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Transcriptional Regulation of *puc* Operon Expression in *Rhodobacter sphaeroides*

ANALYSIS OF THE *CIS*-ACTING DOWNSTREAM REGULATORY SEQUENCE*

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Both site-directed and spontaneous mutagenesis have been used to investigate the role of the *cis*-acting regulatory region between −92 and −1 base pair (bp) of the *puc* operon of *Rhodobacter sphaeroides*. The DNA sequence from −84 to −66 bp upstream of the 5′ end of the start site of *puc* operon transcription is essential for normal *puc* operon expression. This regulatory effect was exerted irrespective of the presence or absence of additional upstream regulatory sequences extending from −629 to −93 bp. It is likely that this region is involved in activator binding. Additionally, two regions of dyad symmetry centered at −42 and −17 bp are shown to be involved in oxygen repression of *puc* operon expression. Mutations within these regions of dyad symmetry were further subdivided on the basis of whether or not the upstream regulatory region was required to observe the mutant phenotype. Based upon these observations we conclude that these regions of dyad symmetry possessing the motif TGT-N_{12}-ACA (where N represents any nucleotide) are involved in repressor binding with the *puc* operon promoter overlapping each of these dyad symmetries.

The *puc* operon of *Rhodobacter sphaeroides* consists of two structural genes, *pucB* and *pucA*, encoding the β- and α-polypeptides, respectively, of the B800–850 light harvesting complex and additional downstream open reading frame(s) that encode gene product(s) involved in the post-transcriptional control of expression and assembly of the β- and α-polypeptides to form the B800–850 complex (1, 2). In a related bacterium, *Rhodobacter capsulatus*, the *puc* operon has been reported to have a similar although not identical organization (3, 4). Formation of the B800–850 complex is variable with respect to the fixed photosynthetic unit. Together with the fixed photosynthetic unit the B800–850 complex has been referred to as the variable photosynthetic unit, which can comprise over 50% of the protein found in the intracytoplasmic membrane (5–9). Intracytoplasmic membrane formation is represented as a series of invaginations arising from and continuous with the cytoplasmic membrane following removal or lowering of the partial pressure of O_2 below threshold levels, approximately 2.5% relative to air (10).

The *puc* operon gives rise to two (0.5- and 2.3-kilobase) transcripts, having a single transcription start site, both of which are severely affected by the presence or absence of O_2, and in the absence of O_2 the relative levels of each transcript are further modulated by light intensity (1, 11). We have subdivided the 629-bp[1] *puc* DNA upstream of the 5′-end (+1) of the *puc* transcripts into two functionally separable *cis*-acting domains, the upstream regulatory sequence (URS) (−629 to −93) involved in enhanced control of operon transcription by oxygen and light, and the downstream regulatory sequence (DRS) (−92 to −1) primarily involved in basal level aerobic transcription as well as anaerobic regulation of transcription (12, 13). Interaction or communication between these two *cis*-acting regions was demonstrated based upon the transcriptional response to oxygen and light and the silencing of point mutations within the DRS when these two domains are uncoupled (12). A region extending from −139 to −93 bp containing overlapping FNR- and IHF-binding sequences separates these two *cis*-acting regions and is suggested to play a role in mediating this interaction (12–14). The IHF-binding sequence is involved in repression of *puc* operon transcription by oxygen as well as modulation of the transcription levels by incident light intensity (14). Recently the gene encoding an FNR homologue in *R. sphaeroides* has been identified in our laboratory.[2]

Recently Eraso and Kaplan (15) reported a new gene locus in *R. sphaeroides*, *prrA*, which was found to be involved in positive regulation of photosynthesis gene expression in response to anaerobiosis. The NH_2-terminal portion of PrrA showed clear similarity to the highly conserved amino termini of response regulators of two-component regulatory systems mediating signal transduction in prokaryotes. PrrA was shown to act (directly or indirectly) at the level of the DRS of the *puc* regulatory region. In *R. capsulatus* a gene designated *regA* was reported (16), to which *prrA* shows a high similarity in both DNA and amino acid sequences. In the present studies, both *in vitro* and *in vivo* mutagenesis were employed to investigate the role of the DRS in *puc* operon expression. As a result, the DRS was itself subdivided into several regulatory regions. One of these domains is similar to those reported to be involved in factor-dependent binding (17). A model is presented that describes the role(s) for each of these regions within the DRS in the regulation of *puc* operon expression.

EXPERIMENTAL PROCEDURES

Bacteria, Plasmids, and Cell Growth—The bacterial strains and plasmids used in this study are presented in Table I. *R. sphaeroides*

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[1] The abbreviations used are: bp, base pair(s); URS, upstream regulatory region; DRS, downstream regulatory region; FNR, fumarate nitrate regulator; IHF, integration host factor; W, watts; Km, kanamycin.

