Isolation and Characterization of trans-Acting Mutations Involved in Oxygen Regulation of puc Operon Transcription in Rhodobacter sphaeroides

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Transcriptional expression of the puc operon in Rhodobacter sphaeroides 2.4.1 is dependent on the partial pressure of oxygen. By using transcriptional fusions in trans of a promoterless fragment derived from the aminoglycoside-3'-phosphotransferase gene of Tn903 to puc operon-specific DNA containing a 629-bp 5' cis-acting regulatory region involved in the expression of puc-specific mRNA, we selected Km' colonies under aerobic conditions. Two broad classes of mutations, trans and cis, which are involved in O₂ control of puc operon transcription, fall into several distinct phenotypic classes. The cis-acting regulatory mutations are characterized in detail elsewhere (J. K. Lee and S. Kaplan, J. Bacteriol. 174:1146-1157, 1992). Two trans-acting regulatory mutants, CL1a and T1a, which are B800-850−Car⁺ and apparently B875⁺, respectively, were shown to derepress puc operon transcription in the presence of oxygen. The mutation giving rise to CL1a has been shown to act at the puc operon-specific cis-acting upstream regulatory region (~629 to ~92). On the other hand, the mutation giving rise to T1a, identifying a second trans-acting regulatory factor(s), appears to act at both the upstream (~629 to ~92) and the downstream (~92 to ~1) regulatory regions of the puc operon as well as at the level(s) of bacteriochlorophyll and carotenoid biosyntheses, as revealed by the presence of the B800-850 complex under chemoheterotrophic growth conditions. Both the B800-850−Car⁺ phenotype and the trans-acting effect on puc operon expression in mutant CL1a were complemented with a 2.2-kb DNA fragment located within the carotenoid gene cluster. Mutant T1a was complemented with a 7.0-kb EcoRI restriction fragment containing the puhA gene and its flanking DNA (6.3 kb) to restore expression of the B875 complex and to suppress the trans-acting effect resulting in the loss of O₂ control. Under chemoheterotrophic conditions, mutant T1a was highly unstable, segregating into a PS− mutant designated T4a.

The puc operon of Rhodobacter sphaeroides consists of the pucBA structural genes (encoding the B800-850-β and -α polypeptides, respectively) and additional DNA sequences which extend approximately 1.8 kb immediately downstream and which encode a gene product(s) apparently involved in the posttranslational regulation or assembly of the pucBA gene products, resulting in the formation of the B800-850 light-harvesting complex (10, 16, 17). In a related bacterium, Rhodobacter capsulatus, involvement of the gene products encoded by the genes pucCDE immediately downstream of pucBA in the formation of the B800-850 complex has also been reported (35). When transcribed, the puc operon of R. sphaeroides yields 0.5- and 2.3-kb puc-specific transcripts. The transcripts share the same 5’ end, which is localized 117 nucleotides upstream of the start of the pucB gene (17) and, as previously demonstrated, transcriptional expression of both transcripts is highly regulated by both oxygen and light (10). For R. capsulatus, two 5’ ends, approximately 125 and 110 nucleotides upstream of the start of pucB, have been reported (38).

Recently, Narro et al. (21) reported cis-acting mutations affecting O₂ regulation of the expression of the R. capsulatus puf operon. The mutations were localized approximately 45 bp upstream (at a region of dyad symmetry) of the 5’ end of the Q transcript. The region of dyad symmetry located upstream of the Q gene was suggested to be a binding site(s) of a protein(s), yet unknown, which may be involved in O₂ regulation of puf operon expression (13). Klug and Jock (14) further suggested that the cis-acting mutation within the Q gene upstream region could result in an altered regulation of puc operon expression by the induction of one or more secondary-site trans-acting mutations. The precise site(s) or mechanism(s) has not been addressed.

Because the B800-850 spectral complex can be gratuitous for photosynthetic growth in R. sphaeroides (20) and additionally because the puc operon shows the greatest extremes in light regulation (11) as well as normal O₂ regulation (11), this operon was chosen for the study of cis- and trans-acting regulatory elements affecting its expression. To understand the oxygen-dependent regulation of puc operon transcription mediated through the action of a trans-acting factor(s), we have used puc-aph translational fusion constructs to isolate trans-acting regulatory mutations which result in derepression of puc operon transcription in the presence of oxygen. Two different trans-acting mutants, CL1a (B800-850−Car⁺) and T1a, (apparently B875⁺), were chosen for further biochemical and genetic analyses of the regulatory mutations involved in the transcriptional regulation of the puc operon by oxygen. We have identified a 2.2-kb DNA fragment within the carotenoid gene cluster which complements both the B800-850− and the Car⁺ phenotypes and suppresses the loss of O₂ control of puc operon expression in mutant CL1a. A 7.0-kb EcoRI restriction fragment containing puhA as well as flanking DNA complements the B875− phenotype and suppresses the loss of O₂ control of puc operon transcription in mutant T1a. Additionally, under chemoheterotrophic conditions, mutant T1a was found to

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form the B800-850 complex and to be genetically very unstable. The studies reported here, together with our initial analysis of the cis-acting DNA sequences involved in the regulation of puc operon transcription (15), provide the first stages in our understanding of the roles of oxygen and light in the regulation of gene expression in R. sphaeroides. The availability of both physical (30) and genetic (31) maps of the R. sphaeroides genome dramatically extend the scope of such studies.

(A preliminary report of this work was presented at Pseudomonas 91 in Trieste, Italy, June 1991.)

MATERIALS AND METHODS

Bacteria, plasmids, and cell growth. All bacterial strains and plasmids used in this study are described in Table 1. R. sphaeroides wild-type strain 2.4.1 and its derivatives were grown as previously described (5). When appropriate, tetracycline, kanamycin, streptomycin, and spectinomycin were added to Sistrom’s minimal medium to final concentrations of 1, 20, 25, 50, and 50 μg/ml, respectively. Phototrophic growth of R. sphaeroides in the presence of tetracycline was accomplished as described previously (2). Cell growth was monitored by use of a Klett-Summerson colorimeter (no. 66 filter).

Escherichia coli JM109, DH5x, and S17-1 were grown at 37°C in Luria medium (24). Ampicillin, tetracycline, kanamycin, streptomycin, and spectinomycin (final concentrations, 50, 20, 25, 50, and 50 μg/ml, respectively) were added to the growth medium for E. coli strains carrying plasmids encoding these drug resistance genes. Plasmids pUC18, pUC19, pBS, pRK415, and pSUP202 were used for cloning.

DNA manipulation and Southern hybridization. Large-scale plasmid DNA was prepared by use of chloramphenicol-amplified Triton X-100 lysates of E. coli and successive equilibrium CsCl gradients (10). Small-scale plasmid DNA was prepared by alkaline sodium dodecyl sulfate lysis (24) or by the Brij (polyethylene glycol hexadecyl ether) lysis method (29). DNA was treated with restriction enzymes and other nucleic acid-modifying enzymes in accordance with manufacturer specifications. DNA fragments were analyzed on agarose gels or polyacrylamide gels, and restriction fragments were isolated as previously described (3). Southern hybridization analysis of genomic DNA was performed as described previously (3, 5). Endogenous plasmid profiles of indicated bacterial strains following SpeI digestion were analyzed by TAFE (transverse alternating-field electrophoresis) gel analysis and compared with those of the wild-type strain (32).

