverse transcriptase (Rous-associated virus-2) were added to the mixture, and the primer extension reaction proceeded at 42°C for 1 h. The DNA synthesized was extracted with phenol-chloroform (1:1), precipitated with ethanol, and suspended in Tris-EDTA buffer. DNA was electrophoresed on a 6% polyacrylamide gel containing 8 M urea with a sequencing ladder, using the same DNA primers. Sequencing gels were dried and exposed to film with an intensifying screen at −80°C.

Measurement of BChl content. Membranes were obtained by sonicating cells grown until mid-logarithmic phase, showing an optical density of 0.5 to 0.6 at 660 nm, followed by differential ultracentrifugation as described previously (30). BChl contents in the membranes were determined using acetone-methanol (7:2) extract as described previously (10). Membrane protein content was determined with a Lowry assay kit (Bio-Rad Laboratories). Then, the relative values of BChl content per membrane protein in each sample were calculated.

Materials. Restriction endonucleases and reverse transcriptase were purchased from Takara. Synthetic oligonucleotide primers were purchased from Life Technologies, Inc. [γ-^{32}P]ATP and [α-^{32}P]dCTP were purchased from Amersham.

RESULTS

Northern blot analysis. Figure 1a shows the structure of the *R. sulfidophilum puf* operon, which consists of six *puf* genes, *pufQ*, *-B*, *-A*, *-L*, *-M*, and *-C* (30). The *puf* operon transcripts were analyzed by Northern blot hybridization with the four *puf*-specific probes (probes A to D). As shown in Fig. 1b, probe A, corresponding to *pufQBA* and part of *pufL* (Fig. 1a), was hybridized with a 0.5-kb mRNA and also weakly with an approximately 3.5-kb mRNA. Probe B, corresponding to *pufC* (Fig. 1a), was hybridized with the 3.5-kb mRNA but not with the 0.5-kb mRNA. These two bands were not detected with probe D, which consisted mainly of the 3′ end of *bchZ* and a small 5′ segment of the *pufQ* gene, but they were detected with probe C, which consisted of *pufB* and part of the *pufQ* gene (Fig. 1b). The transcription of the *puf* operon appeared to be terminated after *pufC* because the signal nucleotide sequence for the typical Rho-independent transcriptional termination

TABLE 2. Effects of oxygen and light on *puf* mRNA levels, *pufQ*- and *pufL-lacZ* expression, and photopigment synthesis[a]

Organism	Growth condition[b]	mRNA content[c]		*pufQ-lacZ*[e]	*pufL-lacZ*[f]	BChl[g]
		0.5 kb	3.5 (2.7) kb[d]			
R. sulfidophilum	HL-ANA	100	100	100	100	100
	LL-ANA	127	114	109	135	128
	DK-AER	86	85	33	35	84
	HL-AER	39	21	2	7	51
R. capsulatus	HL-ANA	100	100	NT[h]	NT	100
	LL-ANA	136	115	NT	NT	130
	DK-AER	30	33	NT	NT	<1
	HL-AER	20	21	NT	NT	<1

[a] Indicated as percentages of the values under anaerobic high-intensity-light conditions (set as 100).
[b] HL-ANA, anaerobic high-intensity light (100 W/m^2); LL-ANA, anaerobic low-intensity light (3 W/m^2); DK-AER, aerobic dark; HL-AER, aerobic high-intensity light (100 W/m^2).
[c] The relative amounts of mRNA were measured by Northern hybridization and quantified by the photoimaging system (Fig. 1c).
[d] 3.5 kb, *R. sulfidophilum puf* mRNA; 2.7 kb, *R. capsulatus puf* mRNA (Fig. 1c).
[e] The relative activities of the *pufQ-lacZ* transcriptional fusion were calculated based on the β-galactosidase activity of pPS100 in *R. sulfidophilum* in Fig. 4. Results are based on the averages of at least three independent assays, and the uncertainty limits in single measurements are within 20%.
[f] The relative activities of the *pufL-lacZ* transcriptional fusion were calculated based on the β-galactosidase activity of pPS001 in *R. sulfidophilum* in Fig. 4. Results are based on the averages of at least three independent assays, and the uncertainty limits in single measurements are within 15%.
[g] Relative BChl contents in membrane preparations were determined as described in Materials and Methods. Results are based on at least three independent assays, with single measurements varying less than 5% from that value.
[h] NT, not tested.

was found downstream of the *puf* operon (30). These observations indicated that the large transcript (3.5 kb) and the small transcript (0.5 kb) in Fig. 1b encode *pufBALMC* and *pufBA*, respectively. The small transcript (0.5 kb) was more abundant than the large transcript (3.5 kb). This difference may be a factor that is thought to adjust the ratio of LH1 peptides to RC proteins, as previously suggested for *R. capsulatus* (7, 25, 47).

