

# Redox property and regulation of PpsR, a transcriptional repressor of photosystem gene expression in *Rhodobacter sphaeroides*

Seung-Hyun Cho,<sup>2</sup> Sang-Hee Youn,<sup>1</sup> Seung-Rock Lee,<sup>3</sup> Hyung-Soon Yim<sup>2</sup> and Sa-Ouk Kang<sup>1,2</sup>

Correspondence  
Sa-Ouk Kang  
kangsaou@snu.ac.kr

<sup>1,2</sup>Laboratory of Biophysics, School of Biological Sciences<sup>1</sup> and Institute of Microbiology<sup>2</sup>, Seoul National University, Seoul 151-742, Republic of Korea

<sup>3</sup>Center for Cell Signalling Research, Division of Molecular Life Sciences, Ewha Women's University, Seoul 120-750, Korea

PpsR from *Rhodobacter sphaeroides* is involved in the repression of photosystem gene expression. The PpsR protein was heterologously overexpressed and purified to homogeneity. Gel mobility shift assay showed that the purified PpsR has DNA-binding activity. SDS-PAGE analysis showed that some portions of PpsR were oxidized, indicating that intramolecular or intermolecular disulphide bonds were formed between the two cysteines in each subunit. When the disulphide bond of PpsR was reduced by DTT, the binding activity of PpsR to the *puc* promoter region distinctly increased. The changes in protein level and DNA-binding activity of PpsR were observed in a conjugant with an extra copy of the *ppsR* gene and in a PpsR-null mutant (PPS1), respectively. Both cysteines in PpsR existed in their reduced form under aerobic, anaerobic-dark and anaerobic-light growth conditions, as determined using thiol-specific chemical modification. In an AppA-null mutant (APP11), the binding activity and the amount of PpsR decreased compared to those of the wild-type and an *appA*-complemented strain, and decreased even more under anaerobic-dark conditions than under aerobic conditions. PpsR had a redox-sensitive property but retained its reduced state in the cell, and its amount was reduced by disruption of AppA.

Received 19 September 2003  
Revised 21 November 2003  
Accepted 24 November 2003

## INTRODUCTION

*Rhodobacter sphaeroides* is a photosynthetic bacterium which is remarkably versatile in its growth modes. It can grow by aerobic respiration, but a reduction in oxygen tension induces differentiation of the inner membrane, leading to the formation of the intracytoplasmic membrane system for the use of light as an energy source. A reduction in light intensity similarly increases the cellular abundance of the photosynthetic apparatus. Therefore, oxygen tension and light intensity were thought to be important factors regulating synthesis and assembly of photosynthetic apparatus (Cohen-Bazire *et al.*, 1957; Aagaard & Siström, 1972). These effects of environmental factors have been shown to be the results of the regulation of photosystem gene transcription, mRNA decay, and the post-translational level of the proteins involved (Gregor & Klug, 1999). Photosystem genes include structural genes (*puf*, *puh* and *puc*) of the light-harvesting system I (LH I) and II (LH II) and reaction centre, and photopigment genes (*bch* and *crt*).

A number of transcriptional regulators of photosystem

gene expression have been identified (Gregor & Klug, 1999). Among them, PpsR was identified as a repressor of photosynthetic synthesis in *R. sphaeroides* (Penfold & Pemberton, 1994; Gomelsky & Kaplan, 1995a). It contains a helix–turn–helix motif at the carboxy terminus and two Per–Arnt–Sim (PAS) domains in its central region, which serve to bind redox cofactors or are involved in oligomerization (Penfold & Pemberton, 1994; Gomelsky *et al.*, 2000; Taylor & Zhulin, 1999). The PpsR homologue from *Rhodobacter capsulatus*, CrtJ (75% homology), was reported to bind to the *bchC* promoter region in a redox-sensitive manner (Ponnampalam & Bauer, 1997). Recently, it was reported that CrtJ could form an intramolecular disulphide bond in aerobically grown cells but not in anaerobically grown cells, and the binding activity to the *bchC* promoter increased fivefold in the presence of a disulphide bond (Masuda *et al.*, 2002). Subsequently, Masuda & Bauer (2002) reported that PpsR also had the same redox property as CrtJ, except that the binding activity to the *puc* promoter increased 2.2-fold in the presence of a disulphide bond.

