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Isolation and Characterization of *Rhodobacter capsulatus* Mutants Defective in Oxygen Regulation of the *puf* Operon

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***cis*-acting mutations that affect regulation of the *Rhodobacter capsulatus puf* operon by oxygen were isolated by placing the mutagenized *puf* regulatory region 5′ to a promoterless Tn*5* *neo* gene, which encodes resistance to kanamycin (Km^r). *R. capsulatus* mutants that failed to show wild-type repression of KM^r by oxygen were selected and analyzed. Four independent clones contained point mutations, three of which were identical, in a region of dyad symmetry located between *puf* operon nucleotide positions 177 and 207, approximately 45 base pairs 5′ to the site of initiation of *puf* transcripts. The phenotypic effects of the aerobically selected mutations were duplicated by single and double point mutations introduced site specifically into the region of dyad symmetry by oligonucleotide-directed mutagenesis. Determinations of the bacterial 50% lethal dose of kanamycin, of aminoglycoside phosphotransferase activity in cell sonicates, and of *neo*-specific mRNA confirmed the diminished responsiveness of the mutants to oxygen and consequently implicated the mutated region in O_2-mediated transcriptional regulation.**

Many microorganisms that can grow under both aerobic and anaerobic conditions have complex regulatory mechanisms to ensure coordinate expression of the appropriate genes for a given growth condition. *Rhodobacter capsulatus* is a purple nonsulfur bacterium capable of chemotrophic growth in aerobic dark conditions and phototropic growth under low oxygen in the presence of light. When the oxygen tension in a culture of *R. capsulatus* decreases, an intracytoplasmic membrane system containing the photosynthetic apparatus is produced. This apparatus consists of two light-harvesting (LH) antenna pigment protein complexes, LH I (B870) and LH II (B800-850), and a photochemical reaction center (RC) where electron transport is initiated.

The genes for the pigment-binding proteins of the LH I and RC complexes, the Q gene, which is involved in bacteriochlorophyll biosynthesis (2, 20), and the X gene, which influences the ratio of antenna complexes (20), are located in a polycistronic operon (6, 39) formerly known as *rxcA* but renamed *puf* (19). While it has been shown that oxygen regulation of the expression of *puf* and the other operons that encode peptides of the photosynthetic apparatus is accomplished primarily at the transcriptional level (6, 12, 22, 38, 39), the regulatory mechanism is not known.

To identify more specifically the sequences involved in gene regulation by oxygen, we isolated *cis*-acting *puf* mutants that show diminished oxygen-mediated repression of *puf* operon expression. The mapping of four independent mutations to a region of dyad symmetry 45 base pairs (bp) upstream of the *puf* transcription initiation site implicates this region in determining the transcriptional response of the *puf* genes to oxygen.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *R. capsulatus* B10 (wild-type strain [27, 33]), U43 (RC$^-$ B870$^-$ B880-850$^-$ [37]), and A3119 (this work) were grown at 35°C in RCV medium (33) supplemented with yeast extract (1 mg/ml). Aerobic growth was in baffled flasks filled to one-fifth of their volume with medium and shaken at 300 rpm (pO_2 of 19 to 20%). Low-oxygen growth was in nonbaffled flasks filled to four-fifths of their volume with medium and shaken at 150 rpm (pO_2 of 1 to 3%). Difco Antibiotic Medium 2 (Penassay base agar [PAA]) plates were used for selection of Km^r mutants. pMLN1 is an RK2-based promoter-cloning vector derived from pTJS133 (31); it contains a promoterless *neo* gene from Tn*5*. pMLN1 and its derivatives were maintained by growth in the presence of 0.5-μg of tetracycline per ml. Details of construction of pMLN1 are provided in Fig. 1. The numbering scheme of Adams et al. (1) for the *puf* operon was employed.

R. capsulatus A3119 was constructed by replacing the wild-type chromosomal DNA between an *Sfi*I site 903 bp upstream of *pufB* (37) and a *Pst*I site in the *pufL* gene (36) with the spectinomycin resistance gene located on an *Eco*RI fragment in plasmid pXJS5425 (18).

