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Genetic Evidence that PpsR from *Rhodobacter sphaeroides* 2.4.1 Functions as a Repressor of *puc* and *bchF* Expression

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The *ppsR* gene (R. J. Penfold and J. M. Pemberton, J. Bacteriol. 176:2869–2876, 1994) from *Rhodobacter sphaeroides* 2.4.1 functions as a transcriptional repressor of *puc* and *bchF* expression. The carboxy terminus of PpsR, containing the putative DNA-binding domain, by itself possesses repressor activity. Intact palindromes having the motif TGT-N_{12}-ACA are required for PpsR activity.

Synthesis and assembly of the photosynthetic apparatus in anoxygenic photosynthetic bacteria are well coordinated (14). A decrease in oxygen tension below a threshold level results in increased expression of numerous photosynthesis (PS) genes encoding photopigment-biosynthetic enzymes and apoproteins for both light-harvesting and reaction center complexes. The incident light intensity is also known to modulate PS gene expression. Recently, substantial progress has been achieved in uncovering some of the molecular species involved in the regulatory network governing PS gene expression (3, 7, 22).

Our search for transcriptional regulators of the *Rhodobacter sphaeroides* 2.4.1 *puc* operon, encoding the structural polypeptides and assembly factor(s) for light-harvesting complex II (8, 13), resulted in identification of the *ppa* gene (9) from cosmid pUI8714. Downstream of *ppa* (Fig. 1A), we found a truncated copy of a gene homologous to the *ppsR* gene of *R. sphaeroides* RS6258 described by Penfold and Pemberton (22). Those investigators showed that PpsR serves as a repressor of photopigment biosynthesis (21). Identification of a putative DNA-binding domain in the C terminus of PpsR, as well as the ability of PpsR to decrease expression from a putative *R. sphaeroides* promoter(s), led to the suggestion that PpsR is a transcriptional repressor (22).

To study the role of PpsR in the regulation of *puc* operon expression, as well as its possible interaction with other members of the regulatory network (9), we cloned the full-length *ppsR* gene from *R. sphaeroides* 2.4.1 by using the truncated gene as a probe (Fig. 1A). The DNA sequence of the *ppsR* gene from strain 2.4.1 (GenBank accession no. L37197) shows 97% identity to the *ppsR* gene from strain RS6258. At the protein level, PpsR from strain 2.4.1 differs from its RS6258 counterpart by only two conserved amino acid substitutions, containing Ile in place of Val-19 and Ser in place of Ala-227 (22).

The plasmids used in this study are described in Table 1. The *ppsR* gene on a 1.6-kb *Pst*I-*Nsi*I fragment was cloned into the *Pst*I site of broad-host-range vector pRK415 to give rise to pPNs (Fig. 1B). When present in strain 2.4.1, pPNs resulted in colonies going from red (in the wild type) to colorless, as was observed by Penfold and Pemberton for RS6258 (21). pCF200Km, containing both the *puc* upstream regulatory sequence and the *puc* downstream regulatory sequence (DRS) transcriptionally fused to *lacZYA'*, was introduced into strains 2.4.1(pPNs) and 2.4.1(pLA2917) to allow monitoring of *puc*::*lacZ* expression. Under aerobic conditions, *puc*::*lacZ* expression in *R. sphaeroides* 2.4.1 is normally lower than under other growth conditions (17). However, an increased dosage of the *ppsR* gene resulted in β-galactosidase activity in strain 2.4.1(pCF200Km, pPNs) that was only ~3% of the activity in strain 2.4.1(pCF200Km, pLA2917), which lacked plasmid-encoded *ppsR* (Fig. 2B). Thus, *ppsR* appeared to repress *puc* transcription in strain 2.4.1.

Because transcription of the *puc* operon is known to be influenced by alterations in photopigment biosynthesis (16, 20) and PpsR has been shown to suppress photopigment biosynthesis (21, 22), we decided to test the effect of PpsR on *puc*::*lacZ* expression in *Paracoccus denitrificans* ATCC 17741, a nonphotosynthetic bacterium related to *R. sphaeroides*. When the *ppsR* gene on pPNs was introduced into strain ATCC 17741(pCF200Km), β-galactosidase activity decreased from 5,575 U to as low as 145 U, i.e., parallel to what was observed in *R. sphaeroides* 2.4.1(pCF200Km). This result suggested that the effect of PpsR on *puc*::*lacZ* expression is direct and further validated the use of strain ATCC 17741 as a heterologous host in these studies.

