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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 Tn*903* 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^r colonies under aerobic conditions. Two broad classes of mutations, *trans* and *cis*, which are involved in O_2 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, CL_{1a} and T_{1a}, 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 CL_{1a} 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 T_{1a}, 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 CL_{1a} were complemented with a 2.2-kb DNA fragment located within the carotenoid gene cluster. Mutant T_{1a} was complemented with a 7.0-kb *Eco*RI 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 O_2 control. Under chemoheterotrophic conditions, mutant T_{1a} was highly unstable, segregating into a PS$^-$ mutant designated T_4.

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 O_2 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 O_2 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 O_2 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* transcriptional 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, CL_{1a} (B800-850$^-$ Car$^-$) and T_{1a} (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 O_2 control of *puc* operon expression in mutant CL_{1a}. A 7.0-kb *Eco*RI restriction fragment containing *puhA* as well as flanking DNA complements the B875$^-$ phenotype and suppresses the loss of O_2 control of *puc* operon transcription in mutant T_{1a}. Additionally, under chemoheterotrophic conditions, mutant T_{1a} 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 extends 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 to 25, 50, and 50 μg/ml, respectively. Photoheterotrophic 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, DH5α, 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 *Spe*I 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 ^{32}P-labeled RNA probes, and quantitation of transcript signals following hybridization were as previously reported (17).

Conjugation technique. Plasmid pRK415- or RSF1010-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 *o*-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-^{32}P]dCTP (800 Ci/mmol) and [α-^{32}P]CTP (400 Ci/mmol) were obtained from Amersham Corp., Arlington Heights, Ill. Isopropyl-β-D-thiogalactopyranoside and *o*-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 Tn*903* (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 *Xho*I-*Eco*RI restriction DNA fragment of the *aph* gene from plasmid pRME1 (8) and cloned the fragment into the multiple cloning region of pRK415 in both orientations relative to *lacP*/*tetP* of the plasmid (Fig. 1A, a and b). When the fragment was in the same orientation as *lacP*/*tetP* of pRK415 in any of the three reading frames relative to the start codon of the *lacZ* α-peptide, *R. sphaeroides* 2.4.1 containing each of the plasmid constructions (Fig. 1A, a) was Km^r at a concentration of 25 μg/ml in Sistrom's minimal medium. When the transcription-translation stop cartridge Ω Sm^r/Sp^r (23) was placed at the border between *lacP*/*tetP* and the 1,070-bp *Xho*I-*Eco*RI fragment (Fig. 1A, c), *R. sphaeroides* containing plasmid pRKR2 was Km^s. The absence of the *aph* promoter on the 1,070-bp *Xho*I-*Eco*RI DNA fragment was confirmed by the Km^s 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 *lacP*/*tetP* (Fig. 1A, b).

We have also shown that a 1,150-bp *Bsp*HI-*Eco*RI restriction DNA fragment of the *aph* gene which contains an additional 80 bp of DNA upstream of the *Xho*I restriction site confers Km^r in *R. sphaeroides* under all conditions, indicating that there is a functional promoter(s) on the 80-bp *Bsp*HI-*Xho*I restriction DNA fragment. Either the 799-bp *Pst*I-*Xmn*I (within *pucB*) or the 699-bp *Pst*I-*Dra*II (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