AppA was identified as a critical component required for the activation of photosystem gene expression in *R. sphaeroides* (Gomelsky & Kaplan, 1995b). A molecular-genetic

Abbreviations: GMSA, gel mobility shift assay; NEM, *N*-ethylmaleimide.

analysis suggested that it would interact with PpsR to regulate photosystem gene expression (Gomelsky & Kaplan, 1997). Recently, it was verified that the FAD cofactor of AppA was essential for the blue-light-dependent sensory transduction of photosystem gene expression (Braatsch *et al.*, 2002). AppA was reported to be able to break the disulphide bond in the oxidized PpsR as well as to form a stable AppA–PpsR<sub>2</sub> complex, but blue light inhibited formation of the AppA–PpsR<sub>2</sub> antirepressor complex (Masuda & Bauer, 2002).

In the present study, we observed that the binding activity of PpsR to the *puc* promoter region was increased by the reduction of the disulphide bond in PpsR, and the mobility of the oxidized (disulphide-bonded) and the reduced PpsR could be distinguished on SDS-PAGE. Furthermore, thiol-specific chemical modification verified that the two cysteine residues in PpsR remain in their reduced form *in vivo* in spite of the presence of oxygen, which is at variance with the report that the disulphide bond of the oxidized PpsR can be formed in the presence of oxygen (Masuda & Bauer, 2002). The fact that the protein expression level and the binding activity of PpsR in APP11 decrease can be explained by the combination of some recent reports.

## METHODS

**Bacterial strains and growth conditions.** *R. sphaeroides* 2.4.1 was kindly donated by J. K. Lee (Sogang University, Korea).

*Escherichia coli* and *R. sphaeroides* strains used in this study are listed in Table 1. *E. coli* strains were grown at 37 °C on Luria–Bertani (LB) medium supplemented, when required, with 10 µg tetracycline ml<sup>-1</sup> or 50 µg ampicillin ml<sup>-1</sup> (final concentrations). *R. sphaeroides* strains were grown at 30 °C on Sistrom's medium A (Cohen-Bazire *et al.*, 1957) containing succinic acid as a carbon source. Anaerobic growth in the dark was maintained in Sistrom's medium A containing 20% (w/v) LB medium and 80 mM dimethyl sulphoxide. Tetracycline (1 µg ml<sup>-1</sup>), kanamycin (50 µg ml<sup>-1</sup>) or trimethoprim (50 µg ml<sup>-1</sup>) was used when required.

### Genetic manipulation methods, conjugation techniques.

General techniques for the isolation and the manipulation of DNA in *R. sphaeroides* or *E. coli* were as described by Sambrook *et al.* (1989). Molecular biological enzymes and reagents used were of the highest quality generally available. All cloned DNA amplified by PCR was sequenced with an automatic sequencer (ALFexpress, Pharmacia). Plasmids used in this study are listed in Table 1. A broad-host-range vector, pRK415, was used for introducing appropriate genes into *R. sphaeroides* (Keen *et al.*, 1988); pGEM-T easy vector (Promega) was used for the cloning of PCR products, and pET15b vector (Novagen) for overexpression. Conjugation was performed according to the methods proposed by Davis *et al.* (1988). To introduce the pRK415 derivative vectors into *R. sphaeroides*, biparental matings with *E. coli* S17-1 as a donor were used (Simon *et al.*, 1983).

**Purification of PpsR from *E. coli*.** Forward and reverse primers, 5'-AGAAGGAAGACATATGCTGGCCGG-3' and 5'-GTCAAAGC-GACCATGGATCCGCG-3', containing *Nde*I and *Bam*HI sites (underlined), respectively, were used to amplify the *ppsR* coding sequence. The PCR product was cloned into pGEM-T easy vector