RNA isolation and Northern (RNA) hybridization. Isolation and quantitation of total RNA from R. sphaeroides, conditions for Northern blot hybridization analysis with 32P-labeled RNA probes, and quantitation of transcript signals following hybridization were as previously reported (17).

Conjugation technique. Plasmid pRK415- or RSFI101-derived plasmids were mobilized into R. sphaeroides by previously described procedures (2).

Preparation of cell extracts and assay of β-galactosidase. R. sphaeroides cultures used for the measurement of β-galactosidase activities were grown chemoheterotrophically or photoheterotrophically by sparging with gas mixtures as described previously (2). Cells grown chemoheterotrophically were harvested at a cell density of 1.0 × 10^8 to 3.0 × 10^8 cells per ml, and cells grown photoheterotrophically were harvested at a cell density of 4.0 × 10^8 to 1.0 × 10^9 cells per ml. Cell breakage with a French press, preparation of crude extracts, and β-galactosidase assays (at 30°C for 5 min) with α-nitrophenyl-β-galactoside hydrolysis were performed as described previously (33). All determinations were made in duplicate and repeated at least three times. Activities were reproducible to ±10 to 15%.

Spectrophotometric assay. Absorption spectra of R. sphaeroides cell-free extracts were analyzed with a Perkin-Elmer Corp. (Norwalk, Conn.) Lambda 4C spectrophotometer. The same concentration of protein (1 mg/ml) was used when the spectral profiles of different strains of R. sphaeroides were examined. Protein was determined by a modified Lowry method with bovine serum albumin as the standard (19).

Materials. Restriction endonucleases and nucleic acid-modifying enzymes were purchased from Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, Md., or New England BioLabs, Inc., Beverly, Mass., and used as specified by the manufacturer. The Klenow fragment of E. coli DNA polymerase I, proteinase K, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. [α-32P]dCTP (800 Ci/mmol) and [α-32P]dCTP (400 Ci/mmol) were obtained from Amersham Corp., Arlington Heights, Ill. Isopropyl-β-D-thiogalactopyranoside and α-nitrophenyl-β-galactoside were obtained from Sigma Chemical Co., St. Louis, Mo. Molecular biology-grade phenol was purchased from Fisher, Pittsburgh, Pa. All other chemicals used in this work were reagent grade.

RESULTS

Isolation of trans-acting mutations involved in puc operon expression. To provide positive selection for the isolation of a regulatory mutation(s) involved in oxygen control of puc operon transcription, we transcriptionally fused a promoterless fragment of the aminoglycoside-3'-phosphotransferase gene from Tn903 (22) to the puc regulatory DNA sequence comprising a 629-bp DNA fragment immediately upstream of the 5' end of the puc-specific transcripts.

We isolated a 1,070-bp Xhol-EcoRI restriction DNA fragment of the aph gene from plasmid pME1 (8) and cloned the fragment into the multiple cloning region of pRK415 in both orientations relative to lacPtetP of the plasmid (Fig. 1A, a and b). When the fragment was in the same orientation as lacPtetP of pRK415 in any of the three reading frames relative to the start codon of the lacZα-peptide, R. sphaerodes 2.4.1 containing each of the plasmid constructions (Fig. 1A, a) was Km' at a concentration of 25 μg/ml in Sistrom’s minimal medium. When the transcription-translation stop cartridge Ω Sm'/Sp' (23) was placed at the border between lacPtetP and the 1,070-bp Xhol-EcoRI fragment (Fig. 1A, c), R. sphaeroides containing plasmid pRK2 was Km'. The absence of the aph promoter on the 1,070-bp Xhol-EcoRI DNA fragment was confirmed by the Km' phenotype displayed by the wild type carrying a derivative of pRK415 containing the same 1,070-bp DNA fragment in the orientation opposite from that of lacPtetP (Fig. 1A, b).

We have also shown that a 1,150-bp BspHI-EcoRI restriction DNA fragment of the aph gene which contains an additional 80-bp of DNA upstream of the Xhol restriction site confers Km' in R. sphaeroides under all conditions, indicating that there is a functional promoter(s) on the 80-bp BspHI-Xhol restriction DNA fragment. Either the 799-bp PstI-XmnI (within pucB) or the 699-bp PstI-DraII (within the 5' leader region of the puc-specific transcripts) restriction DNA fragment was cloned between the transcription-trans-
TABLE 1. Bacterial strains and plasmids