[2] J. Zeilstra-Ryalls and S. Kaplan, unpublished results.

TABLE I
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::Tn7	30
E. coli TG1	K12, Δ(*lac-pro*), *sup*E, *thi*, *hsd*D5/F′*tra*D36, *pro*A$^+$B$^+$, *lac*I^q, *lac*ZΔM15	Amersham Corp.
E. coli DH5α*phe*	*phe*::Tn10*d*Cm of DH5α	15
R. sphaeroides 2.4.1	Wild type	W. R. Sistrom
Plasmids		
pPD19	Ap^r pUC19 *pst*I *Hinc*II: 0.7-kb *Pst*I-*Dra*II *puc* upstream DNA filling in the *Dra*II overhang	This study
pSL301	Ap^r, 3.2-Kb SuperLinker vector containing an extended polylinker region	Invitrogen
pLV106	pUI511 containing *Eco*RI, *Sma*I, *Kpn*I and *Xba*I restriction sites at the *Pst*I site, IncQ/IncP4	12
pRS415	Ap^r, *lacZYA*	21
pCF1010	pLV106 modified to contain unique *Pst*I, *Not*I, *Nsi*I, *Avr*II, *Stu*I, *Bsp*MII, and *Xba*I restriction sites between the 2.0-Kb Ω Sm^R/Sp^R and the 5.1-Kb *lacZYA*′, IncQ/IncP4	This study
pCF400[a] (−629)[b]	pLV106 containing the 0.7-kb *Pst*I-*Dra*II *puc* upstream DNA from pPD19	12
pCF450 (−92)	pLV106 containing the 0.17-kb *Xma*III-*Dra*II *puc* upstream DNA from pPD19	12
pCF801/−68, −67[c]	The same *puc::lacZ* fusion construct as pCF400(−629) except for mutation of GC(−68, −67) to TA as described	This study
pCF851/−68, −67	The same *puc::lacZ* fusion construct as pCF450(−92) except for mutation of GC(−68, −67) to TA as described	This study
pCF802/−83, −82	The same *puc::lacZ* fusion construct as pCF400(−629) except for mutation of GG(−82, −82) to TT as described	This study
pCF852/−83, −82	The same *puc::lacZ* fusion construct as pCF450(−92) except for mutation of GG (−83, −82) to TT as described	This study
pCF4000 (−629)	pCF1010 containing the 0.7-kb *Pst*I-*Dra*II of *puc* upstream DNA derived from pPD19	This study
pCF4500 (−92)	pCF1010 containing the 0.17-kb *Xma*III-*Dra*II of *puc* upstream DNA from pPD19	This study
pCF4060/Δc	The same *puc::lacZ* fusion construct as pCF4000(−629) except for deletion of 32 bp from −87 to −56 as described	This study
pCF4560/Δc	The same *puc::lacZ* fusion construct as pCF4500(−92) except for deletion of 32 bp from −87 to −56 as described	This study
pCF4010/−11	The same *puc::lacZ* fusion construct as pCF4000(−629) except for mutation of C(−11) to T as described	This study
pCF4510/−11	The same *puc::lacZ* fusion construct as pCF4500(−92) except for mutation of C(−11) to T as described	This study
pCF4020/duplication (−34 to −23)	The same *puc::lacZ* fusion construct as pCF4000(−629) except for duplication of 12 bp from −34 to −23 as described	This study
pCF4520/duplication (−34 to −23)	The same *puc::lacZ* fusion construct as pCF4500(−92) except for duplication of 12 bp from −34 to −23 as described	This study
pCF4050/−36	The same *puc::lacZ* fusion construct as pCF4000(−629) except for mutation of C(−36) to T as described	This study
pCF4550/−36	The same *puc::lacZ* fusion construct as pCF4500(−92) except for mutation of C(−36) to T as described	This study
pCF4070/Δd	The same *puc::lacZ* fusion construct as pCF4000(−629) except for deletion of 18 bp from −52 to −35 as described	This study
pCF4570/Δd	The same *puc::lacZ* fusion construct as pCF4500(−92) except for deletion of 18 bp from −52 to −35 as described	This study
pCF4080/−52–50	The same *puc::lacZ* fusion construct as pCF4000(−629) except for mutation of TGT(−52 to −50) to GCG as described	This study
pCF4580/−52–50	The same *puc::lacZ* fusion construct as pCF4500(−92) except for mutation of TGT(−52 to −50) to GCG as described	This study
pCF4100/−37–35	The same *puc::lacZ* fusion construct as pCF4000(−629) except for mutation of ACA(−37 to −35) to CGC as described	This study
pCF4600/−37–35	The same *puc::lacZ* fusion construct as pCF4500(−92) except for mutation of ACA(−37 to −35) to CGC as described	This study

[a] The pCF400/800 series were derived from plasmid pLV106, and the 4000 series were derived from plasmid pCF1010.
[b] The designation in parenthesis indicates whether the entire 629-bp *puc* upstream DNA or the 92-bp *puc* upstream DNA was present.
[c] The designation after the slash indicates the location of the mutational change. Each change is described in the text.

wild type strain 2.4.1 and its derivatives were grown as described previously (18). *R. sphaeroides* cultures used for the measurement of β-galactosidase activities were grown chemoheterotrophically or photoheterotrophically by sparging with gas phase mixtures as described previously (19). Growth of *R. sphaeroides* cultures in the dark with Me_2SO was performed as described previously (14). For isolation of single colonies under photoheterotrophic (100 W/m^2) conditions, agar-embedded cultures in screw-cap tubes were employed.