To gain insight into the functional domains that make up PpsR, we made two deletion constructions. One consisted of PpsR devoid of the C-terminal domain (plasmid pP[R]; Fig. 1B), which contains a helix-turn-helix motif (22). The second consisted of the 108 C-terminal amino acids of PpsR, including the helix-turn-helix motif (plasmid p[Bg]Ns; Fig. 1B). In an experiment in which pCF400Δ (instead of pCF200Km) was used as the source of a *puc*::*lacZ* transcriptional fusion, the β-galactosidase level in strain ATCC 17741(pCF400Δ) containing pPNs in *trans* was substantially, ~430-fold, lower than in strain ATCC 17741(pCF400Δ) containing vector pLA2917 in *trans*. The β-galactosidase levels in strain ATCC 17741 (pCF400Δ) in the presence of pP[R] in *trans* were virtually the same as those measured in the presence of pLA2917, which held true for all of the *lacZ* fusions tested (data not shown). This observation indicated that the lack of the C terminus of PpsR completely abolished repressor activity. However, expression of the C-terminal domain of PpsR resulted in an approximately twofold decrease in β-galactosidase activity in strain ATCC 17741(pCF400Δ, p[Bg]Ns) in comparison with that in strain ATCC 17741(pCF400Δ, pP[R]) (Fig. 2C). We believe that this repression by the C-terminal domain of PpsR

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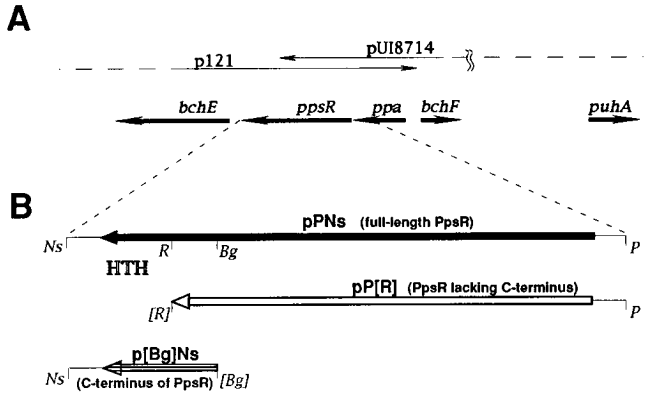

FIG. 1. (A) Genetic map of the region of the *R. sphaeroides* 2.4.1 photosynthesis gene cluster neighboring the *ppsR* gene. Gene designations are based on the following sequence homology: *ppsR* to *ppsR* from *R. sphaeroides* RS6258 (22); *bchE* and *bchF* to the corresponding genes from *R. capsulatus* (EMBL accession no. Z11165). The *ppa* gene (9) is a homolog of *orf-192* from RS6258 (22) and *R. capsulatus* (EMBL accession no. Z11165). *puhA* (18) is contained in pUI8714 (unpublished data) and has been shown to localize the *ppsR* gene from *R. sphaeroides* 2.4.1. (B) Subcloning of the full-length *ppsR* gene from *R. sphaeroides* 2.4.1 and its truncated forms. *Bg*, *Bgl*II; *Ns*, *Nsi*I; *P*, *Pst*I; *R*, *Eco*RI; disrupted restriction sites are in brackets. HTH, helix-turn-helix motif of PpsR (22).

was due to its residual DNA-binding capacity provided by the helix-turn-helix motif.

To localize the region of the *puc* regulatory sequence interacting with PpsR, we tested the effect of PpsR on the *puc* DRS. Plasmid pCF250Km, containing only the *puc* DRS transcriptionally fused to *lacZYA′*, was introduced into Pd(pPNs), Pd(pP[R]), and Pd(p[Bg]Ns). As shown in Fig. 2C, both full-length PpsR and (to a lesser extent) the C-terminal portion of PpsR repressed *puc*::*lacZ* expression. These results indicated that the DRS contains a site for PpsR-mediated repression.