To test the effects of oxygen and light on the expression of the *R. sulfidophilum puf* operon, the levels of the two *puf* mRNA transcripts were analyzed by Northern hybridization under four different growth conditions (Fig. 1c). Total RNAs were isolated from cells grown under anaerobic low-intensity-light (3 W/m^2), anaerobic high-intensity-light (100 W/m^2), aerobic dark, and aerobic high-intensity-light (100 W/m^2) conditions (LL-ANA, HL-ANA, DR-AER, and HL-AER, respectively, in Fig. 1c). The analyses were also performed with *R. capsulatus* (Fig. 1c). Because *R. capsulatus* has a *pufX* instead of a *pufC* gene in the *puf* operon (44), the large transcript of the *puf* operon of *R. capsulatus* is shorter (2.7 kb) than that of *R. sulfidophilum* (3.5 kb) (Fig. 1c) (46, 47). Quantitative values digitized by a photoimaging system are shown in Table 2. The *puf*-specific mRNAs were also monitored by dot blot hybridization, and the results are identical to those of the Northern hybridization analysis (standard deviations are <20%). Under anaerobic conditions, the decrease in light intensity from 100 to 3 W/m^2 resulted in increased levels of the *puf* mRNA (about a 30 and 15% increase for the small and large transcripts, respectively) in both *R. sulfidophilum* and *R. capsulatus* (Table 2). However, the relative levels of *puf* mRNA under aerobic growth conditions were different between these two species. In *R. capsulatus*, the relative levels of the small and large transcripts were about 30 and 20% for aerobic dark and aerobic high-intensity-light conditions, respectively, and only a trace amount of photopigment was observed in such conditions (Table 2). The results were in agreement with previous studies (7, 26, 46, 47) in the sense that not only photopigment synthesis but also the cellular levels of *puf* mRNA were strongly affected by oxygen. On the other hand, the relative level of *puf* mRNA was about 85% for aerobic dark conditions in *R. sulfidophilum* (Table 2), which was consistent with the relative BChl content in cells grown under the same conditions (84%) (Table 2). Thus, in contrast to *R. capsulatus*, a high level of *puf* mRNA was still maintained in *R. sulfidophilum* even under aerobic conditions in the dark, although a significant decrease was observed when high light was used under aerobic conditions (Table 2). The BChl content in *R. sulfidophilum* cells grown under aerobic high-intensity-light conditions was 51% of that under anaerobic high-intensity-light conditions, whereas in *R. capsulatus* the relative BChl content was <1%, although both species contain significant amounts of *puf* mRNA (~20 to 39%) (Table 2). This observation implies that regulation of BChl synthesis differs in these two species.

Primer extension analysis. To determine the possible transcription initiation site of the *R. sulfidophilum puf* operon, we performed RNA 5′-end mapping by primer extension analysis. The ^{32}P-labeled primers (Z1100R, Z0R, and AENDR: see Materials and Methods) were hybridized to the total RNA extracted from cells grown under anaerobic light and aerobic dark conditions (Fig. 2, − and +, respectively). Using a Z0R primer, two 5′ ends of *puf* mRNA were observed. One had a less intense signal corresponding to 11 bp downstream from the *pufQ* start codon (*puf*P3), and the other showed a more intense signal corresponding to 132 bp upstream from the *pufQ* start codon (*puf*P2) (Fig. 2B). Results obtained with the AENDR primer also showed the presence of the two 5′ ends of the *puf* mRNA at the corresponding positions (data not shown). A third 5′ end of the *puf* mRNA was detected by the Z1100R primer (*puf*P1) (Fig. 2A). This site was resolved to a position much farther upstream (332 bp) from the *pufQ* start codon. These 5′ ends of *puf* mRNA are indicated in Fig. 3 as P1, P2, and P3. All three transcripts were detected in both anaerobically and aerobically grown cells, but the relative band intensity between the conditions was different depending on the initiation sites.