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<th>Strains</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
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<tr>
<td>E. coli S17-1</td>
<td>Pro⁻ Res⁺ Mod⁺ recA⁺; integrated plasmid RP4-Tc::Mu-Km::Tn7</td>
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<td>R. sphaeroides 2.4.1</td>
<td>Wild type</td>
<td>W. R. Sistrom</td>
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<td>WT₁</td>
<td>Km' mutant derived from 2.4.1(pPXK-1); wild type-like</td>
<td>This study</td>
</tr>
<tr>
<td>Cl₁</td>
<td>Km' mutant derived from 2.4.1(pPXK-1); less pigmented; RS104 phenotype</td>
<td>This study</td>
</tr>
<tr>
<td>DR₁</td>
<td>Km' mutant derived from 2.4.1(pPXK-1); highly pigmented</td>
<td>This study</td>
</tr>
<tr>
<td>WT₁a</td>
<td>WT₁ cured of mutated pPXK-1⁺; wild type-like</td>
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<td>Cl₁a</td>
<td>Isolated from Cl₁ when cured of pPXK-1; O₂-insensitive expression of the puc operon; B800-850 Car⁻</td>
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<td>DR₁a</td>
<td>Isolated from DR₁ when cured of pPXK-1; trans-acting mutant affecting puc operon transcription; highly pigmented</td>
<td>This study</td>
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<td>CP₁a</td>
<td>Isolated from DR₁ when cured of pPXK-1; reduced B800-850 complex</td>
<td>This study</td>
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<td>T₀a</td>
<td>Isolated from DR₁ when cured of pPXK-1; O₂-insensitive expression of the puc operon; apparently B875⁺</td>
<td>This study</td>
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<td>DCL₁a</td>
<td>Isolated from DR₁ when cured of pPXK-1; O₂-insensitive expression of the puc operon; B800-850 Car⁻</td>
<td>This study</td>
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<td>PUC-ZWT</td>
<td>lacZYA⁺ Sm' Sm' F' inserted at the XmnI site within pucB of the wild type; B800-850 Car⁻</td>
<td>This study</td>
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<td>PUC-ZCL</td>
<td>lacZYA⁺ Sm' Sm' F' inserted at the XmnI site of pucB of Cl₁; B800-850 Car⁻</td>
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<td>Tₙ</td>
<td>Spontaneous mutant derived from T₀⁺; PS⁺ RC⁺ B875⁻ B800-850 Car⁻</td>
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<table>
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<th>Plasmids</th>
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<td>pRRK45</td>
<td>Tc⁺</td>
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<tr>
<td>pSUP202</td>
<td>Ap⁺ lacZYA⁻ Sm'-PrBA; Tc⁺</td>
<td>25</td>
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<td>pRS415</td>
<td>Ap⁺ lacZYA⁺ Sm' PrBA; Tc⁺</td>
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<td>pRS415(lacY)</td>
<td>pRS415 derivative + 2.0-kb ß Sm'/Sm' at the SnaBI site within lacY</td>
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<td>pRRK1</td>
<td>pRRK45 derivative + 1.07-kb Xhol-EcoRI Km' DNA (+); Tc⁺</td>
<td>This study</td>
</tr>
<tr>
<td>pRRK1</td>
<td>pRRK45 derivative + 1.07-kb Xhol-EcoRI Km' DNA (+); Tc⁺</td>
<td>This study</td>
</tr>
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<td>pRRK2</td>
<td>pRRK45 derivative + 2.0-kb ß Sm'/Sm' in the multiple cloning region but upstream of the</td>
<td>This study</td>
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<tr>
<td>pPXK-1</td>
<td>pRRK2 derivative + 0.8-kb PstI-DraI puc DNA between ß Sm'/Sm' and 1.07-kb Xhol-EcoRI Km' DNA; Tc⁺ Sm'/Sm'</td>
<td>This study</td>
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<tr>
<td>pPDF-1</td>
<td>pRRK2 derivative + 0.7-kb PstI-XmnI puc DNA between ß Sm'/Sm' and 1.07-kb Xhol-EcoRI Km' DNA; Tc⁺ Sm'/Sm'</td>
<td>This study</td>
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<tr>
<td>pRRK1</td>
<td>pRRK45 derivative + 1.5-kb EcoRI Km' DNA; Tc⁺</td>
<td>This study</td>
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<td>pCF100</td>
<td>pVh106 EcoRI Smol; 7.1-kb EcoRI-NruI fragment from ß Sm'/Sm' LacZYA⁺; Tc⁺ Sm'/Sm'</td>
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<td>pCF200 (–629)</td>
<td>pVh106 EcoRI Smol; 7.9-kb EcoRI-NruI fragment from ß Sm'/Sm' –puc (0.8-kb PstI-XmnI)-lacZYA⁺; Tc⁺ Sm'/Sm'</td>
<td>15</td>
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<td>pCF250 (–92)</td>
<td>pVh106 EcoRI Smol; 7.37-kb EcoRI-NruI fragment from ß Sm'/Sm' –puc (0.27-kb XmnI–XmnI)-lacZYA⁺; Tc⁺ Sm'/Sm'</td>
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<td>pCF260 (+70)</td>
<td>pVh106 EcoRI Smol; 7.2-kb EcoRI-NruI fragment from ß Sm'/Sm' –puc (0.13-kb DraI-XmnI)-lacZYA⁺; Tc⁺ Sm'/Sm'</td>
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<td>pCF200 Km (–629)</td>
<td>pCF200 derivative + 1.4-kb BamHI Km' DNA' in the NruI site of tet from pCF200; Km' Sm'/Sm'</td>
<td>This study</td>
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<td>pCF250 Km (–92)</td>
<td>pCF250 derivative + 1.4-kb BamHI Km' DNA' in the NruI site of tet from pCF250; Km' Sm'/Sm'</td>
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<td>pWS2</td>
<td>R6K derivative + 109 kb of R. sphaeroides WS8 DNA; Tc⁺ Sm'</td>
<td>27, 37</td>
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<td>Cosmid 487 (pU18487)</td>
<td>pLA2917 derivative + ca. 27 kb of R. sphaeroides 2.4.1.1 DNA containing puf; Tc⁺</td>
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<td>Cosmid 523 (pU18523)</td>
<td>pLA2917 derivative + ca. 22 kb of R. sphaeroides 2.4.1.1 DNA containing pufA and cycA; Tc⁺</td>
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<td>pAS203</td>
<td>pRRK45 derivative + 11 kb of R. sphaeroides 2.4.1 DNA from cosmid 487 containing crt; Tc⁺</td>
<td>32a</td>
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<td>pAS204</td>
<td>pRRK45 derivative + 2.2 kb of R. sphaeroides 2.4.1 DNA from pAS203 containing crt; Tc⁺</td>
<td>32a</td>
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<td>pAS205</td>
<td>pRRK45 derivative + 7.5 kb of R. sphaeroides 2.4.1 DNA from pAS203 containing crt; Tc⁺</td>
<td>32a</td>
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<td>pUI803</td>
<td>pRRK45 derivative + 7.0 kb of R. sphaeroides 2.4.1 DNA from cosmid 523 containing pufA; Tc⁺</td>
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<td>pUI811</td>
<td>pRRK45 derivative + 6.5 kb of R. sphaeroides 2.4.1 DNA from cosmid 523 containing cycA; Tc⁺</td>
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<td>pUI813</td>
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<td>pPXK-93</td>
<td>pPXK-1 derivative; NruI fragment of tetA deleted; Sm'/Sm'</td>
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<td>pUI601::lacO(XmnI)</td>
<td>pUC19 PstI; 2.5-kb PstI fragment of lacZ: ß Sm'/Sm' A' inserted at the XmnI site of pucB; Ap⁺ Sm'/Sm'</td>
<td>This study</td>
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<td>pSUPPUC::lacO(XmnI)</td>
<td>pSUP202 derivative + 9.46-kb PstI fragment of pUI601::lacO(XmnI); Tc⁺ Sm'/Sm'</td>
<td>This study</td>
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* RC⁺, devoid of the reaction center.
* The transcriptional orientation of the inserted DNA fragment is indicated as being either the same as that of the lac promoter (+) or opposite that of the lac promoter (−).
* The 5’ overhangs were made blunt ended with the Klenow fragment of DNA polymerase I before cloning.
lution stop cartridge \( \Omega \) Sm\(^{r}/\)Sp\(^{r}\) and the 1,070-bp *XhoI-EcoRI* promoterless *aph* fragment of pRK415 to generate pPXK-1 or pPDK-1, respectively (Fig. 1B, a and b). Under aerobic growth conditions, the wild type carrying either pPXK-1 or pPDK-1 in *trans* was Km\(^{r}\) while the wild type carrying the larger, promoter-containing Km\(^{r}\) DNA fragment, pRK415 (Fig. 1B, c), as a control was Km\(^{s}\), as expected.

