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appA, a Novel Gene Encoding a *trans*-Acting Factor Involved in the Regulation of Photosynthesis Gene Expression in *Rhodobacter sphaeroides* 2.4.1

MARK GOMELSKY AND SAMUEL KAPLAN*

Department of Microbiology and Molecular Genetics, the University of Texas Medical School at Houston, Houston, Texas 77030

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A new gene, the product of which is involved in the regulation of photosynthesis gene expression in the anoxygenic photosynthetic bacterium *Rhodobacter sphaeroides* 2.4.1, has been identified. The isolation of this gene, designated *appA* (activation of photopigment and *puc* expression), was based on its ability, when provided in extra copies, to partially suppress mutations in the two-component PrrB-PrrA regulatory system. The presence of extra copies of the *appA* gene in either *prrB*, *prrA*, or wild-type strains resulted in an activation of *puc*::*lacZ* expression under aerobic conditions. Constructed AppA null mutants did not grow photosynthetically and were impaired in the synthesis of both bacteriochlorophyll and carotenoids, as well as the structural proteins of the photosynthetic spectral complexes. When grown anaerobically in the dark, these mutants accumulated bacteriochlorophyll precursors. The expression of *lacZ* fusions to several photosynthesis genes and operons, including *puc*, *puf*, and *bchF*, was decreased in the AppA mutant strains in comparison with the wild type. To examine the role of AppA involvement in bacteriochlorophyll biosynthesis, we inactivated an early gene, *bchE*, of the bacteriochlorophyll pathway in both wild-type and AppA$^-$ mutant backgrounds. The double mutant, AppA$^-$ BchE$^-$, was found to be severely impaired in photosynthesis gene expression, similar to the AppA$^-$ BchE$^+$ mutant and in contrast to the AppA$^+$ BchE$^-$ mutant. This result indicated that AppA is more likely involved in the regulation of expression of the *bch* genes than in the biosynthetic pathway per se. The *appA* gene was sequenced and appears to encode a protein of 450 amino acids with no obvious homology to known proteins.

In the anoxygenic photosynthetic bacteria of the genus *Rhodobacter*, synthesis and assembly of the photosynthetic apparatus are regulated primarily by two environmental signals, oxygen and light. High oxygen tension and, to a lesser extent, high light intensities in the absence of oxygen inhibit production of the photosynthetic spectral complexes (4). These complexes consist of light-harvesting I and II (LHI and LHII) antenna complexes, which entrap photons and transmit the excitation energy to the reaction center complex, which in turn converts this energy into chemical energy. Each of the photosynthetic complexes is composed of membrane-bound apoproteins associated with the photopigments bacteriochlorophyll (Bchl) and carotenoids (Crt) (19).

Both oxygen and light act at several levels, including the transcription level, to regulate the abundance of these spectral complexes. It has been shown that the PrrB-PrrA two-component regulatory system in *Rhodobacter sphaeroides* (8, 9), as well as its counterpart RegB-RegA in *R. capsulatus* (16, 27, 30), is required to activate expression of photosynthesis (PS) genes in response to a decrease in oxygen tension. PrrB (RegB) is a histidine kinase which is believed to act through its response regulator, PrrA (RegA), by controlling the extent of phosphorylated regulator. Mutants defective in the PrrB-PrrA two-component regulatory system are unable to fully activate expression of the PS genes upon transition from aerobic to anaerobic growth conditions (8, 9). This ultimately leads to impairment in their photosynthetic growth.

PrrA belongs to the subclass of short response regulators (33), which consist mainly of a phosphorylation domain; therefore, it probably does not interact directly with DNA. If correct, this model then presumes the presence of another downstream member(s) of this regulatory pathway. The DNA-protein complexes bound to the sequences upstream of the different PS genes have been described previously (26, 31), and several identified transcriptional factors were either proposed (13, 29) or shown (24) to interact with these sequences. However, the full spectrum of such factors and the mechanism(s) and hierarchy by and through which their interactions are mediated are yet to be determined.

We have previously described a general method for the isolation and identification of regulatory mutants with defects in PS gene expression based on the use of a *lacZ* transcriptional fusion to the *puc* operon (14). This operon encodes the apoproteins composing the LHII complex, as well as an assembly factor or factors specific to this complex (11, 18, 23). Among others, regulatory mutants containing mutations in the PrrB-PrrA system have been identified (14). Following complementation of these mutants with a cosmid library derived from wild type (WT) *R. sphaeroides* 2.4.1 DNA, we found that some cosmids, although unable to completely compensate for the mutants' defect, could partially compensate for the defect. These cosmids were subsequently shown not to contain either *prrB* or *prrA*. One group of cosmids partially complemented mutations in *prrB* but not those in *prrA*. Analysis of one representative cosmid, pUI8043, revealed that it contains a gene for a heterologous histidine kinase, HupT, which in extra copies was able to substitute for PrrB in the activation of PrrA

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, University of Texas Medical School, 6431 Fannin, Houston, TX 77030. Phone: (713) 794-1742. Fax: (713) 794-1782. Electronic mail address: skaplan@utmmg.med.uth.tmc.edu.

(14). Another group of cosmids, which included cosmid pUI8484, were effective in the partial restoration of PS gene expression in both PrrB and PrrA mutants. This observation led to the assumption that cosmids of the latter subset might carry a regulatory gene(s) which encodes factors either acting downstream of PrrA in the same regulatory pathway or which affect PS gene expression through a regulatory pathway independent of PrrA.

Here we report the identification, cloning, sequencing, and analysis of a new regulatory gene, *appA*, from the cosmid pUI8484. This gene is required for both aerobic and anaerobic expression of genes involved in photopigment production, as well as genes encoding photosynthetic complex apoproteins. Possible sites of action of the *appA* gene product are discussed.

(A preliminary report of this work has been presented previously [12].)

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this work are listed in Table 1.

Growth conditions. *Escherichia coli* strains were grown at 37°C on LB medium (25) supplemented, when required, with the following antibiotics: tetracycline, 10 μg/ml; ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; trimethoprim, 50 μg/ml; streptomycin and spectinomycin, 25 μg/ml each.

R. sphaeroides strains were grown chemo- or photoheterotrophically at 30°C on Sistrom's medium A (4) containing succinate as the carbon source. For anaerobic growth in the dark, LB (20%, vol/vol) and dimethyl sulfoxide (final concentration, 80 mM) were added. Aerobic conditions in liquid cultures were maintained by permanent bubbling of a mixture of 20% O_2, 79% N_2, and 1% CO_2. Semiaerobic conditions were maintained by bubbling of a mixture of 2% O_2, 97% N_2, and 1% CO_2. For anaerobic conditions, screw-cap tubes were used. Photosynthetic conditions were achieved by illuminating tubes with light at ~10 W/m^2. Antibiotics were used, when appropriate, at the following concentrations: tetracycline, 1 μg/ml; kanamycin, 40 μg/ml; streptomycin and spectinomycin, 50 μg/ml each.