**Table 1.** Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference*
<b><i>E. coli</i></b>		
DH5α	F <sup>-</sup> Δ <i>lacU169</i> (φ80 <i>lacZ</i> ΔM15) <i>endA1 rec1 hsdR17 deoR supE44 thi-1 λ<sup>-</sup> gyrA96 relA1</i>	Hanahan (1983)
BL21(DE3)pLysS	F <sup>-</sup> <i>ompT r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup></i> (DE)/pLysS	Novagen
S17-1	Conjugal donor; C600::RP4 2-(Tc::Mu) (Km::Tn7) <i>pro res mod</i> <sup>+</sup> (Tp <sup>r</sup> Sm <sup>r</sup> )	Simon <i>et al.</i> (1983)
<b><i>R. sphaeroides</i></b>		
2.4.1	Wild-type	J. K. Lee
PPS1	2.4.1 <i>ppsR</i> ::ΩKm <sup>r</sup>	S. Kaplan
APP11	2.4.1 Δ <i>appA</i> ::Tp <sup>r</sup>	S. Kaplan
<b>Plasmids</b>		
pGEM-T easy	PCR cloning vector; Ap <sup>r</sup>	Promega
pET15b	N-terminal His-tag expression vector; Ap <sup>r</sup>	Novagen
pRK415	Broad-host-range plasmid; Mob <sup>+</sup> Tc <sup>r</sup>	Keen <i>et al.</i> (1988)
pPPS35	LITMUS28 digested with <i>Nco</i> I– <i>Pst</i> I with the insert of the 1.6 kb <i>Nco</i> I– <i>Nsi</i> I fragment of <i>R. sphaeroides</i> DNA	S. Kaplan
P484Nco50	pUC19 digested with <i>Sph</i> I– <i>Sal</i> I (this site made blunt) with the insert of the 2.6 kb <i>Sph</i> I– <i>Nco</i> I (this site made blunt) fragment of <i>R. sphaeroides</i> DNA	S. Kaplan
pGPUC	pGEM-T easy vector containing the promoter region of <i>puc</i> generated by PCR	This work
pGPSR	pGEM-T easy containing the putative promoter region of <i>ppsR</i> amplified and engineered by PCR	This work
pPPSR35	pPPS35 ( <i>Aat</i> II, <i>Pst</i> I) + 0.5 kb <i>Aat</i> II– <i>Pst</i> I fragment of pGPSR	This work
pET15b::ppsR	His-PpsR expression plasmid; entire <i>ppsR</i> cloned into <i>Nde</i> I/ <i>Bam</i> HI-digested pET15b	This work
pRK415::ppsR	pRK415 ( <i>Hind</i> III, <i>Pst</i> I) + 2 kb <i>Hind</i> III– <i>Nsi</i> I fragment of pPPSR35	This work
pRK415::appA	pRK415 ( <i>Hind</i> III, <i>Sac</i> I) + 2.6 kb <i>Hind</i> III– <i>Sac</i> I fragment of p484Nco50	This work

\*J. K. Lee, Sogang University, Korea; S. Kaplan, University of Texas at Houston, USA.

and subsequently introduced into pET15b between the *Nde*I and *Bam*HI sites to generate pET15b::ppsR. The plasmid was transformed into *E. coli* strain BL21(DE3)pLysE. The His-tagged PpsR protein was overexpressed in LB by induction with 1 mM IPTG at 37 °C for 2 h. Cells were harvested, and the His-tagged PpsR was purified using a Ni<sup>2+</sup>-nitrilotriacetic acid (NTA) Sepharose column according to the guide proposed by the manufacturer (Novagen). The His-tag of PpsR was removed by human thrombin and the detached protein was repurified using a Ni<sup>2+</sup>-NTA Sepharose column and stored at -70 °C for further experiments. The N-terminal amino acid sequence of the purified protein was confirmed with a Procise Protein Sequencing System (Applied Biosystems). During the purification, proteins were analysed by SDS-PAGE according to Laemmli (1970).

**Subcloning of ppsR and appA genes into pRK415.** Forward and reverse primers, 5'-CAGGCGAAGCTTCGCGACATCGCCACCT-3' (*Hind*III site underlined) and 5'-CGCAGGCCTGCAGCGCCG-CCTCAAT-3', were used to amplify the putative promoter region of ppsR from genomic DNA of *R. sphaeroides*. The amplified DNA was inserted into the pGEM-T easy vector, and the product was designated pGPSR. The insert fragment digested with *Aat*II and *Pst*I was ligated with the vector fragment of pPPS35 (~1.6 kb fragment of ppsR) digested with *Aat*II and *Pst*I, and the product was designated pPSR35. The last step was the ligation of the insert of pPSR35 digested with *Hind*III and *Nsi*I and the vector of pRK415 digested with *Hind*III and *Pst*I. This plasmid was designated as pRK415::ppsR. p484Nco50 containing a ~2.7 kb *Nco*I fragment of appA was digested with *Hind*III and *Sac*I, and the insert fragment was ligated with pRK415 also digested with *Hind*III and *Sac*I. This plasmid was designated as pRK415::appA.