Thus, transcriptional repression of the *puc* operon by oxygen resulted in little or no expression of *aph* present on either pPXK-1 or pPDK-1 when cells were grown aerobically, yielding a Km\(^{s}\) phenotype. Under anaerobic, dimethyl sulfoxide (DMSO) growth conditions (5), the wild type carrying either pRK415 or pPXK-1 was Km\(^{r}\). However, pPXK-1 supported the growth of *R. sphaeroides* under anaerobic, dark conditions at kanamycin concentrations of up to 100 \( \mu \)g/ml. On the other hand, the MIC of kanamycin for the growth of the wild type carrying pPDK-1 under anaerobic, dark growth conditions was in the range of 6 to 8 \( \mu \)g/ml. The reasons for the low and high MICs of kanamycin with pPDK-1 and pPXK-1, respectively, under anaerobic, dark conditions are as follows. The start codon of the *aph* gene is preceded by a very poor ribosome binding sequence, which was responsible for the low MIC observed for the wild type carrying pPDK-1 in *trans* under anaerobic, dark growth conditions. In the case of pPXK-1, the presence of the additional upstream ribosome binding sequence provided by *pucB* resulted in the increased expression of the downstream *aph* gene, as observed in certain two-cistron constructs (18). This effect on the translational activity of a downstream *lacZ* gene due to the presence of a ribosome binding sequence provided by *pucB* upstream of the reporter molecule has also been observed with the transcriptional fusion constructs involving *puc::lacZ* (15).

**Isolation and identification of trans-acting mutations af-**
FIG. 3. Absorption spectra of the three phenotypic classes of O₂ control mutants. The numbers in parentheses express the frequencies of occurrence of each type of mutant as the number of Km' mutants per total number of viable cells. The spectra were generated by use of identical amounts of protein (1 mg/ml) from crude cell-free lysates isolated from cells grown under anaerobic, dark, DMSO conditions and harvested at 50 to 100 Klett units. The bar represents an absorbance value of 0.1.

Since Km' could result from mutations either cis or trans to the aph gene, it was necessary to localize each mutation as depicted in the scheme shown in Fig. 2. Plasmid DNA (originally pPXK-1) was isolated from colonies in each of the three classes and ultimately mobilized back into wild-type R. sphaeroides 2.4.1, and the kanamycin sensitivities of the exconjugants were tested under aerobic growth conditions. The exconjugants carrying the plasmids derived from two separate WT colonies (WT₁ and WT₂) had a Km' phenotype, indicating that a mutation(s) leading to Km' was carried on the plasmid (pPXK-1*) in a cis configuration to the Km' gene. On the other hand, exconjugants that received plasmids derived from any of the CL (CL₁ and CL₂) or DR (DR₁ and DR₂) isolates had a Km' phenotype, suggesting that the location of the mutation(s) conferring Km' was on the chromosomal DNA of the original isolate, i.e., CL₁, CL₂, DR₁, or DR₂ (Fig. 2A).

To confirm the location of the mutational site(s) giving rise to a Km' phenotype in WT, CL, and DR colonies more precisely, we cured each of the isolates chosen for further study of its plasmid (Fig. 2B). WT₁a and CL₁a had the same
Absorption spectra (Fig. 4A and B) as WT1 and CL1, respectively (Fig. 3). The spectra of WT2a and CL2a are not shown but were identical to those of WT1a and CL1a, respectively. However, DR1 generated no less than four different phenotypic segregants, DR1a, CP1a, T1a, and DCL1a, during plasmid curing, as was also observed during curing of the plasmid from DR1. Each of the four segregants derived from curing of the plasmid present in DR1 had unique spectral properties (Fig. 4C). T1a appeared to be B875" and was similar to strain RS103 (20). Interestingly, DCL1a was similar to the CL strains (Fig. 4B), which were derived through an entirely different route, suggestive of the interactions between the mutations responsible for oxygen regulation of puc operon expression in CL- and DR-derived strains (see below).

Confirmation that the mutations in strains DR1a, T1a, and DCL1a were located on the chromosome(s) of R. sphaeroides 2.4.1 was carried out as depicted in Fig. 2B. A similar analysis was performed on WT1a and WT2a, 2.4.1, and CL1a and CL2a (Fig. 2B). These data provide convincing evidence that the mutation(s) conferring Km" in either CL1 or CL2 was not cis to the reporter gene but resided within the genome of the derived mutants, indicating the trans-acting nature of the mutation(s). On the other hand, the mutation(s) conferring Km" in either WT1 or WT2 was unambiguously demonstrated to reside on plasmid pPXK-1*. Of the four separate segregants derived from DR1, three had a Km" phenotype and one had a Km" phenotype under aerobic conditions when the normal plasmid construction was reintroduced as outlined in Fig. 2B. CP1(pPXK-1), which had a Km" phenotype under aerobic conditions, was not analyzed further. The cis-acting mutations are the subject of another study and are described in the accompanying paper (15). However, we have shown that the DNA sequence upstream of the puc operon can be divided into two regions, the upstream regulatory sequence (URS), from −629 to −150, and the downstream regulatory sequence (DRS), from −150 to −1. The URS contains sequences involved in O2 repression and light control, and the DRS contains overlapping FNR (fumarate nitrate reductase) and IHF (integration host factor) sequences, a promoter-activator region, and two highly similar sequences of dyad symmetry from −150 to −1. The region from −92 to −1 is sufficient for aerobic and anaerobic expression and derepression of the puc operon, but upstream sequences are essential for the full expression of the puc operon via an interaction of the URS with DRS.

We have chosen CL1a (B800-850 Car") and T1a (apparently B875") for further detailed biochemical and genetic analyses of trans-acting mutations which appear to result in the loss of oxygen control of puc operon transcription. Thus, what is the nature of each mutation leading to an altered regulation of puc operon expression, and how is that mutation related to the loss of a specific light-harvesting activity?

Derepression of puc operon transcription in the presence of oxygen in trans-acting mutants CL1a and T1a. To more accurately assess the extent of the defect leading to the loss of O2 control in mutants CL1a and T1a, we further analyzed biochemically O2 control of puc operon expression.

(i) β-Galactosidase activities in response to the presence of pCF200(−629) and pCF250(−92) in CL1a and T1a in the presence of O2. The puc::lacZ transcriptional fusion construct pCF200(−629) (Fig. 5) contains both the puc URS (−629 to −150; light and oxygen control; 15) and the puc DRS (−150 to −1; anaerobic control; 15), while pCF250 (−92) is confined by the XmalIII (−92) restriction site within the DRS and contains suspected promoter and operator

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**FIG. 4.** Absorption spectra of mutant classes following curing of plasmid pPXK-1 or mutated pPXK-1. Cells were grown phototrophically at 10 W/m² as described in the text. Absorption spectra were obtained as described in the legend to Fig. 3. The spectral profiles of WT1a and CL1a were similar to those of their parental Km" mutants, WT1 and CL1, respectively, in Fig. 3. (C) Four different classes of segregants derived from strain DR1. The bar represents an absorbance value of 0.1.
sequences involved in transcription and anaerobic control of puc operon expression (15). pCF260(+70) and pCF100 contain puc operon sequences which map to the 5′ leader of the puc-specific transcripts and within the B gene of the puc operon, respectively, and were used as negative controls. The number in parentheses following the plasmid designation denotes, in base pairs, the extent of the puc operon sequence relative to the start site of transcription.