Conjugation techniques. Conjugation was performed essentially as described elsewhere (6). Briefly, to introduce plasmids of interest into various *Rhodobacter sphaeroides* strains, biparental matings (with *E. coli* S17-1 as a donor) or triparental matings (with *E. coli* HB101(pRK2013) as a helper strain) were used.

β-Galactosidase assays. The activities of β-galactosidase on colonies were estimated by overlaying plates with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) as described elsewhere (14). β-Galactosidase assays with cell extracts were performed as described elsewhere (22) at least twice, with standard deviations not exceeding 15%. Activity of β-galactosidase is expressed in units, where 1 U corresponds to 1 μmol of *o*-nitrophenyl-β-galactoside (ONPG) hydrolyzed per min per mg of protein.

Spectrophotometric assays. *R. sphaeroides* cell extracts were obtained by sonication of cells grown anaerobically in the dark (anaerobic-dark-grown cells) and assayed as described elsewhere (8) by using samples containing 0.5 mg of protein per ml. Photopigments were extracted with acetone-methanol (7:2, vol/vol) from cell pellets obtained from anaerobic-dark-grown cells essentially as described elsewhere (4).

DNA manipulations and sequence analysis. Standard recombinant DNA techniques (25) and molecular biological enzymes and reagents were used according to the specifications of the manufacturers. Pulsed-field gel electrophoretic analysis of the *R. sphaeroides* genome has been described previously (34). DNA sequencing was performed with an ABI 373A automatic DNA sequencer (Applied Biosystems) at the DNA Core Facility of the Department of Microbiology and Molecular Genetics, University of Texas Medical School at Houston. Templates for sequencing were pUC19-based subclones of the DNA regions of interest. For sequence analysis, the Genetics Computer Group software package of the University of Wisconsin and GenBank/EMBL and SwissProtein data bases were used.

Nucleotide sequence accession numbers. The accession no. L42555 was assigned to the sequence of the DNA fragment containing the *appA* gene. Partial sequence of the *bchE* gene was deposited under the accession no. L37197.

RESULTS

Cloning and characterization of the *appA* gene. Cosmid pUI8484 was originally identified as being able to partially compensate for the defects of regulatory mutants of *R. sphaeroides* designated class I and shown to contain mutations in *prrB* (14, 15). When introduced into this mutant background, pUI8484 resulted in the activation of expression of a *puc*::*lacZ* transcriptional fusion, increase in pigmentation, and some improvement in photosynthetic growth. pUI8484 was subsequently shown to partially complement not only class I mutants but also class II mutants (believed to contain mutations in *prrA*) and other regulatory mutants, designated class III, containing as-yet-unidentified lesions (14). As demonstrated below, this cosmid does not contain either *prrB* or *prrA*. Therefore, we anticipated that pUI8484 might encode a *trans*-acting factor involved in the regulation of PS gene expression and functioning either downstream of PrrA or independently of PrrA.

Using a β-galactosidase assay on colonies of the PrrB mutant D5(pCF200Km) (14) to monitor the level of *puc*::*lacZ* expression, as well as colony pigmentation, we were able to localize the DNA region to a ~2.7-kb *Nco*I fragment (Fig. 1A) responsible for the observed partial complementation. This fragment was functional independently of its orientation with respect to the *tet* and *lac* promoters of vector pRK415 (Fig. 1A). During subcloning, we noted that the integrity of the DNA fragment progressing from either side of the *Eco*RI site of the insert (Fig. 1A) was necessary for its full physiological effect. This indicated that the *Eco*RI site must lie in either the coding or the regulatory region of the locus present in pUI8484. Plasmid p484-NcoI (Fig. 1A), containing the ~2.7-kb *Nco*I fragment, was used for the further characterization of this newly discovered locus.

To estimate the effect of the ~2.7-kb *Nco*I fragment, we introduced plasmid p484-NcoI and, in parallel, control plasmids pLA2917 and pRK4150, into the following strains: the PrrB mutant, D5; the PrrA null mutant, PRRA1; and the WT, 2.4.1. All of these strains also contained the second compatible *lacZ* reporter plasmid, pCF200Km, to monitor *puc* expression. Figure 2A demonstrates the levels of β-galactosidase observed when the above-described strains were grown aerobically. The presence of p484-NcoI in *trans* in all strains tested resulted in a substantial increase in *puc*::*lacZ* expression in comparison with *puc*::*lacZ* expression in the presence of the vector only. Under aerobic conditions, the level of expression of the *puc* operon, as well as most of the PS genes, in WT *R. sphaeroides* is low (18, 22). In the experiments described here, we observed ~220 U of β-galactosidase in the WT strain, 2.4.1, containing pCF200Km and pLA2917 (Fig. 2A). Under identical conditions, β-galactosidase activity in the WT strain containing p484-NcoI, strain 2.4.1(pCF200Km, p484-NcoI), was ~10-fold higher than control levels. Similarly, a 5- to 6-fold increase in *puc*::*lacZ* expression was observed when p484-NcoI was present in D5(pCF200Km) and PRRA1(pCF200Km) (Fig. 2A). Therefore, p484-NcoI encodes a factor which, when present in extra copies in either *prrB*, *prrA*, or WT strains, resulted in the activation of *puc*::*lacZ* expression, even under aerobic conditions.

The additional effect of p484-NcoI on other components of the photosynthetic apparatus is demonstrated in Fig. 3, which presents spectra obtained from anaerobic-dark-grown cells containing either p484-NcoI or the vector alone. Under anaerobic-dark growth conditions, the photosynthetic apparatus of WT *R. sphaeroides* is produced gratuitously and the most abundant photosynthetic spectral complexes are the LHII and LHI complexes. In comparison with the WT strain, 2.4.1, the PrrB mutant, D5(pLA2917), produced substantially lower levels of both spectral complexes, and the PrrA mutant, PRRA1 (pRK4150), produced only trace amounts of the two complexes (Fig. 3). However, upon introduction of p484-NcoI into both D5 and PRRA1, the abundance of both the LHI and LHII complexes increased substantially (Fig. 3). Nonetheless, p484-NcoI was unable to restore photosynthetic complexes to