**Antibody preparation.** Purified PpsR was subjected to SDS-PAGE, and the protein band was cut from the gel and homogenized. This preparation was used to immunize mice. Mice were initially immunized subcutaneously with 5 µg of the protein. Two booster injections were given at 2-week intervals and bleeding was done 3–5 days after last injection. The blood was agglutinated for 1 h at 37 °C and centrifuged at 14 000 g for 15 min. The supernatant, mouse antiserum to PpsR, was obtained and stored at -70 °C.

**Analyses of protein from *R. sphaeroides*.** Cells were harvested by centrifugation at 14 000 g for 10 min and washed with 20 mM Tris/HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM DTT and 10% (v/v) glycerol. After resuspension in the same buffer, cells were disrupted by sonication with a Microson XL-2000 (Heat System Ultrasonics). The supernatant obtained after centrifugation at 14 000 g for 20 min was used as crude extract. The protein concentration was determined by the Lowry method, with bovine serum albumin as a standard. The expression level of PpsR in crude extract was analysed by Western blotting, using mouse antiserum against PpsR as a primary antibody and alkaline-phosphatase-linked sheep anti-mouse IgG as a secondary antibody. For the colorimetric detection of alkaline phosphatase, *p*-nitro blue tetrazolium chloride and 5-bromo-4-chloro-indol-3-ol dihydrogen phosphate (ester) mono-*p*-toluidinium salt were used as substrates.

**Gel mobility shift assay (GMSA).** To prepare the *puc* promoter region, PCR was carried out using the forward primer 5'-TTTT-TGCAGCAGCGAGAGGCTG-3' and the reverse primer 5'-AAAT-CGACGGTTTGCCTGTAGG-3'. The amplified DNA fragment was cloned into pGEM-T easy vector and the construct was designated pGPUC. This plasmid was digested with *Eco*RI and the insert fragment was gel-purified. About 200 ng of purified fragment was end-labelled with Klenow fragment using digoxigenin (DIG)-dUTP (Roche) and dATP as substrates for 30 min at 37 °C. The labelled probes were clarified by ethanol precipitation and stored at -20 °C for further experiments.

GMSA was performed under the following conditions: 4–40 fmol probe DNA was incubated with either purified PpsR (0.2 ng–2 µg) or crude extract (2–10 µg) at 30 °C in 15 µl binding buffer containing 50 mM Tris/HCl (pH 7.0), 1 mM EDTA, 150 mM NaCl, 1–5 µg calf thymus DNA or 1 µg poly(dI-dC) nonspecific competitor DNA, and 10% (v/v) glycerol. After 30 min incubation, the reaction mixture was loaded onto a 4.5% (w/v) non-denaturing Tris/acetate/EDTA-buffered polyacrylamide gel and electrophoresed at room temperature.