Deletion analysis of the *puf* operon promoter. In order to analyze the *R. sulfidophilum puf* operon promoter, a nested set of deletions was constructed in the region of DNA upstream from the *pufL* gene. The deleted DNA fragments were cloned into the promoter testing vector, pCF1010 (28), to construct *puf-lacZ* transcriptional fusions (Fig. 3). These constructs were

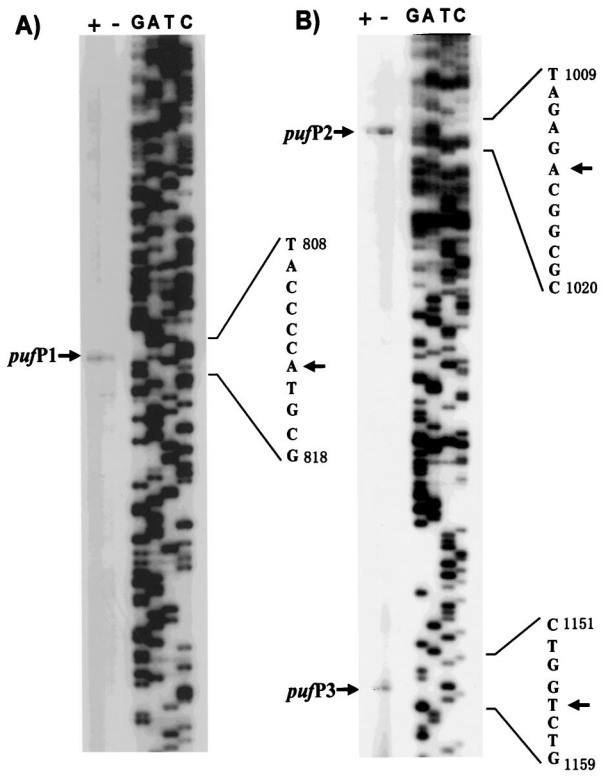


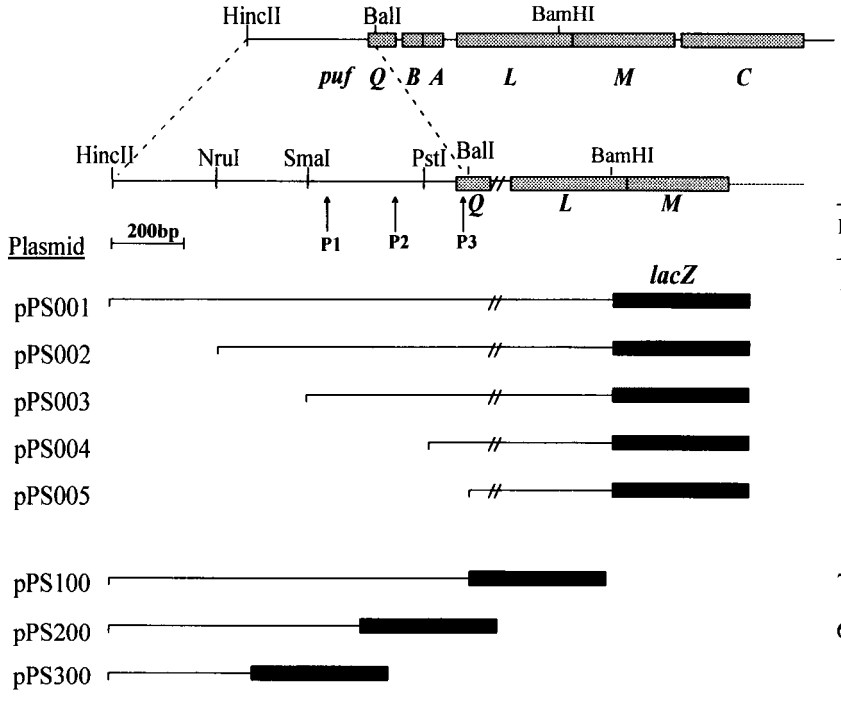
FIG. 2. Primer extension mapping of the 5′ end of *puf* operon transcripts. Total cellular RNA isolated from *R. sulfidophilum* grown under aerobic dark conditions (+) and anaerobic light conditions (−) was hybridized with 5′-end-labeled Z1100R primer (A) or Z0R primer (B) (see Materials and Methods). Lanes G, A, T, and C show sequence ladders using the same synthetic primers. The numbers of 5′ and 3′ ends shown in the nucleotide sequences are the nucleotide numbering in the GenBank sequence with accession no. AB020784.