Palindromes with a TGT-N_{12}-ACA consensus are positioned in the 5′ regulatory regions of some PS genes in *R. sphaeroides*, as well as in *R. capsulatus*, and for some time have been considered potential sites for repressor binding (2, 4, 15, 17). In a gel shift assay, the *bchC* regulatory sequence from *R. capsulatus*, containing two such palindromes, was shown to interact with a protein from crude extracts of this bacterium in a sequence-specific and oxygen-dependent manner (19). Two TGT-N_{12}-ACA motifs are situated in the *puc* DRS (17). Lee and Kaplan isolated spontaneous mutations, G-26→A and A-12→C, in the most proximal TGT-N_{12}-ACA motif within the *puc* DRS which result in ~4.7- and ~6.9-fold derepression of *puc*::*lacZ* expression in aerobically grown *R. sphaeroides* 2.4.1 (17). To test whether the observed derepression could be PpsR mediated, we introduced plasmids pCF300Δ and pCF302Δ, containing, respectively, the −26 and −12 mutations in an otherwise intact *puc* regulatory sequence, into *P. denitrificans* ATCC 17741.

We found that *puc*::*lacZ* expression in strain ATCC 17741(pCF300Δ, pP[R]) did not differ from that in strain ATCC 17741(pCF400Δ, pP[R]). However, in strain ATCC 17741(pCF302Δ, pP[R]), expression was ~1.8-fold higher than in strain ATCC 17741(pCF400Δ, pP[R]) (Fig. 2C), which is most likely due to the increased strength of the *puc* promoter caused by the mutation A-12→C. In the presence of pPNs in *trans*, the level of β-galactosidase in strains ATCC 17741 (pCF400Δ), ATCC 17741(pCF300Δ), and ATCC 17741 (pCF302Δ) decreased dramatically but to a different extent in each strain. The repressed level of β-galactosidase in strain ATCC 17741(pCF300Δ, pPNs) was about fivefold higher and that in ATCC 17741(pCF302Δ, pPNs) was about sixfold higher than the level found in ATCC 17741(pCF400Δ, pPNs) (Fig. 2C). Thus, for strain ATCC 17741(pCF300Δ, pPNs), repression was ~5-fold less effective than in ATCC 17741(pCF400Δ, pPNs), and for ATCC 17741(pCF302Δ, pPNs), repression was ~3.3-fold less effective when the ~1.8-fold difference in promoter strength is taken into account. Since the mutations in the proximal TGT-N_{12}-ACA motif decreased PpsR-mediated repression, we propose that PpsR does interact with the prox-

TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
pUI8714	pLA2917-derived cosmid from *R. sphaeroides* 2.4.1 cosmid library, Tc^r	6
pLA2917	Tc^r Km^r IncP cosmid vector	1
p121	pBS/*Sma*I + ~4.6-kb *Sma*I insert containing *ppsR* gene from *R. sphaeroides* 2.4.1	J. K. Lee
pRK415	Tc^r *lacZ*α IncP	11
pPNs	pRK415/*Pst* + 1.6-kb *Pst*I-*Nsi*I fragment containing *ppsR* gene in same orientation as *tet* and *lacZ* genes	This work
pP[R]	pPNs/*Eco*RI (deletion of *Eco*RI fragment containing 3′ end of *ppsR* gene), PolIk[a] + dNTP[b] (to create stop codon after amino acid 406 of PpsR), ligase	This work
pP[Bg]Ns	pPNs/*Bgl*II, PolIk + dNTP, ligase (to create *Cla*I site for next manipulation)	This work
p[Bg]Ns	pP[Bg]Ns/*Hin*dIII + *Cla*I, PolIk + dNTP, ligase (to create LacZ′::′PpsR translational fusion containing several first residues of LacZ fused to amino acids 357–464 of PpsR C terminus	This work
pCF200Km	Sm^r/Sp^r Km^r IncQ, *puc* (URS^c + DRS)::*lacZYA′*	17
pCF250Km	Sm^r/Sp^r Km^r IncQ, *puc* (DRS)::*lacZYA′*	17
pCF400Δ	Tc^s derivative (deletion of *Hin*dIII fragment) of pCF400	This work
pCF300Δ	Tc^s derivative (deletion of *Hin*dIII fragment) of pCF300	This work
pCF302Δ	Tc^s derivative (deletion of *Hin*dIII fragment) of pCF302	This work
pCF400	Sm^r/Sp^r Tc^r IncQ, *puc* (URS + DRS)::*lacZYA′*	17
pCF300	Same as pCF400 but with point mutation G-26→A in DRS	17
pCF302	Same as pCF400 but with point mutation A-12→C in DRS	17
pLX200	Sm^r/Sp^r IncQ, *bchF*::*lacZYA′*	This work
pUI1830Δ	Tc^s derivative (deletion of *Hin*dIII fragment) of pUI1830	This work
pUI1830	Sm^r/Sp^r Tc^r IncQ, *pufB*::*lacZYA′*	L. Gong

[a] PolIk, Klenow fragment of DNA polymerase I.
[b] dNTP, deoxynucleoside triphosphate.
[c] URS, upstream regulatory sequence.