Escherichia coli strains JM109, DH5α*phe*, TG1, or S17–1 were grown at 37 °C in Luria medium (20). 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 pUC19, pBS, pSL301, pRS415 (21), and pLV106 (12) were used for cloning.

DNA Manipulation and Sequence Analysis—Large and small scale plasmid DNA preparations were prepared as described previously (11, 20, 22). Treatment of DNA with restriction enzymes and other nucleic acid-modifying enzymes was performed in accordance with manufacturer specifications. DNA fragments were analyzed on agarose gels or polyacrylamide gels, and restriction fragments were isolated as described previously (23).

DNA sequence analysis was performed with phage M13 clones as described previously (11, 18) or with an ABI 373A automatic DNA

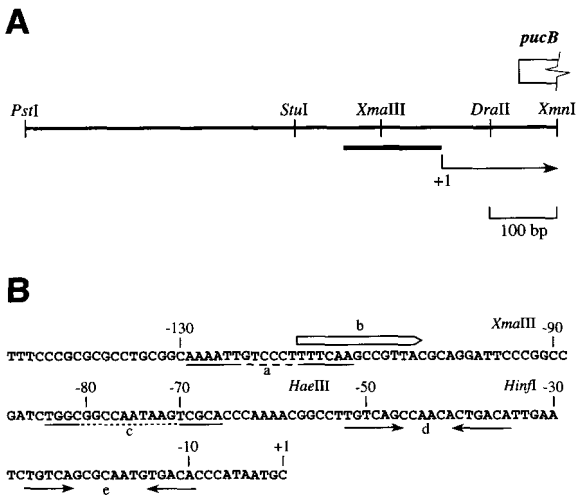


FIG. 1. *A*, restriction map of the *puc* upstream DNA. +1 is the 5′-end of the *puc*-specific transcripts. The DNA region corresponding to the sequence shown in *B* is *underlined*. *B*, DNA sequence from −148 to +1. An IHF-binding sequence (*b*) overlapping a putative consensus sequence for FNR (*a*) is followed by the putative activator-binding site(s) (*c*). The two regions of dyad symmetry are distal (*d*) and proximal (*e*).

sequencer (Applied Biosystems Inc., Foster City, CA) at the DNA Core Facility of the Department of Microbiology and Molecular Genetics (University of Texas Health Science Center, Houston, TX).

Conjugation Techniques—Plasmid pLV106- or pCF1010-derived plasmids were mobilized into *R. sphaeroides* 2.4.1 by previously described procedures (19).

Site-directed Mutagenesis of the puc Regulatory DNA—The oligonucleotide-directed *in vitro* mutagenesis system (Amersham Corp.) was used according to the manufacturer's specifications. The presence of the desired mutations was confirmed by dideoxy sequencing of the mutated DNA molecules.

Construction of pCF1010 and Cloning of the puc::lacZ Transcriptional Fusion Constructs—pCF1010 was constructed from pLV106 through a series of clonings. The vector contains seven unique restriction sites (*Pst*I, *Not*I, *Nsi*I, *Avr*II, *Stu*I, *Bsp*MII, and *Xba*I sites derived from pSL301) situated between a 2.0-kilobase Ω cartridge harboring a spectinomycin/streptomycin-resistance gene (from pHP45) and a 5.1-kilobase DNA fragment containing *lacZYA′* (from pRS415). Transcriptional fusions between *puc* and *lacZ* within the vector can be performed by one-step cloning of a *puc* regulatory DNA fragment (from pUC19, pBS, or M13 clones) into the multiple cloning site of pCF1010. Cloning of the *puc* upstream DNA fragments to form transcriptional fusions of *puc* to *lacZ* within the restriction sites of pLV106 was performed as described (12).

Preparation of Cell Extracts and Assay of β-Galactosidase—Cell harvest, preparation of crude extracts, and β-galactosidase assays were performed as described (24). All experiments involving β-galactosidase assays were conducted at least two times.

