Identification and in vivo characterization of PpaA, a regulator of photosystem formation in *Rhodobacter sphaeroides*

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A regulatory protein, PpaA, involved in photosystem formation in the anoxygenic phototrophic proteobacterium *Rhodobacter sphaeroides* has been identified and characterized in vivo. Based on the phenotypes of cells expressing the *ppaA* gene in extra copy and on the phenotype of the *ppaA* null mutant, it was concluded that PpaA activates photopigment production and *puc* operon expression under aerobic conditions. This is in contrast to the function of the PpaA homologue from *Rhodobacter capsulatus*, AerR, which acts as a repressor under aerobic conditions [Dong, C., Elsen, S., Swem, L. R. & Bauer, C. E. (2002). *J Bacteriol* 184, 2805–2814]. The expression of the *ppaA* gene increases several-fold in response to a decrease in oxygen tension, suggesting that the PpaA protein is active under conditions of low or no oxygen. However, no discernible phenotype of a *ppaA* null mutant was observed under anaerobic conditions tested thus far. The photosystem gene repressor PpsR mediates repression of *ppaA* gene expression under aerobic conditions. Sequence analysis of PpaA homologues from several anoxygenic phototrophic bacteria revealed a putative corrinoid-binding domain. It is suggested that PpaA binds a corrinoid cofactor and the availability or structure of this cofactor affects PpaA activity.

INTRODUCTION

*Rhodobacter sphaeroides* is a facultative phototrophic proteobacterium with a broad spectrum of metabolic and energetic capabilities. It can catalyse oxidative phosphorylation using oxygen or, in the absence of oxygen, alternative electron acceptors. Under anaerobic conditions it can also use cyclic photosynthetic electron transfer to drive photosynthesis (Jackson, 1988; McEwan, 1994). The photosystem (PS) of *R. sphaeroides* consists of two light-harvesting complexes (LHI and LHII) and a photochemical reaction centre (RC). The LH complexes trap light energy and transfer the excitation energy to the RC, the site of primary photochemistry. This initiates light-driven cyclic electron transfer, the process that generates a proton electrochemical potential. The LH complexes and the RC are pigment–protein complexes and in *R. sphaeroides* these pigments are carotenoids and bacteriochlorophyll *a*. Most of the genes required for the synthesis of these pigment–protein complexes are clustered together on chromosome I of *R. sphaeroides* (Suwanto & Kaplan, 1989; Choudhary & Kaplan, 2000). These genes include *bch* and *crt* operons (encoding enzymes involved in bacteriochlorophyll and carotenoid biosynthesis, respectively), the *puf* operon and *puhA* gene (encoding the structural and assembly proteins of the LHI complex and RC), and the *puc* operon (encoding the structural and assembly proteins of the LHII complex).

Oxygen is a critical environmental signal that regulates formation of the PS in *R. sphaeroides*. In the presence of oxygen, formation of the PS is inhibited. A decrease in oxygen tension stimulates expression of the PS genes and subsequent synthesis of PS components and formation of a functional PS. Several regulatory factors controlling PS gene expression in response to oxygen have been identified (reviewed by Pemberton et al., 1998; Zeilstra-Ryalls et al., 1998; Gregor & Klug, 1999; Oh & Kaplan, 2001). Under conditions of low or no oxygen, light intensity and quality affects the abundance of photosynthetic complexes and PS
gene expression. Much less is understood about light-dependent regulation of PS formation in *R. sphaeroides* (Zeilstra-Rylls et al., 1998; Braatsch et al., 2002; Masuda & Bauer, 2002).

Two major regulatory systems involved in the activation of PS gene expression upon a decrease in oxygen availability are (i) the two-component regulatory system, PrrB/PrrA, where PrrA is a response regulator and PrrB is its cognate sensor kinase (Lee & Kaplan, 1992b; Eraso & Kaplan, 1994, 1995) and (ii) the anaerobic activator Fnr (Zeilstra-Ryalls & Kaplan, 1995). Both the PrrB/PrrA system and Fnr are global regulators; in addition to activating PS genes, they control expression of a number of other genes in an oxygen-dependent manner (Comolli et al., 2002; Laratta et al., 2002; Zeilstra-Rylls & Kaplan, 1998).