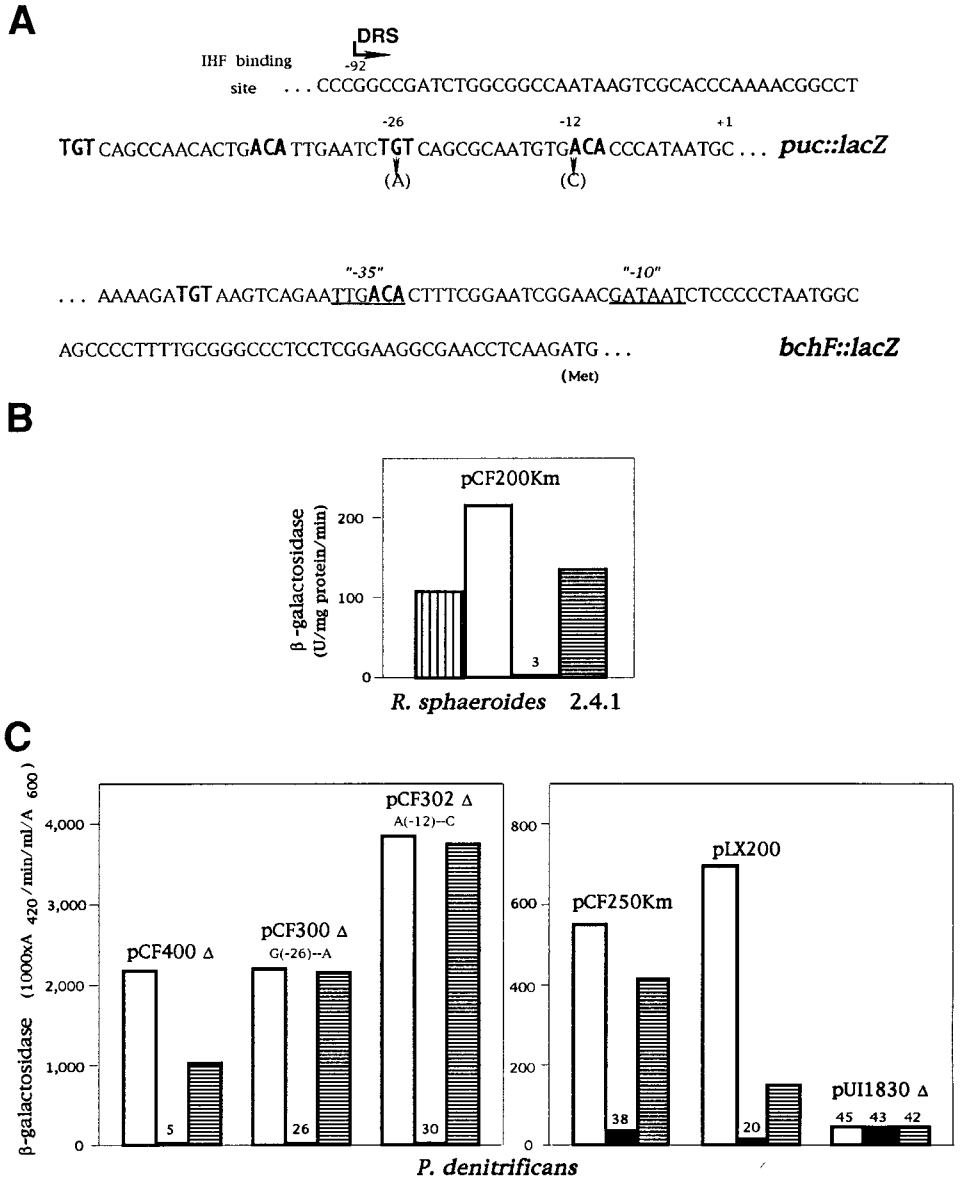

FIG. 2. (A) Sequences corresponding to the regulatory regions of the *puc* operon and the *bchF* gene from *R. sphaeroides* 2.4.1 relevant to the *puc*::*lacZ* and *bchF*::*lacZ* fusion constructions. The TGT-N_{12}-ACA consensus is in boldface. The +1 above the *puc* regulatory sequence corresponds to the transcriptional start point (18). The sequences of the "−35" and "−10" regions of the putative σ^{70} type promoter of *bchF* are underlined; the putative first-translated codon is shown as (Met). (B) β-Galactosidase activity in aerobically grown (A_{600}, <0.12) (17) *R. sphaeroides* 2.4.1(pCF200Km), containing in *trans* a second plasmid (pLA2917 [no PpsR], vertically striped bar; pP[R] [PpsR lacking C terminus], open bar; pPNs [full-length PpsR], filled bar; p[Bg]Ns [C terminus of PpsR], horizontally striped bar). (C) β-Galactosidase activity, measured as described in reference 23, in aerobically grown *P. denitrificans* harboring two plasmids; the first contained the appropriate *lacZ* fusion (shown above the bars), and the second was pP[R], pPNs, or p[Bg]Ns. Cultures (10 ml) were grown in 125-ml flasks on Sistrom's succinate medium (5) at 31°C with vigorous shaking. Samples of the cultures from each set were collected at approximately the same optical density (in the A_{600} range of 0.32 to 0.45), and β-galactosidase activity was assayed. The bars in panels B and C represent mean values of at least two experiments. Standard errors did not exceed 25% for values below 10 and 13% for the remaining values. Values below 100 are shown above the corresponding bars.

imal motif. However, the presence of the intact distal TGT-N_{12}-ACA motif in the *puc* DRS (Fig. 2A), either by itself or in combination with the partially impaired proximal motif, was still sufficient for repression.

To increase the sensitivity of the experimental protocol, we tested the effect of the C-terminal domain of PpsR on *puc*::*lacZ* expression of pCF300Δ and pCF302Δ. In contrast to strain ATCC 17741(pCF400Δ), where the presence of p[Bg]Ns resulted in about twofold repression, virtually no repression by p[Bg]Ns was observed when strain ATCC 17741(pCF300Δ) or ATCC 17741(pCF302Δ) was used (Fig. 2C). Thus, these results stress the importance of the intact proximal TGT-N_{12}-ACA motif for DNA binding by the C-terminal domain of PpsR. Moreover, together with the data described above, they suggest that PpsR interacts with both palindromes of the *puc* DRS.