Materials—Restriction endonucleases and nucleic acid-modifying enzymes were purchased from Life Technologies, Inc., or New England Biolabs, Inc. (Beverly, MA) 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. [α-^{35}S]dATP was obtained from Amersham Corp. Isopropyl-β-D-thiogalactopyranoside and *o*-nitrophenyl β-D-galactopyranoside were obtained from Sigma. Oligonucleotides used for construction of mutants were made by Genosys Biotechnologies, Inc. (The Woodlands, TX) or by the Microbiology and Molecular Genetics Core Facility (University of Texas Health Science Center, Houston, TX). DNA sequencing kits and Sequenase™ were obtained from U.S. Biochemical Corp. Molecular biology grade phenol was purchased from Fisher. All other chemicals used in this work were reagent grade.

RESULTS

A Putative Binding Site for a Transcription Activator in the DRS—Some general features of the *cis*-acting regulatory region involved in *puc* operon expression are presented in Fig. 1*A*. The importance of the 35-bp sequence, immediately downstream of the IHF-binding sequence (14), between the *Xma*III (−92) and *Hae*III (−57) restriction sites (Fig. 1*B*) has been recognized previously (12). A similar sequence and spacing to that found in *R. sphaeroides* ($^{-84}$TGGC-N$_{10}$-TCGCA^{-66}) is observed in *R. capsulatus*, TGGC-N$_8$-CCGCA, and is located between −80 and −40 bp upstream of the 5′-ends of the *puc* transcripts (25, 26). To further assess the role of this sequence in *puc* operon expression several alterations in this sequence were introduced by site-directed mutagenesis, and the results of these changes were examined by following the expression of *lacZ* transcription fusions.

In the first constructions, two consecutive bases, GG (−83, −82) or GC (−68, −67) were changed to TT and TA, respectively. Each of the altered DNA fragments corresponding to the DRS (−92 to −1) with or without the URS (−629 to −93) were transcriptionally fused to *lacZ*, each fusion construct was mobilized into *R. sphaeroides* 2.4.1, and β-galactosidase activities were determined under the conditions described.

From the data presented in Fig. 2 it is obvious that the changes at −68 and −67 had a severalfold effect upon *puc* operon expression in both the presence and absence of the URS. Further, and most importantly when compared with wild type, the effect was observed regardless of the environmental conditions under which *puc* operon expression was measured. On the other hand, mutations at −83 and −82 showed little alteration in *puc* expression in the presence of the URS and little if any change in the absence of the URS.

A third mutation, deleting three helical turns, *i.e.* 32 bp between −87 and −56 as depicted in Fig. 3 and designated Δc, was introduced in the DRS. This deletion encompasses the region of $^{-84}$TGGC-N$_{10}$-TCGCA^{-66} as well as 3 and 10 bp upstream and downstream, respectively. The mutated DRS was transcriptionally fused to *lacZ* in the presence and absence of the URS and β-galactosidase activities determined as described in the legend to Fig. 4. The removal of the DNA sequence extending from −87 to −56 resulted in a 3–8-fold decrease in *puc* operon expression under the conditions employed in both the presence and absence of the URS, when compared with the wild type construction.

One further point is worth noting regarding the level of derepression when *puc* operon expression is measured in cells growing aerobically and under dark/Me$_2$SO conditions. When the URS is present, both the Δc mutation and the mutations at −68 and −67 still show an approximate 6-fold derepression, which is about 30% of that observed for the corresponding wild type constructions. Thus, despite the overall damaging effect upon *puc* operon expression, these mutations largely retain the ability of the *puc* operon to respond to the removal of O_2.

Selection of cis-Acting Mutations Involved in Oxygen and Light Control of puc Transcription—Previously, two *cis*-acting mutations affecting oxygen regulation of *puc* operon expression were isolated by exploiting a *puc::aph* transcriptional fusion (12). The mutants were selected because of their increased resistance to Km in the presence of O_2. One mutation mapped to −26 (G → A) and the second to −12 (A → C). Both mutations were silent in the absence of the URS, and both showed increased LacZ expression in the presence of O_2. Other differences were also noted and will be discussed later.

Because these mutations mapped to one of the two regions of dyad symmetry (Fig. 1*B*) a similar approach (12, 13) to the isolation of additional mutants displaying increased resistance to Km was undertaken using even higher concentrations of Km (40 and 80 μg/ml). Four independent isolates of the same *cis*-acting mutation at −11 (C → T) were isolated at a Km concentration of 40 μg/ml (Fig. 3). At 80 μg/ml Km two additional mutations were isolated: the same at −11 (C → T) as above and a duplication of the 12-bp DNA sequence between −34 and −23