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli S17-1	Pro$^-$ Res$^-$ Mod$^+$ *recA*; integrated plasmid RP4-Tc::Mu-Km::Tn*7*	25
R. sphaeroides		
2.4.1	Wild type	W. R. Sistrom
WT_1	Km^r mutant derived from 2.4.1(pPXK-1); wild type-like	This study
CL_1	Km^r mutant derived from 2.4.1(pPXK-1); less pigmented; RS104 phenotype	This study
DR_1	Km^r mutant derived from 2.4.1(pPXK-1); highly pigmented	This study
WT_{1a}	WT_1 cured of mutated pPXK-1*; wild type-like	This study
CL_{1a}	Isolated from CL_1 when cured of pPXK-1; O_2-insensitive expression of the *puc* operon; B800-850$^-$ Car$^-$	This study
DR_{1a}	Isolated from DR1 when cured of pPXK-1; *trans*-acting mutant affecting *puc* operon transcription; highly pigmented	This study
CP_{1a}	Isolated from DR1 when cured of pPXK-1; reduced B800-850 complex	This study
T_{1a}	Isolated from DR_1 when cured of pPXK-1; O_2-insensitive expression of the *puc* operon; apparently B875$^-$	This study
DCL_{1a}	Isolated from DR1 when cured of pPXK-1; O_2-insensitive expression of the *puc* operon; B800-850$^-$ Car$^-$	This study
PUC-ZWT	*lacZY*::Ω Sm^r/Sp^r *A'* inserted at the *Xmn*I site within *pucB* of the wild type; B800-850$^-$	This study
PUC-ZCL	*lacZY*::Ω Sm^r/Sp^r *A'* inserted at the *Xmn*I site of *pucB* of CL_{1a}; B800-850$^-$ Car$^-$	This study
T_4	Spontaneous mutant derived from T_{1a}; PS$^-$ RC$^-$[a] B875$^-$ B800-850$^-$ Car$^-$	This study
Plasmids		
pRK415	Tc^r	9
pSUP202	pBR325-Mob$^+$ Ap^r Cm^r Tc^r	25
pRS415	Ap^r *lacZYA*	26
pRS415Ω(*lacY*)	pRS415 derivative + 2.0-kb Ω Sm^r/Sp^r at the *Sna*BI site within *lacY*	This study
pRKR1	pRK415 derivative + 1.07-kb *Xho*I-*Eco*RI Km^r DNA (+)[b]; Tc^r	This study
pRKL1	pRK415 derivative + 1.07-kb *Xho*I-*Eco*RI Km^r DNA (−)[b]; Tc^r	This study
pRKR2	pRKR1 derivative + 2.0-kb Ω Sm^r/Sp^r in the multiple cloning region but upstream of the 1.07-kb *Xho*I-*Eco*RI Km^r DNA; Tc^r Sm^r/Sp^r	This study
pPXK-1	pRKR2 derivative + 0.8-kb *Pst*I-*Dra*II *puc* DNA between Ω Sm^r/Sp^r and 1.07-kb *Xho*I-*Eco*RI Km^r DNA; Tc^r Sm^r/Sp^r	This study
pPDK-1	pRKR2 derivative + 0.7-kb *Pst*I-*Xmn*I *puc* DNA between Ω Sm^r/Sp^r and 1.07-kb *Xho*I-*Eco*RI Km^r DNA; Tc^r Sm^r/Sp^r	This study
pRKM1	pRK415 derivative + 1.5-kb *Eco*RI Km^r DNA; Tc^r Km^r	This study
pCF100	pLV106 *Eco*RI *Sma*I; 7.1-kb *Eco*RI-*Nru*I fragment from Ω Sm^r/Sp^r-*lacZYA'*; Tc^r Sm^r/Sp^r	15
pCF200(−629)	pLV106 *Eco*RI *Sma*I; 7.9-kb *Eco*RI-*Nru*I fragment from Ω Sm^r/Sp^r-*puc* (0.8-kb *Pst*I-*Xmn*I)-*lacZYA'*; Tc^r Sm^r/Sp^r	15
pCF250(−92)	pLV106 *Eco*RI *Sma*I; 7.37-kb *Eco*RI-*Nru*I fragment from Ω Sm^r/Sp^r-*puc* (0.27-kb *Xma*III-*Xmn*I)-*lacZYA'*; Tc^r Sm^r/Sp^r	15
pCF260(+70)	pLV106 *Eco*RI *Sma*I; 7.2-kb *Eco*RI-*Nru*I fragment from Ω Sm^r/Sp^r-*puc* (0.1-kb *Dra*II-*Xmn*I)-*lacZYA'*; Tc^r Sm^r/Sp^r	15
pCF200Km(−629)	pCF200 derivative + 1.4-kb *Bam*HI Km^r DNA[c] in the *Nru*I site of *tet* from pCF200; Km^r Sm^r/Sp^r	This study
pCF250Km(−92)	pCF250 derivative + 1.4-kb *Bam*HI Km^r DNA[c] in the *Nru*I site of *tet* from pCF250; Km^r Sm^r/Sp^r	This study
pWS2	R68.45 derivative + 109 kb of *R. sphaeroides* WS8 DNA; Tc^r Nm^r	27, 37
Cosmid 487 (pUI8487)	pLA2917 derivative + ca. 27 kb of *R. sphaeroides* 2.4.1 DNA containing *puf*; Tc^r	6
Cosmid 523 (pUI8523)	pLA2917 derivative + ca. 22 kb of *R. sphaeroides* 2.4.1 DNA containing *puhA* and *cycA*; Tc^r	6
pAS203	pRK415 derivative + 11 kb of *R. sphaeroides* 2.4.1 DNA from cosmid 487 containing *crt*; Tc^r	32a
pAS204	pRK415 derivative + 2.2 kb of *R. sphaeroides* 2.4.1 DNA from pAS203 containing *crt*; Tc^r	32a
pAS205	pRK415 derivative + 7.5 kb of *R. sphaeroides* 2.4.1 DNA from pAS203 containing *crt*; Tc^r	32a
pUI803	pRK415 derivative + 7.0 kb of *R. sphaeroides* 2.4.1 DNA from cosmid 523 containing *puhA*; Tc^r	24a
pUI811	pRK415 derivative + 6.5 kb of *R. sphaeroides* 2.4.1 DNA from cosmid 523 containing *cycA*; Tc^r	24a
pUI813	pRK415 derivative + 1.6 kb of *R. sphaeroides* 2.4.1 DNA from cosmid 523; Tc^r	24a
pPXK-93	pPXK-1 derivative; *Nru*I fragment of *tetA* deleted; Sm^r/Sp^r	This study
pUI601::*lac*Ω(*Xmn*I)	pUC19 *Pst*I; 2.5-kb *Pst*I (−)[a] fragment of *pucBA* + 6.96-kb *lacZY*::Ω Sm^r/Sp^r *A'* inserted at the *Xmn*I site of *pucB*; Ap^r Sm^r/Sp^r	This study
pSUPPUC::*lac*Ω(*Xmn*I)	pSUP202 derivative + 9.46-kb *Pst*I fragment of pUI601::*lac*Ω(*Xmn*I); Tc^r Sm^r/Sp^r	This study

[a] RC$^-$, devoid of the reaction center.
[b] 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 (−).
[c] The 5′ overhangs were made blunt ended with the Klenow fragment of DNA polymerase I before cloning.