Mutagenesis and selection for mutants. *Sac*I and *Xba*I linkers were blunt-end ligated onto the 5′ and 3′ ends, respectively, of an 857-bp DNA fragment containing the sequence at the 5′ end of the *puf* operon from bp 46 (1) to the *Aha*II site located at bp 902 (approximately 30 bp upstream of the start codon of the *pufB* gene). The resulting fragment was inserted into pUC19. Hydroxylamine mutagenesis was carried out as described by Birch and Cullum (8). Mutagenized plasmids showed 1 to 8% viability, based on transformation efficiency, when compared with transformations of samples of unmutagenized DNA.

Mutagenized *puf* DNA was inserted into pMLN1 as *Sac*I-*Xba*I fragments and introduced into *Escherichia coli* MC1061 (11) by transformation (13). Three plates, the first containing individual colonies of Tc^r *E. coli* transformants (donor), the second containing a lawn of *R. capsulatus* B10 (recipient), and the third containing a lawn of *E. coli* HB101 (9) carrying pRK2073 (mobilizing plasmid) (25), were replica plated onto a PAA plate and incubated at 35°C for approximately 5 h to allow transfer of pMLN1 derivatives to *R. capsulatus* by conjugation (14, 21). Mating efficiency was 85%. After mating, each plate containing the three mixed cultures were replica plated onto PAA plates containing 50,

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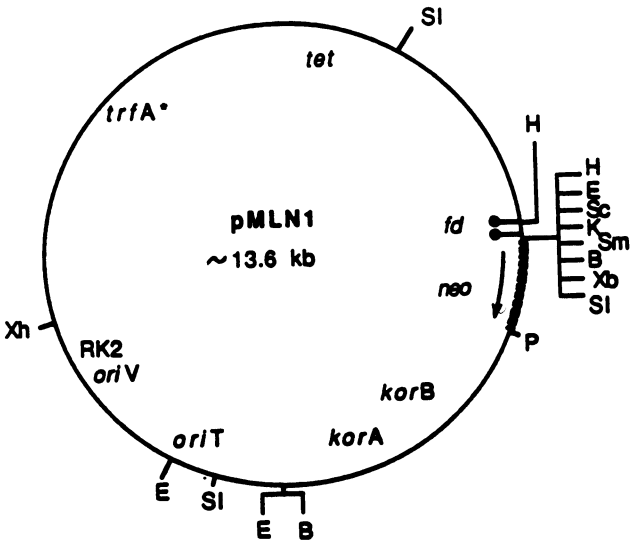

FIG. 1. pMLN1 is an RK2-based promoter-probe vector derived from pTJS133 (27). A 900-bp *Pst*I fragment containing the promoterless *neo* gene from Tn*5* (hatched region) (4) encoding APH(3'), which confers Km^r, was inserted at the *Pst*I site of pTJS133; two copies of the bacteriophage *fd* terminator (⊷) (5, 16) were inserted at the *Hin*dIII site upstream of the promoterless *neo* gene to prevent transcription initiated at vector DNA sequences from interfering with the assay of transcription originating from *puf* DNA inserts. DNA fragments (857 bp) containing the 5' end of the *puf* operon from bp 46 to the *Aha*II site located at bp 902 (1) were isolated from pUC19 after hydroxylamine mutagenesis as *Sac*I-*Xba*I fragments and inserted into pMLN1. The arrow indicates the direction of transcription. Polylinker sites were derived from pUC19 (34). The locations of restriction sites are approximate. Restriction site abbreviations: *Bam*HI, B; *Eco*RI, E; *Hin*dIII, H; *Kpn*I, K; *Pst*I, P; *Sac*I, Sc; *Sal*I, Sl; *Sma*I, Sm; *Xba*I, Xb; *Xho*I, Xh.

80, or 100 μg of kanamycin per ml and incubated at 35°C for 2 days under aerobic conditions to select for mutations that resulted in Km^r during aerobic growth of *R. capsulatus*. Km^r *E. coli* strains do not grow under these conditions since the *puf* promoter does not function in *E. coli* (C. W. Adams, S. N. Cohen, J. T. Beatty, and M. E. Forrest, unpublished data). Control matings performed with plasmids containing the unmutagenized *Sac*I-*Xba*I fragment showed little or no colony growth on 50 μg of kanamycin per ml.