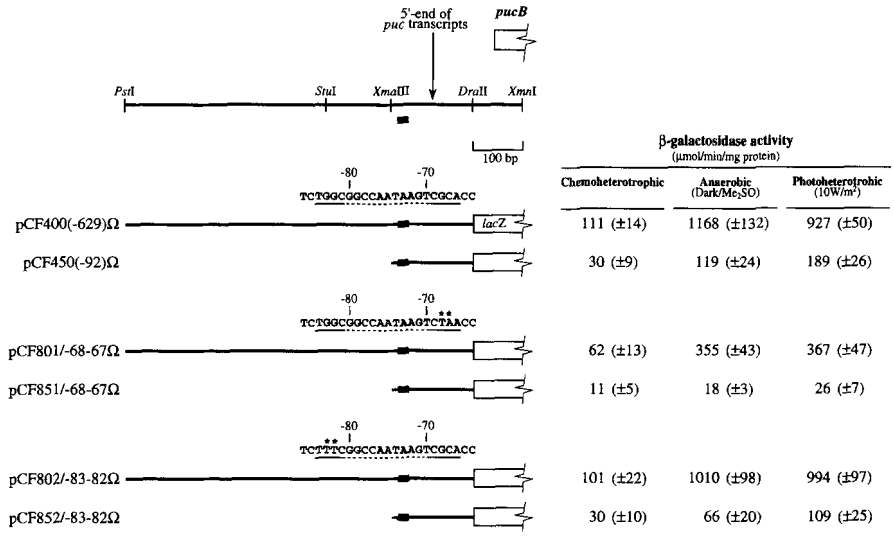


FIG. 2. **β-galactosidase activities associated with transcriptional fusions of the *lacZ* gene to *puc* upstream DNA containing either wild type or mutated forms of the putative activator-binding sequence within the DRS.** The *puc* DNA region corresponding to the 19 bp from −84 to −66 is *underlined below* the restriction map and also shown as a *thick line* for each *puc::lacZ* fusion. Mutated bases are denoted with *asterisks*. The *puc::lacZ* fusion constructs were present *in trans* in *R. sphaeroides* 2.4.1 grown chemoheterotrophically (30% O_2, 1% CO_2, 69% N_2) or anaerobically in the dark with Me_2SO. Photoheterotrophic cultures grown at 10 W/m^2 were harvested between 40 and 60 Klett units (1 Klett unit = ~0.75 × 10^7 cells). Deviations for β-galactosidase measurements are shown in *parentheses*.

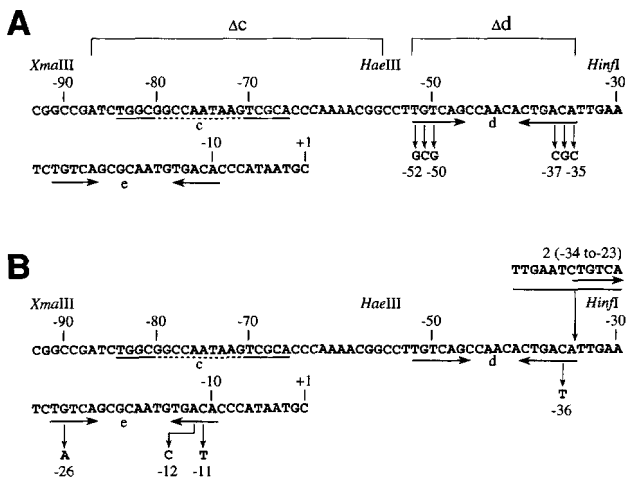


FIG. 3. *A*, the *puc* DNA sequence extending from −93 to +1 illustrating the region mutated through oligonucleotide-directed *in vitro* mutagenesis. Δ denotes deletion of the area confined by the *brackets above* the *sequence*. TGT (−52 to −50) and ACA (−37 to −35) were mutated to GCG and CGC, respectively. *B*, the *puc* DNA sequence extending from −93 to +1 illustrating the DNA region mutated by selection of regulatory mutations employing the *puc::aph* fusion. *cis* mutations at −36 and −11 are shown *below* the DNA sequence together with the previously described −26 and −12 mutations (12). Duplication of the 12-bp DNA sequence from −34 to −23 is illustrated *above* the region of duplication.

(Fig. 3). The 12-bp duplication includes the left half of the proximal region of dyad symmetry plus the gap sequence between the two regions of dyad symmetry (Fig. 3).