introduced into *R. sulfidophilum* cells and assayed for β-galactosidase activity levels under anaerobic low-intensity-light (3 W/m^2), anaerobic high-intensity-light (100 W/m^2), aerobic dark, and aerobic high-intensity-light (100 W/m^2) conditions (LL-ANA, HL-ANA, DR-AER, and HL-AER, respectively, in Fig. 3). The largest fragment, which extended from 584 bp upstream of *puf*P1 to *pufL* (pPS001), showed the highest *lacZ* expression under anaerobic low-intensity-light conditions. β-Galactosidase activity of the plasmid was reduced by aeration and high-light illumination, suggesting that the transcriptional activity assayed with this *lacZ* fusion was repressed by oxygen and high-intensity light. High-light repression of *lacZ* activity was more apparent under aerobic conditions than under anaerobic conditions for this construct. Deletion of 303 and 540 bp from the 5′ end of the largest fragment (pPS002 and pPS003, respectively) resulted in different levels of *lacZ* activity. A comparison of the activities with the largest fragment (pPS001) indicates that the values were reduced under anaerobic low-light, anaerobic high-light, and aerobic dark conditions (~30 to 50%) but increased under aerobic high-light conditions (~280 to 480%).

The transcriptional activity was detected in the construct pPS004, which contained one of the three 5′ ends (*puf*P3). Removal of all the sites of the mRNA 5′ ends, yielding the construct pPS005, caused a very low level of *lacZ* expression (approximately 50-fold reduction relative to the most active fragment, pPS001). Deletion of 1.4 kb from the 3′ end of the largest fragment, yielding the construct designated pPS100, resulted in reduced *lacZ* activity (~25 to 60%) although the fragment contained the same 5′ end and all three sites of the mRNA 5′ ends. The fragment of pPS200 containing only one of the three 5′ ends (*puf*P1) showed significant *lacZ* activity, but the removal of an additional 282 nucleotides with *puf*P1, yielding the plasmid pPS300, caused no *lacZ* activity. The in vivo expression activities observed with these vectors support the presence of at least two possible promoters (*puf*P1 and *puf*P3) based on the results of the primer extension analysis. The effect of light intensities on *lacZ* activity in some constructs was the opposite under aerobic conditions from what it was under anaerobic conditions. The transcriptional activities of *pufQ-lacZ* (pPS100) and *pufL-lacZ* (pPS001) under four different growth conditions are summarized in Table 2 as values relative to those for anaerobic high-light conditions. The relative BChl contents in cells grown under four different growth conditions were in general agreement with the *puf* mRNA contents, although most of the BChl in the membrane could be present in the *puc*-encoded LH2 complex. However, the *puf* operon expression measured by the *pufQ*- or *pufL-lacZ* transcriptional fusions was not directly correlated with the BChl levels (Table 2). This may be due to the lack of upstream sequences on these plasmid-borne fusions. A readthrough transcription from the upstream region may be present in the *R. sulfidophilum puf* operon (see Discussion).

Decay rate of *puf* mRNA in *R. sulfidophilum*. In order to examine the posttranscriptional control of *puf* operon expression in *R. sulfidophilum*, the half-lives of both small (0.5-kb) and large (3.5-kb) *puf* transcripts were determined under anaerobic and aerobic growth conditions by Northern hybridization with the ^{32}P-labeled probe C (Fig. 1a) corresponding to *pufB* and part of the *pufQ* gene. Results are shown in Fig. 4. The half-lives of 3.5-kb *puf* mRNA were about 10 and 13 min in aerobic and anaerobic conditions, respectively, whereas the half-lives of 0.5-kb mRNA isolated from aerobically and anaerobically grown cultures did not show a significant difference, with values of about 40 min. We also performed corresponding measurements with *R. capsulatus* (data not shown). The results obtained were similar to those of the previous study, which showed that the half-lives of the larger *puf* transcript (2.7 kb) were about 3 and 8 min in aerobically and anaerobically grown cultures, respectively, and that those of the smaller RNA (0.5 kb) were about 30 min under both aerobic and anaerobic conditions (23). The larger transcript was degraded about twice as fast under aerobic conditions in *R. capsulatus* but only about 1.3 times faster under aerobic than under anaerobic conditions in *R. sulfidophilum*. The stability of *puf* mRNA was only weakly influenced by oxygen tension in *R. sulfidophilum*.