In addition to the activation of PS gene expression in response to decreased oxygen tension, a repressor PpsR downregulates PS gene expression in the presence of oxygen (Penfold & Pemberton 1991, 1994). PpsR binds to the target sequences, TGTN12ACA (where N is a nucleotide), located upstream of *puc* and several *crt* and *bch* operons (Gomelsky & Kaplan, 1995a; Gomelsky et al., 2000). The DNA binding activity of PpsR is regulated in part through oxidation/reduction of the thiol groups of its cysteine residues (Masuda et al., 2002; Masuda & Bauer, 2002) and in part through the anti-repressor AppA (Gomelsky & Kaplan, 1995c, 1997, 1998; Braatsch et al., 2002; Gomelsky & Klug, 2002; Masuda & Bauer, 2002).

The gene encoding AppA anti-repressor was identified by Gomelsky & Kaplan (1995c) in a genetic screen, in which cosmids representing the *R. sphaeroides* library were identified that upregulated PS gene expression in the PrrB or PrrA mutants when provided *in trans*. Here we describe the characterization of the second gene identified in this screen. In our preliminary reports this gene was designated *ppa* for photopigment and *puc* activation (Gomelsky & Kaplan, 1994, 1995a; Horne et al., 1997; Zelistra-Rylls et al., 1998). It will be further referred to as *ppaA*.

### METHODS

#### Strains, plasmids and growth conditions.

All strains and relevant plasmids used in this study are shown in Table 1. *R. sphaeroides* strain 2.4.1 and its derivatives, as well as *Paracoccus denitrificans*, were grown on Sistrom’s minimal medium A with succinate as carbon source (Cohen-Bazire et al., 1957). Unless indicated otherwise, 60 ml cultures of *R. sphaeroides* were grown at 30 °C in 100 ml glass culture tubes. The following gas mixtures were vigorously bubbled through the culture: 30 % O₂, 69 % N₂, 1 % CO₂ (high oxygen tension); 3 % O₂, 96 % N₂, 1 % CO₂ (low oxygen tension); 98 % N₂, 2 % CO₂ (no oxygen, anaerobic photosynthetic conditions; these cultures were illuminated with white light at 10 W m⁻²). Growth and genetic manipulations with strain PPA1 were conducted under anaerobic/dark conditions in presence 0-3 % (v/v) DMSO and 10 % (v/v) LB medium (Sambrook et al., 1989). *P. denitrificans* was grown in culture flasks as described previously (Gomelsky & Kaplan, 1995a). *E. coli* strains were grown in LB medium. When appropriate, media were supplemented with antibiotics at the following final concentrations: 1 μg tetracycline (Tc) ml⁻¹ for *R. sphaeroides* and 10 μg ml⁻¹ for *E. coli* kanamycin (Km), spectinomycin (Sp) and streptomycin (Sm), 50 μg ml⁻¹ for *R. sphaeroides* and *P. denitrificans* and 25 μg ml⁻¹ for *E. coli*. *Saccharomyces cerevisiae* L40 (Stratagene) was grown in minimal medium supplemented with appropriate nutrients and antibiotics according to specifications of the manufacturer of the MatchMaker kit (Stratagene).

#### Spectroscopy and enzyme analyses.

Pigments were extracted from *R. sphaeroides* frozen cell pellets with acidic acetone/methanol (7:2, v/v). Pigments extracted from cell mass of equal protein content were assayed by UV-visible spectroscopy. Assays of β-galactosidase and catechol-2,3-dioxygenase activities in *R. sphaeroides* and *P. denitrificans* were performed as described previously (Gomelsky & Kaplan, 1995a; Dryden & Kaplan, 1990). β-Galactosidase activity in yeast was measured according to the protocol of the manufacturer of the MatchMaker kit (Stratagene).

#### Genetic manipulations

Standard recombinant DNA techniques were applied (Sambrook et al., 1989). Plasmid mobilization into *R. sphaeroides* and *P. denitrificans* was carried out from *E. coli* S17-1 as described previously (Gomelsky & Kaplan, 1995a).

#### Construction of the PPA1 mutant.

A deletion/substitution mutation in *ppaA* from *R. sphaeroides* 2.4.1 was constructed by replacing the internal NcoI fragment with an fTKm² cartridge from pU11637 (Gomelsky & Kaplan, 1995b) to generate plasmid p714BgHAnc²::Km (see Fig. 1b). The mob region and Tc² gene from pSUP202 were cloned into p714BgHAnc²::Km and the resulting plasmid, p714BgHAnc²::Km::mob, was transferred into *R. sphaeroides* 2.4.1 by conjugation. Several Km² Tc² exconjugants were selected under anaerobic/dark conditions in the presence of DMSO. The DNA structure of the mutants was confirmed by Southern hybridization. One mutant strain was designated PPA1 and used subsequently.