As reported in the accompanying paper (15), pCF200 (−629) and pCF250(−92) showed low background levels of β-galactosidase activity in trans in the wild type under aerobic conditions because of the O2-dependent nature of puc operon expression. Under identical conditions, pCF200 (−629) in CL1α showed high, approximately 40-fold, derepression of β-galactosidase activity compared with that of pCF200(−629) in the wild type. However, pCF250(−92) showed no derepression in oxygen control of β-galactosidase activity in CL1α. These results strongly suggest that the mutation affecting O2 control of puc operon transcription in CL1α not only is trans-acting but also acts on or requires for its activity the 536-bp PstI-XmaIII (−629 to −92) puc upstream DNA.

The second trans-acting mutant, T4a, was also examined for its ability to affect the expression of the lacZ gene present on pCF200(−629) and pCF250(−92) under aerobic conditions. Unfortunately, under aerobic conditions, T4a is genetically very unstable and mutates at a high frequency to a PS− phenotype; the mutant is designated T4d. T4d is virtually devoid of any of the light-harvesting complexes (BB800-850− B875−) as well as the reaction center complex and all pigmentation (Car−) under anaerobic, dark DMSO conditions (see below and Fig. 10A). To maintain a pure culture of T4d in the presence of O2, we placed a puc::aph transcriptional fusion in trans on pPXK-93 into T4a and cultured T4a(pPXK-93) aerobically in the presence of kanamycin. Although T4a(pPXK-93) cells can still spontaneously mutate to T4d(pPXK-93) cells under aerobic conditions, T4a(pPXK-93) will be killed by kanamycin because puc operon expression is turned off in the T4d mutant strain. Each of the puc:: lacZ transcriptional fusion plasmids (IncQ or IncP4) and pPXK-93 (IncP1) were maintained together in T4a in the presence of kanamycin under aerobic conditions, and β-galactosidase activities were monitored. When present in T4a, both pCF200(−629) and pCF250(−92) in the presence of oxygen showed high levels of β-galactosidase activities, indicating that the mutation involved in O2 control of puc operon transcription in T4a involves a second, different trans-acting factor which, in addition to acting on the 536-bp PstI-XmaIII upstream puc DNA, also acts on the immediately downstream puc DNA (−92 to −1).

(ii) Northern hybridization analysis of puc-, puf-, and puhA-specific transcripts in CL1α, CL12a, and the wild-type strain were grown under both aerobic and photosynthetic (10 W/m2) conditions, and the mRNA levels for the puf, puhA, and puc operons were determined to directly assess the effect of the mutation present in CL1α. Under aerobic conditions, the level of the 0.5-kb puc-specific transcript (17) in CL1α (Fig. 6, lane 3) was at least five- to sevenfold higher than that in the wild type grown identically (Fig. 6, lane 1). After prolonged exposure of the X-ray film, we were able to observe the presence of the 2.3-kb puc-specific transcript in CL1α (Fig. 6, lane 3) versus the wild type (Fig. 6, lane 1, and data not shown), suggesting that O2 control of puc operon expression in CL1α acts on both the 2.3-kb puc-specific

![Image](http://jb.asm.org/)

FIG. 6. Northern blot hybridization analysis of puc operon expression in R. sphaeroides 2.4.1 (lanes 1 and 2) and CL1α (lanes 3 and 4). The RNAs were prepared from each of the strains grown chemoheterotrophically (lanes 1 and 3) or photoheterotrophically at 10 W/m2 (lanes 2 and 4) as described in the text. An RNA probe corresponding to an XmaIII restriction DNA fragment extending from 211 nucleotides upstream of pucB to the third base of the second-to-last amino acid of pucA was used.
transcript and the 0.5-kb puf-specific transcript. Under photosynthetic (10 W/m²) conditions, however, the 0.5-kb puf-specific transcripts in both CL1a and the wild type (Fig. 6, lanes 1 and 2, respectively) were present at levels similar to one another, i.e., approximately 70- and 115-fold higher than the level of the 0.5-kb puf-specific transcript in the wild type under aerobic conditions (Fig. 6, lane 1). Thus, the effect of the mutation in CL1a appears to occur primarily under aerobic conditions.

Each of the three puf-specific transcripts (0.5, 0.7, and 2.7 kb; 3) in CL1a grown under aerobic and photosynthetic conditions was present at levels almost identical (within 30%) to those of each of the same three puf-specific transcripts in the wild type under the corresponding growth conditions (Fig. 7A). This result suggests that the transacting mutation in CL1a is not involved in the regulation of puf operon transcription by oxygen.

The puhA-specific transcript in the wild type was approximately eightfold more abundant under photosynthetic conditions than under aerobic conditions, as reported previously (4) (Fig. 7B, lanes 1 and 2). However, in CL1a, the puhA-specific transcript was present at similar levels regardless of the growth conditions. Interestingly, these levels were about 3.5-fold higher than that in the wild type under aerobic conditions but severalfold lower than that in the wild type grown photosynthetically (Fig. 7B, lanes 3 and 4). Thus, the steady-state level of the chemoheterotrophically derived 1.13-kb puhA-specific transcript in CL1a was derepressed compared with that in the wild type, although the level of the puhA-specific transcript in CL1a under chemoheterotrophic conditions was about 50% that in the wild type under the same conditions. Whether this effect on puhA operon expression by the mutation in CL1a is direct or indirect remains to be determined.

(iii) Construction of PUC-ZWT and PUC-ZCL, containing chromosomally localized puc::lacZ transcriptional fusions. Mutant CL1a containing pCF200(−629) in trans had β-galactosidase levels about 40-fold higher than those in the wild type under aerobic conditions (Fig. 5). However, Northern hybridization analysis of the puc-specific transcripts in CL1a revealed only five- to sevenfold derepression in puc operon expression. This latter measure of derepression is in the range of that observed in the wild type making the transition from aerobic to high-light photosynthetic conditions (10).

This discrepancy could be due to (i) copy number effects, (ii) differences in the local DNA structures of the puc upstream regulatory regions present on the plasmid versus the chromosome, (iii) the stability of the puc-specific transcripts in CL1a under aerobic conditions, or (iv) any combination of the above. To address this question, we interrupted the chromosomal copy of the puc operon by the insertion of a puc::lacZ transcriptional fusion through homologous recombination with the wild type and CL1a to generate strains PUC-ZWT and PUC-ZCL, respectively (Fig. 8A). We then proceeded to measure the β-galactosidase activities in each of the two strains under both aerobic and photosynthetic conditions (Fig. 8B).