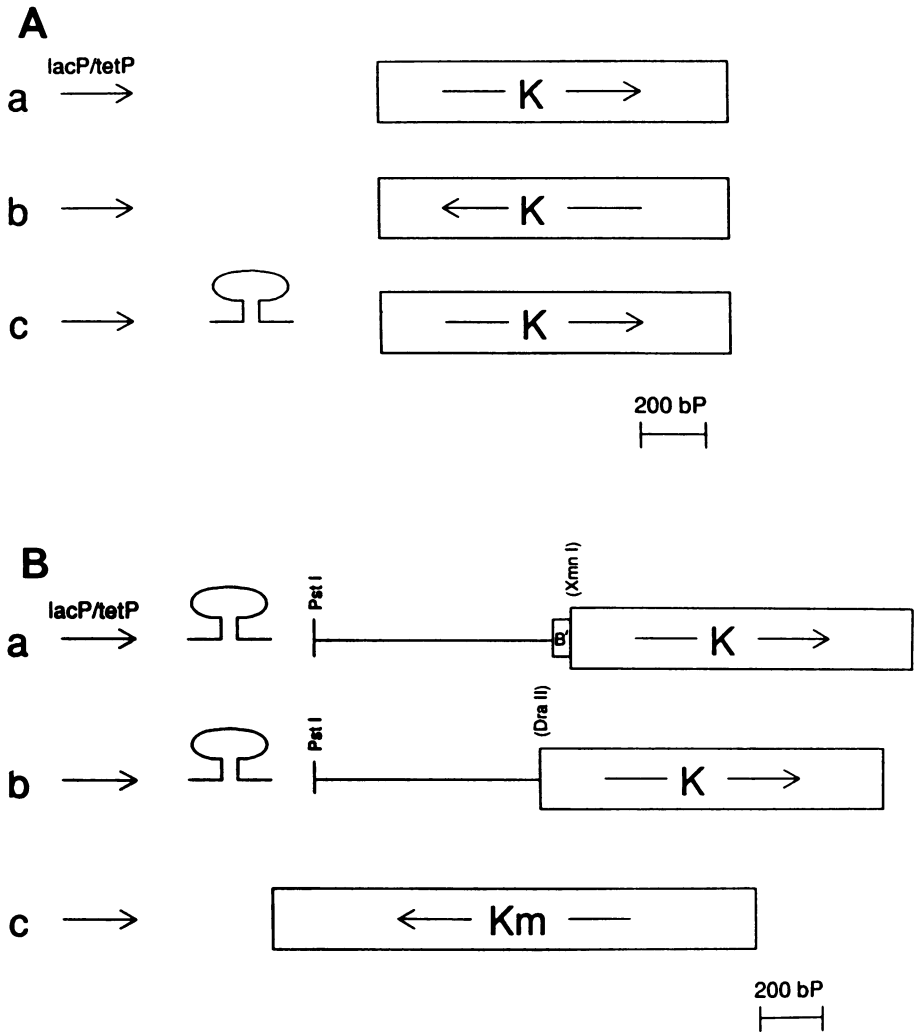


FIG. 1. (A) Cloning of a *Xho*I-*Eco*RI fragment (K) (1,070 bp) of the Km^r gene from pRME1 into the multiple cloning region of pRK415 in both orientations relative to *lacP/tetP* of the plasmid (pRKR1 [a] and pRKL1 [b]). A transcription-translation stop cartridge, Ω Sm^r/Sp^r was cloned into the multiple cloning region between *lacP/tetP* and the *Xho*I-*Eco*RI fragment of pRKR1 (a), to yield pRKR2 (c). (B) Cloning of a *Pst*I-*Xmn*I DNA fragment (799 bp) containing the *puc* regulatory region, including 629 bp of upstream DNA from the 5′ end of the *puc*-specific transcripts in addition to 117 bp of DNA from the 5′ leader region of the transcripts and 54 bp of DNA from *pucB*, or a *Pst*I-*Dra*II DNA fragment (699 bp) containing the 629-bp *puc* regulatory region DNA and only 70 bp of DNA corresponding to the 5′ leader region of the transcripts between Ω Sm^r/Sp^r and the promoterless Km^r fragment of pRKR1 to generate pPXK-1 (a) in the former or pPDK-1 (b) in the latter. pRKM1 (c) contains the entire Km^r fragment from pRME1 but no *puc*-specific DNA sequences.

lation stop cartridge Ω Sm^r/Sp^r and the 1,070-bp *Xho*I-*Eco*RI promoterless *aph* fragment of pRKR2 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^s while the wild type carrying the larger, promoter-containing Km^r DNA fragment, pRKM1 (Fig. 1B, c), as a control was Km^r, 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, dark dimethyl sulfoxide (DMSO) growth conditions (5), the wild type carrying either pRKM1 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 μg/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 μg/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-

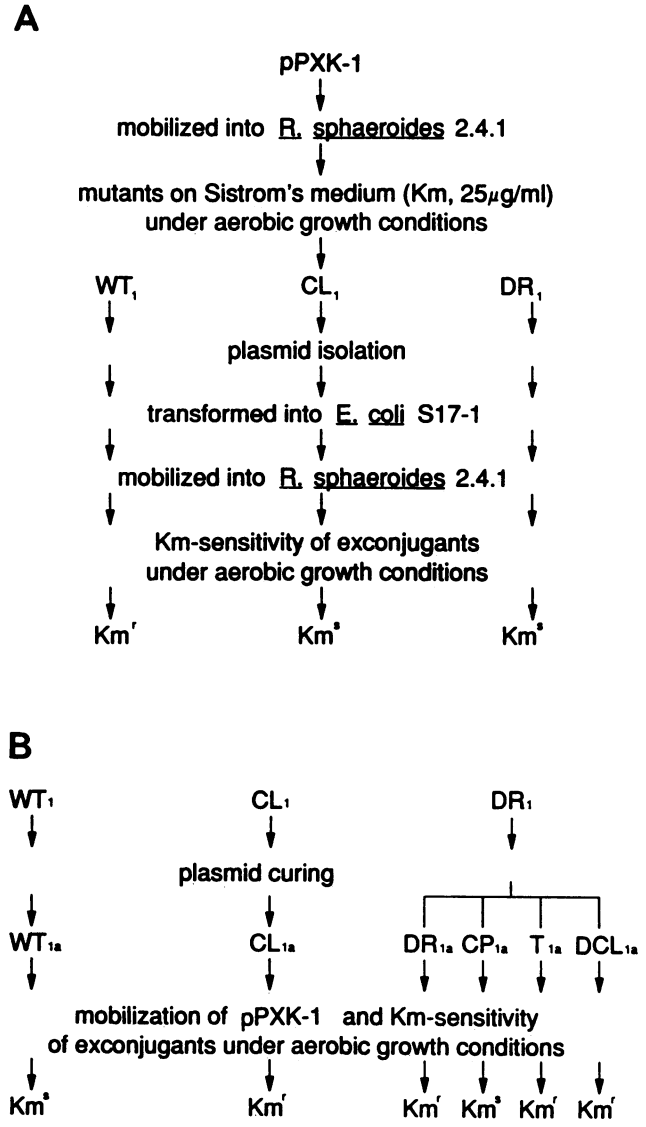


FIG. 2. Flow chart outlining the experimental scheme used to isolate and localize Km^r mutations, *cis* or *trans*, involved in O_2 control of *puc* operon transcription (A) and confirmation of the mutations and their localization within strains cured of their plasmids (pPXK-1 or mutated pPXK-1*) (B).

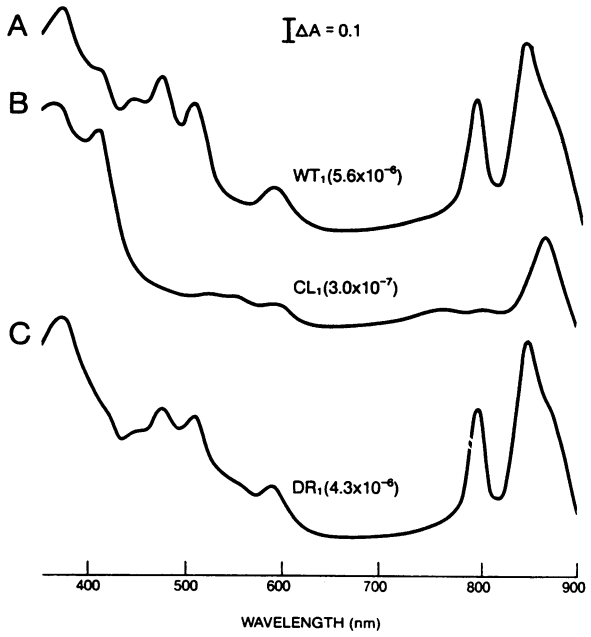


FIG. 3. Absorption spectra of the three phenotypic classes of O_2 control mutants. The numbers in parentheses express the frequencies of occurrence of each type of mutant as the number of Km^r mutants per total number of viable cells. The spectra were generated by use of identical amounts of protein (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.