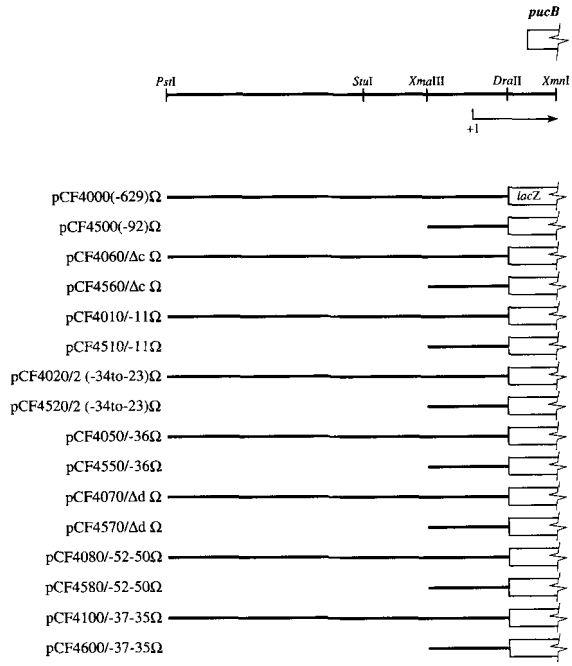
Another approach was employed to isolate a different mutation at −36 (C → T) (see Fig. 3). In this case increased resistance to Km was selected in cells growing at 100 W/m^2 light intensity. Normally, these cells containing the *puc::aph* fusion (12, 13) are resistant to up to 2.5 μg/ml Km. At 3 W/m^2 these same cells are resistant to 25 μg/ml Km. Thus, mutants resistant to 100 μg/ml Km at 100 W/m^2 were isolated. Only one *cis*-acting mutation (at −36) was obtained, and the remainder were shown to be *trans*-acting mutations, which are not described here.

All of these mutations residing in the DRS were transcriptionally fused to *lacZ* in the presence or absence of the URS and β-galactosidase activities measured under various conditions of growth as depicted in Fig. 4. Upon examination of the results, several points are noteworthy. As expected for the mutations at −11 and the duplication of the sequence from −34 to −23, the level of LacZ expression in the presence of O_2 is greater, ~5–6 fold, than the corresponding wild type. What was unexpected is that the mutation at − 36 yielded LacZ activity 5-fold greater than wild type in the presence of O_2, although it was selected for in response to altered expression in the presence of high light under anaerobic conditions. However, unlike the former two mutations, the change at −36 did lead to increased expression either under conditions of anaerobic dark or anaerobic light growth, when compared with the wild type. When the DRS was uncoupled from the URS, each of these three mutations resulted in different levels of expression of β-galactosidase. The mutation at −36 was essentially silent, and the duplication showed decreased expression. However, the mutation at −11 showed increased LacZ expression in the presence or absence of O_2.

Site-directed Mutations of the Distal Region of Dyad Symmetry—Of the five independently isolated mutations involving both the distal and proximal regions of dyad symmetry, four involved the proximal region (see Fig. 1), with only one, namely −36, involving the distal region (see Fig. 1). Using site-directed mutagenesis we created three additional mutations in the distal region of dyad symmetry as follows: Δd, involving the removal of the entire distal region of dyad symmetry; −52 to −50, involving the change of TGT to GCG; and −37 to −35, involving the change of ACA to CGC (see Fig. 3). These last two mutations were created because of the obvious symmetry in DNA sequence between the distal and proximal regions of dyad, *i.e.* TGT-N_{12}-ACA. Each mutation within the DRS was followed with respect to its effect on *lacZ* expression in the presence or absence of the URS as described previously (Fig. 4).

In the complete absence of the distal region of dyad symmetry (Δd) the level of β-galactosidase in the absence of the URS is near background. Even in the presence of the URS, LacZ levels are low, and there appears to be no increased expression upon removal of O_2.

Also evident is the fact that changes at positions −52 to −50 and −37 to −35 lead to increased LacZ activity in the presence of O_2, and in the case of −52 to −50 increased expression in the absence of O_2 is observed when the URS is present. In the absence of the URS, only the changes at −37 to −35 show increased β-galactosidase in the presence or absence of O_2, whereas the changes at −52 to −50 are silent when these mutations are compared with the wild type. However, both sets of changes continue to show derepression under an aerobic to anaerobic transition. Thus, it appears that both regions of dyad symmetry play a role in the repression of *puc* operon expression



	β-galactosidase activity (μmol/min/mg protein)		
	Chemoheterotrophic	Anaerobic (Dark/Me_2SO)	Photoheterotrophic (10W/m^2)
pCF4000(-629)Ω	93 (±9)	1538 (±209)	1451 (±54)
pCF4500(-92)Ω	30 (±7)	185 (±9)	176 (±19)
pCF4060/Δc Ω	39 (±5)	241 (±31)	n.d.
pCF4560/Δc Ω	11 (±4)	21 (±4)	n.d.
pCF4010/-11Ω	584 (±59)	1742 (±136)	n.d.
pCF4510/-11Ω	155 (±16)	701 (±31)	n.d.
pCF4020/2 (-34to-23)Ω	631 (±66)	1710 (±137)	n.d.
pCF4520/2 (-34to-23)Ω	29 (±6)	69 (±6)	n.d.
pCF4050/-36Ω	553 (±25)	2285 (±182)	2282 (±120)
pCF4550/-36Ω	37 (±4)	207 (±20)	275 (±23)
pCF4070/Δd Ω	39 (±2)	87 (±27)	n.d.
pCF4570/Δd Ω	14 (±5)	10 (±2)	n.d.
pCF4080/-52-50Ω	688 (±59)	2137 (±97)	n.d.
pCF4580/-52-50Ω	39 (±4)	148 (±14)	n.d.
pCF4100/-37-35Ω	810 (±142)	1784 (±181)	n.d.
pCF4600/-37-35Ω	125 (±24)	526 (±65)	n.d.