Influence of *regA* mutation on the control of transcription of *R. sulfidophilum puf* operon. We previously constructed a *regA* deletion mutant (RESA1) from *R. sulfidophilum* and showed that the RegA-RegB regulatory cascade has an important role for the expression of photosynthesis genes in this bacterium, as in the *Rhodobacter* species (29). To test the effects of the regulatory mutant on the promoter activity of *puf* operon in *R. sulfidophilum*, we assayed the β-galactosidase activity of various *puf-lacZ* fusions in RESA1. As shown in Fig. 5, the effects of β-galactosidase activity caused by *regA* mutation fell into three types. In one type, the transcriptional *pufL-lacZ* fusions containing all three promoters (pPS001, pPS002, and pPS003 [Fig. 3]) exhibited nearly baseline levels of *lacZ* activity in RESA1 (Fig. 5A for pPS001; data for pPS002 and pPS003 are not shown). In contrast, a construct containing only the *puf*P3 promoter (pPS004 [Fig. 3]) retained the β-galactosidase activity in the *regA* mutant as much as in the wild-type cells (Fig. 5B). The third type is a *pufQ-lacZ* transcriptional fusion from



β-galactosidase activity
(μmol/min/mg protein)

LL-ANA	HL-ANA	DK-AER	HL-AER
1250 (50)	920 (20)	325 (50)	66 (10)
555 (55)	315 (30)	99 (3)	322 (15)
632 (70)	435 (70)	120 (10)	191 (20)
125 (5)	187 (15)	137 (20)	17 (1)
21 (6)	24 (3)	15 (5)	7 (1)
730 (15)	668 (50)	221 (20)	16 (2)
606 (60)	443 (30)	87 (5)	94 (5)
<1	<1	<1	<1

FIG. 3. Deletion analysis of *R. sulfidophilum puf* operon. 5′ and 3′ deletions were constructed by using DNA-restricted and PCR-generated fragments (see Materials and Methods). These deletions were fused to the *lacZ* gene to construct various *puf-lacZ* transcriptional fusions. β-Galactosidase activities associated with the transcriptional fusions on plasmids present in *R. sulfidophilum* cells were determined under different growth conditions (LL-ANA, anaerobic low-intensity light [3 W/m^2]; HL-ANA, anaerobic high-intensity light [100 W/m^2]; DK-AER, aerobic dark; HL-AER, aerobic high-intensity light [100 W/m^2]). Values in parentheses are the standard deviations of at least three independent assays. No activity was detected for the sequence without insertion of the *lacZ* fusion (vector only). The positions of three 5′ ends of *puf* mRNA determined in experiments in Fig. 2 are indicated by arrows (P1, *puf*P1; P2, *puf*P2; P3, *puf*P3).

which the 3′ end was deleted from a DNA fragment of pPS001 (pPS100 [Fig. 3]) and which showed a low level of β-galactosidase activity even in the mutant while still containing the same 5′ end of pPS001 (16 and 28% of those in wild-type cells under anaerobic and aerobic conditions, respectively [Fig. 5C]).