fecting oxygen regulation of *puc* operon transcription. To isolate regulatory mutations involved in O_2 control of *puc* operon transcription (Fig. 2A), we spread 10^7 to 10^8 cells of *R. sphaeroides* 2.4.1(pPXK-1) on Sistrom's minimal medium plates containing kanamycin at 25 μg/ml, substantially above the MIC for this construction. Following 2 to 3 days of incubation at 30°C under aerobic growth conditions, three differently colored colony types appeared at the frequencies shown in Fig. 3. We observed as the predominant Km^r classes (i) wild-type colored (WT) colonies; (ii) colorless colonies (CL), very much like *R. sphaeroides* RS104 (12); and (iii) highly pigmented, dark red (DR) colonies. We grouped the mutant colonies into these three classes (WT, CL, and DR) for the sake of convenience and spectrally analyzed two representative colonies (WT_1 and WT_2, CL_1 and CL_2, and DR_1 and DR_2) from each class for growth under anaerobic, dark DMSO conditions. WT_2, CL_2, and DR_2 showed spectra identical to those of WT_1, CL_1, and DR_1 (Fig. 3). Each isolate within the three classes was PS^+, and the absorption spectrum obtained for each strain grown photoheterotrophically at 10 W/m^2 was similar to the corresponding spectrum obtained for each strain in Fig. 3. WT_1 showed a wild-type-like spectrum, while the spectrum of CL_1 was very similar to that of RS104, failing to show both B800-850 and carotenoids. DR_1, although apparently more pigmented than the wild type, also had an absorption spectrum similar to that of WT_1.

Since Km^r 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 (WT_1 and WT_2) had a Km^r phenotype, indicating that a mutation(s) leading to Km^r was carried on the plasmid (pPXK-1*) in a *cis* configuration to the Km^r gene. On the other hand, exconjugants that received plasmids derived from any of the CL (CL_1 and CL_2) or DR (DR_1 and DR_2) isolates had a Km^s phenotype, suggesting that the location of the mutation(s) conferring Km^r was on the chromosomal DNA of the original isolate, i.e., CL_1, CL_2, DR_1, or DR_2 (Fig. 2A).

To confirm the location of the mutational site(s) giving rise to a Km^r phenotype in WT, CL, and DR colonies more precisely, we cured each of the isolates chosen for further study of its plasmid (Fig. 2B). WT_{1a} and CL_{1a} had the same

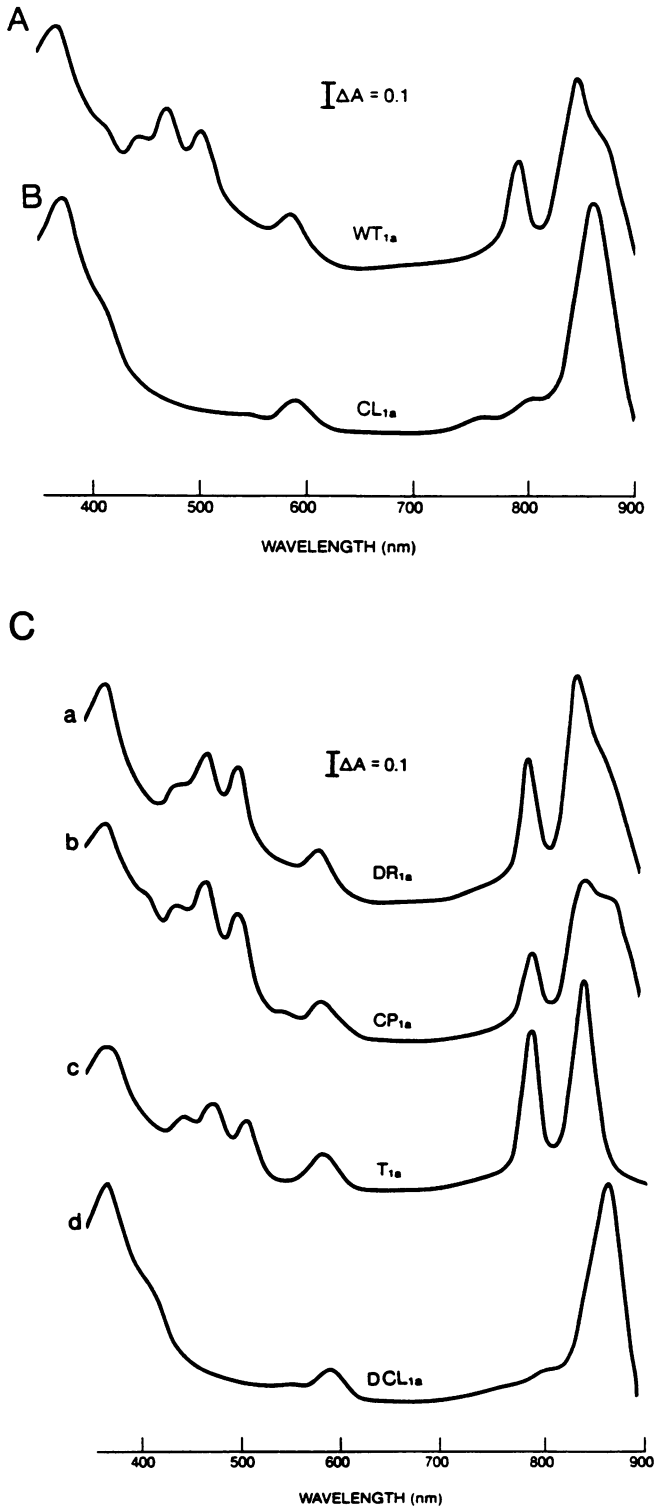


FIG. 4. Absorption spectra of mutant classes following curing of plasmid pPXK-1 or mutated pPXK-1*. Cells were grown photoheterotrophically at 10 W/m^2 as described in the text. Absorption spectra were obtained as described in the legend to Fig. 3. The spectral profiles of WT_{1a} and CL_{1a} were similar to those of their parental Km^r mutants, WT_1 and CL_1, respectively, in Fig. 3. (C) Four different classes of segregants derived from strain DR_1. The bar represents an absorbance value of 0.1.

absorption spectra (Fig. 4A and B) as WT_1 and CL_1, respectively (Fig. 3). The spectra of WT_{2a} and CL_{2a} are not shown but were identical to those of WT_{1a} and CL_{1a}, respectively. However, DR_1 generated no less than four different phenotypic segregants, DR_{1a}, CP_{1a}, T_{1a}, and DCL_{1a}, during plasmid curing, as was also observed during curing of the plasmid from DR_2. Each of the four segregants derived from curing of the plasmid present in DR_1 had unique spectral properties (Fig. 4C). T_{1a} appeared to be B875$^-$ and was similar to strain RS103 (20). Interestingly, DCL_{1a} 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 DR_{1a}, T_{1a}, and DCL_{1a} 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 WT_{1a} and WT_{2a}, 2.4.1, and CL_{1a} and CL_{2a} (Fig. 2B). These data provide convincing evidence that the mutation(s) conferring Km^r in either CL_1 or CL_2 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^r in either WT_1 or WT_2 was unambiguously demonstrated to reside on plasmid pPXK-1*. Of the four separate segregants derived from DR_1, three had a Km^r phenotype and one had a Km^s phenotype under aerobic conditions when the normal plasmid construction was reintroduced as outlined in Fig. 2B. CP_{1a}(pPXK-1), which had a Km^s 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 O_2 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 the DRS.