To confirm the requirement of the TGT-N_{12}-ACA motif for PpsR-mediated repression, we tested the effect of PpsR on the expression of two additional PS operons, namely, *puf*, which encodes the structural polypeptides for light-harvesting complex I and the reaction center L and M polypeptides (12), and *bchF*, which encodes an enzyme involved in bacteriochloro-

phyll biosynthesis (4). The *puf* regulatory region does not contain a TGT-N_{12}-ACA motif (10), and therefore we were not surprised that *pufB*::*lacZ* expression in *P. denitrificans* ATCC 17741 was not affected by PpsR (plasmid pUI1830Δ; Fig. 2C). Conversely, the sequence upstream of the *bchF* gene contains the TGT-N_{12}-ACA motif overlapping a putative σ^{70} type promoter (Fig. 2A). As anticipated, the presence in *P. denitrificans* ATCC 17741, in *trans*, of either full-length PpsR or the C-terminal domain of PpsR repressed *bchF*::*lacZ* expression ~35- and ~4.4-fold, respectively (plasmid pLX200; Fig. 2C).

As mentioned above, PpsR lacking the C terminus did not affect the expression of *puc*::*lacZ* fusions in *P. denitrificans* ATCC 17741. However, in aerobically grown cultures of *R. sphaeroides* 2.4.1(pCF200Km, pP[R]), the level of β-galactosidase activity was found to be about twofold higher than in strain 2.4.1(pCF200Km, pLA2917) (Fig. 2B). The observed derepression of *puc*::*lacZ* expression in strain 2.4.1 (pCF200Km) suggests that the plasmid pP[R]-encoded truncated protein interfered with the activity of the chromosome-encoded intact PpsR protein. This could indicate the potential importance of interactions between PpsR molecules, although other interpretations are possible.

In conclusion, by using transcriptional fusions and a heterologous expression system, we demonstrated that PpsR functions as a transcriptional repressor of *puc* and *bchF* expression in *R. sphaeroides* 2.4.1. The C terminus of PpsR, containing the putative DNA-binding domain, by itself possesses sequence-specific repressor activity. TGT-N_{12}-ACA motifs are most likely the target for PpsR binding.

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