DISCUSSION

Three possible *puf* operon promoters in *R. sulfidophilum*. In this study, the *R. sulfidophilum puf* operon and its promoters were analyzed to clarify the mechanism of the oxygen-independent expression of photosynthetic genes. Primer extension analysis showed that there are three 5′ ends of *puf* mRNA (*puf*P1, *puf*P2, and *puf*P3 in Fig. 2). By employing deletion analyses, two of the three 5′ ends of *puf* mRNA were mapped as the possible transcription initiation sites at positions 332 bp upstream and 11 bp downstream of the *pufQ* start codon (*puf*P1 and *puf*P3, respectively [Fig. 2 and 3]), because the deletion of *puf*P1 or *puf*P3 from the inserted fragment of *lacZ* fusions resulted in significant reduction of the *lacZ* activity (Fig. 3). The other 5′ end found in the primer extension analysis (*puf*P2; 132 bp upstream from *pufQ*) was not directly supported as a transcription initiation site by the deletion analysis because of the lack of appropriate restriction sites. In *R. capsulatus*, two *puf* transcription initiation sites have been reported and mapped to positions 316 and 129 bp upstream of the *pufQ* start codon. The nucleotide sequence alignment of *puf* operon promoter regions of *R. capsulatus* and *R. sulfidophilum* revealed that the two *puf* transcription initiation sites of *R. capsulatus* are located near the corresponding sites of *puf*P1 and *puf*P2 of *R. sulfidophilum* (separated by 9 and 4 bp, respectively) (data not shown) (1, 5). The regions are highly conserved between these two species, suggesting that the *puf*P2 of *R. sulfidophilum* is also a transcription initiation site, as in *R. capsulatus*.

Oxygen regulation on the *puf* operon expression in *R. sulfidophilum*. Northern blot hybridization experiments showed that the mRNA levels of the *R. sulfidophilum puf* operon were mostly maintained even under aerobic dark conditions (Fig. 1c), which was consistent with the relative BChl content in the cells (Table 2). This observation is in contrast to that in the *Rhodobacter* species (Fig. 1c) (7, 26, 46, 47), indicating that the aerobic synthesis of the photosynthetic apparatus in *R. sulfidophilum* seems to be mostly due to the *puf* operon expression, which is weakly sensitive to oxygen and regulated at the transcriptional level.

Deletion analysis showed that one of the possible promoters, *puf*P3, was expressed independently of oxygen (Fig. 3, pPS004). The activity of the promoter can contribute to the aerobic expression of the *puf* operon. However, the activity of the major promoter for the *puf* operon transcription located far upstream from the *pufB* gene, *puf*P1, showed fivefold-lower levels under aerobic dark than under anaerobic high-intensity-light conditions (Fig. 3, pPS200). Probably, as a result, the transcriptional activities of both *pufQ-lacZ* (pPS100) and *pufL-lacZ* (pPS001) fusions were repressed by oxygen by a factor of three when the activities were compared between the aerobic dark and anaerobic high-intensity-light conditions (Table 2 and Fig. 3). On the other hand, only a 15% decrease in the amount of mRNA was observed under aerobic dark conditions (Table 2). A similar difference was also observed under aerobic high-intensity-light conditions. The transcriptional *pufQ-lacZ* and *pufL-lacZ* activities showed very low levels under such condi-

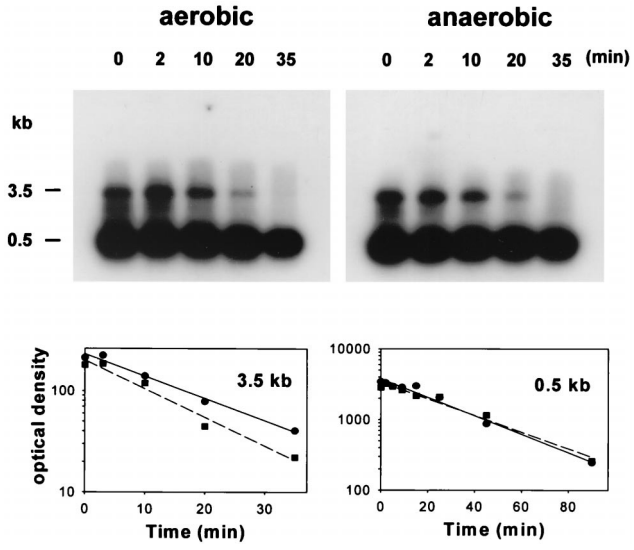


FIG. 4. *puf* mRNA decay kinetics in *R. sulfidophilum*. Three micrograms of total RNA isolated from rifampin-treated *R. sulfidophilum* cells grown under aerobic dark and anaerobic light conditions was loaded on each lane. Hybridization was performed with the ^{32}P-labeled probe C (Fig. 1a). The optical density of the bands was plotted against the time of RNA isolation (squares, aerobic conditions; circles, anaerobic conditions). The half-lives calculated from these blots for the 3.5-kb mRNA were about 10 min under aerobic conditions (dashed line) and about 13 min under anaerobic conditions (solid line). For the 0.5-kb mRNA, the half-lives were about 40 min under both aerobic conditions (dashed line) and anaerobic conditions (solid line).