We have chosen CL_{1a} (B800-850$^-$ Car$^-$) and T_{1a} (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 CL_{1a} and T_{1a}. To more accurately assess the extent of the defect leading to the loss of O_2 control in mutants CL_{1a} and T_{1a}, we further analyzed biochemically O_2 control of *puc* operon expression.

(i) β-Galactosidase activities in response to the presence of pCF200(−629) and pCF250(−92) in CL_{1a} and T_{1a} in the presence of O_2. 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 *Xma*III (−92) restriction site within the DRS and contains suspected promoter and operator

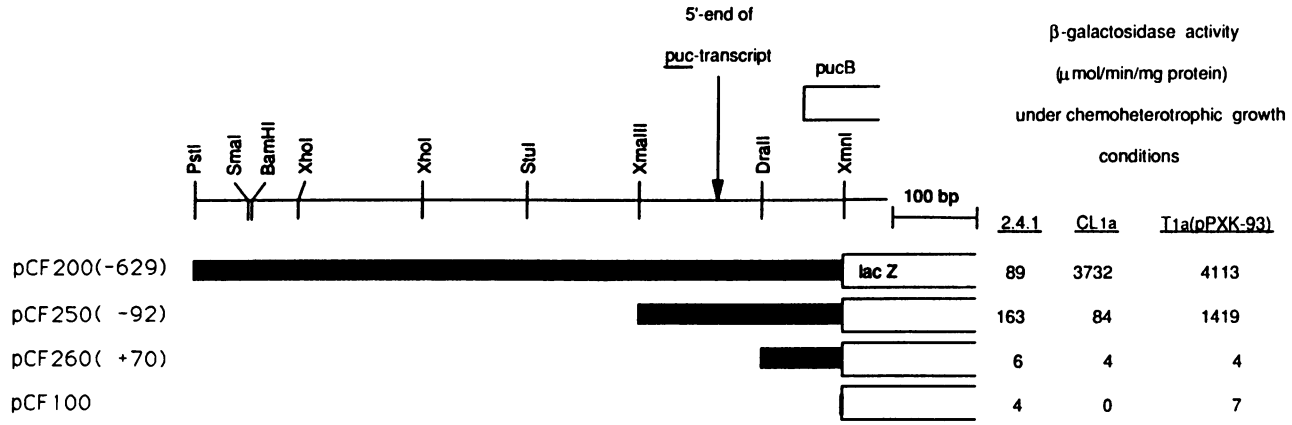


FIG. 5. β-Galactosidase activities of pCF200(−629), pCF250(−92), pCF260(+70), and pCF100 in *R. sphaeroides* 2.4.1 and its *trans*-acting mutants CL_{1a} and T_{1a} under chemoheterotrophic conditions (30% O_2–1% CO_2–69% N_2). T_{1a} was grown chemoheterotrophically with pPXK-93 in *trans* in the presence of kanamycin to prevent the accumulation of T_4 mutant cells (see the text).

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 O_2-dependent nature of *puc* operon expression. Under identical conditions, pCF200 (−629) in CL_{1a} 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 CL_{1a}. These results strongly suggest that the mutation affecting O_2 control of *puc* operon transcription in CL_{1a} not only is *trans*-acting but also acts on or requires for its activity the 536-bp *Pst*I-*Xma*III (−629 to −92) *puc* upstream DNA.

The second *trans*-acting mutant, T_{1a}, 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, T_{1a} is genetically very unstable and mutates at a high frequency to a PS^- phenotype; the mutant is designated T_4. T_4 is virtually devoid of any of the light-harvesting complexes ($B800\text{-}850^-$ $B875^-$) 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 T_{1a} in the presence of O_2, we placed a *puc*::*aph* transcriptional fusion in *trans* on pPXK-93 into T_{1a} and cultured T_{1a}(pPXK-93) aerobically in the presence of kanamycin. Although T_{1a}(pPXK-93) cells can still spontaneously mutate to T_4(pPXK-93) cells under aerobic conditions, T_4(pPXK-93) will be killed by kanamycin because *puc* operon expression is turned off in the T_4 mutant strain. Each of the *puc*::*lacZ* transcriptional fusion plasmids (IncQ or IncP4) and pPXK-93 (IncP1) were maintained together in T_{1a} in the presence of kanamycin under aerobic conditions, and β-galactosidase activities were monitored. When present in T_{1a}, both pCF200(−629) and pCF250(−92) in the presence of oxygen showed high levels of β-galactosidase activities, indicating that the mutation involved in O_2 control of *puc* operon transcription in T_{1a} involves a second, different *trans*-acting factor which, in addition to acting on the 536-bp *Pst*I-*Xma*III 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 CL_{1a}. CL_{1a} and the wild-type strain were grown under both aerobic and photosynthetic (10 W/m^2) conditions, and the mRNA levels for the *puf*, *puhA*, and *puc* operons were determined to directly assess the effect of the mutation present in CL_{1a}. Under aerobic conditions, the level of the 0.5-kb *puc*-specific transcript (17) in CL_{1a} (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 CL_{1a} (Fig. 6, lane 3) versus the wild type (Fig. 6, lane 1, and data not shown), suggesting that O_2 control of *puc* operon expression in CL_{1a} acts on both the 2.3-kb *puc*-specific

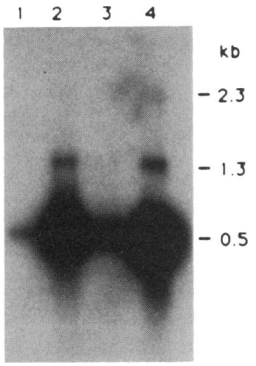


FIG. 6. Northern blot hybridization analysis of *puc* operon expression in *R. sphaeroides* 2.4.1 (lanes 1 and 2) and CL_{1a} (lanes 3 and 4). The RNAs were prepared from each of the strains grown chemoheterotrophically (lanes 1 and 3) or photoheterotrophically at 10 W/m^2 (lanes 2 and 4) as described in the text. An RNA probe corresponding to an *Xma*III 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.