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
E. coli		
DH5α*phe*	F$^-$ φ80Δ*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA endA hsdR supE44 thi gyrA relA phe*::Tn*10d*Cm^r	8
S17-1	C600::RP4-2 (Tc::Mu)(Km::Tn7) *thi pro hsdR recA* Tra$^+$	32
HB101	*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	25
R. sphaeroides		
2.4.1	WT	W. R. Sistrom
D5	2.4.1 *prrB*	14
PRRA1	2.4.1 *prrA*::ΩSm^r/Sp^r	8
APP1	2.4.1 *appA*::ΩKm^r	This work
APP11	2.4.1 Δ*appA*::Tp^r	This work
BCHE	2.4.1 *bchE*::ΩKm^r	This work
APPE	APP11 *bchE*::ΩKm^r	This work
Plasmids		
pCF200Km	Sm^r/Sp^r Km^r IncQ, *puc*(URS + DRS)::*lacZYA′*	22
pCF250Km	Sm^r/Sp^r Km^r IncQ, *puc*(DRS)::*lacZYA′*	22
pLX200	Sm^r/Sp^r IncQ, *bchF*::*lacZYA′*	13
pUI1830Δ	Sm^r/Sp^r IncQ, *pufB*::*lacZYA′*	13
pUI1063	Sm^r/Sp^r Tc^r IncQ, *hemA*::*lacZYA′*	J. H. Zeilstra-Ryalls, University of Texas—Houston
pLA2917	Tc^r Km^r IncP cosmid vector	1
pRK415	Tc^r *lacZ*α IncP	17
pRK4150	pRK415 with inactivated *lacZ*α gene	13
pRK2013	Km^r Inc ColE1 Mob	10
pSUP202	Ap^r Cm^r Tc^r Inc ColE1 Mob	32
pUC19	Ap^r *lacZ*α	35
pUI1637	Ap^r Km^r (source of ΩKm^r cartridge)	8
pSUP5TpMCS	Ap^r Tc^r Tp^r (source of Tp^r cartridge)	C. MacKenzie, University of Texas—Houston
pUI8484	pLA2917-derived cosmid containing the *appA* gene	7
p484-Sa	pUI8484 (*Sac*I, ligase)[a] deletion of all internal *Sac*I fragments of the insert DNA of pUI8484	This work
p484-RH	pRK415 (*Eco*RI, *Hin*dIII) + 2.3-kb *Eco*RI-*Hin*dIII fragment of p484-Sa	This work
p484-BR	pRK415 (*Bam*HI, *Eco*RI) + 3.0-kb *Bam*HI-*Eco*RI fragment from p484-Sa	This work
p484NcoI	pUC19 (*Sal*I, Pol[b]) + 2.7-kb blunt-end *Nco*I fragment of p484-Sa (*appA* is in opposite orientation relative to *lacZ*α)	This work
p484-NcoI	pRK415 (*Hin*dIII, *Sac*I) + 2.7-kb *Hin*dIII-*Sac*I fragment of p484NcoI	This work
p484-Nco5	pUC19 (*Sal*I, Pol[b]) + 2.7-kb blunt-end *Nco*I fragment of p484-Sa (*appA* is in the same orientation as *lacZ*α)	This work
p484-Nco5	pRK415 (*Hin*dIII, *Sac*I) + 2.7-kb *Hin*dIII-*Sac*I fragment of p484Nco5	This work
p484SaH	pUC19 (*Hin*dIII, *Sac*I) + 5.6-kb *Hin*dIII-*Sac*I fragment of p484-Sa	This work
p484SaH::Tp^R	p484SaH (*Mun*I, *Stu*I) + 1.6-kb *Eco*RI-*Dra*I fragment containing Tp^r cartridge of pSUP5TpMCS; Δ*appA*::Tp^r	This work
p484SaH::Tp^R::mob	p484SaH::Tp^R (*Ssp*I) + 5.1-kb *Pvu*II fragment of pSUP202 containing Tc^r and *mob* loci; Δ*appA*::Tp^r, Mob	This work
p484SaH::Km^R	p484SaH (*Stu*I) + 2.0-kb *Dra*I fragment containing ΩKm^r cartridge of pUI1637; *appA*::Km^r	This work
p484SaH::Km^R::mob	p484SaH::Km^R (*Ssp*I) + 4.1-kb *Mcs*I fragment of pSUP202 containing Tc^r and *mob* loci, *appA*::Km^r, Mob	This work
p121	pBluescript KS containing *ppa*, *ppsR*, and *bchE* genes from *R. sphaeroides* 2.4.1	13
p121-6	p121 (*Kpn*I, *Not*I, Pol[b] ligase)	This work
p121-6::Km^R	p121-6 (*Nco*I, Pol[b]) + 2.0-kb *Dra*I fragment containing ΩKm^r cartridge of pUI1637, *bchE*::Km^r	This work
p121-6::Km^R::mob	pSUP202 (*Ssp*I) + 2.5-kb *Ecl* 136I fragment of p121-6::Km^R; *bchE*::Km^r, Mob	This work

[a] Enzymes used to treat the plasmids are shown in parentheses.
[b] Pol, T4 DNA polymerase plus deoxynucleoside triphosphates.

WT levels in either D5 or PRRA1, i.e., it only partially complements mutations in *prrB* and *prrA*.

The effect of p484-NcoI on both LHII and LHI spectral complexes under anaerobic conditions indicates that p484-NcoI encodes a factor involved in the regulation of expression of not only the *puc* operon, but presumably other PS genes as well. For the *puc* operon, the effect of this factor is exerted at the transcriptional level. We have designated this factor AppA, for activation of photopigment and *puc* expression.

Sequence analysis of the *appA* gene. The DNA sequence of the ~2.0-kb *Nco*I-*Mlu*I fragment derived from p484-NcoI and containing the *Eco*RI site referred to earlier (Fig. 1A) has been determined (Fig. 4). One possible open reading frame (ORF), which showed a codon usage with a bias for the third position,