Oligonucleotide-directed mutations were made as described by Kunkel et al. (24) except that a gapped duplex step (23) was included. In pMLN84, the *puf* 5' fragment was mutagenized to change the C at position 184 to T, using the oligonucleotide 5'-GGATCGCCGCACCGGGGAAGG-3', which is complementary to the coding sequence at bp 174 to 194. In pMLN8493, both C-184 and C-193 were changed to T's, using the oligonucleotide 5'-GCGCGGCGAATCGCCG CACCGGGGAA-3', which is complementary to the coding sequence at bp 176 to 201. The mutagenized DNA fragments were sequenced prior to inserting them into pMLN1 to confirm that the desired mutations were present.

Sequencing. Fragments containing mutagenized *puf* DNA segments were inserted into M13mp18 and M13mp19 (29) and sequenced by the method of Sanger et al. (30), using synthesized oligonucleotides and/or a 17-mer universal sequencing primer (New England BioLabs, catalog no. 1211). The Klenow fragment of DNA polymerase I (Boehringer Mannheim Biochemicals) was used for extension reactions. The noncoding strand was initially screened for hydroxylamine-induced mutations (C-to-T transitions) by sequencing with only A and T reactions. Both strands of the segments in which mutations were found were sequenced.

Assay for APH(3') activity. Cultures of *R. capsulatus* were grown aerobically to an optical density at 660 nm of 0.3 and split into two 40-ml aliquots; one was used as the aerobic sample, and the other was grown under low oxygen for 4 h. Cells were disrupted by sonication, and the supernatant fraction was assayed for aminoglycoside phosphotransferase [APH(3')] activity by a phosphocellulose binding assay (17). Reaction mixtures contained 10 μl of kanamycin (250 μg/ml), 20 μl of [γ-^{32}P]ATP solution (0.5 μM ATP), and 10 μl of cell extract. Samples were incubated for 1 h at 37°C, rapidly spotted onto phosphocellulose filters, and boiled for 1 min to stop the reaction. The filters were washed three times for 15 min each in 50°C deionized water and dried for 30 min in a vacuum oven at 80°C, and the radioactivity was counted in 10 ml of Aquasol. Controls consisted of extracts of *R. capsulatus* lacking a plasmid and reaction mixtures in which the cell extract was omitted. Protein determinations were made by the Bradford method (10), using Bio-Rad protein assay reagent (Bio-Rad Laboratories).

RNA isolation and quantitation. For RNA isolation and quantitation, cultures were grown and treated as described above except that the low-oxygen sample was cultured for 20 min prior to isolation of RNA as previously described (32).

S1 nuclease mapping of 5'-ends. Mapping of 5' ends of mRNA was performed by the method of Berk and Sharp (7) as previously described (1). Double-stranded DNA probes were end labeled by standard methods (26).

RESULTS

Identification of mutants defective in repression of *puf* expression by oxygen. Cells in *R. capsulatus* colonies growing on plates under aerobic conditions are sufficiently limited in oxygen to induce *puf* operon expression (our unpublished data). However, we reasoned that individual cells would be exposed to fully aerobic growth conditions when initially plated, and thus *puf* expression would be repressed. Mutations that render *puf* regulatory regions insensitive to oxygen repression should allow colony formation on kanamycin plates when the mutated *puf* region is fused to the *neo* gene.

Mutagenized *Sac*I-*Xba*I DNA fragments containing the 5' region of the *puf* operon were inserted into pMLN1 upstream of the promoterless *neo* gene and introduced into *R. capsulatus* by triparental mating; cells were spread on plates containing kanamycin (50, 80, or 100 μg/ml). Seven putative mutant clones that grew reproducibly on plates containing tetracycline and aerobically on at least 50 μg of kanamycin per ml were obtained. Plasmid DNA was isolated from these and reintroduced into *R. capsulatus*. In each case, plasmid recipients grew aerobically on the same kanamycin concentration as that used for the initial isolation of the mutant.