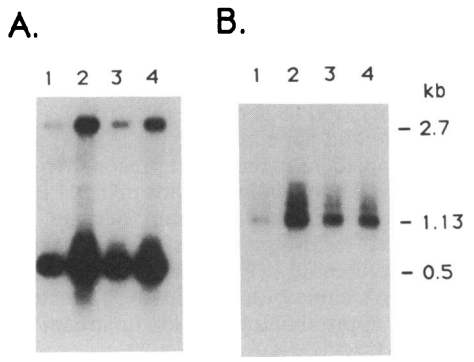


FIG. 7. Northern blot hybridization analysis of *puf* (A)- and *puhA* (B)-specific transcripts with RNA from the wild type (2.4.1) (lanes 1 and 2) and CL_{1a} (lanes 3 and 4) grown chemoheterotrophically (lanes 1 and 3) or photoheterotrophically at 10 W/m^2 (lanes 2 and 4) as described in the text. The RNA probes were derived from the *Sty*I fragment of *pufBA* (3) and the *Sph*I-*Xho*I fragment of *puhA* (4).

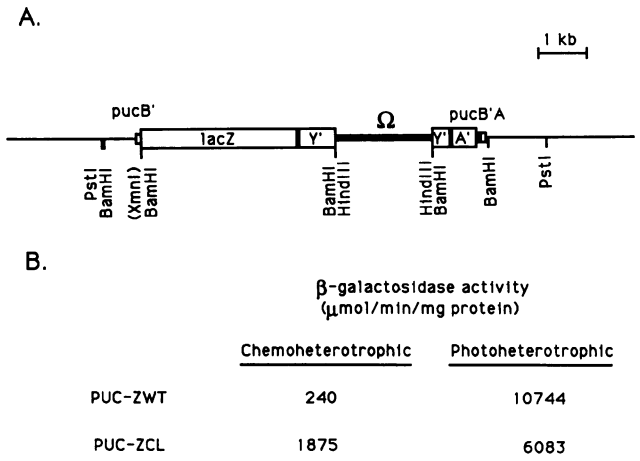


FIG. 8. (A) Structures of PUC-ZWT and PUC-ZCL constructed by insertion of *lacZY*::Ω Sm^r/Sp^r *A'* through homologous recombination at the *Xmn*I restriction site within *pucB* of the wild type (2.4.1) and CL_{1a}, respectively. (B) β-Galactosidase activities of PUC-ZWT and PUC-ZCL grown under chemoheterotrophic (30% O_2–1% CO_2–69% N_2) and photoheterotrophic (10 W/m^2; 95% N_2–5% CO_2) conditions as described in the text.

transcript and the 0.5-kb *puc*-specific transcript. Under photosynthetic (10 W/m^2) conditions, however, the 0.5-kb *puc*-specific transcripts in both CL_{1a} and the wild type (Fig. 6, lanes 4 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 *puc*-specific transcript in the wild type under aerobic conditions (Fig. 6, lane 1). Thus, the effect of the mutation in CL_{1a} appears to occur primarily under aerobic conditions.

Each of the three *puf*-specific transcripts (0.5, 0.7, and 2.7 kb; 3) in CL_{1a} 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 *trans*-acting mutation in CL_{1a} 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 CL_{1a} 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 CL_{1a} was derepressed compared with that in the wild type, although the level of the *puhA*-specific transcript in CL_{1a} under photoheterotrophic conditions was about 50% that in the wild type under the same conditions. Whether this effect on *puhA* operon expression by the mutation in CL_{1a} is direct or indirect remains to be determined.

(iii) Construction of PUC-ZWT and PUC-ZCL, containing chromosomally localized *puc*::*lacZ* transcriptional fusions. Mutant CL_{1a} 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 CL_{1a} 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 CL_{1a} 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 CL_{1a} 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 pUI601 (10) was interrupted at the *Xmn*I restriction site with a 7.0-kb *Sma*I-*Nru*I fragment of *lacZY*::Ω Sm^r/Sp^r *A'* from pRS415Ω(*lacY*) for *puc-lacZY*::Ω Sm^r/Sp^r *A'* to be used to generate pUI601::*lac*Ω(*Xmn*I). The *Pst*I restriction fragment containing a 0.75-kb sequence upstream of *pucBA* to 1.3 kb downstream of *pucBA* and with *lacZY*::Ω Sm^r/Sp^r *A'* inserted at the *Xmn*I site in *pucB* was moved into the *Pst*I site of pSUP202 (a suicide vector in *R. sphaeroides*) to generate pSUPPUC::*lac*Ω(*Xmn*I). This plasmid was transformed into *E. coli* S17-1 and mobilized into *R. sphaeroides* 2.4.1 and CL_{1a}, and Sm^r/Sp^r Tc^s double crossovers were isolated as previously described (17). Five of the 520 Sm^r/Sp^r recombinants observed in 2.4.1 were Tc^s, while 2 of 190 Sm^r/Sp^r recombinants were Tc^s following mating of the donor strain with CL_{1a}. All of the five *R. sphaeroides* Sm^r/Sp^r Tc^s 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^r/Sp^r Tc^s CL_{1a} derivatives were spectrally the same as CL_{1a} (B800-850$^-$ Car$^-$). One representative strain from each of the five wild-type recombinant strains and one from each of the two CL_{1a} 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/m^2) 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 CL_{1a}, 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 *Pst*I restriction site limiting the 5′ end of *puc* DNA on pCF200(−629). However, all previous studies revealed that DNA sequences upstream of *pucBA* to the *Pst*I 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 CL_{1a} background in photoheterotrophically grown cells; although a number of explanations come to mind, we have no results directly bearing on this observation.