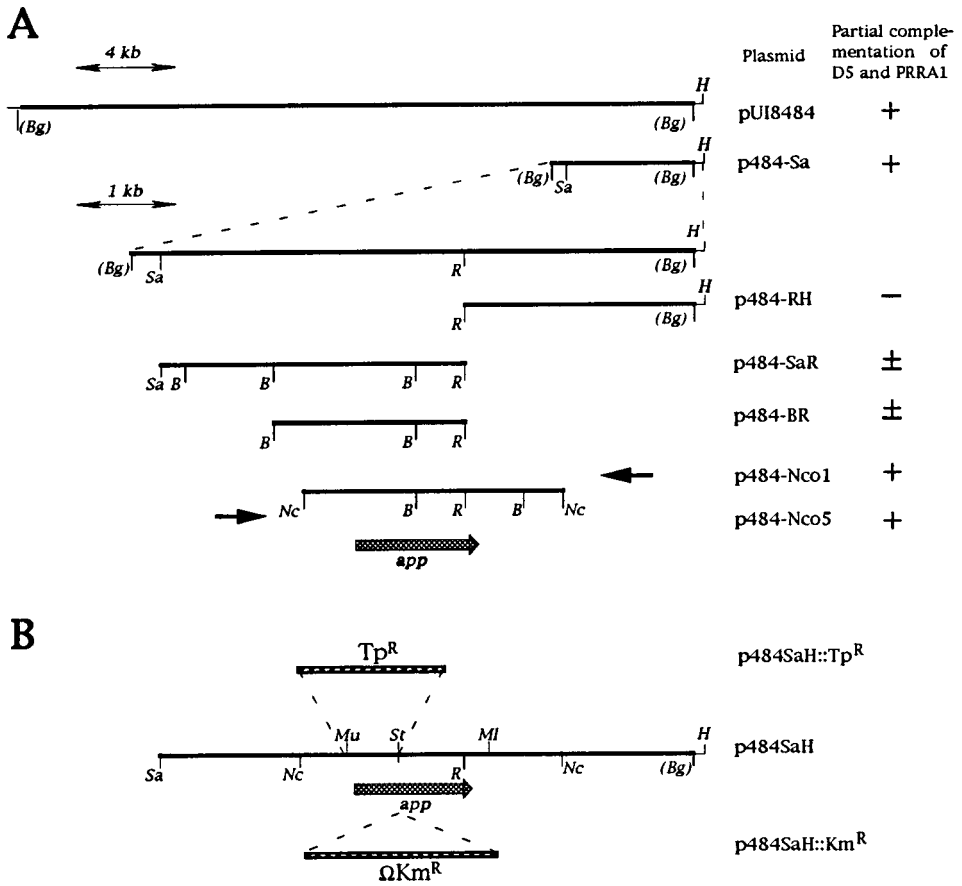

FIG. 1. Cloning (A) and inactivation (B) of the *appA* gene. Thick lines correspond to *R. sphaeroides* DNA, and thin lines correspond to DNA of the vector pLA2917. Abbreviations: *B*, *Bam*HI; *H*, *Hin*dIII; *Ml*, *Mlu*I; *Mu*, *Mun*I; *Nc*, *Nco*I; *R*, *Eco*RI; *Sa*, *Sac*I; *St*, *Stu*I; (*Bg*), *Bgl*II site of pLA2917 used for gene bank construction. Only restriction sites relevant to cloning and inactivation are shown. Solid arrows indicate the directions of the *tet* and *lac* promoters of the vector pRK415.

typical of *R. sphaeroides*, was identified. The gene corresponding to this ORF was therefore designated *appA*. Preceding the putative start codon (Fig. 4, position 501) of the *appA* gene there is a G+A-rich sequence which may function as a site for ribosome binding. No other ORFs were found in the immediate vicinity of the *appA* gene (sequence data, not shown). The *Eco*RI site (Fig. 4, position 1776) is located toward the 3′ end of *appA*; therefore, plasmid p484-BR, which retained partial AppA activity (Fig. 1A), is predicted to encode a protein lacking the 23 carboxy-terminal amino acids.

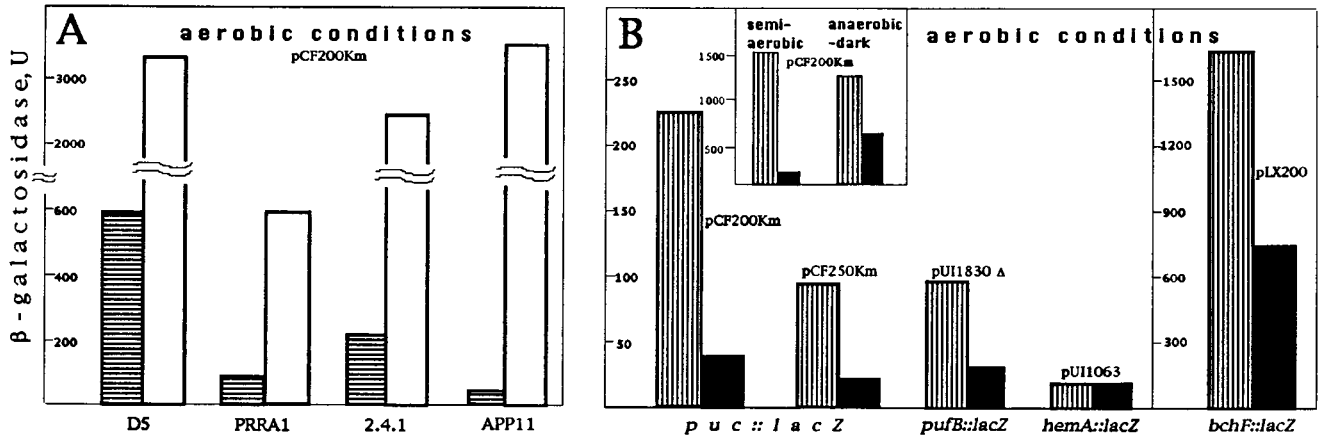

FIG. 2. Expression of *lacZ* fusions. (A) Expression of *puc*::*lacZ* from plasmid pCF200Km in aerobically grown strains of *R. sphaeroides* carrying a second plasmid, vector pLA2917 (pRK4150 for PRRA1) (striped bars) or the *appA*-containing plasmid, p484-NcoI (clear bars). (B) Expression of *lacZ* fusions to various PS genes in aerobically grown *R. sphaeroides* strains 2.4.1 (striped bars) and APP11 (black bars). The insert represents *puc*::*lacZ* expression from plasmid pCF200Km under semiaerobic and anaerobic-dark growth conditions.

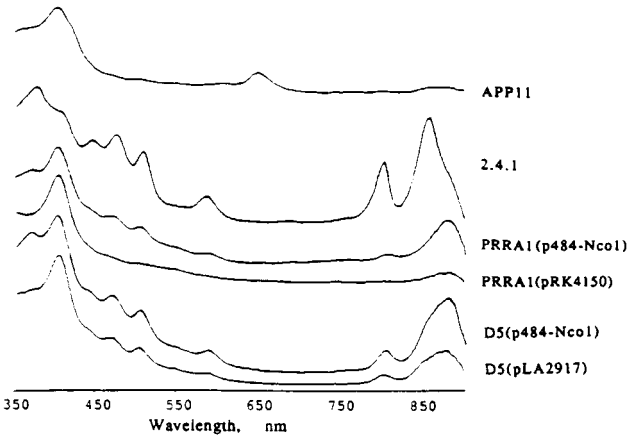

FIG. 3. Absorbance spectra of anaerobic-dark-grown cells. Spectra of 2.4.1 and APP11 are reduced 2.5-fold in order to permit visual comparison with the other spectra. The absorbance maximum of Bchl associated with the LHI complex is 875 nm, and those of Bchl associated with the LHII complex are 800 and 850 nm. Colored Crt absorb in the range of ~450 to 550 nm.

