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Molecular Genetic Analysis Suggesting Interactions between AppA and PpsR in Regulation of Photosynthesis Gene Expression in *Rhodobacter sphaeroides* 2.4.1

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The AppA protein plays an essential regulatory role in development of the photosynthetic apparatus in the anoxygenic phototrophic bacterium *Rhodobacter sphaeroides* 2.4.1 (M. Gomelsky and S. Kaplan, J. Bacteriol. 177:4609–4618, 1995). To gain additional insight into both the role and site of action of AppA in the regulatory network governing photosynthesis gene expression, we investigated the relationships between AppA and other known regulators of photosynthesis gene expression. We determined that AppA is dispensable for development of the photosynthetic apparatus in a *ppsR* null background, where PpsR is an aerobic repressor of genes involved in photopigment biosynthesis and *puc* operon expression. Moreover, all suppressors of an *appA* null mutation thus far isolated, showing improved photosynthetic growth, were found to contain mutations in the *ppsR* gene. Because *ppsR* gene expression in *R. sphaeroides* 2.4.1 appears to be largely independent of growth conditions, we suggest that regulation of repressor activity occurs predominately at the protein level. We have also found that PpsR functions as a repressor not only under aerobic but under anaerobic photosynthetic conditions and thereby is involved in regulating the abundance of the light harvesting complex II, depending on light intensity. It seems likely therefore, that PpsR responds to an integral signal (e.g., changes in redox potential) produced either by changes in oxygen tension or light intensity. The profile of the isolated suppressor mutations in PpsR is in accord with this proposition. We propose that AppA may be involved in a redox-dependent modulation of PpsR repressor activity.

Rhodobacter sphaeroides is a photosynthetic (PS) bacterium which belongs to the α3-subdivision of the *Proteobacteria* and is remarkably versatile in its growth capabilities. In the presence of oxygen it derives energy from aerobic respiration. However, when the oxygen tension drops below certain threshold levels, the bacterium develops a PS apparatus, which allows the use of light as an energy source. Oxygen is, therefore, the major environmental stimulus regulating development of the PS apparatus. Light is another important stimulus regulating the cellular abundance of the PS apparatus depending on available light intensity (2, 15).

The PS apparatus involved in light energy transfer is comprised of the reaction center and two light-harvesting (LH I and LH II) complexes. The reaction center complex functions as the "converter" by which light energy is transformed into chemical energy, and the LH complexes function as antennae for harvesting photons and ultimately delivering their energy to the reaction center. All PS complexes are localized in the specialized intracytoplasmic membrane and are composed of structural proteins in association with the photopigments, bacteriochlorophyll (Bchl) and the carotenoids (Crt).

The regulation of development of the PS apparatus in *R. sphaeroides* 2.4.1 appears to be a well-orchestrated multilevel process. This is not surprising considering that formation of the PS apparatus requires major rearrangements in both cellular metabolism and morphology. The development of the PS apparatus involves regulation of the expression of photosynthesis genes which encode the structural and assembly proteins for PS complex formation, as well as of genes which encode enzymes for both Bchl and Crt biosynthesis (*bch* and *crt*, respectively). The form of the Crt end product, i.e., spheroidene versus spheroidenone, also plays a role in determining the final cellular abundance of the LH II complex and thus the ratio of the LH I and LH II complexes (29). Although substantial progress has been achieved over the past several years, knowledge of many important regulatory components and relationships governing this process is still incomplete.

The Prr (also known as Reg) regulatory system belongs to the class of two-component, sensory kinase-response regulator systems (22) and functions to activate expression of most photosynthesis genes when oxygen tension decreases below threshold levels. Prr mutants are impaired in the development of the PS apparatus, at least partially, because of an inability to increase photosynthesis gene expression in response to anaerobiosis (5, 6, 13, 21, 25). The response regulator PrrA (RegA) does not appear to contain a DNA binding motif, and the mechanism of activation of photosynthesis genes transcription by PrrA is unknown.

The PpsR protein is another transcription regulator (11, 23, 24). It contains a DNA-binding domain and functions as an aerobic repressor of many *bch* and *crt* genes and operons as well as the *puc* operon encoding the structural proteins of the LH II complex. PpsR is proposed to bind to the motif TGT-N_{12}-ACA (where N is a nucleotide), which either overlaps or lies downstream of promoters for PpsR-controlled genes (11, 16, 17).

It was recently shown that the anaerobic activator FnrL, a homolog of *Escherichia coli* Fnr, is involved, directly and/or indirectly, in regulation of expression of some photosynthesis genes, including the *puc* operon (30, 31). Besides the major regulatory factors, described above, photosynthesis gene ex-

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TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Genotype and description	Source or reference
Strains		
R. sphaeroides		
2.4.1	Wild type	W. R. Sistrom
APP11	2.4.1 Δ*appA*::Tp^r	10
PRRA1	2.4.1 *prrA*::ΩSm^r/Sp^r	5
JZ1678	2.4.1 *fnrL*::ΩSm^r/Sp^r	31
PPS1	2.4.1 *ppsR*::ΩKm^r	This work
APS1	APP11 *ppsR*::ΩKm^r	This work
PS6	APP11 *ppsR′* (Q144-stop)	This work
P. denitrificans		
ATCC 17741	Wild type	American Type Culture Collection
Plasmids		
pCF200Km	Sm^r/Sp^r Km^r IncQ *puc*::*lacZ*	17
pLX41	Sm^r/Sp^r IncQ *ppsR*::*lacZ*	This work
pLX14	Sm^r/Sp^r IncQ *bchF*::*lacZ*	This work
pLX14P	Sm^r/Sp^r IncQ *bchF*::*lacZ ppsR*	This work
pRK415	Tc^r *lacZ*α IncP, vector	14
p484-Nco5	pRK415::*appA*	10
pUI1621	pRK415::*prrA*	5
pUI1971	pRK415::*fnrL*	31
pUI8461, pUI8714	Cosmids from the *R. sphaeroides* 2.4.1 genomic library	4

pression may be subject to additional fine-tuning by a number of proteins, e.g., PrrC (6), TspO (28), Ppa (9), and HvrA (1), etc., which function through either known or unknown transcription regulators and will not be considered further here.