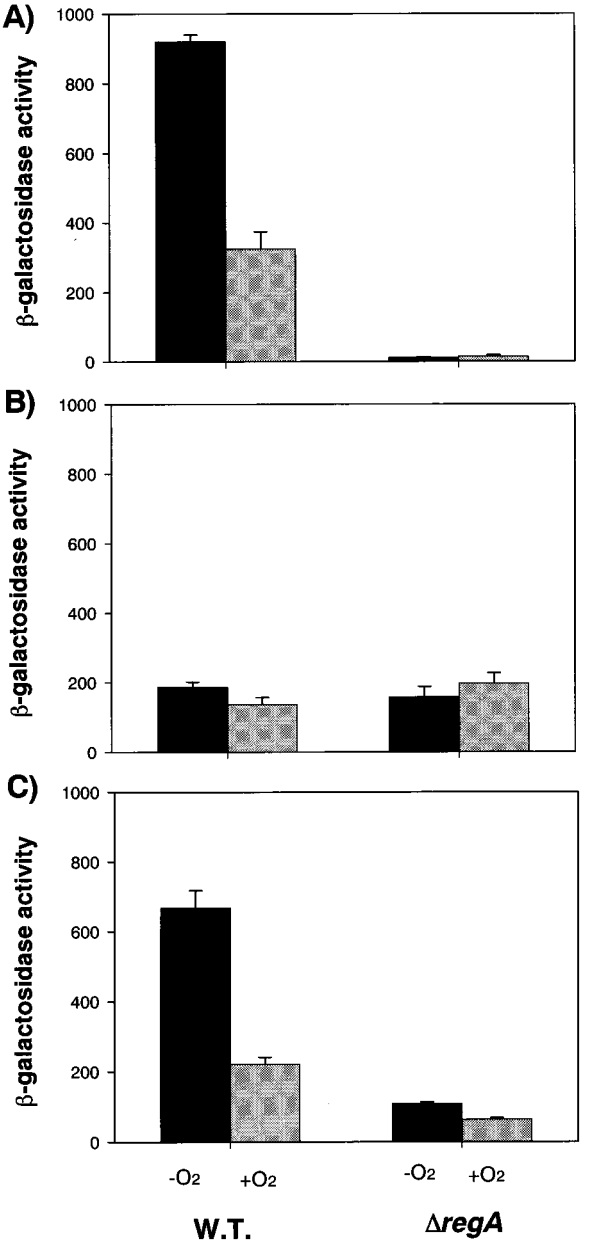


FIG. 5. Measurement of *puf* operon transcriptional activity in wild-type (W.T.) and *regA*-disrupted *R. sulfidophilum* strains. Bars represent β-galactosidase activities associated with the *puf-lacZ* transcriptional fusions, pPS001 (A), pPS004 (B), and pPS100 (C) (Fig. 3), in cells grown under anaerobic high-intensity light (100 W/m^2) ($-O_2$) or aerobic dark conditions ($+O_2$). Data for the wild type are taken from the results shown in Fig. 3. Units of β-galactosidase activity represent micromoles of ONPG hydrolyzed per minute per milligram of total protein.

tions (2 and 7%, respectively, compared to values for anaerobic high-intensity-light conditions) (Table 2). However, the amounts of the two *puf* mRNA transcripts were 39 and 21% for the small and large transcript, respectively, when those two growth conditions were compared (Table 2). Thus, the *puf*-specific transcriptional activity does not directly reflect the level of *puf* mRNA under aerobic growth conditions (Table 2). The transcriptional overlap (readthrough transcription) must be a factor causing the absence of direct correlation between the level of chromosomally derived *puf* mRNA and transcriptional activity of DNA segments inserted into the *lacZ* fusion plasmid. Since the stop codon of *bchZ* overlaps the start codon of *pufQ* in *R. sulfidophilum* (30) and the *puf* promoters (at least *puf*P1) are located in the *bchZ* gene (Fig. 3), the readthrough should exist between the transcription for *bchZ* and that for the *puf* operon, as shown for *R. capsulatus* (40, 43).