#### Construction of the PPAxC mutant.

A *ppaxC* mutation was constructed in three steps. (1) A frame-shift mutation, *ppaA(Xc)*, in the 5’ region of the *ppaA* gene (i.e. deletion of a single nucleotide, T958; accession no. L37197) was constructed. The frame-shift mutation was made by digestion of plasmid p121 (Gomelsky & Kaplan, 1995a) with restriction enzyme XcmI followed by end repair using T4 polymerase and subsequent ligation, resulting in plasmid p121Xc. The *ppaA(Xc)* mutation was then cloned into the mobilizable suicide vector pSUP202ac. This vector is a derivative of pSUP202 (Simon et al., 1983) containing a sacB gene encoding levan sucrose from plasmid pLO1 (Lenz et al., 1994). (2) The *ppaA(Xc)*-containing plasmid was introduced into strain PPA1 and Tc² single crossover recombinants were selected. (3) The single crossover recombinants were grown for several generations with no antibiotics to allow for resolution of single crossovers. They were subsequently plated on agar medium containing 7-5 % sucrose. Sucre-resistant colonies (indicative of a loss of the *sacB* gene) were isolated and double crossover recombinants were identified as Km² Tc². The structures of these mutants were analysed by PCR and by Southern blot hybridization. One of the mutants containing the *ppaA(Xc)* mutation, which was confirmed by DNA sequencing, was designated PPAXC and used subsequently.

#### Protein–protein interactions.

Yeast two-hybrid system MatchMaker (Stratagene) was used to assay for PpaA–PpsR interactions. Plasmid pYEpPSR, expressing the full-length PpsR protein translationally fused to the B42 transactivation domain (‘prey’), was constructed based on vector pYESTrp2. A second plasmid, pZPpaA, expressing the PpaA-
protein translationally fused to the DNA-binding domain of the E. coli LexA protein ('bait'), was constructed based on vector pHybLex/Zeo. As a positive control for protein–protein interactions, plasmid pZppsR expressing the lexA::ppsR gene fusion was constructed based on vector pHybLexZeo. The plasmids were introduced into S. cerevisiae L40.

RNA extraction and quantitative RT-PCR. R. sphaeroides cells in the early exponential phase (OD 

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>R. sphaeroides</strong></td>
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<tr>
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<td>2.4.1 prrA::ΩSm/Sp'</td>
<td>Eraso &amp; Kaplan (1994)</td>
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<td>2.4.1 (puc::lacZ)</td>
<td>Lee &amp; Kaplan (1992b)</td>
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<td>2.4.1 ftrL::ΩSm'/Sp'</td>
<td>Zeilstra-Ryalls &amp; Kaplan (1995)</td>
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<td>2.4.1 bchE::ΩKm'</td>
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<td>Dryden &amp; Kaplan (1990)</td>
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<td>Vector for two-hybrid system, B42-transactivation domain</td>
<td>Stratagene</td>
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<tr>
<td>pZppsR</td>
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RNA extraction and quantitative RT-PCR. R. sphaeroides cells in the early exponential phase (OD600 0.16–0.20) were collected into centrifugation bottles containing shaved ice. Rifampicin was added to a final concentration of 200 μg ml⁻¹ to halt transcription initiation. Cells were pelleted by a brief centrifugation at high speed and cell pellets were frozen at −80°C until further processing. The cell lysis buffer from the RNAEasy midikit (Qiagen) and an equal volume of sterile zirconium beads were added to the frozen pellets. Cells were disrupted by 1 min of shaking in a Mini-BeadBeater (Biospec Products). RNA was extracted from the supernatants of cell lysates by use of the RNAEasy midikit and tested for the lack of contamination with genomic DNA by quantitative real-time PCR. cDNA from DNA-free...
RNA was made using SuperScript II reverse transcriptase (Stratagene). The following primers were designed for gene amplification: puc, Puc2AB-F (5'-GGCGTCCGTGGTTC-3') and Puc2AB-R (5'-GGCGTCCGTGGTTC-3') and PufAB-R (5'-ATCACCGCAGGAGGAAAC-3'); bchF, BchF2-F (5'-GGCGTCCGTGGTTC-3') and BchF2-R (5'-GGCGTCCGTGGTTC-3'); crtA, CrtA-F (5'-ATCACCGCAGGAGGAAAC-3'); gom, Gomelsky & Kaplan, 1995b, c). The thick line corresponds to R. sphaeroides DNA, the thin line corresponds to vector DNA. (b) Localization of the puc gene and pucA(Xc) mutation. Drawn to the same scale as panel (a). ∩ signifies the consensus sequence of the PpsR binding site, TGTN12ACA. (c) Structures of the puc null mutants, PPA1 and PPAx. The putative start and direction (or lack of thereof) of transcription is shown by horizontal lines ending with arrowheads (or short vertical lines). Bg, BglII; H, HindIII; Nc, Ncol; No, NotI; Sa, SacI; Sm, Smal. (Bgl) BglII site of the vector impaired during cosmid construction.