Sequence analysis of the *Sac*I-*Xba*I *puf* DNA inserts of the seven isolates (Fig. 2) showed that four independent clones contained C-to-T point mutations in a region of dyad symmetry located between nucleotide positions 177 and 207, approximately 45 bp upstream from the *puf* operon transcription start site (1, 3). Three of the mutations were at bp 184, while the fourth was at bp 193. No mutations were found in the *puf* DNA segment of the remaining three clones.

Further analysis (see results below) revealed that isolates having the same point mutation showed some variation in the level of *neo* gene expression. The basis for this variation has not been determined. However, to determine whether the mutations identified in the region of dyad symmetry were

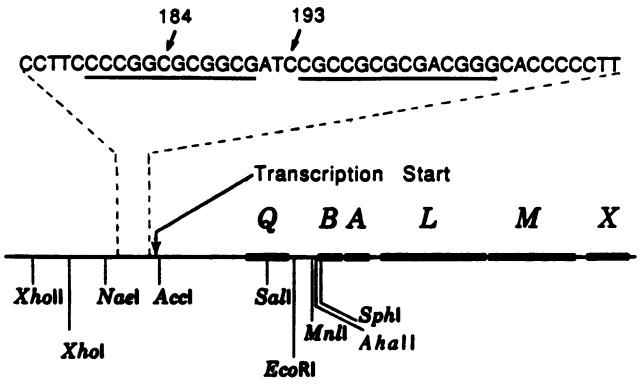

FIG. 2. Genetic and partial restriction map of the *puf* operon (1). Thick lines indicate the Q and X genes and genes encoding subunits of the LH I and RC complexes. The expanded region showed the location of the point mutations that affected the repression of *puf*-linked *neo* gene expression under aerobic conditions. The underlined sequences indicate a region of dyad symmetry.

entirely sufficient to account for the observed expression of *puf*-linked Km^r during aerobic growth, oligonucleotide-directed mutations were introduced at bp 184 or at bp 184 and 193 in DNA isolated from the wild-type *puf* operon.

Effect of mutations on expression of the *puf*-linked *neo* gene. The 50% lethal dose (LD_{50}) of kanamycin for both hydroxylamine-induced and oligonucleotide-directed mutagenesis-derived mutants was compared with the LD_{50} for the *neo*-linked wild-type *puf* operon regulatory region (Table 1). Strains containing point mutations in the *puf* upstream region showed LD_{50}s that were two- to fourfold higher than that for the strain containing the wild-type *puf* upstream region. A double mutation in the region of dyad symmetry (pMLN8493) conferred sixfold higher Km^r.

Direct assay of APH(3′) (Table 1) showed that *R. capsulatus* B10 strains carrying mutated plasmids had 5- to 10-fold greater enzyme activity under aerobic growth conditions than the wild-type strain; the double mutant, pMLN8493, was the least repressed by oxygen, showing an activity 22 times that of the wild-type strain. Additionally, all of the mutants showed increased APH(3′) activity after 4 h of growth under low-oxygen conditions; single mutations resulted in a two- to threefold increase and the double mutant showed a fourfold increase over the wild-type activity. The APH(3′) activity was induced 14-fold in the wild-type strain when it was shifted from aerobic to low-oxygen growth, while the observed increase in the mutant strains ranged from three- to sixfold.

Effect of mutations on synthesis of *puf*-controlled mRNA. The mutated 857-bp DNA fragments containing the *puf* operon regulatory region and *pufQ* were ligated at the *Eco*RI site in *pufQ* to a DNA fragment containing the genes *pufQ* through *pufX*, thereby reconstituting an intact *puf* operon with the potential for altered response to oxygen control. These DNA fragments were inserted into the polylinker site of a pMLN1 derivative in which the *neo* gene had been removed. When these plasmids were introduced into *R. capsulatus* U43 by conjugal mating to complement the RC^- $B870^-$ phenotype of *R. capsulatus* U43, the resulting plasmids showed variable phenotypes and plasmid DNA rearrangement. Therefore, analysis of the effect of the *puf* mutations on the synthesis of *puf*-controlled mRNA was done with plasmids containing *puf-neo* gene fusions.