FIG. 4. **β-galactosidase activities associated with transcriptional fusions of the *lacZ* gene to the *puc* upstream DNA containing wild type and various mutations associated with the putative activator(s)-binding region and the regions of dyad symmetry as described in Fig. 3.** Each mutation can be identified by noting the precise change to the *immediate right* of each plasmid designation. The *solid lines* indicate the presence of either the entire 629-bp upstream sequence or the 92-bp upstream sequence. The *puc::lacZ* fusion constructs were present *in trans* in *R. sphaeroides* 2.4.1 grown chemoheterotrophically (30% O_2, 1% CO_2, 69% N_2) or anaerobically in the dark with Me_2SO. Photoheterotrophic cultures at 10 W/m^2 were harvested between 20 and 30 Klett units. Deviations of the β-galactosidase activities are shown in *parentheses*. *n.d.*, results not determined here.

in the presence of O_2 as well as in derepressed expression of the *puc* operon in response to the removal of O_2. Also, mutations localized to these regions of dyad symmetry respond differently, depending upon whether or not the DRS and URS are coupled or uncoupled.

DISCUSSION

The *puc* operon upstream DNA between the *Xma*III (−92) and the *Hae*III (−57) restriction sites immediately downstream of the overlapping FNR- and IHF-binding sequences was shown previously to play an important role in the expression of the *puc* operon under both aerobic and photoheterotrophic conditions when the DRS was uncoupled from the URS (12). For the purposes of this discussion expression of *lacZ* is considered to be a relative measure of *puc* operon transcription. The results reported here support further the importance of this DNA sequence centered at approximately −75. We propose that this region serves as a binding site for a positively acting transcriptional regulator (14). The fact that the presence of this region results in an increased expression of the *puc* operon whether or not O_2 is present would be anticipated for a generalized transcriptional activator. The mutation at −68 and −67 appears to have a lesser effect in the presence of oxygen than in its absence. This may reflect, as discussed below, the ability of the hypothetical activator to contact RNA polymerase whose promoter binding ability would be strongly affected by the presence of repressor when O_2 is present.

Although the results of this study were focused on the DRS, the question naturally arises as to the role or roles of the URS since there are absolute differences in the expression of the *puc* operon reported here and elsewhere (12–15) dependent upon the presence or absence of the URS. One possibility is that a second promoter is present in the URS in addition to a promoter within the DRS. There are several observations arguing against that possibility. First and foremost all attempts to detect *puc* operon transcripts or 5′ ends derived from DNA sequences upstream of the reported unique 5′ end (1) have proven unsuccessful despite the method of RNA preparation. Second, the response regulator PrrA having a major effect upon *puc* operon expression has been interpreted to exert its effect upon *puc* operon expression through the DRS. Third, the silencing of certain specific mutations (*e.g.* at −12, −26 (12), −52 to −50, and −36 reported here) when the URS is uncoupled from the DRS is difficult to explain solely on the basis of two promoters. *lacZ* fusions to either the *Stu*I or *Xma*III sites located approximately 225 and 92 bp, respectively, upstream of the start site of transcription, result in only background levels of *lacZ* expression under various conditions of growth. For example, in the presence of high oxygen the wild type level of LacZ is ~163 units of β-galactosidase/mg of protein, whereas the *Xma*III fusion yields ~4 units, and the *Stu*I fusion yields ~9 units. Similarly, under dark/Me_2SO conditions the wild type yields ~1533 units and the *Xma*III and *Stu*I fusions yield ~8 and 11 units, respectively. These low levels of β-galactosidase from the upstream fusions are similar to those observed when *lacZ* is positioned immediately downstream of an Ω cartridge without any obvious promoter sequences positioned in between. Similar experiments conducted with cells growing under low light intensities have given similar results. It would be necessary to assume that changes in the DRS could affect the upstream promoter, leaving us back where we started. Finally, the exceptionally low levels of residual *puc* operon expression as well as the absence of any increased expression following the removal of O_2 in the Δd mutation lying entirely within the DRS supports the existence of a single promoter within the DRS. Therefore, without evidence to the contrary we will continue with the working hypothesis that the interaction of the URS with the DRS is through the mediation of additional *trans*-acting factors. Our laboratory has defined several potential factors and, as described earlier, an FNR homologue.[2]

Where is the promoter within the DRS located? Previously (12, 14) we proposed that the regulatory region of the *puc* operon possessed the properties of both σ^{54}- and σ^{70}-type promoters (27). In either instance RNA polymerase can cover DNA coordinates between −50 and +20. In light of the effects of the Δd deletion on *puc* operon expression we suggest that the most upstream portion of the promoter has been removed. This interpretation is complicated by the fact that the Δd deletion has resulted in the removal of less than two helical turns of the DNA, placing the putative activator binding region out of phase with the promoter. However, the Δc mutation that removes the putative activator binding sequence does not diminish *puc* operon expression to the extent of the Δd mutation, and more

importantly, the Δc mutation leaves ± 0_2 control largely intact. Therefore, we propose that the promoter for the *puc* operon overlaps both the distal and proximal regions of dyad symmetry. This is supported by recent work by Gomelsky and Kaplan.[3] A similar although not identical case has been observed in the regulatory region of the *bchCXYZ* operon in *R. capsulatus* (28).