The deduced *appA* gene product consists of 450 amino acids and is most likely a cytoplasmic protein. A search for homologies in the data bases did not reveal any obvious similarity to known proteins. No functional domains, including DNA binding motifs, could be readily found in the derived sequence for AppA, although two structural features of AppA seem worth mentioning: an unusual cysteine-rich cluster (C-5X-CC-4X-C-6X-CC, where X is an amino acid) at the carboxy-terminal portion, as well as a very hydrophilic carboxy terminus composed of a large number of basic amino acids. The relevance of these features to the function of AppA is not known at this time. However, since only partial activity of *appA′*, which was truncated at the *Eco*RI site, was observed, we propose that the carboxy-terminal region of the protein is important for full activity.

Genetic analysis of *appA*. In order to obtain greater insight into the role of *appA*, we constructed two *appA* null mutations. The *appA* disruptions were made by the insertion of either ΩKm^r or Tp^r cartridges as depicted in Fig. 1B. The ΩKm^r cartridge was inserted into the *Stu*I site of *appA* positioned approximately in the middle of the gene, and the *appA*::ΩKm^r construct is predicted to encode the amino-terminal half of the AppA protein (Fig. 1B). The Tp^r cartridge was inserted so that it replaced the *Mun*I-*Stu*I fragment which included the potential ribosome binding site and 5′ coding region of the *appA* gene (Fig. 4). The latter construction, Δ*appA*::Tp^r (Fig. 1B), is likely not to result in the formation of any AppA protein. Each of these mutations was crossed into the genome of 2.4.1, and representative putative double crossovers, APP1 containing *appA*::ΩKm^r and APP11 containing Δ*appA*::Tp^r, were selected. The structure of each of the double crossovers was confirmed by pulsed-field gel electrophoresis and Southern blot hybridization (data not shown). These data suggested that the *appA* gene is present in the ~85-kb *Dra*I-fragment of chromosome I and is positioned ~110 kb from the junction of the ~275-kb and 410-kb *Ase*I fragments (34). Therefore, the location of the *appA* gene on chromosome 1 of *R. sphaeroides* 2.4.1 is at kb ~1285. It is worth mentioning that the *appA* gene resides ~1.2 Mb apart from the PS gene cluster of *R. sphaeroides* 2.4.1, which encompasses most of the structural and some of the regulatory genes related to PS. The location of *appA* is also distant, over 130 kb apart from the *prrB-prrA* regulatory locus (8).

Phenotype of the *appA* null mutants. Mutants APP1 and APP11 formed virtually colorless colonies and were unable to grow under photosynthetic conditions. To determine the reason(s) for impairment in photosynthetic growth, we analyzed spectra obtained from stationary-phase, anaerobic-dark-grown cultures of each of these mutant strains. Because the spectra of APP1 and APP11 were indistinguishable, as were other characteristics associated with these mutant strains (data not shown), we focused our attention on mutant APP11. As can be seen from Fig. 3, under anaerobic-dark conditions, APP11 produced only trace amounts of the photosynthetic complexes. A compound with an absorbance maximum at ~655 nm was found to accumulate to relatively high levels in extracts of APP11 but not in 2.4.1. Figure 5 shows the spectra obtained following extraction of the photopigments with acetone-methanol from APP11 and 2.4.1. The level of Bchl present in APP11 was approximately ninefold lower than that present in 2.4.1. The spectrum of the compound absorbing at ~655 nm in the aqueous extract was shifted, to ~645 nm, in the acetone-methanol extract. This peak is similar to the absorbance maximum of chlorophyllide *a* (3), one of the Mg-containing tetrapyrrole precursors of Bchl.

As shown in Fig. 2A, an increased dosage of *appA* in *trans* substantially increased expression of the *puc*::*lacZ* fusion in mutants D5 and PRRA1, as well as in the WT, under aerobic conditions. Therefore, we anticipated that in mutant APP11 *puc*::*lacZ* expression would be correspondingly decreased. To test this hypothesis, we introduced into APP11 two *puc*::*lacZ* fusions, pCF200Km and pCF250Km. The former plasmid contains a fusion to *lacZ* of the entire regulatory region upstream of *puc*. This region comprises two domains, the so-called upstream (bp −629 to −92 upstream of the transcription start point) and downstream (bp −92 to +1) regulatory sequences, URS and DRS, respectively (22). The latter plasmid, pCF250Km, contains a fusion to *lacZ* of only the DRS (22). Under aerobic conditions, the levels of β-galactosidase were reduced in APP11 versus 2.4.1 by approximately four- to fivefold, independently of whether the entire regulatory region, URS plus DRS (Fig. 2B, plasmid pCF200Km), or only the DRS (Fig. 2B, plasmid pCF250Km) was present. Therefore, the DRS contains the site through which AppA exerts its effect on *puc* transcription. Under semiaerobic or anaerobic-dark growth conditions, β-galactosidase activity showed an increase in both APP11 and 2.4.1 (Fig. 2B, insert) in comparison with activity under aerobic conditions. However, *puc*::*lacZ* expression from pCF200Km in mutant APP11 was significantly—2.2- to 10-fold, depending upon growth conditions—lower than it was in 2.4.1. Therefore, the *appA* gene product appears to be required for full activation of expression of the *puc* operon in response to decreased oxygen tension or anaerobiosis.

To determine the effect of the *appA* null mutation on expression of other PS genes, we examined *lacZ* transcriptional fusions to the *puf* operon, which encodes apoproteins of LHI and RC complexes, and to the genes which encode enzymes involved in photopigment biosynthesis, *bchF* and *hemA* (Fig. 6). We found that, under aerobic conditions, *puf*::*lacZ* and *bchF*::*lacZ* expression in mutant APP11 was severalfold lower than that in 2.4.1, whereas *hemA*::*lacZ* expression was virtually unchanged (Fig. 2B). These results indicate that *appA* plays a role in the regulation of expression of at least several PS genes and operons.