S1 nuclease protection mapping was used to compare the initiation sites for the *puf-neo* gene fusion transcripts synthesized in the mutant versus wild-type plasmids. For these experiments, total RNA was extracted from *R. capsulatus* A3119 containing mutant or wild-type plasmids; since A3119 has a chromosomal deletion extending from a locus 903 bp upstream of *pufB* to a *Pst*I site in *pufL*, probes that are 5′ end labeled at the *Ava*II site upstream of *pufQ* would detect only *puf* transcripts encoded by the plasmids. Identical clusters of 5′ ends were observed for RNA derived from mutant or wild-type *puf-neo* constructs and extracted from bacterial strains grown under either high- or low-oxygen conditions (Fig. 3). These 5′ ends mapped to the same location as the previously identified site of initiation of *puf* operon transcription (1).

The concentration of mRNA encoded by the wild-type *puf*-neo gene fusion in *R. capsulatus* B10 peaked 20 min after a shift from high to low oxygen (data not shown); therefore, samples for *puf-neo* mRNA quantitation were taken at that time. Figure 4 shows a typical slot blot of total cellular RNA hybridized to a *neo* gene probe. Quantitation of data from such hybridizations by liquid scintillation counting (Table 2 indicated that under high-oxygen growth, the amounts of *neo*-hybridizable RNA from the mutant constructs was two-

TABLE 1. LD_{50} values and APH(3′) activities for *puf-neo* fusion constructs

Plasmid[a]	APH(3′) activity[b]					Position(s) of mutated base	LD_{50}[c]
	Aerobic	Low O_2	A	B	C		
None							~2
pMLN2[d]	460 ± 110	6,490 ± 1,310			14	None	20
pMLN510	4,540 ± 190	15,520 ± 4,150	10	2	3	184	90
pMLN805	2,290 ± 310	14,400 ± 1,210	5	2	6	193	60
pMLN101	3,370 ± 1,210	19,890 ± 4,680	7	3	6	184	50
pMLN102	3,440 ± 350	16,480 ± 3,670	8	3	5	184	90
pMLN84	3,550 ± 250	17,710 ± 890	8	3	5	184	70
pMLN8493	9,940 ± 400	27,260 ± 2,730	22	4	3	184, 193	130

[a] Plasmids with 500, 800, and 100 series numbers were selected by growth of *R. capsulatus* B10 on kanamycin at 50, 80, and 100 μg/ml, respectively. The mutations in pMLN84 and pMLN8493 were made by oligonucleotide-directed mutagenesis.

[b] APH(3′) is expressed as counts per minute per microgram[c] protein per hour. The data represent the averages of APH(3′) activity from three different cell extracts. Controls consisted of B10 lacking a plasmid and B10 carrying pMLN1 (approximately 40 cpm/μg of protein per h, which was subtracted from the data shown above). A, Ratio of the aerobic APH(3′) activity of the mutant to that of the wild-type strain; B, ratio of the low-O_2 APH(3′) activity of the mutant to that of the wild-type strain; C, ratio of low-O_2 to aerobic APH(3′) activity (induction) for each strain.

[c] LD_{50} indicates the Km^r (micrograms per milliliter) was lethal to 50% of colonies tested on PAA plates under aerobic growth conditions. Average of three determinations.

[d] Wild type.