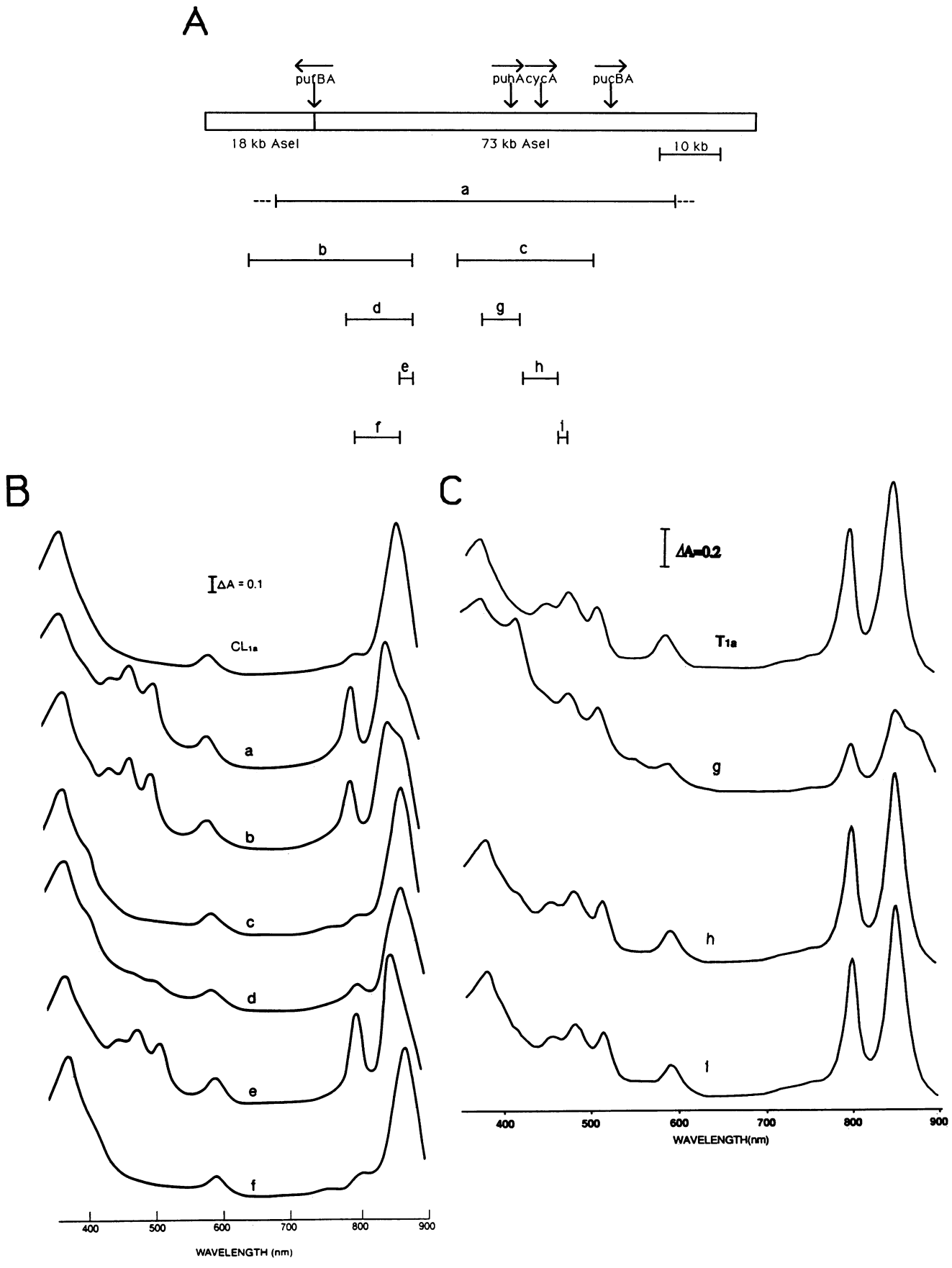
Complementation of CL_{1a} and T_{1a}. Since both CL_{1a} and T_{1a} appear to possess different mutations, as judged by their phenotypic properties, we first set out to localize each mutation following the introduction of various segments of *R. sphaeroides* chromosome I (30). Once a suspected region 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 CL_{1a}. R′ plasmid pWS2 (27, 37) (Fig. 9A, a) harboring approximately 109 kb of *R. sphaeroides* WS8 DNA containing the *puf*, *puhA*, *cycA*, and *puc* operons complemented CL_{1a}, restoring both the B800-850 complex and the normal carotenoid profile (Fig. 9B, a). The DNA region complementing CL_{1a} 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 *puhA* 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), CL_{1a} was complemented with pAS204 but not with pAS205. Furthermore, the spectrum derived from CL_{1a} (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 *puhA*, approximately 11 kb upstream of the *puf* operon (Fig. 9A).

Since the B800-850$^-$ Car$^-$ phenotype of CL_{1a} 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 CL_{1a} under aerobic (30% O_2, 1% CO_2, 69% N_2) conditions and compared with the β-galactosidase activity of pCF200(−629) in CL_{1a} 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 *Nru*I site of the *tet* gene of pCF200(−629) was interrupted with the Km^r 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 CL_{1a}(pCF200Km, pRK415) was approximately the same as that of strain CL_{1a} (pCF200)(−629) (Fig. 5), as expected. On the other hand, the introduction of cosmid 487 or pAS204 (Fig. 9A) together with pCF200Km(−629) in CL_{1a} 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 CL_{1a} involved in the loss in control by O_2 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 CL_{1a} 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 CL_{1a} background.

(ii) Complementation of T_{1a}. T_{1a} 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, T_{1a} containing pWS2 or cosmid 523 was genetically very unstable and segregated into several distinct,

FIG. 9. Complementation of CL_{1a} and T_{1a}. (A) Location of each plasmid used for complementation. (a) pWS2. (b) Cosmid 487. (c) Cosmid 523. (d) pAS203. (e) pAS204. (f) pAS205. (g) pUI803. (h) pUI811. (i) pUI813. (B) Absorption spectra (10 W/m^2) of CL_{1a} carrying plasmids a to f in *trans*. (C) Absorption spectra (10 W/m^2) of T_{1a} 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).


A
pufBA
puhAcycA
pucBA
18 kb AseI
73 kb AseI
10 kb
a
b
c
d
g
e
h
f
i
B
ΔA = 0.1
CL1a
a
b
c
d
e
f
400
500
600
700
800
900
WAVELENGTH (nm)
C
ΔA=0.2
T1a
g
h
f
400
500
600
700
800
900
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 T_{1a}, the exconjugants did not segregate into several phenotypic classes and the resulting exconjugants were quite stable. This observation lends credibility 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 T_{1a}, resulting in the restoration of the B875 complex to the wild-type level (Fig. 9C, g). Additionally, T_{1a}(pUI803) was genetically stable under aerobic conditions, and no T_4-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 T_{1a} with pUI803 (Tc^r; IncP1), we examined the β-galactosidase activities of pCF200Km(−629) and pCF250Km(−92) (Km^r; IncQ or IncP4) in the presence of pUI803 in *trans* in T_{1a} under aerobic (30% O_2, 1% CO_2, 69% N_2) conditions. In the absence of pUI803, both pCF200(−629) and pCF250(−92) were shown to yield derepressed levels of β-galactosidase activity under aerobic conditions when present in *trans* in T_{1a} (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 T_{1a}, compared with the results obtained with T_{1a}(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 T_{1a} 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 *Eco*RI 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 T_{1a} mutation.

Formation of the B800-850 complex in T_{1a} under aerobic conditions. The absorption spectrum of T_{1a}(pPXK-93) grown chemoheterotrophically (30% O_2–1% CO_2–69% N_2) was examined and compared with that of the wild type under the same growth conditions. T_{1a}(pPXK-93) formed substantial amounts of the B800-850 complex in the presence of oxygen (Fig. 10B).