**RESULTS**

**Identification of R. sphaeroides PpaA as an activator of photopigment production and puc expression**

Cosmid pUI8714 was identified in the genetic screen for activators of PS gene expression in R. sphaeroides described previously (Gomelsky & Kaplan, 1995b). When provided in trans in the PrRB or PrRA null strains, it increased puc expression and pigmentation (Fig. 1a). We localized the
mineral DNA locus on cosm id pUI8714 responsible for this phenotype to a 0·9 kb Smal–NotI fragment (Fig. 1a). Sequence analysis of the Smal–NotI fragment revealed one putative ORF that was originally designated ppa (Gomelsky & Kaplan, 1994, 1995a; Horne et al., 1997; Zeilstra-Ryalls et al., 1998) and will be further referred to as ppaA. The ppaA gene is predicted to encode a basic protein (PI 9·5) of 264 aa. The ppaA gene is located in the PS gene cluster immediately upstream of ppsR, which encodes a PS gene repressor (Fig. 1b).

To assess the effect of ppaA in extra copy, we extracted photopigments from the PRRA1 strain containing in trans either the ppaA gene on plasmid pSmNo or vector pRK415. At high oxygen tension, when only traces of photopigments are present, the increase in carotenoids and bacteriochlorophyll was observed in strain PRRA1(pSmNo) compared to PRRA1(pRK415) (Fig. 2a). At low oxygen tensions, the several-fold increase in photopigments abundance in strain PRRA1(pSmNo) compared to PRRA1(pRK415) was also evident (Fig. 2b).

**Fig. 2.** Effect of ppaA in extra copy on photopigment abundance: thin line, PRRA1(pSmNo); thick line, PRRA1(pRK415). (a) High oxygen tension (20 ml cultures grown in 500 ml flasks with vigorous shaking); (b) low oxygen tension (100 ml cultures grown in 125 ml flasks with slow shaking). The absorbance peak characteristic of bacteriochlorophyll is at 765 nm; absorbance peaks characteristic of carotenoids are at 470–530 nm. ppaA in extra copy increased puc::lacZ expression over threefold in mutant PRRA1(pCF200Km) grown at high oxygen tension (Fig. 3a). A similar increase in puc::lacZ expression was observed in the wild-type-like strain, PUCZ-WT, containing a single copy of the puc::lacZ fusion integrated into a chromosome (Fig. 3a). We tested whether ppaA in extra copy affects expression of the PS genes other than puc, i.e. puf and crta (encoding one of the enzymes of carotenoid biosynthesis) and found that it does not (Fig. 3b).

**Relationship between PpaA and known regulators of PS gene expression**

**PpaA and PrrBA system.** The experimental design employed for isolation of the ppaA gene suggested that PpaA acts independently of the PrrBA signal transduction pathway (Fig. 2 and 3a). We proceeded to investigate the relationship between PpaA and other known regulators of PS gene expression in *R. sphaeroides*.

**PpaA and FnrL.** We tested the effect of ppaA in extra copy on puc::lacZ expression in the FnrL null mutant, JZ1678(pCF200Km). ppaA in extra copy activated puc expression in this mutant, suggesting that its effect is independent of FnrL (Fig. 3a).

**PpaA and bacteria chlorophyll biosynthetic pathway.** Bacteriochlorophyll, or an intermediate in the bacteriochlorophyll biosynthetic pathway, influences expression of PS genes in a related bacterium, *Rhodobacter capsulatus* (Rodig et al., 1999; Abada et al., 2002). To investigate whether the effect of PpaA depends on the bacteriochlorophyll biosynthetic pathway in *R. sphaeroides*, we introduced ppaA in extra copy into the BchE null mutant, BCHE, impaired in one of the early steps of bacteriochlorophyll biosynthesis. PpaA increased puc::lacZ expression in mutant BCHE (Fig. 3a). Therefore, neither the presence of bacteriochlorophyll nor an intact bacteriochlorophyll biosynthetic pathway (following the BchE catalysed step) are required for the activity of PpaA.

**PpaA and AppA-PpsR system.** ppaA in extra copy activated puc::lacZ expression in the AppA null mutant, APP11(pCF200Km), suggesting that its effect is independent of the anti-repressor (Fig. 3a).