By providing p484-Ncol in *trans* in APP11 or APP1, we could restore both *puc*::*lacZ* expression (Fig. 2A) and production of photosynthetic complexes (data not shown) to levels resembling those characteristic of 2.4.1(p484-Ncol). This observation

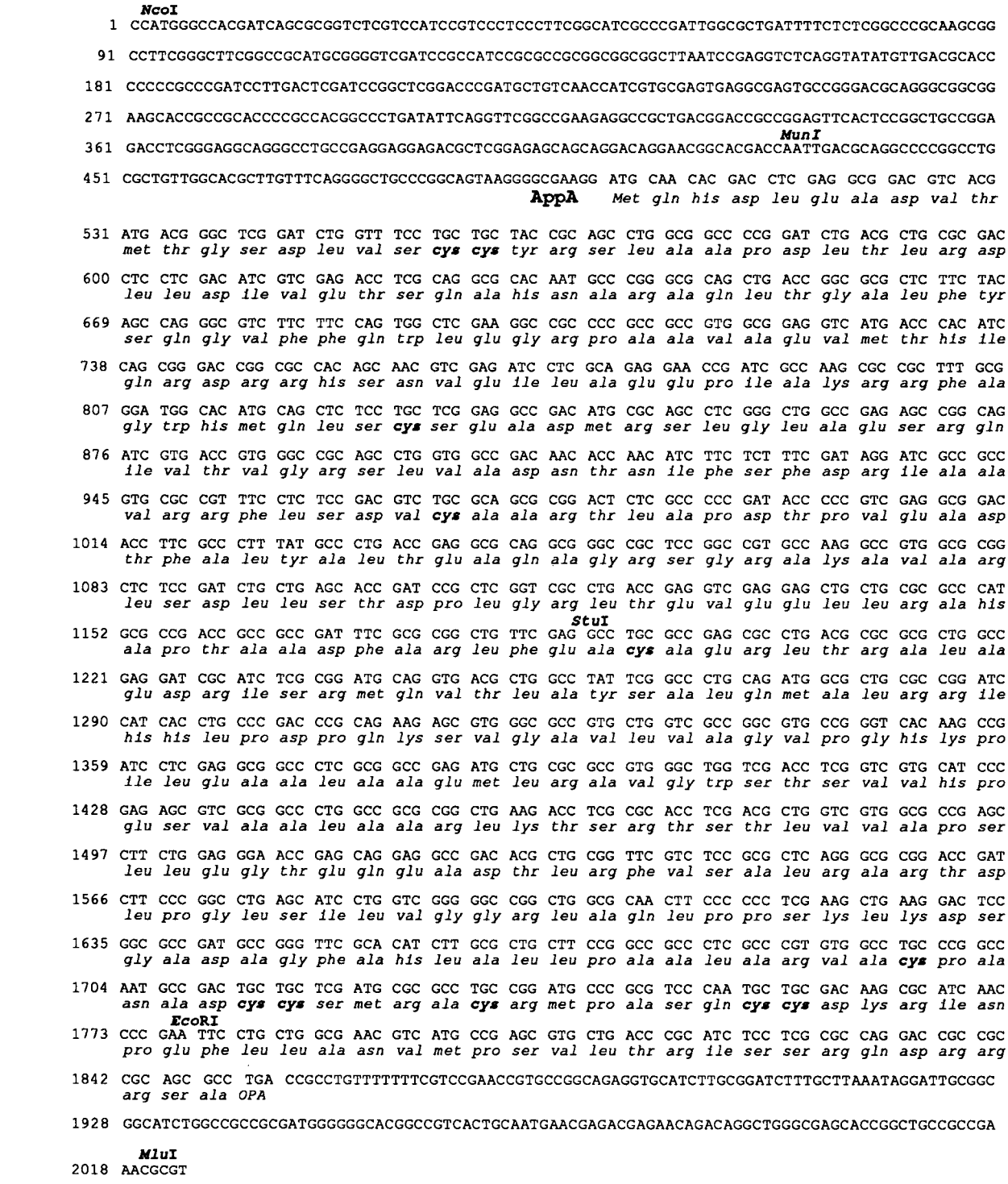

FIG. 4. Nucleotide sequence of the ~2.0-kb *Nco*I-*Mlu*I fragment and deduced amino acid sequence of the *appA* gene product (GenBank accession no. L42555). Restriction sites mentioned in the text are presented. Cysteine residues are shown in boldface type.

suggests that the phenotype of APP11, as well as APP1, resulted from the inactivation of a single gene, *appA*.

Role of AppA in Bchl biosynthesis. It has been observed for both *R. sphaeroides* and *R. capsulatus* that interference with the biosynthesis of Bchl results in a decline in Crt accumulation and *puc* operon expression (2, 20, 21, 28). These observations point to a link between the accumulation of Bchl, or an intermediate in the Bchl biosynthetic pathway, and expression of other PS genes, including *puc* and *crt*. Since the *appA* mutation resulted in a dramatic decrease in Bchl production and the apparent accumulation of Bchl precursors (Fig. 5), it was essential to determine whether the phenotype of APP11 was

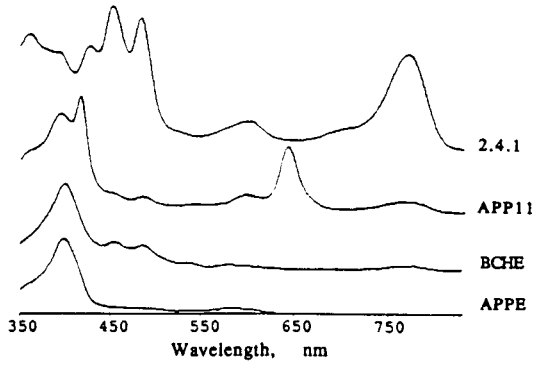

FIG. 5. Absorbance spectra of photopigments extracted from anaerobic-dark-grown cells.

merely a consequence of a partial block in Bchl biosynthesis. To answer this question, we constructed a Bchl$^-$ mutant blocked at a step in Bchl biosynthesis preceding chlorophyllide *a*. If AppA was somehow involved in the biosynthesis of Bchl per se, after the step of chlorophyllide *a* formation, then a block at an earlier biosynthetic step could (i) prevent formation of the Bchl precursor(s) accumulated in APP11 and (ii) abolish the effect of the *appA* null mutation on the expression of *puc* and *crt*. In other words, the phenotype of a Bchl$^-$ AppA$^-$ double mutant should be indistinguishable from that of a Bchl$^-$ mutant with respect to *puc* and *crt* expression.

We chose to disrupt the *bchE* gene (Fig. 6), whose product is involved in the conversion of Mg-protoporphyrin monomethyl ester into protochlorophyllide (3). The position of the *bchE* gene in the PS gene cluster of *R. capsulatus* is immediately downstream of *orf1469* (*crtJ*) (3). The latter gene has a homolog in *R. sphaeroides*, namely, the *ppsR* gene (29). We have described (13) cloning of this region of the DNA from *R. sphaeroides* 2.4.1 (Fig. 7A). The DNA sequence immediately downstream of *ppsR* (Fig. 7B) was found to be homologous to the corresponding region of the *bchE* gene from *R. capsulatus* (GenBank accession no. Z11165). The derived gene products are ~76% identical at the amino acid level. (Fig. 7C). On the basis of the very high degree of homology, we felt confident in assuming that in 2.4.1 the gene downstream of *ppsR* is *bchE*. This assumption is supported by previous reports of the localization of *R. sphaeroides bchE* mutations to approximately the same region (5).