The pucB gene on pUL601 (10) was interrupted at the XmnI restriction site with a 7.0-kb Smal-NruI fragment of lacZ::O Sm′/Sp′ A′ from pRS415(lacY) for puc-lacZ::Y′ Sm′/Sp′ A′ to be used to generate pUL601::lacZ(XmnI). The PsrI restriction fragment containing a 0.75-kb sequence upstream of pucB to 1.3 kb downstream of pucB with lacZ::O Sm′/Sp′ A′ inserted at the XmnI site in pucB was moved into the PsrI site of pSUP202 (a suicide vector in R. sphaeroides) to generate pSUPPUC::lacZ(XmnI). This plasmid was transformed into E. coli S17-1 and mobilized into R. sphaeroides 2.4.1 and CL1a, and Sm′/Sp′ Tc double crossovers were isolated as previously described (17). Five of the 520 Sm′/Sp′ recombinants observed in 2.4.1 were Tc⁺, while 2 of 190 Sm′/Sp′ recombinants were Tc⁺ following mating of the donor strain with CL1a. All of the five R. sphaeroides Sm′/Sp′ Tc⁺ recombinant strains from 2.4.1 were B800-850⁻, as was expected because of the disruption of pucB as well as the downstream sequences, while the two Sm′/Sp′ Tc⁺ CL1a derivatives were spectrally the same as CL1a (B800-850⁻ Car⁻). One representative strain from each of the five wild-type recombinant strains and one from each of the two CL1a recombinant strains were chosen for further analysis and designated PUC-ZWT and PUC-ZCL, respectively (Fig. 8A). The construction of each strain as depicted in Fig. 8A.
was confirmed by detailed Southern hybridization analysis (data not shown).

(iv) β-Galactosidase activities of PUC-ZWT and PUC-ZCL. The β-galactosidase activities of the chromosome-localized puc::lacZ fusions present in the wild type and mutant strains were measured under aerobic and photosynthetic (10 W/m²) conditions. PUC-ZWT had a β-galactosidase level of approximately 10,000 μmol/min/mg of protein under photosynthetic conditions (Fig. 8B), a level which was approximately 45-fold higher than that in the same construction under aerobic conditions, which itself was approximately threefold higher than that in the comparable construction in trans. On the other hand, the β-galactosidase level in PUC-ZCL under aerobic and photosynthetic conditions was about 7.4-fold higher than and only 73% that in PUC-ZWT under the corresponding growth conditions, respectively. Thus, under aerobic conditions the data are in good agreement with those from the earlier Northern hybridization analysis measuring the levels of the puc-specific transcripts in the wild type and CL1a, implying that differences in the copy number or local DNA structure of the puc upstream sequences could affect the expression of differentially localized puc::lacZ fusions. We still cannot rule out the possibility of the presence of an additional cis-acting regulatory site(s) within DNA sequences upstream of the PstI restriction site limiting the 5’ end of puc DNA on pCF200(−629). However, all previous studies revealed that DNA sequences upstream of pucB4 to the PstI site were sufficient for the regulated expression of the puc operon. One additional point worth noting is the decreased expression of β-galactosidase in the CL1a background in phototrophically grown cells; although a number of explanations come to mind, we have no results directly bearing on this observation.

**Complementation of CL1a and T1a.** Since both CL1a and T1a appear to possess different mutations, as judged by their phenotypic properties, we first set out to localize each mutation following the introduction of various segments of *R. sphaeroides* chromosome I (30). Once a suspected region was localized following restoration of the missing spectral complex, we then measured the β-galactosidase activity of the puc::lacZ fusion (IncQ or IncP4) in trans in the mutant strains containing the complementing fragment present on a second plasmid containing a different incompatibility function.

(i) **Complementation of CL1a.** R’ plasmid pWS2 (27, 37) (Fig. 9A, a) harboring approximately 109 kb of *R. sphaeroides* WS8 DNA containing the *puf*, *puaA*, *cycA*, and *puc* operons complemented CL1a, restoring both the B800-850 complex and the carotenoid profile (Fig. 9B, a). The DNA region complementing CL1a was narrowed to an approximately 27-kb *R. sphaeroides* 2.4.1 DNA fragment carried on cosmid 487, which contains the *puf* operon and the carotenoid gene cluster (Fig. 9B, b), while cosmid 523, carrying approximately 22 kb of *R. sphaeroides* 2.4.1 DNA including the *puaA* and *cycA* operons, showed no complementation (Fig. 9B, c). The responsible 27-kb DNA fragment of cosmid 487 was further narrowed to an 11-kb DNA fragment on pAS203, which contains much of the carotenoid gene cluster (Fig. 9A and B, d). However, pAS203 did not restore the B800-850 complex or carotenoids to their wild-type levels. There are several potential explanations, but the most likely is an imbalance of critical interacting components. This explanation is supported by the fact that when pAS203 was subcloned to generate pAS205 (7.5 kb of DNA within the carotenoid gene cluster; Fig. 9A, f) and pAS204 (2.2 kb of DNA adjacent to the insert present in pAS205; Fig. 9A, e), CL1a was complemented with pAS204 but not with pAS205. Furthermore, the spectrum derived from CL1a (pAS204) showed wild-type levels of both the B800-850 complex and carotenoids (Fig. 9B, e). The 2.2 kb of *R. sphaeroides* DNA cloned on pAS204 was located between *puf* and *puaA*, approximately 11 kb upstream of the *puf* operon (Fig. 9A).

Since the B800-850 Car− phenotype of CL1a was complemented with pAS204, the β-galactosidase activity of pCF200(−629) (IncQ or IncP4) carrying puc::lacZ was measured in the presence of pAS204 (IncP1) in CL1a under aerobic (30% O₂, 1% CO₂, 69% N₂) conditions and compared with the β-galactosidase activity of pCF200(−629) in CL1a carrying pRK415 as a control. For maintenance of the two plasmids together with antibiotic selection, the two plasmids should carry different antibiotic resistance determinants. To this end, the Nrl1 site of the tet gene of pCF200(−629) was interrupted with the Km’ gene fragment from pUC4K to generate pCF200Km(−629). On the other hand, pRK415, cosmid 487, and pAS204 carried the tet gene, providing compatible antibiotic selection. The β-galactosidase activity (3.597 μmol/min/mg of protein) of strain CL1a(pCF200Km, pRK415) was approximately the same as that of strain CL1a (pCF200)(−629) (Fig. 5), as expected. On the other hand, the introduction of cosmid 487 or pAS204 (Fig. 9A) together with pCF200Km(−629) in CL1a resulted in a loss of 13 or 70%, respectively, of the β-galactosidase activity (3.145 or 1.092 μmol/min/mg of protein, respectively). These data confirm that the 2.2-kb DNA fragment designated “e” in Fig. 9A is able to overcome the effect of the mutation in CL1a involved in the loss in control by O₂ of puc operon expression as well as to restore the B800-850 complex and Car−. Furthermore, in a comparison of the results obtained with cosmid 487 and pAS204, the most likely explanation is that the copy number of the cosmid is 1 or 2 and that the copy number of pAS204 is ≈4 or 6. This is also the likely explanation for the fact that the ultimate level of lacZ, even with pAS204 in trans, was not fully reduced to the wild-type aerobic level of ≈100 (Fig. 5). The use of two different plasmids, one to monitor LacZ expression and the second containing *R. sphaeroides* DNA complementary to the CL1a mutation, is at best difficult. Furthermore, the lack of any detailed knowledge of what promoter (vector or insert DNA) is being used to express the insert containing the complementing DNA further complicates this experiment. Thus, we are not surprised that we only reduced LacZ expression by 70% under aerobic conditions in the CL1a background.