Does PpaA work through PpsR? Testing for the effect of ppaA in extra copy in the PpsR null mutant, PPS1, would be unfeasible because expression of puc and photopigment biosynthesis genes in this mutant is already derepressed. Furthermore, mutant PPS1 is genetically unstable in the presence of oxygen, apparently because bacteriochlorophyll intermediates produce toxic reactive oxygen species (Gomelsky & Kaplan, 1997). Therefore, we tested for putative PpaA-PpsR relationships using two alternative approaches. (i) We reconstituted the PpsR-mediated repression of puc in a heterologous host. (ii) We tested for direct
interactions between PpaA and PpsR using a yeast two-hybrid system.

(i) Does PpaA affect PpsR-mediated repression of puc expression in a heterologous host? Paracoccus denitrificans, a non-photosynthetic relative of R. sphaeroides, is capable of expressing PS genes when these are introduced in trans (Pemberton & Harding, 1987). P. denitrificans lacks specific regulators of PS gene expression, which makes it a convenient host to examine PS gene regulation (Gomelsky & Kaplan, 1995a, 1997). We introduced into P. denitrificans a puc::lacZ fusion (in plasmid pCF400A) and the second plasmid, carrying ppsR in combination with either intact ppaA (plasmid pSmNs) or inactivated ppaA (plasmid pSmNsXc) (Fig. 1b). Plasmid pSmNsXc differs from pSmNs by a single base pair deletion that results in a frame-shift mutation in the 5′ coding region of ppaA. This mutation, designated ppaA(Xc), completely inactivates ppaA as judged by the inability of ppaA(Xc) in extra copy to affect puc::lacZ expression in R. sphaeroides (data not shown). As anticipated, the level of β-galactosidase in P. denitrificans containing pSmNs was low, 6 ± 15 Miller units, indicative of strong repression by PpsR of puc::lacZ expression. An identical level of expression was observed for plasmid pSmNsXc. Hence, in this experimental design, we could not detect an effect of ppaA on PpsR-mediated repression of puc expression.

We proceeded to test whether PpaA alone can activate puc::lacZ expression in P. denitrificans. To this end, plasmid pSmNo carrying the ppaA gene was introduced into P. denitrificans containing plasmid pCF400A. β-Galactosidase activity in P. denitrificans was equal to 1751 ± 120 Miller units measured as described previously (Gomelsky & Kaplan, 1995a). A similar value, 1689 ± 30 Miller units, was obtained when vector pRK415 was present in place of pSmNo. Therefore, PpaA alone does not affect puc expression in P. denitrificans.

(ii) Do PpaA and PpsR interact? We tested for a possible PpaA-PpsR interaction by using the yeast two-hybrid system. Strain S. cerevisiae L40 expressing PpsR as ‘bait’ and PpaA as ‘prey’ (plasmids pYppsR and pZppaA), remained auxotrophic for histidine, and the β-galactosidase level was unchanged [3.2 U (mg protein)−1] when compared to a negative control, strain L40(pYESTrp2, pZppaA). These data argue against direct protein–protein contacts between PpaA and PpsR.

The data presented above argue against involvement of PpaA in the PpsR-mediated repression. However, they need to be taken with caution because it is possible that a critical cofactor required for PpaA activity was not present in the heterologous host (see Discussion).

**Construction of the ppaA mutants**

The role of PpaA in regulation of PS gene expression was further investigated by constructing ppaA null mutants. Two mutant strains, PPA1 and PPAXc, were constructed. PPA1 contains an ßKm′ cassette that replaced an internal ppaA fragment between the two NcoI sites (Fig. 1c). The PPA1 strain had a phenotype that was very similar to the phenotype of the PpsR mutant, PPS1 (Gomelsky & Kaplan, 1997), i.e. highly pigmented and genetically unstable in the presence of oxygen (data not shown). Penfold & Pemberton (1994) suggested that the ppsR gene promoter is located between the Ncol sites used for ßKm′ cassette insertion. It is likely that an ßKm′ cassette in strain PPA1 has a polar effect on the downstream ppsR gene, thus making PPA1 in effect a double ppaA ppsR mutant. This hypothesis was confirmed by greatly diminished levels of the PpsR protein in the PPA1 mutant (immunoblot data not shown).