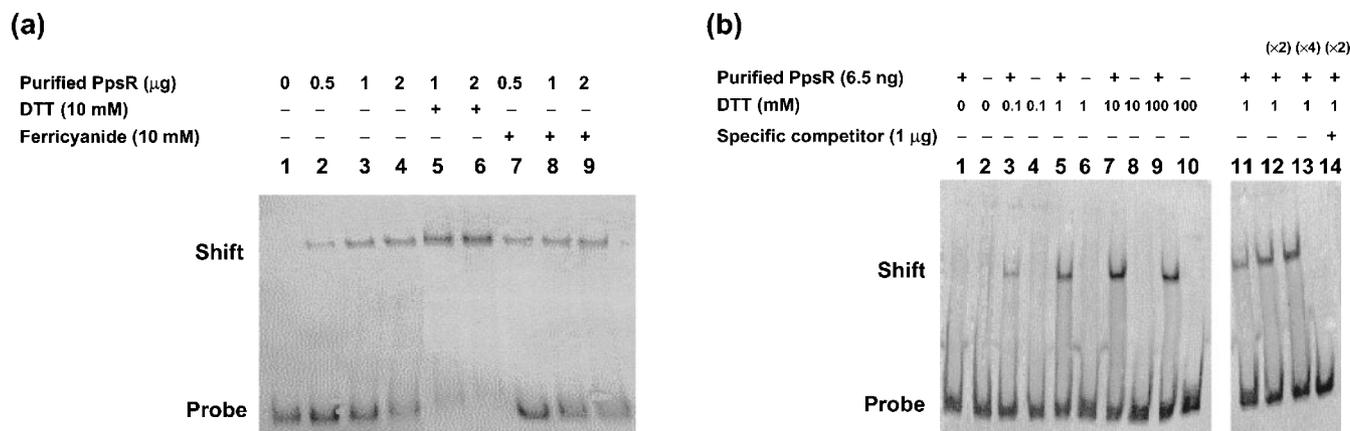
**Measurement of *in vivo* redox states of PpsR.** The redox states of the two cysteines in PpsR in living cells were estimated by the method of thiol-specific chemical modification following acidic precipitation of protein. The cultured cells were treated with 10% trichloroacetic acid to avoid thiol oxidation and then sonicated. The precipitate was washed several times with acetone. The pellet was dissolved in 100 mM Tris/HCl (pH 8.0), 1 mM EDTA and 0.1% (w/v) SDS, and divided into three aliquots. The first one was treated with a final concentration of 70 mM iodoacetamide. The second one was first reduced by a final concentration of 10 mM DTT and then alkylated with iodoacetamide. The third one, with no modification, was used as a reference. These samples were loaded onto the SDS-PAGE gel without β-mercaptoethanol in the sample buffer and analysed by Western blotting. In some experiments, *N*-ethylmaleimide (NEM) was replaced by iodoacetamide at pH 6.8.

## RESULTS

### Analyses of purified PpsR under reduced or oxidized conditions

PpsR was heterologously overexpressed and purified to homogeneity. To estimate whether the purified PpsR could bind to the promoter region of *puc* (light harvesting II structural gene), one of the binding sites, GMSA was performed, as shown in Fig. 1(a). The DNA fragment used in GMSA was the 304 bp *puc* promoter region that contains two PpsR-binding sites. A shifted band with a slow electrophoretic mobility was formed when a DNA fragment containing the *puc* promoter region was incubated with the purified PpsR. As the concentration of the purified PpsR increased, so did the amount of the PpsR-*puc* promoter complex (lanes 1–4). These results indicate that the heterologously expressed PpsR is isolated in a properly folded form and the purified PpsR does bind to the *puc* promoter region.

It was reported that a homologue of PpsR, CrtJ, in *R. capsulatus* was redox-sensitively bound to the *bchC* promoter region, exhibiting a 4.5-fold higher binding activity under oxidizing conditions (20 mM potassium ferricyanide) than under reducing conditions (10 mM sodium dithionite) (Ponnampalam & Bauer, 1997). Therefore, we investigated whether the binding of PpsR to the *puc* promoter region is redox-sensitive. As shown by the GMSA in Fig. 1(a), preincubation of PpsR with DTT (lanes 5–6) enhanced the intensity of the shifted bands but preincubation with ferricyanide did not (lanes 7–9). To confirm the effect of DTT on PpsR, GMSA was carried out with increasing concentrations of DTT. As shown in Fig. 1(b), the intensity of the shifted band increased (lanes 3, 5, 7 and 9) with the increase of DTT concentration. Lanes 11–14 in Fig. 1(b)