(ii) **Complementation of T1a.** T1a was also complemented with pWS2 and cosmid 523 but not with cosmid 487, resulting in the restoration of the B875 complex (data not shown). However, T1a containing pWS2 or cosmid 523 was genetically very unstable and segregated into several distinct.

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**Fig. 9.** Complementation of CL1a and T1a. (A) Location of each plasmid used for complementation. (a) pWS2. (b) Cosmid 487. (c) Cosmid 523. (d) pAS203. (e) pAS204. (f) pAS205. (g) pUB803. (h) pUB811. (i) pUB813. (B) Absorption spectra (10 W/m²) of CL1a carrying plasmids a to f in trans. (C) Absorption spectra (10 W/m²) of T1a carrying plasmids g to i in trans. Absorption spectra were obtained as described in the legend to Fig. 3. The bar represents an absorbance value of 0.1 (B) or 0.2 (C).
OXYGEN REGULATION OF THE puc OPERON IN R. SPHAEROIDES

A

B

C

WAVELENGTH (nm)

WAVELENGTH (nm)
colored colony types. These results suggest that the presence of a substantial block of DNA results in an altered gene(s) dosage or balance, which in turn results in a selective advantage of specific mutant types. These cells were not analyzed further. Cosmid 523 was subcloned on pRK415 to generate pUI803, pUI811, and pUI813 (Fig. 9A, g, h, and i, respectively). When these plasmids were mobilized into T1a, the exconjugants did not segregate into several phenotypic classes and the resulting exconjugants were quite stable. This observation lends credence to the above-described interpretation, i.e., gene imbalance. Of the three plasmids, only pUI803 containing puhA and approximately 6.3 kb of flanking DNA was shown to complement T1a, resulting in the restoration of the B875 complex to the wild-type level (Fig. 9C, g). Additionally, T1a(pUI803) was genetically stable under aerobic conditions, and no T1a-type segregants were observed in successive aerobic cultures of this strain. This is an important observation and will be discussed later.

Since we were able to complement T1a with pUI803 (Tc'; IncP1), we examined the β-galactosidase activities of pCF200Km(−629) and pCF250Km(−92) (Km'; IncP4 or IncP4) in the presence of pUI803 in trans in T1a under aerobic (30% O2, 1% CO2, 69% N2) conditions. In the absence of pUI803, both pCF200(−629) and pCF250Km(−92) were shown to yield derepressed levels of β-galactosidase activity under aerobic conditions when present in trans in T1a (Fig. 5). pUI803 suppressed the observed derepression of β-galactosidase activity (1,530 or 467 μmol/min/mg of protein) when either pCF200Km(−629) or pCF250Km(−92), respectively, was present in trans in T1a, compared with the results obtained with T1a(pPXK-93) and either pCF200(−629) or pCF250(−92) (3,323 or 1,587 μmol/min/mg of protein, respectively). The final levels of β-galactosidase in the former were approximately 30% to 40% those in the latter. Thus, the trans-acting mutation present in T1a which leads to derepression of puc operon transcription under aerobic conditions by acting on both the URS and the DRS of the puc operon is confined to the 7.0-kb EcoRI restriction fragment containing puhA and flanking DNA, amounting to approximately 6.3 kb. Again, it is essential to point out that these complementation experiments were performed with strains containing two different vectors in trans to the T1a mutation.

Formation of the B800-850 complex in T1a under aerobic conditions. The absorption spectrum of T1a(pPXK-93) grown chemoheterotrophically (30% O2-1% CO2-69% N2) was examined and compared with that of the wild type under the same growth conditions. T1a(pPXK-93) formed substantial amounts of the B800-850 complex in the presence of oxygen (Fig. 10B).

DISCUSSION

We exploited the O2-regulated dependency of puc operon transcription in the isolation of both trans- and cis-acting regulatory mutations involved in puc operon expression. By introducing a transcriptional fusion comprising the amino-glycoside-3'-phosphotransferase gene at the downstream junction of puc regulatory DNA sequences (629 bp [URS and DRS] located upstream of the 5' ends of the puc-specific transcripts) into R. sphaeroides 2.4.1 and selecting for Km', we were able to isolate regulatory mutations affecting puc operon expression. Both trans- and cis-acting mutations involved in O2 control of puc operon transcription were isolated and analyzed further. Detailed analysis of the cis-acting mutations together with other cis-acting elements involved in O2 and light control of puc operon transcription is described in the accompanying paper (15).

Two different fusion constructions, pPDK-1 and pPXK-1, were used for the isolation of regulatory mutations involved in puc operon expression, and both yielded similar classes of regulatory mutants. However, when the 536-bp PstI-XhoI restriction DNA fragment of puc upstream DNA containing the URS (O2 and light control; 15) was removed from pPXK-1, the resulting plasmid, pXXK-1, in trans in the wild type showed only WT-like Km' colonies and the DR and CL phenotypes were not expressed. This result strongly suggests that the trans-acting mutations present in the DR and CL mutant classes must involve an interaction(s) between the putative trans-acting factor(s) and the puc URS.

The two trans-acting mutants, CL1a and T1a, studied here have absorption spectra which are very similar to those observed of RS104 (B800-850· Car−) and RS103 (B875−) (12), respectively, which have been shown to be defective in the assembly of their light-harvesting complexes. However, when pPXK-1 was mobilized into both RS104 and RS103, both exconjugants had a Km' phenotype under aerobic conditions. These results indicate that the mutations present in both RS104 and RS103 are not trans acting and are not involved in O2 control of puc operon transcription. There

![Absorption spectra of T1a (10 W/m²) and T4 (anaerobic, dark growth with DMSO).](http://jb.asm.org/)
fore, assembly of these complexes can be distinguished from the loss of O₂ control of gene expression. Conversely, the introduction of a DNA fragment shown to complement CL₁₉ did not result in complementation of RS104. However, since RS104 could be complemented with pWS2 (16), the mutation is of DNA cluster. Previously it was demonstrated that pWS2 complemented the B875 complex and that this extrinsic factor(s) is required for the assembly of the B875 complex. Therefore, it appears that the mutation affecting B875 complex formation and present in RS103 must be different from the mutation present in T₁₉a. However, we suspect that these two mutations are closely linked.

Although T₁₉a showed no apparent B875 complex in a room temperature absorption spectrum, a low-temperature spectral analysis of T₁₉a revealed the presence of the B875 complex in T₁₉a at approximately 3.1% the wild-type level (16). Thus, it is possible that a single mutation in T₁₉a affects both O₂ control and B875 complex assembly, but the latter effect is probably the result of a partially polar mutation whose primary effect is on O₂ control. Recent results from our laboratory (see below) suggest that the mutation in CL₁₉a is also polar. We designate the altered gene involved in O₂ control in T₁₉a oxyB.