To avoid a polar effect on ppsR, a ppaA(Xc) frame-shift mutant allele was introduced into the R. sphaeroides genome replacing the wild-type ppaA gene and yielding strain PPAXc. Because the ppaA(Xc) mutation lies upstream of the

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**Fig. 3.** Effect of ppaA in extra copy on PS gene expression in strains grown at high oxygen tension (20 ml cultures grown in 500 ml flasks at vigorous shaking). Filled bars, pSmNo; clear bars, vector pRK415. (a) puc::lacZ expression. All strains except for PUCZ-WT contained plasmid pCF200Km. (b) put::lacZ (plasmid pUI1830Δ) and crtA::lacZ (plasmid pUI2711) expression. Genotypes of the strains are shown below the charts.
putative promoter/operator region of ppsR, we anticipated that it would have no effect on ppsR gene expression (Fig. 1c). To test this prediction, we quantified the PpsR protein levels in mutant PPAXc by immunoblot with the PpsR-specific antibody. The PpsR levels in strain PPAXc were essentially identical to those in the wild-type, i.e. 81 ± 14 % at high oxygen tension, 106 ± 20 % at low oxygen tension (Fig. 4) and 90 ± 17 % at no oxygen (anaerobic photosynthetic conditions), where 100 % represents a value of the wild-type at a given growth condition.

**Analysis of the ppaA null mutant**

Under the conditions tested, the phenotype of the PPAXc mutant did not differ significantly from that of the wild-type. To evaluate the effect of PpaA inactivation on PS formation at various oxygen tensions, we compared (i) photopigment content and (ii) PS gene expression in the PPAXc mutant and the wild-type.

(i) The photopigment content in PPAXc and the wild-type strain, 2.4.1, grown at low or no oxygen were very similar. However, at high oxygen tension, when photopigments are present only at the background levels, photopigment levels in the PPAXc strain were lower than in the wild-type (Fig. 5a). This suggests that PpaA is required to maintain the low basal level of photopigments at high oxygen tension and is consistent with the stimulatory effect of ppaA in extra copy on photopigment production (Fig. 2a).

(ii) Expression of a subset of the PS genes in the PPAXc mutant and the wild-type was assessed by quantitative RT-PCR (Fig. 6). The lack of PpaA resulted in a moderate, approximately 50 %, reduction in puc expression under conditions of high and low oxygen tension, but no change under anaerobic conditions. This observation is consistent with the activation effect of ppaA in extra copy on puc expression (Fig. 3). Under high oxygen tension, the lack of PpaA also resulted in a moderate decrease in puf expression. Interestingly, expression of bchl was higher in the PPAXc mutant under low oxygen conditions. Expression of crta was not affected by the lack of PpaA. We conclude that PpaA has either no or only a moderate effect on expression of the PS genes tested. It also appears that the effect of PpaA on gene expression and photopigments is evident only in the presence of oxygen.

**ppaA gene expression**

To gain further insight into the physiological role of PpaA, we investigated ppaA gene expression. The expression profiles of the wild-type strain grown under various oxygen tensions were obtained by genome-wide R. sphaeroides DNA microarrays (genechips). ppaA gene expression in the wild-type increased approximately fivefold upon lowering oxygen tension from high to low and increased an additional two-fold upon transition from low to no oxygen (anaerobic photosynthetic conditions) (Fig. 7a). The genechip data were confirmed by using a ppaA::xylE transcriptional fusion (plasmid pLX200, Table 1). Expression of ppaA::xylE in R. sphaeroides was approximately 27-fold higher in cells grown anaerobically compared to cells grown at high oxygen tension (Fig. 7b). The ppaA expression studies indicate that the ppaA gene product is likely to be more abundant under conditions of low or no oxygen than in the presence of high oxygen.

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**Fig. 4.** Quantitative immunoblot analysis of PpsR protein levels. A representative immunoblot derived from cells grown at low oxygen tension is shown. Lanes: 1, the PpsR null mutant, PPS1, (2-4 μg soluble protein loaded); 2, 4, 6, strain 2.4.1 (2-4, 1-2, 0-6 μg soluble protein, respectively); 3, 5, 7, strain PPAXc (2-4, 1-2, 0-6 μg soluble protein, respectively).

**Fig. 5.** Effect of ppaA inactivation on photopigment abundance. Thin line, PPAXc; thick line, 2.4.1. (a) High oxygen tension. (b) Low oxygen tension. The protein content of cells, from which photopigments were extracted, is one half of that presented in panel (a). An absorption peak at 370 nm observed in the PPAXc mutant is probably due to the accumulation of tetrapyrrole precursor(s) in the bacteriochlorophyll biosynthetic pathway.
We investigated the mechanism responsible for increased ppaA gene expression following a decrease in oxygen tension. The intergenic region between ppaA and the divergently transcribed bchF gene contains two putative PpsR binding sites, TGTN12ACA (Fig. 1b). It is conceivable that PpsR represses ppaA gene expression under high oxygen tension. To test for the involvement of PpsR in ppaA gene expression, we introduced ppsR in plasmid pPNs into P. denitrificans carrying ppaA::xylE on plasmid pLX200. Expression of ppaA::xylE in the presence of plasmid pPNs decreased to approximately 2.5% of that in the presence of vector pRK415. This establishes that PpsR is directly involved in repression of ppaA gene expression (Fig. 7c).