DISCUSSION

We exploited the O_2-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 aminoglycoside-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^r, we were able to isolate regulatory mutations affecting *puc* operon expression. Both *trans*- and *cis*-acting mutations involved in O_2 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 O_2 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 *Pst*I-*Xma*III restriction DNA fragment of *puc* upstream DNA containing the URS (O_2 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^r 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, CL_{1a} and T_{1a}, 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^s 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 O_2 control of *puc* operon transcription. There-

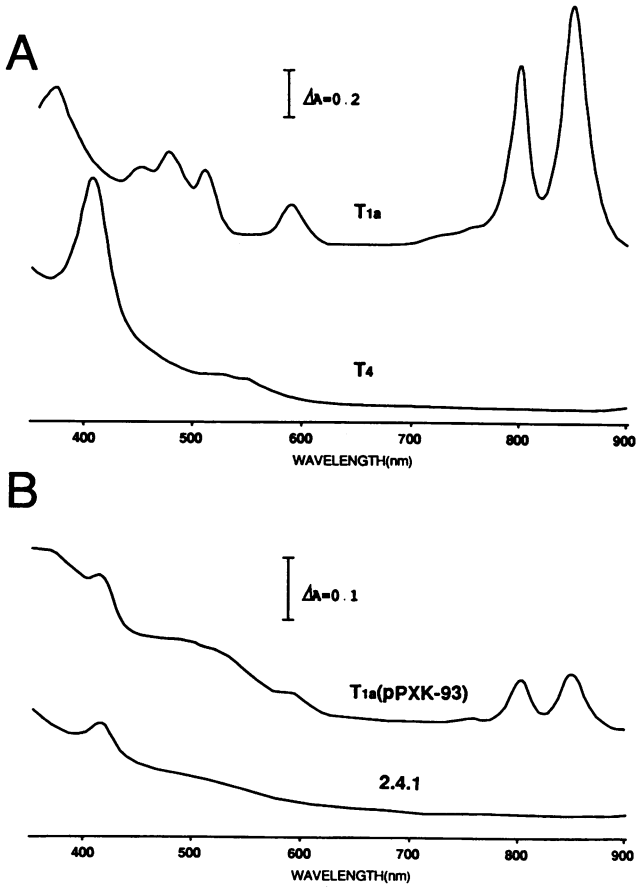


FIG. 10. (A) Absorption spectra of T_{1a} (10 W/m^2) and T_4 (anaerobic, dark growth with DMSO). (B) Absorption spectra of T_{1a}(pPXK-93) and the wild type (2.4.1) under chemoheterotrophic growth conditions as described in the text. Absorption spectra were obtained as described in the legend to Fig. 3. The bar represents an absorbance value of 0.2 (A) or 0.1 (B).

fore, assembly of these complexes can be distinguished from the loss of O_2 control of gene expression. Conversely, the introduction of a DNA fragment shown to complement CL_{1a} did not result in complementation of RS104. However, since RS104 could be complemented with pWS2 (16), the mutation in RS104 appears to be localized to the photosynthetic gene cluster. Previously (28), RS103 was shown to be complemented with cosmid 523, which includes the 7.0-kb *Eco*RI DNA fragment of pUI803 demonstrated to complement T_{1a}. We also know from that study (28) that RS103 contains a mutation encoding a factor(s) extrinsic to the structural gene components of the B875 complex and that this extrinsic factor(s) is required for the assembly of the B875 complex. Thus, it appears that the mutation affecting B875 complex formation and present in RS103 must be different from the mutation present in T_{1a}. However, we suspect that these two mutations are closely linked.

Although T_{1a} showed no apparent B875 complex in a room temperature absorption spectrum, a low-temperature spectral analysis of T_{1a} revealed the presence of the B875 complex in T_{1a} at approximately 3.1% the wild-type level (16). Thus, it is possible that a single mutation in T_{1a} affects both O_2 control and B875 complex assembly, but the latter effect is probably the result of a partially polar mutation whose primary effect is on O_2 control. Recent results from our laboratory (see below) suggest that the mutation in CL_{1a} is also polar. We designate the altered gene involved in O_2 control in T_{1a} *oxyB*.

DCL_{1a}, derived from DR_1, was spectrally almost identical to mutant CL_{1a}. In addition, DCL_{1a} showed derepressed expression of β-galactosidase activity when pCF200(−629) but not pCF250(−92) was present in *trans* and, like CL_{1a}, it was complemented with pAS204 to the restoration of the B800-850 complex and carotenoids. These close biochemical and genetic characteristics of both DCL_{1a} and CL_{1a}, 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 CL_{1a} was genetically stable under aerobic conditions, T_{1a} was very unstable, spontaneously generating a second mutant class, designated T_4, at a high frequency. This nonpigmented, PS^- mutant (T_4), however, readily reverted to T_{1a} under photosynthetic (10 W/m^2) conditions. In turn, T_{1a} derived from T_4 under photoheterotrophic conditions segregated to T_4 again, completing the cycle under aerobic conditions. When efforts were made to complement T_4, 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 T_{1a} phenotype (7). Additionally, when *puc*::*lacZ* transcriptional fusions were moved into the T_4 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 T_4 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 T_4. 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 CL_{1a} and T_{1a}, 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 O_2-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 T_{1a} appears to interact with both the URS and the DRS of the operon (15), while the second *trans*-acting factor, lacking in CL_{1a}, appears to interact with only the URS of the *puc* operon. The former was designated *oxyB*, and the mutation in CL_{1a} 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/m^2) conditions (15) was approximately 20% 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(pRKRP1) or PUC705-BA(pRKLP1) (17), which 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/m^2) 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 *puc* upstream DNA which titrate some positively acting factor, since the copy numbers of both the IncQ [pCF200(−629)] and the IncP1 (pRKRP1 and pRKLP1) 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 *lacZY*::Ω Sm^r/Sp^r *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 (17). 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 O_2 repression in the presence of *oxyA* in *trans* when the chromosomal copy of the *puc* operon was interrupted. Since pAS204 restored the O_2 repression of the *puc* operon on pCF200(−629) in *trans* in CL_{1a} 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 O_2 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) in

trans in PUC705-BA and PUC-Pv (16). pCF200(−629) and pCF250(−92) in each of these mutants showed approximately 50 to 40%, respectively, of the activity of the corresponding plasmids in *trans* in the wild type under photosynthetic (10 W/m^2) 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 CL_{1a} affected both *crtB* and *crtK*, thus leading to both a Car$^-$ B800-850$^-$ phenotype and a lack of O_2 expression of *puc* operon expression. These two effects have now been separated, and *oxyA* has been shown only to affect O_2 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 T_{1a} under aerobic conditions could be related to the ability of T_{1a} to express both bacteriochlorophyll and the apoproteins of the B800-850 complex in the presence of oxygen. Thus, *oxyB* would be a more global regulator of photosynthetic gene expression than *oxyA*. As a result, the synthesis of bacteriochlorophyll in the presence of O_2 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 CL_{1a} or T_{1a} can be compromised when the essential gene is part of a large but incomplete block of genes. Thus, some form of pleiotrophy 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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