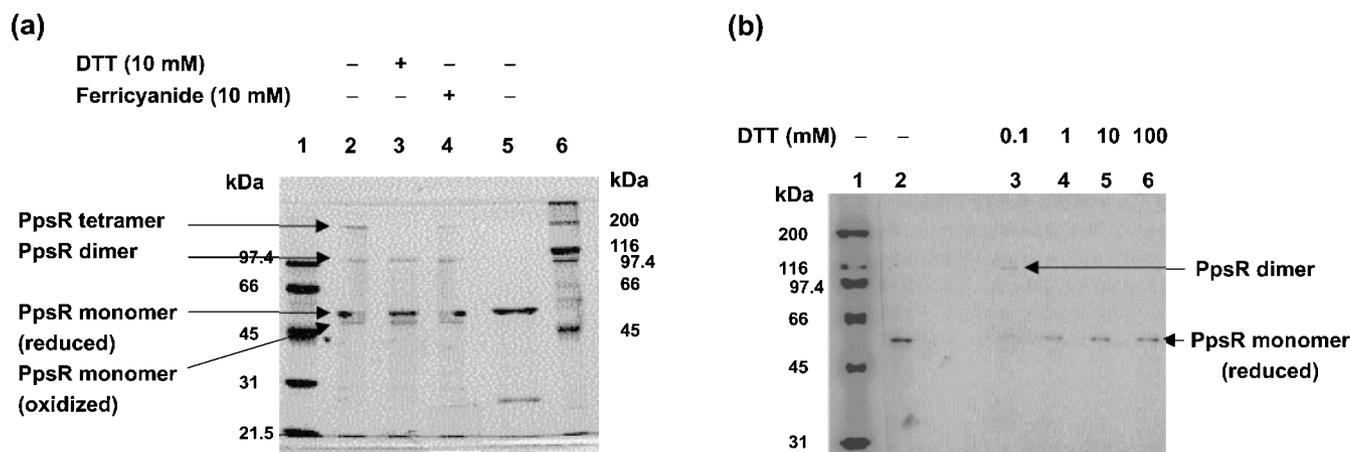


**Fig. 1.** (a) EMSA under various redox conditions. A DIG-labelled probe of the *puc* promoter region was incubated with increasing amounts (0, 0.5, 1 and 2  $\mu\text{g}$ ) of purified PpsR. Lanes: 1–4, no treatment; 5–6, incubation with 10 mM DTT for 1 h; 7–9, incubation with 10 mM potassium ferricyanide for 1 h. (b) EMSA under different DTT concentrations. A DIG-labelled probe of the *puc* promoter region was incubated with purified PpsR at various DTT concentrations. Lanes: 1, 3, 5, 7 and 9, incubation with various DTT concentrations; 2, 4, 6, 8 and 10, no PpsR; 11–14, ( $\times 2$ ) and ( $\times 4$ ) denote two- and fourfold quantity of PpsR, respectively.

show the increase of band shift caused by the addition of PpsR and the disappearance of band shift caused by an excess of unlabelled specific competitor DNA.

To observe the effect of redox reagents on purified PpsR, samples incubated under various redox conditions were subjected to SDS-PAGE using non-reducing SDS-sample buffer. As shown in Fig. 2(a), denatured PpsR existed in several oligomeric forms built of disulphide bonds: tetramer

with intermolecular disulphide bonds, dimer with intermolecular disulphide bond, and monomer with or without intramolecular disulphide bond (lane 2). DTT treatment resulted in an increase of reduced monomer and disappearance of tetramer as shown in lane 3. However, the oligomeric state of PpsR was not changed by the reaction of ferricyanide, as shown in lane 4. Due to the diffusion of  $\beta$ -mercaptoethanol contained in the SDS-sample buffer, the additional reduced monomer of PpsR appeared at the



**Fig. 2.** (a) SDS-PAGE under various redox conditions. Lanes: 1 and 6, molecular mass markers; 2 and 5, no treatment; 3, incubation with 10 mM DTT for 1 h; 4, incubation with 10 mM potassium ferricyanide for 1 h. In lanes 3 and 4, dialysis was carried out overnight after chemical treatment at 4 °C; in lanes 2–4, non-reducing SDS-sample buffer was used; in lanes 1, 5 and 6, reducing SDS-sample buffer containing  $\beta$ -mercaptoethanol was used. (b) SDS-PAGE under different DTT concentrations. Lanes: 1, molecular mass markers; 2, purified PpsR; 3–6, purified PpsR incubated with various DTT concentrations. In lanes 3–6, non-reducing SDS-sample buffer was used; in lanes 1 and 2, reducing SDS-sample buffer containing  $\beta$ -mercaptoethanol was used.