We previously identified the AppA protein as a critical player in the ability of *R. sphaeroides* 2.4.1 to develop the PS apparatus. Disruption of the *appA* gene results in decreased expression of the photosynthesis genes and impairs production of both pigments and proteins comprising the PS complexes. *appA* present in extra copy either in wild type or the AppA null mutant increases photosynthesis gene expression, even under aerobic conditions when expression is normally low (10). AppA does not appear to contain a DNA binding motif, nor is it similar to known regulators of gene expression. Moreover, AppA shows no substantial similarity to any known protein in databases. We therefore attempted to shed light on its role and site of action in the regulatory network by exploring the relationships between AppA and other known transcription factors.

The present study revealed a link between AppA and repressor PpsR, which was further confirmed by suppressor analysis of the *appA* mutation and coexpression of AppA and PpsR in a heterologous host. This analysis has led to the identification of critical amino acid residues in PpsR and of how this protein may function in photosynthesis gene expression.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids, which are relevant to this work and mentioned in the text, are listed in Table 1. Other, intermediate strains and plasmids are described below, in the appropriate part of this section.

Growth conditions. *E. coli* strains were grown at 37°C on Luria-Bertani (LB) medium (19) supplemented, where required, with the following antibiotics at final concentrations: tetracycline, 10 μg/ml; ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and streptomycin plus spectinomycin, 25 μg/ml each.

R. sphaeroides and *Paracoccus denitrificans* were grown at 30°C on Sistrom's medium A (2) containing succinate as carbon source. For β-galactosidase assays in *R. sphaeroides*, liquid cultures were maintained by permanent bubbling with the following gas mixtures: 20% O_2–79% N_2–1% CO_2 (aerobic conditions); 2% O_2–97% N_2–1% CO_2 (semi-aerobic conditions); and 98% N_2–2% CO_2 (PS conditions at a light intensity of 10 W/m^2). For monitoring PS growth, we used fully filled screw-cap tubes which were illuminated with light at various intensities: 100 W/m^2 (high), 10 W/m^2 (medium), or 3 W/m^2 (low). Anaerobic growth in the dark was maintained in Sistrom's medium A containing 20% LB medium and 80 μM dimethyl sulfoxide. For β-galactosidase assays in *P. denitrificans*, liquid cultures (10 ml) were grown in 125-ml flasks under vigorous shaking. Antibiotics were used, where appropriate, at the following final concentrations: tetracycline, 1 μg/ml; kanamycin, 50 μg/ml; streptomycin plus spectinomycin, 50 μg/ml each.

Conjugation techniques. Conjugation was performed essentially as described previously (3). To introduce plasmids of interest into various *R. sphaeroides* and *P. denitrificans* strains, biparental (with *E. coli* S17-1 [26] as a donor) or triparental [with *E. coli* HB101(pRK2013) (7) as a helper strain] matings were used.

β-Galactosidase assays. In *R. sphaeroides*, β-galactosidase was assayed in cell extracts as described earlier (17). The activity of β-galactosidase in *R. sphaeroides* is expressed in units (U), where 1 U corresponds to 1 μmol of *o*-nitrophenyl-β-galactoside hydrolyzed per min per mg of protein. In *P. denitrificans*, β-galactosidase was assayed in whole cells treated with chloroform and sodium dodecyl sulfate (SDS) as described previously (17). The activity of β-galactosidase in *P. denitrificans* is expressed in Miller units (MU), where 1 MU corresponds to 1 μmol of *o*-nitrophenyl-β-galactoside hydrolyzed per min per unit of optical density at 600 nm. All assays were performed at least twice with standard deviations not exceeding 15%.

Spectrophotometric assays. *R. sphaeroides* cell extracts were obtained by sonication of photosynthetically grown cells and assayed as described previously (17) by using samples containing equal amounts of protein. PS growth of *R. sphaeroides* strains was monitored with a Klett-Summerson photometer with filter no. 66 and is expressed in Klett units, where 1 Klett unit is approximately equal to 10^7 cells ml^{-1}.

DNA manipulations and sequence analysis. Standard recombinant DNA techniques (19) and molecular biological enzymes and reagents were used according to the specifications of the manufacturers. DNA sequencing was performed on an ABI 377 automatic DNA sequencer (Applied Biosystems) at the DNA Core Facility of the Department of Microbiology and Molecular Genetics at the University of Texas Medical School at Houston.