The *bchE* gene was disrupted by an insertion of the ΩKm^r cartridge into an internal *Nco*I site (Fig. 7A). The *bchE* null mutations were constructed in both the 2.4.1 and APP11 genetic backgrounds, giving rise to strains BCHE and APPE, respectively. The structure of these mutants was confirmed by both pulsed-field electrophoresis and Southern blot hybridization (data not shown). The photopigment extracts from the anaerobic-dark-grown cells of mutant BCHE (Fig. 5) showed no Bchl, and the mutant was unable to grow under photosynthetic conditions, as anticipated.

When the spectra of APPE and APP11 were compared, we observed that the peak at ~645 nm characteristic of mutant APP11 was absent in the spectrum of APPE (Fig. 5). This was in accord with our assumption that the compound giving rise to this peak is an Mg-tetrapyrrole intermediate in Bchl biosynthesis which arises after the step catalyzed by BchE. Further, we compared Crt accumulation and *puc* expression in the two Bchl-deficient strains, BCHE and APPE. The decrease in Crt accumulation was more pronounced in APPE than it was in BCHE, as evident from the spectra of anaerobic-dark-grown cells (Fig. 5). The level of aerobic expression of a *puc*::*lacZ* fusion from pCF200Km in BCHE was ~190 U, i.e., similar to the ~220 U observed for the WT (Fig. 2B), whereas in APPE β-galactosidase activity was only ~35 U, i.e., similar to the ~38 U observed for APP11 (Fig. 2B). These data unambiguously demonstrated that the effect of the *appA* null mutation present in APPE was still apparent in the *bchE* background. Therefore, the function of the *appA* gene product cannot be assigned to an enzymatic step in the biosynthesis of Bchl, occurring after the formation of chlorophyllide *a*. It seems more likely that the block in Bchl biosynthesis observed to occur in the AppA mutant is due to decreased expression of *bch* genes.

DISCUSSION

In this report, we have presented the cloning, sequencing, and characterization of a *trans*-acting factor, *appA*, involved in the regulation of PS gene expression in *R. sphaeroides*. The identification of *appA* was based on its ability, when provided in extra copies, to partially complement mutants with defects in the PrrB-PrrA two-component regulatory system, i.e., to function as a suppressor of the *prrB* and *prrA* mutations.

In the PrrB-PrrA signal transduction pathway, PrrB functions as a sensor kinase which modulates the phosphorylation state of the downstream regulator PrrA in response to changes in oxygen tension and/or redox potential. When PrrA is in an active state, it increases expression of the PS genes by an as-yet-unknown mechanism(s). Both PrrA and PrrB mutants are unable to increase, to WT levels, expression of the PS genes in response to a decrease in oxygen tension. Hence, they produce either no (8) or low (9, 14) levels of the photosynthetic complexes required for photosynthetic growth. Because PrrA appears not to contain an identifiable DNA binding domain, there may be another component(s) of the PrrB-PrrA regula-

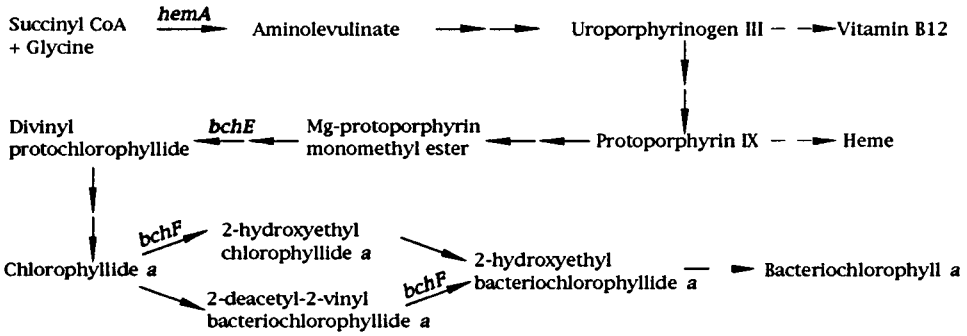

FIG. 6. Scheme for the biosynthesis of major tetrapyrroles in *Rhodobacter* spp. Only genes discussed in the text are shown.