DCL₁₉a, derived from DR₁, was spectrally almost identical to mutant CL₁₉a. In addition, DCL₁₉a showed derepressed expression of β-galactosidase activity when pCF200(−629) was present in trans and, like CL₁₉a, it was complemented with pAS204 to the restoration of the B800-850 complex and carotenoids. These close biochemical and genetic characteristics of both DCL₁₉a and CL₁₉a, which were isolated independently through two separate routes, suggests a possible interaction between the genes leading to the expression of the trans-acting factor(s) present in the DR and CL mutants.

Whereas CL₁₉a was genetically stable under aerobic conditions, T₁₉a was very unstable, spontaneously generating a second mutant class, designated T₂₉a, at a high frequency. This nonpigmented, PS₄’ mutant (T₂₉a), however, readily reverted to T₁₉a under photosynthetic (10 W/m²) conditions. In turn, T₂₉a derived from T₂₉a under photoheterotrophic conditions disappeared to T₁₉a again, complementing the T₁₉a gene under aerobic conditions. When efforts were made to complement T₂₉a, the second site mutation could be complemented in trans with a DNA fragment mapping over 1,000 kb away from the puc operon, resulting in the restoration of the original T₁₉a phenotype (7). Additionally, when puc::lacZ transcriptional fusions were moved into the T₂₉a chromosome, there was no expression of β-galactosidase activity under any growth conditions (16). This result indicates that the lack of B800-850 complex formation observed in T₂₉a must be due to the total repression or lack of puc operon transcription, even under anaerobic conditions. It remains to be determined whether the lack of expression of other photosynthetic genes is also controlled at the transcriptional level in mutant T₂₉a. Additionally, the results reveal the presence of an additional trans-acting factor(s) which is required for puc operon transcription and which is encoded by a gene(s) located outside the photosynthetic gene cluster. Finally, this gene(s) would appear to possess positive regulatory activity. Initial DNA sequence information (7) revealed strong amino acid sequence homology at the amino-terminal end of the derived sequence to a number of two-component regulatory systems.

Since both the deficiency in the photosynthetic apparatus and the locations of the trans-acting mutations in CL₁₉a and T₁₉a, as judged by the locations of the complementing DNA fragments, were different, there must be at least two separate trans-acting factors involved in the O₂-regulated control of puc operon transcription. Although both trans-acting factors are involved in the repression of puc operon transcription in the presence of oxygen, the factor lacking in T₁₉a appears to interact with both the URS and the DRS of the operon (15), while the second trans-acting factor, lacking in CL₁₉a, appears to interact with only the URS of the puc operon. The former was designated oxyB, and the mutation in CL₁₉a is designated oxyA.

Interestingly, the specific activity of β-galactosidase, approximately 2,000 μmol/min/mg of protein, observed when pCF200(−629) was present in the wild type under photosynthetic (10 W/m²) conditions (15) was approximately 100% the specific activity, 10,000 μmol/min/mg of protein, observed in PUC-ZWT under identical conditions. Thus, single-copy expression of the puc::lacZ fusion (chromosomal location) is approximately fivefold higher than multicopy expression of a similar construction in an otherwise wild-type background. This observation explains the results of the Northern hybridization analyses of PUC705-BA(pRK101) or PUC705-BA(pRK415); these were shown to contain the 0.5-kb puc-specific transcript at about 25% the level observed in the wild type under photosynthetic (100 W/m²) conditions. This low level of β-galactosidase activity in trans when compared with that in cis could be due to the presence of additional copies of the upstream DNA which titrate some positive acting factor, since the copy numbers of both the IncQ pCF200(−629) and the IncP(pRK101 and pRK415) plasmids fall within the range of four to six in R. sphaeroides 2.4.1 (2, 33). However, another possible explanation involves the effects of DNA structural differences between plasmid and chromosomal locations.

When pAS204 was mobilized into PUC-ZCL, it restored the normal carotenoid phenotype but not the B800-850 complex. This result is readily explained because the insertion of lacZc:·31 Sm/Sp' A' into the pucB gene in PUC-ZCL interrupts the pucB gene and the expression of the downstream region of the operon, which is essential for B800-850 complex assembly. In addition, the β-galactosidase activity of PUC-ZCL(pAS204) under aerobic conditions was essentially the same as that of the control, PUC-ZCL(pRK415); i.e., there was little or no restoration of O₂ repression in the presence of oxyA in trans when the chromosomal copy of the puc operon was interrupted. Since pAS204 restored the O₂ repression of the puc operon on pCF200(−629) in trans in CL₁₉a under aerobic conditions, we tentatively suggest that a second function of the DNA sequences downstream of pucBA in the puc operon is the regulation of puc operon expression, in addition to the posttranslational control of B800-850 complex formation. This suggestion is in agreement with a recent conclusion of Tichy et al., working with R. capsulatus (34). The results additionally suggest that the gene product of oxyA encoded by pAS204 may interact with the gene product(s) encoded by sequences downstream of pucBA to exert its action. A detailed analysis of this interaction is under way. The involvement of the downstream (from pucBA) gene product(s) in O₂ and light control of puc operon expression may also be inferred from the analysis of β-galactosidase activities expressed from pCF200(−629) and pCF250(−92).
trans in PUC705-BA and PUC-Pv (16). pCF200(--629) and pCF250(--92) in each of these mutants showed approximately 50 to 40% of the activity of the corresponding plasmids in trans in the wild type under photosynthetic (10 W/m²) conditions (16). In either of the mutants there was a lack of expression of the downstream puc operon sequences.

Wood and Kaplan (36) have shown that oxyA is identical to crtK of R. capsulatus (1) and that oxyA has apparently nothing to do with the pathway for carotenoid biosynthesis. It appears that the original mutation in CL1αa affected both crtB and crtK, thus leading to both a Car" B800-850" phenotype and a lack of O₂ expression of puc operon expression. These two effects have now been separated, and oxyA has been shown only to affect O₂ control of puc operon expression. Because oxyA is clearly involved in oxygen control and not carotenoid biosynthesis, we believe that the designation oxyA is more appropriate.

The genetic instability of mutant T₁α under aerobic conditions could be related to the ability of T₁α to express both bacteriochlorophyll and the apoproteins of the B800-850 complex in the presence of oxygen. Thus, oxyB could be a more global regulator of photosynthetic gene expression than oxyA. As a result, the synthesis of bacteriochlorophyll in the presence of O₂ in light is a potentially lethal situation; thus, any secondary mutations alleviating this compromising situation have an enormous survival value, reflected in our ability to readily isolate segregants which lacked all apparent expression of photosynthetic gene activity. In passing, it should also be noted that the complementation of either CL1αa or T₁α can be compromised when the essential gene is part of a large but incomplete block of genes. Thus, some form of pleiotropy or genetic imbalance can mask these critical findings. This complexity seems almost typical of the regulation of photosynthetic gene expression in R. sphaeroides.

Finally, this study and other studies of puc operon expression have now revealed the presence of numerous cis-acting upstream regulatory elements and several linked trans-acting elements, the possible existence of downstream regulatory elements, and finally, additional trans-acting elements mapping over 1,000 kb away from the puc operon.

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