Interestingly, the genechip data suggest that expression of the ppsR gene is independent of oxygen tension (Fig. 7a). This correlates well with the ppsR::lacZ data presented by us previously (Gomelsky & Kaplan, 1998). Therefore, ppaA and ppsR appear have independent promoters and the promoter upstream of ppaA does not contribute significantly to expression of the downstream ppsR gene.

The PpaA protein family

Corrected sequence of the PpaA homologue from R. sphaeroides strain RS630. The locus upstream of ppsR had previously been identified by Penfold & Pemberton (1994) in R. sphaeroides strain RS630 and designated ppsS (GenBank accession no. L19596). The N-terminal sequence corresponding to PpsS from strain RS630 differed significantly from that of PpaA from strain 2.4.1. It was surprising that two strains of R. sphaeroides would possess such different proteins. We resequenced DNA upstream of ppsR in strain RS630. The newly obtained DNA sequence from strain RS630 (accession no. L19596) is 99.5% identical to the sequence of ppaA from R. sphaeroides strain 2.4.1.
2.4.1 resulting in a 100% identical PpaA protein. Apparently an error in subcloning was involved when initial sequencing was done.

**PpaA homologues.** To gain an insight into the potential function of the PpaA protein, we analysed sequences of PpaA homologues from several species of anoxygenic phototrophic proteobacteria. All species of this group, whose PS gene cluster sequences are available, contain homologues of the ppaA gene. The PpaA homologues share 26–40% identity to each other (Fig. 8). This is consistent with a conserved role for PpaA in PS formation.

**A putative corrinoid-binding domain.** The central region of *R. sphaeroides* PpaA, residues 66–238, shows significant similarity to the central domains of the two ORFs from *Myxococcus xanthus*, ORF11 and ORF10 (Fig. 8). The PpaA sequence was used as a query in PSI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/index.html; Altschul et al., 1997). After four iterations, significant similarities (scores > 40, E-values < 1 x e^-4) were revealed to proteins containing corrinoid cofactors, e.g. B12-dependent isoflavone synthase from various micro-organisms, including *E. coli*, and mammals, di- and trimethylamine corrinoid proteins from *Methanosarcina* species, and demethylase corrinoid protein from *Acetobacterium dehalogenans* (Fig. 8). Furthermore, a B12-binding domain, pfam02310, was identified (score 34-8, E-value 0-003) using the SMART search engine (http://smart.embl-heidelberg.de; Schultz et al., 2000).

The conserved sequence pattern, DxHxxG(41)xSxL(26-30)xGG, where x is an amino acid, has been found in a large subset of B12-binding enzymes (reviewed by Ludwig & Jarrett, 1997). Where x is an amino acid, has been found in a large subset of B12-binding enzymes (reviewed by Ludwig & Jarrett, 1997). Three residues, D, H and S, form the so-called ligand triad, with H directly co-ordinating cobalt (Fig. 8). With the exception of L, most of the residues involved in corrinoid binding (Drennan et al., 1994; Stubbe, 1994) are either present in the PpaA homologues and ORF10 and ORF11, or substituted for similar residues (Fig. 8). The presence of E in place of the conserved D in the putative corrinoid-binding pockets of the PpaA homologues is noteworthy. The studies by Amarantunga et al. (1996) and Jarrett et al. (1996) showed that substitution of D for E in the B12 binding pocket of the *E. coli* methionine synthase MetH does not abolish B12 binding. Therefore, sequence analysis strongly suggests that the PpaA proteins bind a corrinoid cofactor(s). The implications of this finding are discussed below.

**DISCUSSION**

Production of the PS in *R. sphaeroides* is controlled by a number of regulatory circuits that respond to changes in oxygen availability and under conditions of low or no oxygen, the intensity and spectrum of light. In this work, a new regulator encoded by the *ppaA* gene was isolated based on its ability in extra copy to activate photopigment production and *puc* expression under aerobic conditions. A non-polar mutation in *ppaA* resulted in somewhat decreased pigment production and lower *puc* expression under aerobic conditions, as expected. The *ppaA* gene product apparently functions independently of the PrrBA-, FnrL- and AppA-PspR regulatory pathways and its effect is moderate compared to these major regulators. Expression of the *ppaA* gene is repressed under high oxygen by the PpsR repressor, but it increases as oxygen tension drops and reaches the highest value under anaerobic conditions.