Construction of the PpsR null mutant, PPS1, and the AppA PpsR double mutant, APS1. For disruption of the *ppsR* gene, the *Sma*I(405)-*Bgl*II(2422) fragment (positions refer to GenBank sequence L37197) from cosmid pUI8714 (11) was cloned into pUC19 (27). An ΩKm^r cartridge from pUI1637 (5) was introduced into the unique *Nru*I(1409) site, ~60 bp downstream from the *ppsR* start codon. The construct was then linearized with *Ssp*I and ligated with the 4.2-kb *Msc*I fragment from pSUP202 containing the Tc^r gene as well as the *mob* region (26). The resulting plasmid, p714SmH::Km^r::*mob*, was mobilized from *E. coli* S17-1 into *R. sphaeroides* 2.4.1 to generate the PpsR null mutants and into strain APP11 to generate the AppA PpsR double mutants.

Attempts to isolate similar double cross-over recombinants (Km^r Tc^s) under aerobic conditions failed because of the instability of the PpsR null mutant, which generated a variety of phenotypes. Therefore the double cross-over recombinants were selected independently under anaerobic-dark and anaerobic-light (PS) conditions. The correctness of the *ppsR* disruptions in independent isolates from both conditions was verified by Southern hybridization (19). From several independent double cross-over recombinants which showed an identical phenotype, one representative of each type was chosen for further analysis and designated PPS1 (PpsR null mutant) or APS1 (AppA-PpsR double mutant).

Isolation and characterization of the photosynthesis-competent pseudorevertants of strain APP11. Strain APP11 was plated under PS conditions (10 W/m^2), and photosynthesis-competent pseudorevertants, which appeared after 4 days of incubation at a frequency of ~10^{-7}, were isolated and streak purified. Chromosomal DNA from each clone was isolated as previously described (19). It was digested with *Nsi*I and *Nco*I and subjected to agarose gel electrophoresis. The ~1.4- to 1.6-kb fragments were recovered from the gel and ligated with vector LITMUS28 (Pharmacia) digested with *Nco*I and *Pst*I. The resulting mixture was used for transformation of strain DH5α*phe* (5) and plated onto LB agar containing ampicillin and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Approximately 200 to 600 white colonies from each pseudorevertant were collected and used for colony hybridization (19) with an ~1.5-kb *Nsi*I-*Nco*I labeled fragment containing the wild-type *ppsR* gene. Plasmids from those clones which gave a positive signal were isolated and used for DNA sequencing with primers specific to LITMUS28 as well as internal primers corresponding to the *ppsR* sequence.

Construction of *lacZ* fusions. The ~1.4-kb *Nru*I fragment from p121 (11) was inserted into the unique *Xba*I site (which was made blunt) of pLX1 (12). This insert generated the *ppsR*::*lacZ* fusion (plasmid pLX41) when present in one orientation and the *bchF*::*lacZ* fusion (plasmid pLX14) when present in the opposite orientation. Cloning the *Bsp*EI-*Xma*I fragment from p121 into the *Bsp*EI site of pLX14 restored the full-size *ppsR* gene. The resulting plasmid, pLX14P, therefore contained the *bchF*::*lacZ* fusion and the *ppsR* gene in *cis*. All of the *lacZ* constructs were verified by DNA sequencing.

RESULTS

Relationships between AppA and anaerobic activators of photosynthesis gene expression in *R. sphaeroides* 2.4.1. PrrA and AppA null mutants are similar (but not identical) in that

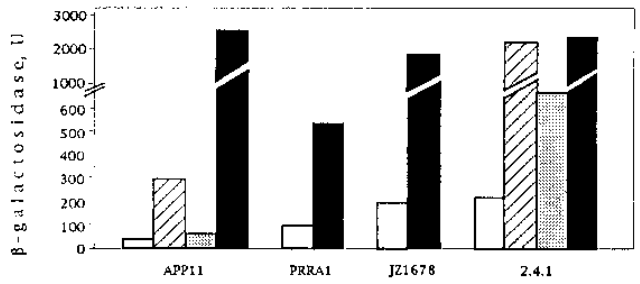

FIG. 1. Expression of the *puc*::*lacZ* fusion from plasmid pCF200Km in aerobically grown strains of *R. sphaeroides* carrying a second plasmid: vector pRK415 (open bars); the *appA*-containing plasmid, p484-Nco5 (solid bars); the *prrA*-containing plasmid, pUI1621 (striped bars); or the *fnrL*-containing plasmid, pUI1971 (stippled bars).

neither mutant develops appreciable levels of PS complexes because of impairment in the production of structural proteins and photopigments. In both mutants, the defects are shown to be, at least partially, at the level of expression of the photosynthesis genes, with the AppA null mutant being less severely impaired. Increased dosage of *appA* can partially compensate for mutation in *prrA* but does not restore PS growth (10).

To further explore the relationships between the response regulator PrrA and AppA, we followed the effect of extra copies of *prrA* (plasmid pUI1621) introduced in *trans* into an AppA null mutant, APP11. It has been shown that increased expression of the *prrA* gene in wild type or a PrrA null mutant is sufficient to induce formation of PS complexes under aerobic conditions (5). However, extra copies of *prrA* present in *trans* in mutant APP11 grown aerobically or anaerobically did not result in the formation of PS complexes or of additional PS complexes, respectively (data not shown). A similar effect was observed when the *trans*-dominant *prrB78* allele (a histidine kinase [6]), which also results in formation of PS complexes under aerobic conditions, was introduced into mutant APP11. Therefore, activation by the Prr regulatory system appears to be insufficient to overcome the defect imposed by an *appA* null mutation. We further examined how activation by the Prr regulatory system affected photosynthesis gene transcription in mutant APP11. We observed that, under aerobic conditions, *puc*::*lacZ* expression in APP11(pUI1621) was ~10-fold higher compared to the control, APP11(pRK415), yet was much lower than in wild type, 2.4.1(pUI1621) (Fig. 1) or PRRA1(pUI1621) (5). Interestingly, extra copies of *prrA* in *trans* resulted in the same fold increase, ~10-fold, in *puc*::*lacZ* expression in both wild type and APP11. These results suggest that, although activation by the Prr-dependent pathway cannot overcome the defects imposed by the *appA* null mutation, this two-component system is able to activate photosynthesis gene expression independently of a functional AppA.