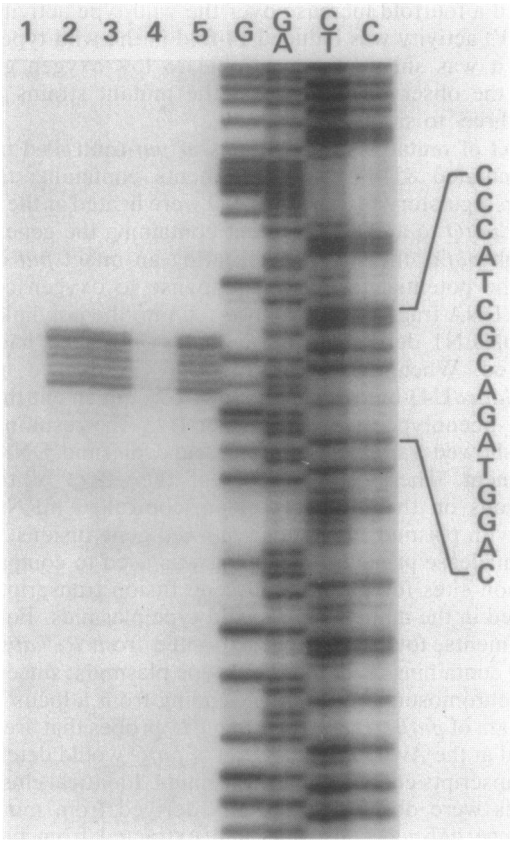

FIG. 3. S1 nuclease protection mapping of 5′ ends from mutant and wild-type *puf* operon constructs. Double-stranded DNA probes derived from plasmid pMLN2 or pMLN8493 were 5′ end labeled at an *Ava*II site (see reference 1, Fig. 4, bp 387) and digested at the *Sac*I site in the polylinker region of the plasmids. Ten nanograms of probe was hybridized to 6 μg of *E. coli* tRNA (lane 1) or 6 μg of total RNA extracted from *R. capsulatus* A3119 containing pMLN8493 (lanes 2 and 3) or pMLN2 (lanes 4 and 5) and grown under high-oxygen conditions (lanes 2 and 4) or low-oxygen conditions (lanes 3 and 5). All samples were treated with 1,200 U of S1 nuclease. A portion of the wild-type probe was cleaved chemically by the sequencing technique of Maxam and Gilbert (28). The sequence notation on the right corresponds to the sequence of the coding strand.

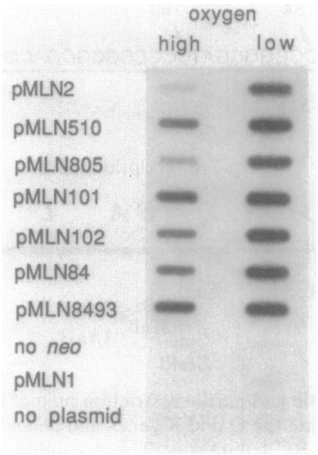

FIG. 4. Autoradiogram of a typical slot blot of total RNA isolated from strains grown under high- or low-oxygen conditions, as described in the text. *neo*-hybridizable mRNA was analyzed by blotting 4 μg of total RNA onto GeneScreenPlus hybridization transfer membranes (DuPont, NEN Research Products) and hybridization, following the protocol recommended by the manufacturer, to the 900-bp *Pst*I fragment from the Tn*5* *neo* gene, which was radiolabeled by random priming (15). The membranes were then exposed to X-ray film (Kodak XAR5) for approximately 3 h; the radioactive bands were cut out and quantitated by liquid scintillation counting in 7 ml of Ready Protein$^+$ (Beckman Instruments, Inc.).

to eightfold greater than for the strain carrying a plasmid containing the wild-type *puf* upstream region; the double mutant showed the greatest amount of *neo*-specific RNA synthesis. Under low-oxygen growth, the mutants had about the same concentration of *neo* RNA as that in the wild-type strain. All of the mutants except pMLN8493 showed at least another twofold increase in *neo*-hybridizable counts when the cultures were shifted from high to low oxygen.

DISCUSSION

We isolated and characterized mutations that affect oxygen responsiveness of the region 5′ to the *puf* operon structural genes. Four independent mutants, three of which contained mutations at the same base pair, were obtained by selecting *R. capsulatus* strains that failed to show wild-type repression of Km^r under aerobic growth conditions following linkage of the mutagenized *puf* regulatory region to a Tn*5*-derived *neo* gene. All of the mutations obtained were located in a region of dyad symmetry found about 45 bp upstream of the *puf* transcription start site. Under aerobic growth conditions, the mutant *puf-neo* gene fusions showed diminished repression of the *puf*-linked *neo* gene as assayed by LD_{50} on kanamycin, APH(3′) activity, and mRNA synthesis.