Comparison of the 90-bp *puc* upstream DNA of *R. sphaeroides* with that of *R. capsulatus* (25, 26) reveals a high degree of sequence homology from −84 to −66 (putative activator binding regions(s)) and −52 to −28 (distal dyad symmetry and its immediate 7-bp downstream DNA) as well as from −9 to −1. Only one sequence of dyad symmetry was found in the *puc* upstream DNA from *R. capsulatus* (25, 26).

Penfold and Pemberton (29) recently identified a gene, *ppsR*, which alone is sufficient for photopigment suppression in *R. sphaeroides*. The *ppsR* gene encodes a protein with a DNA-binding domain within the COOH-terminal region. These authors suggested that the PpsR protein recognizes the motif TGT-N_{12}-ACA, found to reside within the immediate *cis*-acting sequences of some but not all photopigment genes. We proposed (12) that these same sequences within the DRS of the *puc* operon are involved in factor binding (17). In a more direct approach to this hypothesis Gomelsky and Kaplan[3] have determined that the *ppsR* gene product interacts with these regions of dyad symmetry and that point mutations within these sequences can alter PpsR-mediated repression of *puc* operon expression.

We now possess genetic evidence that the region of dyad symmetry possessing the TGT-N_{12}-ACA motif has repressor binding activity.[3] Because these same regions of dyad symmetry occupy the region −10 to −52, this sequence may also contain the promoter for the *puc* operon. The net effect of mutations within these sequences could simultaneously reflect alterations in both repressor binding and promoter strength. For example, point mutations having increased *puc* operon expression in the presence of O_2 may reflect diminished repressor binding without any effect upon the *puc* promoter. However, we cannot rule out the possibility that other mutations could lead to increased promoter strength without affecting repressor binding. Intermediate alternations between these extremes are more than likely. It is also likely that each region of dyad symmetry is capable of binding repressor, since mutations mapping to both regions lead to increased *puc* operon expression in the presence of O_2.

If we assume that under anaerobic conditions repressor binding to the region(s) of dyad symmetry is no longer involved in regulating expression of the *puc* operon, then the mutations at −12, −36, and −52 to −50 may reflect changes in promoter accessibility, the former two directly representing changes in promoter sequence and −52 to −50 indirectly through their effect on the −36 region by virtue of a change in DNA structure. Since these changes are only observed in the presence of the URS, we conclude that the presence of the URS is important for enhanced accessibility of RNA polymerase to the *puc* operon promoter.

There are two mutations, at −11 and −37 to −35, which in addition to displaying increased *puc* operon expression in the presence of O_2 do so whether or not the URS is coupled to the DRS. Each of these changes occurs in the proximal arm of both the distal and proximal regions of dyad symmetry. Several interpretations, which are not mutually exclusive, are possible. These mutations might signify asymmetric binding of the repressor to the region(s) of dyad symmetry. Or these sequences may lie within and therefore define the *puc* operon promoter.

At this point it is worth noting that mutations in the URS sequence located between −197 and −190 led to a 7-fold increase in *puc* operon expression in the presence of O_2.[4] This observation is most readily explained if direct interactions, mediated by protein factors, between the URS and DRS can occur. Interactions between the URS and the DRS are further strengthened by the results obtained with the duplication of the region from −34 to −23. In the absence of the URS, RNA polymerase interactions would be destablized and activity would be low, as seen. However, in the presence of the URS and other protein factors proper alignment of those regions might be favored. Any explanations involving transcription initiation from sequences within the URS would still require additional mechanisms for such changes in the URS resulting in silencing of repressor binding within the unaffected DRS.

It is obvious that much remains to be learned regarding the *cis*-acting regulatory sequences involved in *puc* operon expression. If we are correct in our assumptions that the regions of dyad symmetry within the DRS serve both to bind repressor (and perhaps other regulatory effectors) and RNA polymerase, then the mutational effects observed here represent the outcome of a subtle interplay between these disparate activities. Therefore, it may be possible to view each of these unique roles through the use of specific genetic backgrounds. For example, in a PpsR minus background it may be possible to undertake an investigation of the *puc* operon promoter, all else being equal.

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