We further investigated the relationships between AppA and FnrL, another anaerobic activator involved in the regulation of at least some of the photosynthesis genes, including *puc* (30, 31). Extra copies of *appA* increased *puc* expression several fold in either wild type or an FnrL null mutant, JZ1678 (Fig. 1), which suggested that FnrL is not critically involved in the role of AppA. Extra copies of *fnrL* contained in pUI1971 increased *puc*::*lacZ* expression in wild type ~4.5-fold and to a much lesser extent, ~1.6-fold, in APP11 (Fig. 1). Therefore, *fnrL* retains some ability to affect *puc*::*lacZ* expression regardless of the *appA* allele. The extent of activation by *fnrL* in extra copy in an *appA* null background could implicate AppA in having a minor role in controlling FnrL activity under aerobic conditions. However, should any such effect of AppA on FnrL exist, it cannot account for the observed effect of AppA on photosynthesis gene expression.

Relationships between AppA and PpsR. The relationships between AppA and the aerobic transcription repressor, PpsR, are not readily testable employing the same experimental design as described above for PrrA and FnrL because of the nature of the *ppsR* null mutation (see below). We therefore investigated the AppA-PpsR relationships by introducing a *ppsR* null mutation into both the wild-type and the *appA* null genetic backgrounds.

The constructed PpsR null mutant, PPS1, is extremely unstable under aerobic conditions producing tiny, deeply pigmented (dark red) colonies which readily give rise to a variety of faster growing secondary mutants. Oxygen intolerance of the PpsR mutant may be caused by toxicity of Bchl and its precursors in the presence of oxygen as well as by the apparent energetic burden resulting from the gratuitous synthesis of the PS apparatus. The spectra produced by mutant PPS1 grown under anaerobic PS conditions at different light intensities are shown in Fig. 2 along with the corresponding spectra of wild type. At high light intensity, mutant PPS1 produced markedly more LH II complex (absorption maxima at 800 and 850 nm) compared to wild type. However, the abundances of LH I complex (absorption maximum at 875 nm) were similar in both strains. Quantitation of the LH I and LH II complexes is presented in Table 2. At high light intensity, the LH II complex from strain PPS1 was ~4.5-fold greater than that present in 2.4.1, whereas the LH I complex from strain PPS1 was ~0.9 times that of the amount of the LH I complex in 2.4.1. This finding is consistent with the derepressed expression of the *puc* operon (encoding the structural proteins for the LH II complex) as well as *bch* and *crt* genes and operons (encoding enzymes for photopigment production). Therefore, PpsR functions as a repressor of *puc*, *bch*, and *crt* not only under aerobic conditions, as was proposed previously (23, 24), but under anaerobic PS conditions as well. The difference in the amount of the LH II complex between PPS1 and 2.4.1 decreases at lower light intensities (Table 2).

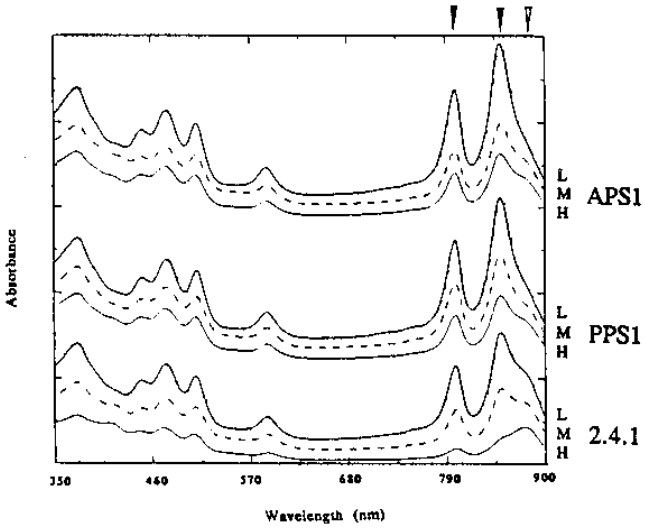

FIG. 2. Absorbance spectra of *R. sphaeroides* strains grown anaerobically at various light intensities: H, high (100 W/m^2); M, medium (10 W/m^2), or L, low (3 W/m^2). Crude extracts containing equal amounts of protein were used to obtain these spectra. Arrowheads indicate peak absorbance maxima for LH I (open arrowhead) and LH II (solid arrowheads) complexes.

TABLE 2. Distribution of LH I and LH II complexes from strains 2.4.1, PPS1, and APS1 grown photosynthetically at different light intensities

Light intensity (W/m^2)	LH complex	Relative amount of LH complexes in strains[a]:		
		2.4.1	PPS1	APS1
High (100)	LH I	1[b]	0.87	0.86
	LH II	1	4.46	4.46
Medium (10)	LH I	1	0.56	0.60
	LH II	1	1.72	1.67
Low (3)	LH I	1	0.61	0.60
	LH II	1	1.47	1.59

[a] Relative amount of LH complexes was calculated (20) based on spectra from Fig. 2.
[b] A given LH complex from the wild type, 2.4.1, grown at a given light intensity was set equal to 1 arbitrary unit. Other values were normalized with respect to values from 2.4.1.