Previous studies using *R. capsulatus* have shown that decay of mRNA is an important factor in controlling *puf* operon expression (1, 18, 21, 23, 24). In this study, we measured the decay time of *puf* operon transcripts under aerobic and anaerobic conditions. For *R. sulfidophilum*, the large *puf* mRNA was less stable by a factor of 0.77 (Fig. 4) under aerobic conditions than under anaerobic conditions. This number, however, was 0.38 (data not shown) in *R. capsulatus* (23). The difference in relative stability of this molecule between the two bacterial species seems to contribute to the high content of the *puf* mRNA under aerobic conditions in *R. sulfidophilum*. However, this contribution is not sufficient to explain the higher content of *puf* mRNA than that expected from the *puf* promoter activity (Table 2).

Light regulation of *puf* operon expression in *R. sulfidophilum*. Light regulation of *puf* operon expression is apparent under aerobic conditions in *R. sulfidophilum* (Fig. 1c and Table 2). *pufQ*- and *pufL-lacZ* activities were highly repressed by high-intensity light under aerobic conditions (~6 to 20% of those under aerobic dark conditions). Similarly, high-light illumination also resulted in lower levels of chromosomally derived *puf* mRNA under such conditions (~24 to 45% of those under aerobic dark conditions). Since the major fraction of *puf* mRNA seems to be derived from the readthrough transcription under aerobic conditions as discussed above, the readthrough activity may also be repressed to some extent by high-intensity light under aerobic conditions.

The effect of light on the *puf* operon-specific promoters was

analyzed by deletion analysis, and the results were a little complicated. The strains containing pPS004 covering only *puf*P3 showed the high-light repression under aerobic conditions, whereas pPS200, covering only *puf*P1, and pPS003, from which the 5′ end of 540 bp was deleted from the largest fragment (pPS001), did not exhibit the light repression (Fig. 3). Plasmid pPS002, from which 303 bp was deleted from the 5′ end of pPS001, showed a unique phenotype in that high-intensity light activated the promoter activity under aerobic conditions (Fig. 3). These observations suggest that there are several light-dependent *cis* regulatory sites on the *puf* operon promoters. It was also suggested that the *Hinc*II-*Nru*I region (584 to 281 bp upstream from the first transcription initiation site, respectively) is an important regulatory site responsible not only for the elevation of the *puf* operon transcription responding to anaerobiosis but also for the light regulation of the *puf* operon expression (Fig. 3). Unidentified regulatory factors may bind to this region and interact with RNA polymerase mediated by other protein factors.

RegA-RegB regulatory system for *puf* operon expression in *R. sulfidophilum*. As mentioned previously, two promoters in the *R. capsulatus puf* operon have been reported. The *R. capsulatus* upstream promoter corresponding to *puf*P1 of *R. sulfidophilum* is highly expressed and regulated (1, 5). It was recently reported that the response regulator RegA binds to this promoter region (8, 16). It is clear from *puf-lacZ* fusion analysis that RegA is involved in activating *puf* operon transcription in *R. sulfidophilum* through association with the upstream region of the *Pst*I site located between *puf*P1 and *puf*P3 (Fig. 5A and B). The promoter activity of *puf*P1 may be regulated by the RegA-RegB regulatory system in *R. sulfidophilum*, as in *R. capsulatus* (8, 16).

Comparison of activities obtained with pPS100 with those of pPS001 indicates that deletion of the *Bal*I-*Bam*HI region located between *pufQ* and *pufL* (Fig. 3) resulted in reduced *lacZ* activity (~25 to 60%) although the fragment contained the same 5′ end and all possible promoters. In addition, deletion of this region recovered some *puf* operon transcriptional activity in a *regA* mutant (Fig. 5A and C), suggesting that the region has a possible *cis* site which is involved in the transcriptional control of *puf* operon expression by the RegA-RegB regulatory system. Further genetic characterization of the *R. sulfidophilum puf* operon should be useful in clarifying the details of the control mechanisms of *puf* operon expression in purple bacteria.

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