Previous deletion analysis has indicated that *cis*-acting elements involved in the oxygen-regulated expression of the *puf* operon are located within a region extending 70 bp upstream from the *puf* operon transcriptional start (1, 3), and Bauer et al. have noted that sequences located between bp 204 and 218 show homology to the consensus sequence for promoters transcribed by RNA polymerase utilizing the *ntrA* sigma subunit (3). The point mutations described here mapped to a region of dyad symmetry that overlaps the DNA segment that has homology to the *ntrA* consensus sequence. Deletion of the region of dyad symmetry at bp 179 to 208 or the change of base pairs 225 and 227 from A's to G's greatly

TABLE 2. *neo*-hybridizable RNA

Plasmid in *R. capsulatus*	Amt of hybridizable RNA[a]					Mutated base(s)
	Aerobic (cpm)	Low O_2 (cpm)	A	B	C	
pMLN2[b]	180 ± 50	1,360 ± 270			7	None
pMLN510	1,130 ± 310	3,130 ± 970	6	2	3	184
pMLN805	300 ± 40	1,260 ± 230	2	1	4	193
pMLN101	720 ± 90	1,580 ± 80	4	1	2	184
pMLN102	470 ± 40	1,360 ± 160	3	1	3	184
pMLN84	700 ± 180	1,650 ± 180	4	1	2	184
pMLN8493	1,420 ± 160	1,640 ± 280	8	1	1	184, 193

[a] Counts per minute represent the averages of data from slot blots of three different RNA preparations and have been corrected for background hybridization by subtracting the counts (approximately 240 cpm) that hybridized to RNA isolated from the control strain carrying pMLN1. A, Ratio of the amount of *neo* mRNA in the mutant to that in the wild-type strain under aerobic conditions; B, ratio of the amount of *neo* mRNA in the mutant to that in the wild-type strain under low-O_2 conditions; C, ratio of the low-O_2 amount to the aerobic amount of *neo* mRNA for each strain.

decreases expression of the *puf* operon (1). Since constructs carrying mutations at positions 184 and/or 193 failed to show wild-type repression of *puf* expression during growth under aerobic conditions, we postulate that the region of dyad symmetry that contains the mutated sites is required for both transcription and oxygen regulation.

S1 mapping of 5′ ends of mRNA showed identical transcript start sites for wild-type and mutant constructs (Fig. 3). Under low-oxygen conditions, mRNA concentrations in the mutant strains were approximately the same as the wild-type concentrations while APH(3′) activity was two- to fourfold that of the wild type. The concentration of mRNA encoded by the wild-type *puf-neo* gene fusion peaked 20 min after a shift to low oxygen, while the APH(3′) activity continued to increase for up to 8 h after the shift to low-oxygen growth (unpublished data). This difference may reflect differences in the stability of the *neo* gene protein product versus the *puf-neo* mRNA.

Sequence analysis of pMLN510, pMLN101, and pMLN102 showed that all of these plasmids had mutations at bp 184. The point mutation of pMLN84 was introduced by oligonucleotide-directed mutagenesis. However, the LD_{50} on kanamycin, the concentration of *neo* mRNA, and the APH(3′) activity for these plasmids was not the same; the reason for the variation in the phenotypes remains unclear. The concentrations of *neo*-specific mRNA obtained for pMLN101 under aerobic or low-oxygen growth conditions were approximately the same as those observed for pMLN84, while pMLN102 had a lower and pMLN510 had a higher mRNA concentration than pMLN84. Additional mutations have not been detected by sequence analysis in the 857-bp *puf* inserts of pMLN102 and pMLN510.

The double mutant, pMLN8493, showed a concentration of *neo* mRNA under aerobic growth conditions that was about the same as the mRNA concentration obtained for pMLN2 (which contains the wild-type *puf* regulatory region) under low-oxygen growth conditions. Moreover, mRNA did not increase significantly when pMLN8493 was shifted to low oxygen, suggesting that the two point mutations in this construct render *puf* transcription fully or almost fully constitutive. The remaining mutants showed a higher concentration of *neo* mRNA than the wild-type strain under aerobic conditions; however, they still were induced to some extent when shifted to low oxygen. While the *cis*-acting mutations we have isolated clearly affect regulation of *puf* operon gene expression by oxygen, more precise analysis of the quantitative aspects of their effects may require introduction of the mutated sites into the *R. capsulatus* chromosome, where they will be free from the influence of copy number effects and superhelicity differences between plasmid and chromosome and can be studied in the context of the recently discovered overlapping transcription that reads into the *puf* operon from the *bchA* gene (35).

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