When we disrupted the *ppsR* gene in mutant APP11, to our surprise, the phenotype of the resulting double mutant, designated APS1, appeared to be indistinguishable from that of PPS1 with regard to oxygen intolerance, pigmentation, growth, and spectral characteristics (Fig. 2; Table 2). Therefore, an impairment in photopigment biosynthesis as well as *puc* operon expression, characteristic of the *appA* null mutation, was apparently suppressed by disruption of *ppsR*. This finding suggests that AppA and PpsR could belong to the same regulatory pathway, with AppA acting upstream of PpsR.

Suppressor analysis of the *appA* null mutation. To explore in greater detail the involvement of AppA in photosynthesis gene expression, we undertook a suppressor analysis of the *appA* mutation. Two approaches were employed in a search for suppressors. The first involved isolation of spontaneous secondary mutations by screening for improved PS growth of strain APP11. The second approach was aimed at an identification of a gene(s) which, when provided in extra copies in *trans*, would at least partially complement the *appA* mutation. Both approaches could prove useful in elucidating the site of AppA action.

(i) Isolation and analysis of the photosynthesis-competent pseudorevertants of the *appA* mutation. Strain APP11 plated on agar and exposed to light (10 W/m^2) under anaerobic conditions readily yielded photosynthesis-competent pseudorevertants. Under aerobic conditions, these pseudorevertants appeared to be of varying pigmentation, from dark red to pink, but always significantly more pigmented than the virtually colorless mutant APP11. Eleven independent pseudorevertants, representing a variety of pigmentation types, were chosen for further analysis.

One pseudorevertant strain, designated PS6, resembled APS1 and PPS1 in that it formed tiny, dark red colonies and was extremely unstable under aerobic conditions. Moreover, the spectra of strain PS6 grown photosynthetically at different light intensities (data not shown) were identical to the spectra of strain APS1 (Fig. 2). DNA sequence analysis of the cloned *ppsR* gene from strain PS6 revealed a single point mutation which resulted in a premature stop codon in PpsR from strain PS6 (Table 3). Hence, the similarity between the phenotypes of strain PS6 and of the double mutant APS1 derived from the same genetic background, i.e., an inactive *ppsR* gene.

We then proceeded to clone and sequence the *ppsR* locus from the 10 additional independent pseudorevertants. Each pseudorevertant turned out to contain a point mutation in *ppsR* (Table 3). Among the mutations found, four represented amino acid substitutions from L to P (Table 3). Proline is known to affect protein secondary structure; hence, the PpsR proteins in those four pseudorevertants may be structurally and therefore functionally defective. Two substitutions (K422-E and C424-A) were localized within the helix-turn-helix motif of PpsR, which is involved in DNA binding and essential for repressor activity (11, 23). Four other analyzed pseudorevertants revealed the following substitutions in PpsR: H90-R and C251-R, with the latter being isolated three times (see Discussion for details). Therefore, all eleven of the photosynthesis-competent pseudorevertants of the *appA* null mutation contained mutations in *ppsR*. The fact that we observed a variety of phenotypes in these pseudorevertants suggests that the PpsR repressor proteins were impaired to various extents, from a complete inactivation (e.g., in strain PS6) to a less severe impairment in other mutant strains (8).

(ii) Identification of cosmids capable of partial complementation of mutant APP11. We introduced a cosmid library of ~800 clones (4) into APP11. Two screens were employed. One was based on enhanced pigmentation, compared to APP11, of the aerobically grown exconjugants, and the other was based on the ability of the exconjugants to grow photosynthetically. Both screens resulted in the identification of the same two cosmids, pUI8714 and pUI8461, in addition to the *appA*-containing cosmid (10). Both cosmids mapped to the photosynthesis gene cluster, i.e., the chromosomal region which encompasses most genes for photopigment biosynthesis as well as genes for structural and assembly proteins corresponding to the PS complexes.

When pUI8714 and pUI8461 were tested in *trans* in APP11, we discovered that PS complexes (at high or medium light intensities) were not restored to levels characteristic of the wild type. In all cases only the abundance of the LH I complex was slightly increased, with little observable formation of the LH II complex (Fig. 3A). PS growth of APP11(pUI8714) or APP11 (pUI8461) differed from the growth of APP11(pRK415) by having a shorter lag phase and a higher growth rate (Fig. 4B). However, these cosmids failed to support growth of APP11 at low light intensity where the LH II complex is essential (data

TABLE 3. Positions in *ppsR* of suppressor mutations of the AppA null mutant

Designation of the suppressor mutation	Nucleotide substitution	Amino acid substitution	Occurrence of mutation	Proposed effect on PpsR
PS6	c(1785[a])-t	Q144[b]-stop	1	Truncation at the N terminus
PS3	t(1675)-c	L107-P	1	Disruption of secondary structure
PS33	t(2098)-c	L248-P	1	Disruption of secondary structure
PS8	t(2389)-c	L345-P	1	Disruption of secondary structure
PS24	t(2563)-c	L403-P	1	Disruption of secondary structure
PS36	a(2619)-g	K422-E	1	Decreased DNA binding
PS40	t(2625)-c	C424-A	1	Decreased DNA binding/ altered redox center (?)
PS18, 31, 34	t(2106)-c	C251-R	3	Altered redox center (?)
PS23	a(1624)-g	H90-R	1	Altered redox center (?)