In trying to understand the function of the PpaA protein we encountered the following paradoxes: (i) *ppaA* expression is highest under anaerobic conditions, therefore one would anticipate that the *ppaA* gene product is active under these conditions. However, no distinct phenotype related to PS formation of the *ppaA* mutant was observed under the anaerobic conditions tested. Only in the presence of oxygen was the phenotype of the *ppaA* null mutant expressed. (ii) The *R. sphaeroides* PpaA protein acts as an apparent activator of *puc* expression and photopigment production under aerobic conditions. In contrast, the PpaA homologue from *R. capsulatus*, AerR (ORF192), was recently reported to function as an aerobic repressor of a subset of PS genes (Dong et al., 2002). In a search for clues regarding PpaA function, we turned to the protein sequence analysis. Based on the alignment of several PpaA homologues, we found that PpaA possibly contains a corrinoid-binding domain. This means that the activity of PpaA may depend on the availability, structure or redox status of the bound corrinoid cofactor. There is as yet no direct evidence of corrinoid binding by PpaA; however, a substitution of the conserved H-residue, a putative co-ordinating ligand for cobalt (Fig. 8), inactivated PpaA function (unpublished data).

What are the implications of possible corrinoid binding to PpaA? One possibility is that PpaA is an enzyme, or a subunit of a multisubunit enzymic complex. Some bacteriochlorophyll biosynthetic reactions are corrinoid-dependent (Gough et al., 2000), therefore PpaA may be involved in bacteriochlorophyll biosynthesis. However, the *ppaA* null mutant shows only minor impairment in bacteriochlorophyll biosynthesis and only under aerobic conditions (Fig. 5). Furthermore, the *ppaA* gene in extra copy activated PS gene expression independently of the bacteriochlorophyll pathway in the BCHE mutant (Fig. 3). These observations make PpaA an unlikely candidate for a bacteriochlorophyll biosynthetic enzyme.

An alternative possibility is that PpaA is a bona fide regulator of gene expression whose activity depends on a corrinoid cofactor. This would suggest that PpaA is involved in co-ordination of PS gene expression with the availability or status of corrinoid. Because vitamin B12 is required for bacteriochlorophyll biosynthesis, a co-ordination of PS gene expression with availability of B12 would seem reasonable. Interestingly, evidence from *R. capsulatus* suggests that
intact $\text{B}_{12}$ biosynthetic pathway is needed for correct regulation of PS gene expression (Pollich et al., 1993; Pollich & Klug, 1995; Rödig et al., 1999).

To our knowledge, no precedent of a corrinoid-binding sensory protein has yet been documented, whereas other cofactors, e.g. haems, flavins, iron–sulfur clusters, have been
shown to perform sensory functions, in addition to catalytic functions (Beinert & Kiley, 1999; Christie & Briggs, 2001; Gilles-Gonzalez, 2001; Braatse et al., 2002; Masuda & Bauer, 2002; Gomelsky & Klug, 2002). Two groups hypothesized that corrinoids might regulate activities of transcription factors (Roof & Roth, 1992; Sheppard & Roth, 1994; Cervantes & Murillo, 2002). The latter report is especially relevant because it considers a possibility that DNA-binding activity of M. xanthus ORF10 (CarA) might be corrinoid-dependent. The central region of ORF10 shares significant similarity with the PpaA proteins (Fig. 8). ORF10 is a transcriptional regulator, whose DNA-binding motif resembles a well characterized, MerR-like, helix–turn–helix domain (Cervantes & Murillo, 2002). In contrast to ORF10, no DNA-binding motif could be identified in PpaA or its homologues. However, the R. capsulatus AerR protein was shown to bind to DNA in vitro when present at high concentrations and to assist in DNA bending. It is worth mentioning that the predicted high pl values of the PpaA homologues might contribute to the relatively non-specific binding to negatively charged DNA. If DNA binding by the PpaA homologues is proven to be physiologically meaningful, it would strengthen the hypothesis that these proteins are corrinoid-dependent transcription regulators. It is possible that the differences in the original observations and interpretations of functions of the R. sphaeroides PpaA and R. capsulatus AerR proteins will turn out to be minor when a deeper understanding of the functions of these proteins is achieved.

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