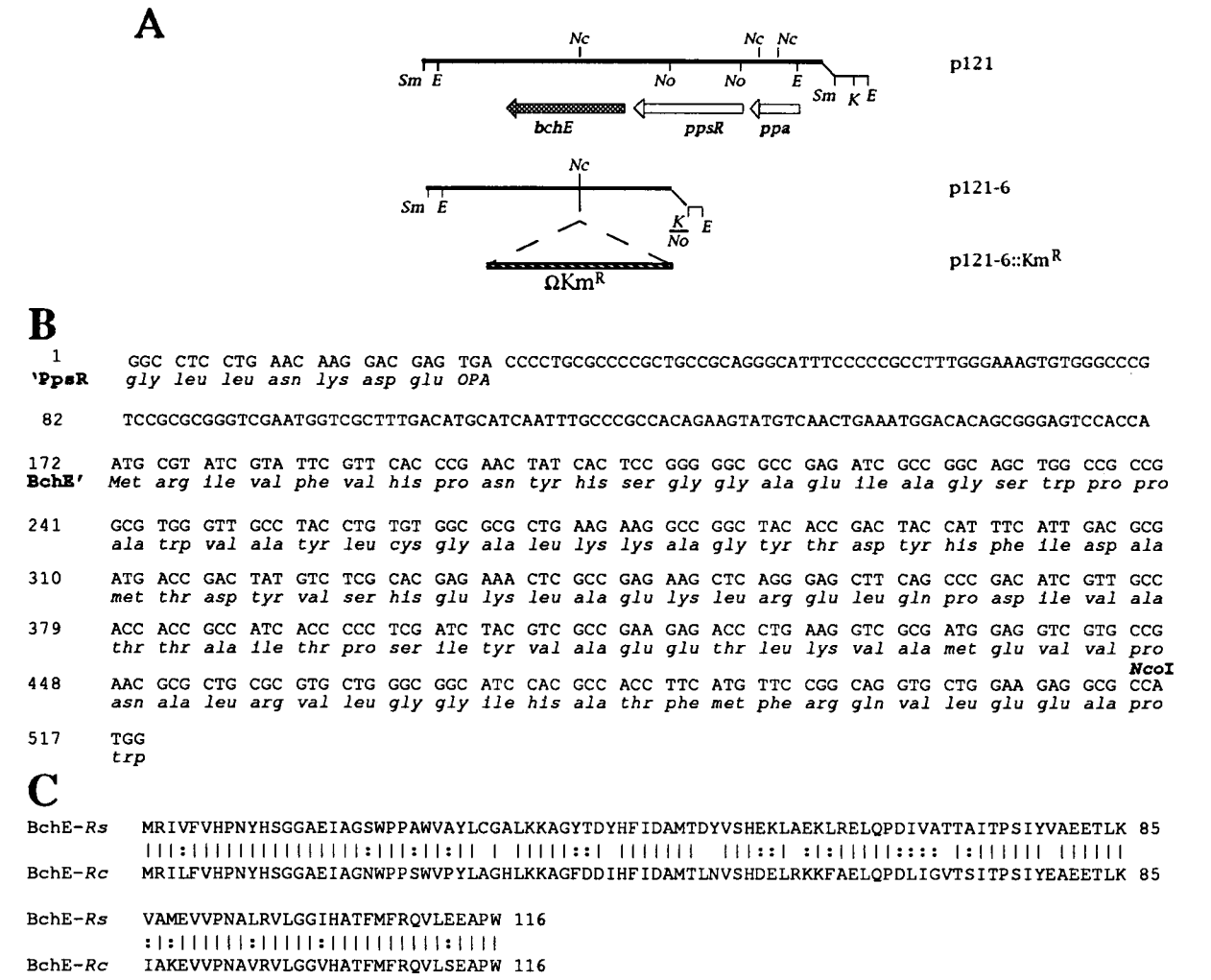

FIG. 7. Cloning and sequence of the 5′ end and inactivation of the *bchE* gene from *R. sphaeroides* 2.4.1. (A) Cloning and inactivation of the *bchE* gene. *E*, *Ecl* 136II; *K*, *Kpn*I; *Nc*, *Nco*I; *No*, *Not*I; *Sm*, *Sma*I. $\frac{K}{No}$ denotes the site disrupted during manipulations. (B) Nucleotide and deduced amino acid sequences of the *ppsR-bchE* region (GenBank accession no. L37197).(C) Amino acid sequence alignment of N termini of the BchE proteins from *R. sphaeroides* 2.4.1 (*Rs*) and *R. capsulatus* (*Rc*). Vertical lines connect identical residues, and colons connect conservative changes.

tory pathway. This component(s) would deliver the signal downstream, to the level of transcription of the PS genes. Despite the crucial role of the PrrB-PrrA regulatory cascade, it is not unreasonable to consider that there may be other, independently functioning regulatory networks involved in the oxygen- and/or redox dependent control of PS gene expression.

The ability of the *appA* gene in extra copies to partially suppress both *prrB* and *prrA* mutations suggests that AppA might act downstream of PrrA in the same regulatory cascade. We have shown that, similar to PrrA, AppA is required for normal expression of at least several PS genes. However, other evidence indicates that AppA, if a factor downstream of PrrA, cannot be the only mediator acting through PrrA. We base this assumption on two observations: first, the *appA* and *prrA* mutations result in different phenotypes (e.g., in contrast to what is observed with APP11, no Mg-tetrapyrrole intermediates are accumulated in PRRA1 [Fig. 3]); second, the *prrA* gene in multiple copies is still capable of enhancing PS gene expression in an *appA* null background (15).

There exists the alternative possibility that AppA may belong to a regulatory pathway different from PrrB-PrrA. Such a pathway might exist in parallel with and independently of the PrrB-PrrA pathway, or it might be convergent upon the latter pathway and therefore not completely independent from it. One of the members of such a regulatory pathway is PpsR (13, 28). This protein contains a helix-turn-helix motif, which is required for its repressor activity towards the *puc*, *bch*, and *crt* operons (13, 29). There is also a chance that the role of AppA in PS gene expression is relatively remote and mediated through its influence on cellular redox potential. The presence of multiple cysteine residues, especially a cysteine cluster at the carboxy terminus, might suggest that AppA either is sensitive to or is capable of sensing changes in cellular redox potential.

Another possibility, which we explored, pertaining to the involvement of AppA in PS gene expression was that AppA is directly involved in Bchl biosynthesis. This assumption seemed plausible because, similar to Bchl biosynthetic mutants, mutant APP11 was impaired in Bchl production and accumulated an intermediate(s) in Bchl biosynthesis. Such an involvement could have a secondary effect on expression of the PS genes, as has been previously documented (2, 20, 21, 28), although the precise mechanism is unknown.

To test this possibility, we constructed a null mutation in the *R. sphaeroides bchE* gene in both a WT genetic background

and the AppA mutant background. The *bchE* mutation was expected to block the biosynthesis of Bchl at the step preceding the site of AppA involvement (Fig. 6). This was directly confirmed by the disappearance in the double mutant, APPE (Fig. 5), of the Mg-tetrapyrrole precursor(s) which was accumulated in mutant APP11 (Fig. 5). However, the effect of the *appA* null mutation, as judged by *puc* expression and Crt accumulation, remained evident independently of Bchl production. This invalidates the hypothesis that AppA has a direct role in the Bchl biosynthetic pathway. Thus, the involvement of AppA in Bchl biosynthesis most likely resides at the level of the regulation of *bch* gene expression.

One of the sites in the Bchl biosynthesis pathway most likely to be subject to regulation by AppA is at the conversion of chlorophyllide *a*, since this appears to be the most abundant Bchl precursor accumulated in mutant APP11 (Fig. 5). In support of this assumption is our observation that expression of the *bchF* gene, encoding chlorophyllide *a* hydratase, is decreased in APP11 (Fig. 2B). Further, our data (15) suggest that the accumulation in mutant APP11 of protoporphyrin IX, an earlier precursor of Bchl and heme (Fig. 6), is not limiting the flow of tetrapyrrole intermediates toward Bchl. Hence, AppA specifically regulates the Bchl branch of tetrapyrrole synthesis.

In conclusion, we have identified a new regulatory gene, *appA*, which is essential for photosynthetic growth of *R. sphaeroides* 2.4.1. This gene specifies yet another locus on chromosome I of *R. sphaeroides* 2.4.1, outside the PS gene cluster and the *prrB-prrA* region, which is involved in the regulation of PS gene expression. The predicted amino acid sequence of AppA bears some interesting features, e.g., an unusual cysteine cluster at the carboxy terminus. However, the derived amino acid sequence does not allow us to reveal the function of AppA by homology to any known proteins. Experiments to assess the functional domain(s) of AppA are currently under way. Further, we intend to explore the possible interactions of AppA with other known regulators of PS gene expression. Together, these and other studies may provide the insights necessary to an understanding of the role(s) of AppA in regulation of PS gene expression in *R. sphaeroides* 2.4.1.

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