[a] Position refers to the L37197 sequence deposited in GenBank.
[b] Position refers to amino acid residues in the PpsR protein.

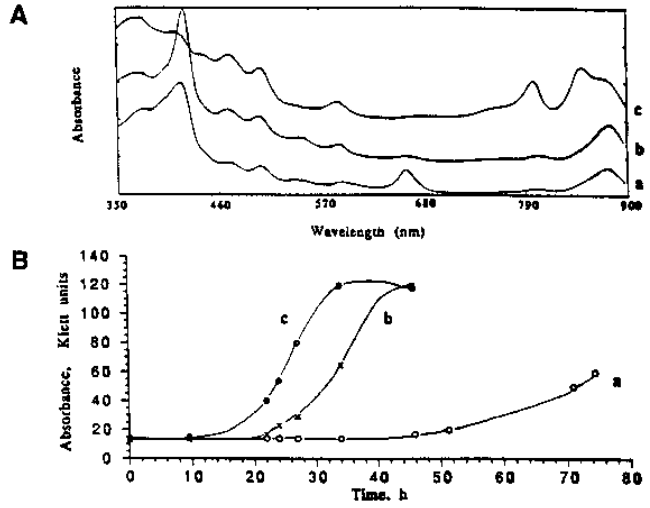

FIG. 3. Absorbance spectra (A) and photosynthetic growth (B) at 10 W/m^2 of strains APP11(pRK415) (a), APP11(pUI8461) (b), and APP11(p484-Nco5) (c). Both the spectrum and growth curve of strain APP11(pUI8714) were qualitatively similar to the spectrum and growth curve of APP11(pUI8461) (8).

not shown). Therefore pUI8714 and pUI8461 could apparently compensate for the defects inherent in the AppA null mutant only to a limited extent. Analysis of these cosmids will be presented elsewhere.

The above-described analysis of the relationships between AppA and other regulators of photosynthesis gene expression as well as the suppressor analysis of the *appA* null mutation are consistent with AppA acting antagonistically to PpsR.

***ppsR* gene expression.** To test the possibility that AppA antagonizes PpsR-mediated repression by decreasing the level of *ppsR* gene expression, a *ppsR*::*lacZ* fusion was constructed (Fig. 4A). We followed the dependence of *ppsR*::*lacZ* expression under aerobic conditions on the presence or absence of the *appA* allele by measuring β-galactosidase activity in the AppA null mutant, wild type, and wild type containing extra copies of *appA*. We found that *ppsR*::*lacZ* expression did not differ significantly among these strains, and in fact was somewhat elevated when an increased dosage of *appA* was present: APP11 < 2.4.1 < 2.4.1(p484-Nco5) (Fig. 4B). These results

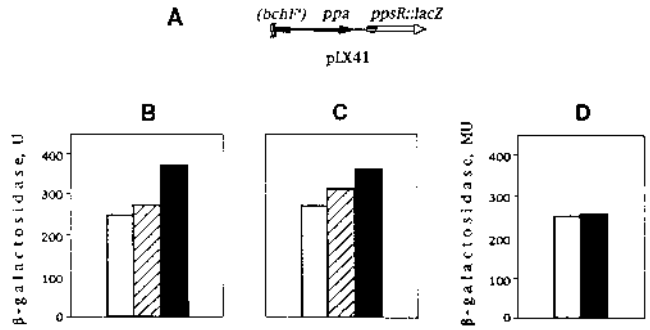

FIG. 4. (A) Depiction of the *ppsR*::*lacZ* fusion in plasmid pLX41. (B) Expression of *ppsR*::*lacZ* in aerobically grown strains APP11(pRK415) (open bar), 2.4.1(pRK415) (striped bar), and 2.4.1(p484-Nco5) (solid bar). (C) Expression of *ppsR*::*lacZ* in 2.4.1 under the following growth conditions: aerobic (open bar), semi-aerobic (striped bar), and photosynthetic (10 W/m^2) (solid bar). (D) Expression of *ppsR*::*lacZ* in aerobically grown strains of *P. denitrificans*, Pd(pRK415) (open bar) and Pd(p484-Nco5) (solid bar).

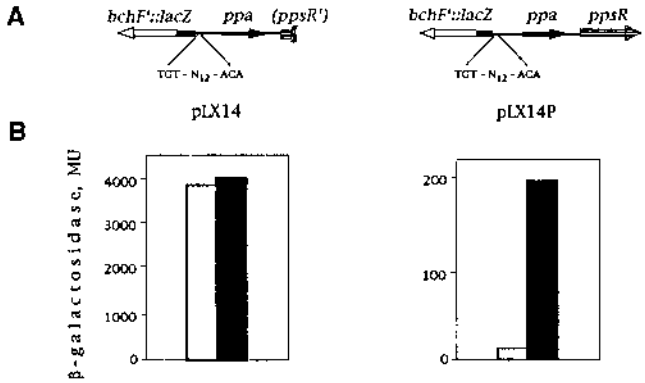

FIG. 5. (A) Depiction of the *bchF*::*lacZ* fusion contained in plasmids pLX14 and pLX14P. pLX14P carries the *ppsR* gene, while pLX14 does not. (B) Expression of *bchF*::*lacZ* from pLX14 (left) or pLX14P (right) in *P. denitrificans* strains, Pd(pRK415) (open bar) or Pd(p484-Nco5) (solid bar).

appear to preclude the possibility that AppA is involved in decreasing *ppsR* expression.

