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 Tn5 neo gene, which encodes resistance to kanamycin (Km'). *R. capsulatus* mutants that failed to show wild-type repression of Km' 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 oxygen and consequently implicated the mutated region in O₂-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 phototrophic 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 rccA 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 (pO₂ 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 (pO₂ of 1 to 3%). Difco Antibiotic Medium 2 (Penassay base agar [PAA]) plates were used for selection of Km' mutants. pMLN1 is an RK2-based promoter-cloning vector derived from pTJS133 (31); it contains a promoterless neo gene from Tn5. 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 SfiI site 903 bp upstream of pufB (37) and a PstI site in the pufL gene (36) with the spectinomycin resistance gene located on an EcoRI fragment in plasmid pXJS5425 (18).

**Mutagenesis and selection for mutants.** SacI and XbaI 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 KpnI 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 SacI-XbaI fragments and introduced into *Escherichia coli* MC1061 (11) by transformation (13). Three plates, the first containing individual colonies of *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 pKK2073 (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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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′ during aerobic growth of *R. capsulatus*. Km′ *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 *SacI-XbaI* 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*′ fragment was mutagenized to change the C at position 184 to T, using the oligonucleotide 5′-GGATCGCAGCGATCGCCGAGG-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′-GGCGCGTGAATTCTGCGGCAAGG-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→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-32P]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).

**SI 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 *SacI-XbaI* 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 *SacI-XbaI* *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...
entirely sufficient to account for the observed expression of puf-linked Km' 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 (LD₅₀) of kanamycin for both hydroxyamine-induced oligonucleotide-directed mutagenesis-derived mutants was compared with the LD₅₀ for the neo-linked wild-type puf operon regulatory region (Table 1). Strains containing point mutations in the puf upstream region showed LD₅₀s 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'.

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 EcoRI 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 poly linker 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 PstI site in pufL, probes that are 5' end labeled at the AvaII 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. LD₅₀ values and APH(3’) activities for puf-neo fusion constructs

<table>
<thead>
<tr>
<th>Plasmid*</th>
<th>LD₅₀**</th>
<th>APH(3’) activityb</th>
<th>Position(s) of mutated base</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>~2</td>
<td>None</td>
<td>20</td>
</tr>
<tr>
<td>pMLN2(ab)</td>
<td>460 ± 110</td>
<td>6,490 ± 1,310</td>
<td>14</td>
</tr>
<tr>
<td>pMLN510</td>
<td>4,540 ± 190</td>
<td>15,520 ± 4,150</td>
<td>10, 2, 3</td>
</tr>
<tr>
<td>pMLN805</td>
<td>2,290 ± 310</td>
<td>14,400 ± 1,210</td>
<td>5, 2, 6</td>
</tr>
<tr>
<td>pMLN101</td>
<td>3,370 ± 1,210</td>
<td>19,890 ± 4,680</td>
<td>7, 3, 6</td>
</tr>
<tr>
<td>pMLN102</td>
<td>3,440 ± 350</td>
<td>16,480 ± 3,670</td>
<td>8, 3, 5</td>
</tr>
<tr>
<td>pMLN84</td>
<td>3,550 ± 250</td>
<td>17,710 ± 890</td>
<td>8, 3, 5</td>
</tr>
<tr>
<td>pMLN8493</td>
<td>9,940 ± 400</td>
<td>27,260 ± 2,730</td>
<td>22, 4, 3</td>
</tr>
</tbody>
</table>

* 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.

* APH(3') is expressed as counts per minute per microgram 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 rpm/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-O₂ APH(3') activity of the mutant to that of the wild-type strain; C, ratio of low-O₂ to aerobic APH(3') activity (induction) for each strain.

** LD₅₀ indicates the Km' (micrograms per milliliter) was lethal to 50% of colonies tested on PAA plates under aerobic growth conditions. Average of three determinations.

* Wild type.
to eightfold greater than for the strain carrying a plasmid 
containing the wild-type puf operon 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 oxy-
gen 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′ under aerobic growth conditions following linkage of the mutated puf regulatory region to a 
Tn5-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 LD50 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

**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 AvaiII site (see reference 1, Fig. 4, bp 387) and digested at the 
Sacl site in the polynucleotide region of the plasmids. Ten nanograms of 
probe was hybridized to 6 μg of *E. coli* rRNA (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.** Autoradiograph of a typical slot blot of total RNA iso-
lated 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 hybrid-
ization, following the protocol recommended by the manufacturer, 
to the 900-bp PstI fragment from the Tn5 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 C (Beckman Instruments, Inc.).

**TABLE 2.** neo-hybridizable RNA

<table>
<thead>
<tr>
<th>Plasmid in <em>R. capsulatus</em></th>
<th>Amt of hybridizable RNA*</th>
<th>Mutated base(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic (cpm)</td>
<td>Low O2 (cpm)</td>
</tr>
<tr>
<td>pMLN2*</td>
<td>180 ± 50</td>
<td>1,360 ± 270</td>
</tr>
<tr>
<td>pMLN510</td>
<td>1,130 ± 310</td>
<td>3,130 ± 970</td>
</tr>
<tr>
<td>pMLN805</td>
<td>300 ± 40</td>
<td>1,260 ± 230</td>
</tr>
<tr>
<td>pMLN101</td>
<td>720 ± 90</td>
<td>1,580 ± 80</td>
</tr>
<tr>
<td>pMLN102</td>
<td>470 ± 40</td>
<td>1,360 ± 160</td>
</tr>
<tr>
<td>pMLN84</td>
<td>700 ± 180</td>
<td>1,560 ± 180</td>
</tr>
<tr>
<td>pMLN8493</td>
<td>1,420 ± 160</td>
<td>1,640 ± 280</td>
</tr>
</tbody>
</table>
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 mRNAs 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 change 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 in pMLN84 was introduced by oligonucleotide-directed mutagenesis. However, the LD90 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 puff 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 puff 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 puff 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 puff operon from the bchA gene (35).

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LITERATURE CITED