In light of these results we then deemed it necessary to follow expression of *ppsR* in the wild type under different growth conditions with the *ppsR*::*lacZ* expression system (Fig. 4C). We found that *ppsR*::*lacZ* expression showed little dependence on growth mode. There was an ~1.4-fold increase in the level of β-galactosidase under PS conditions compared to aerobic conditions, which suggests an increased cellular concentration of PpsR under PS conditions. This observation could imply that photosynthesis gene expression should be lower under anaerobic conditions. However, photosynthesis gene expression is known to be substantially higher under anaerobic as opposed to aerobic conditions. Therefore, PpsR repressor activity per se would appear to be regulated mainly at the posttranscriptional level, presumably at the protein level.

Expression of AppA and PpsR in a heterologous host. To confirm that AppA antagonizes PpsR-mediated repression, we expressed *appA* and *ppsR* separately and together in the heterologous host, *P. denitrificans*. This bacterium is related to *R. sphaeroides* but is nonphotosynthetic and therefore provides an advantage for studying specific relationships between selected *R. sphaeroides* genes involved in photosynthesis. We have previously demonstrated the validity of this alternative expression system for the study of photosynthesis gene expression (11, 12). *P. denitrificans* does not possess PpsR repressor activity (11). The presence of an activity equivalent to AppA in *P. denitrificans* had not yet been tested. However, because increased dosage of *appA* profoundly affected photosynthesis gene expression in *R. sphaeroides* (Fig. 1), we anticipated that the effect of the *R. sphaeroides appA* gene in *trans* in *P. denitrificans* would be noticeable, even if *P. denitrificans* contained an *appA* homolog.

In order to monitor the repressor activity of PpsR, a *bchF*::*lacZ* fusion was employed, where *bchF* represents one of the photosynthesis genes whose transcription is under the control of the PpsR repressor (11). We have demonstrated in *R. sphaeroides* that *bchF*::*lacZ* expression in the AppA null mutant is lower compared to the expression in wild type (10). Two plasmids, pLX14 and pLX14P, were constructed so that they contain the same *bchF*::*lacZ* fusion but differ only by the presence of the *ppsR* gene in *cis* in pLX14P but not in pLX14 (Fig. 5A). The level of β-galactosidase in *P. denitrificans* containing pLX14 and the second compatible vector pRK415, Pd(pLX14, pRK415), was ~210-fold higher than that in Pd(pLX14P,

pRK415) (Fig. 5B). This result is readily explained by the fact that the PpsR works as an efficient repressor in *P. denitrificans* and is similar to what we observed previously (11). When the *appA*-containing plasmid p484-Nco5 was introduced in *trans* into Pd(pLX14P), the β-galactosidase level increased substantially, ~11-fold, compared to Pd(pLX14P) containing only vector pRK415 (Fig. 5B). In control experiments we showed that AppA does not affect expression of the *bchF*::*lacZ* fusion in the absence of PpsR (Fig. 5B), nor does it affect expression of the *ppsR* gene in *P. denitrificans* (Fig. 4D). A promoterless *lacZ* gene resulted in ~16 MU of activity in either Pd(pRK415) or Pd(p484-Nco5). Hence, the effect of AppA was specific to expression of *bchF*::*lacZ* in the presence of PpsR. This provides additional confirmation that AppA can antagonize PpsR-mediated repression.

DISCUSSION

This study describes a molecular genetic approach to unravel the role of the AppA protein in the development of the PS apparatus in *R. sphaeroides* 2.4.1. We have attempted to localize the site of AppA action in the regulatory network by investigating the relationships between AppA and other known transcriptional regulators: the Prr (Reg) two-component regulatory system and FnrL, both of which are involved in the anaerobic activation of the expression of photosynthesis genes. We also examined the relationship of AppA to the PpsR protein, the known aerobic repressor of *bch*, *crt*, and *puc* operon transcription.

These studies reveal a link between AppA and PpsR. This linkage is based on several lines of evidence. First, while the AppA null mutant requires an extremely long lag phase to adapt to PS growth, and once growing, growth is poor due to the low levels of PS complexes, inactivation of the *ppsR* gene rescued these PS defects. Moreover, the phenotype of the constructed AppA-PpsR double mutant appeared to be identical to the phenotype of the PpsR null mutant. Second, eleven spontaneous photosynthesis-competent pseudorevertants of the *appA* null mutation, analyzed so far, contained suppressor mutations in *ppsR*. The phenotypes of these pseudorevertants varied widely, all the way from that which was identical to APS1 (in strain PS6) to those corresponding to a presumed partial inactivation of PpsR (8). Although we do not have formal proof that each isolated pseudorevertant acquired only one suppressor mutation, i.e., in *ppsR*, the high frequency of pseudorevertants is characteristic of a single spontaneous mutation. Further, some of the pseudorevertants showed phenotypes indistinguishable from or similar to the phenotype of the strain in which we constructed a disruption in *ppsR*, namely APS1. Finally, coexpression of AppA and PpsR in *P. denitrificans* resulted in decreased repression of the PpsR-regulated *bchF*::*lacZ* fusion. Collectively, this evidence strongly suggests that AppA can affect photosynthesis gene expression through the PpsR regulatory pathway and that AppA seems to antagonize PpsR function.

To reveal whether or not AppA affects *ppsR* gene expression, and therefore the intracellular concentration of repressor, we tested the dependence of *ppsR* gene expression on the *appA* allele. We demonstrated that *ppsR*::*lacZ* expression in the AppA null mutant does not differ substantially compared to *ppsR*::*lacZ* expression in wild type or wild type with extra copies of *appA*. Therefore, the effect of AppA does not take place at the level of *ppsR* gene expression.

We further showed that *ppsR* gene expression in wild type is generally unaffected by growth conditions, which leads us to suggest that the extent of PpsR-mediated repression may depend upon the activity of the PpsR protein. Therefore, how could the repressor activity of PpsR be regulated? There are several possibilities, including the direct interaction of AppA with PpsR or through the interactions of other mediators. These questions are outside the scope of this study and need further investigation. However, some predictions concerning the modulation of PpsR repressor activity can be extrapolated from the existing data.

The PpsR homolog from *Rhodobacter capsulatus* was proposed to act as an aerobic repressor of the *puc* operon as well as certain *bch* and *crt* genes and operons (24). In a previous report, we suggested that the role of the *R. sphaeroides* repressor of *puc* operon expression may extend beyond aerobic-anaerobic regulation (18). Our present data provide evidence that indeed *R. sphaeroides* PpsR is functional under anaerobic PS conditions. Comparisons of the levels of PS spectral complexes found in the PpsR null mutant with those of the wild type, both grown photosynthetically at the same light intensity, reveals characteristic differences, i.e., the PpsR null mutant produced more LH II complex (Fig. 2; Table 2). This suggests that, in wild type, PpsR is also involved in the regulation of *puc* (and most likely *bch* and *crt*) under anaerobic PS conditions. Moreover, the differences in LH II complex abundance between the PpsR null mutant and wild type is more pronounced at high light intensity compared to medium and low light intensity. This implies that PpsR is part of the regulatory mechanism which determines LH II complex abundance under anaerobic conditions when light regulation is operative. Under low light intensity, because LH II complex abundance is fully derepressed, PpsR may be inactive. Because PpsR is capable of acting in response to several environmental stimuli, i.e., oxygen tension and light intensity, it may be connected to several different sensors or, most likely, it responds to a downstream intrinsic signal (e.g., such as redox potential) which we can imagine to reflect both changes in oxygen tension and light intensity.

Such reasoning gains support from the spectrum of suppressor mutations mapping in PpsR and giving rise to photosynthesis-competent pseudorevertants of the AppA null strain (Table 3). Besides mutations which resulted in protein inactivation (Q144-stop) or disruption of protein secondary structure (L107-P, L248-P, L345-P, L403-P) or which affected DNA binding (K422-E), there were only a limited number of additional substitutions with less obvious consequences for PpsR activity. These changes affected one conserved histidine (H90-R) and the only two cysteine residues present in PpsR (C251-R and C424-A) (23). Both of these cysteines are conserved in the PpsR homologs from *R. sphaeroides* RS630 and *R. capsulatus* (ORF469). The substitution C424-A may affect DNA binding because it lies within the helix-turn-helix domain of PpsR (23) which we have previously shown to be sufficient for PpsR-mediated repression (11). However, the nature of the amino acid substitutions is such that it is tempting to propose that the primary function of C251, C424, and perhaps H90 involves redox sensing, e.g., through metal (or other ligand) binding. The isolation and characterization of the PpsR protein and its mutant forms should prove helpful in verification of the above prediction.

Recent biochemical analysis of the AppA protein has revealed that its amino terminus binds a flavin which could function as a redox center (8). The carboxy-terminal domain of AppA contains an unusual cysteine-rich motif (10) which may also serve as a redox center or metal binding domain. When the data described previously (10) are considered together with those presented here, the following proposed scheme of regulation of the AppA-PpsR branch emerges. AppA is directly

responsive to changes in redox potential which result from changes in oxygen tension or light intensity, which are themselves interpreted by other cellular components (30). These alterations in redox potential are then transfered by AppA to the PpsR repressor either directly or indirectly. As a result, PpsR repressor activity decreases, i.e., the affinity of PpsR for the TGT-N_{12}-ACA motif located upstream of *puc* decreases, as well as a subset of *bch* and *crt* genes and operons, and of other genes presumably controlled by PpsR (8). This gives rise to increased expression of the corresponding genes and operons whose products are involved in development of the PS apparatus. In the absence of AppA, the development of the PS apparatus is blocked at several points, e.g., biosynthesis of Bchl, Crt, and the LH II complex structural proteins. This regulatory impairment imposed by the absence of AppA cannot be overcome by other mechanisms which are known to activate photosynthesis gene expression in response to anaerobiosis, e.g., the Prr (Reg) two-component regulatory system or FnrL, i.e., the repressor effect of PpsR is normally dominant under both aerobic and anaerobic conditions. When *appA* is placed in extra copy in the wild type, it results in an artificial decrease in PpsR repressor activity, even under aerobic conditions, and therefore results in derepression of the photosynthesis genes and in development of the PS apparatus. In the absence of PpsR, e.g., in the PpsR null mutant, AppA is dispensable for the PS growth.

Whether or not PpsR is the only target of AppA has yet to be determined. For example, cosmids pUI8461 and pUI8714 partially compensated for the PS defects of the AppA null mutant without an increase in the amount of the LH II complex, i.e., without an apparent decrease in PpsR-mediated repression of the *puc* operon. This predicts another, PpsR-independent, alteration in development of the PS apparatus in the AppA null mutant.

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