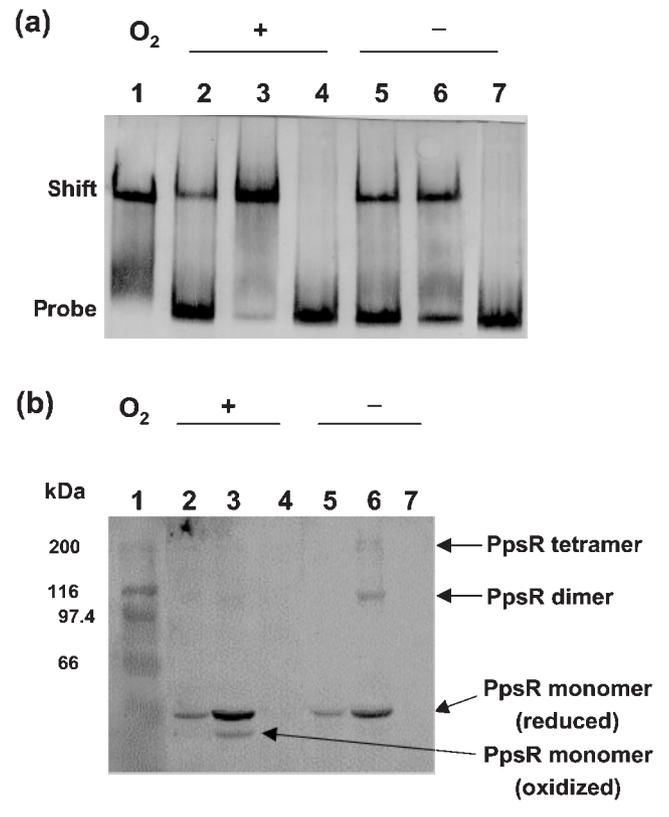
edges of lanes 2 and 4 in Fig. 2(a). As shown in lanes 1–4 of Fig. 2(b), the reduced monomer of PpsR also increased with increasing DTT concentration. There are only two cysteines in PpsR, so these two amino acid residues must be involved in the intermolecular or intramolecular disulphide bond formation although they are separated by 173 amino acid residues in the primary amino acid sequence. These two residues were reported to be well conserved and to form a disulphide bond in CrtJ in the presence of oxygen (Masuda *et al.*, 2002). From these results, we conclude that the two cysteines in the purified PpsR are a mixture of thiol forms and residues involved in intramolecular or intermolecular disulphide bonds, and can bind to the *puc* promoter region when each thiol group in its two cysteines is in the reduced state.

### Characterization of PpsR in *R. sphaeroides*

To monitor the activity of PpsR in a PpsR-null mutant (PPS1) and a conjugant with pRK415::*ppsR*, GMSA and Western blotting were carried out. As shown in the GMSA of Fig. 3(a), the shifted bands were present in the conjugants with pRK415 (lanes 2 and 5) and pRK415::*ppsR* (lanes 3 and 6) at the same position as the band formed by purified PpsR in lane 1, but no shifted bands were observed in PPS1 (lanes 4 and 7). In addition, probe DNA was shifted by incubating with crude extract prepared from anaerobic-dark conditions (lanes 5 and 6). A greater quantity of probe was shifted by incubating crude extracts of the conjugant with pRK415::*ppsR* than that of the conjugant with pRK415 (lanes 2, 3, 5 and 6). These results indicate that it is actively working PpsR in crude extract that shifts the *puc* promoter region in GMSA. The Western blot (Fig. 3b) showed the presence of PpsR in the conjugant with pRK415::*ppsR* (lanes 3 and 6) and controls (lanes 2 and 5), but not in PPS1 (lanes 4 and 7). These results are in good agreement with those of GMSA. As for the purified PpsR, which is heterologously expressed, the oxidized monomer and oligomers of PpsR were also detected in crude extracts of *R. sphaeroides* as shown in Fig. 3(b). The difference in PpsR level between aerobically and anaerobically grown cells conjugated with pRK415::*ppsR* containing the PpsR gene under control of the *lac* promoter might result from increased induction of *lac* promoter in the presence of oxygen.

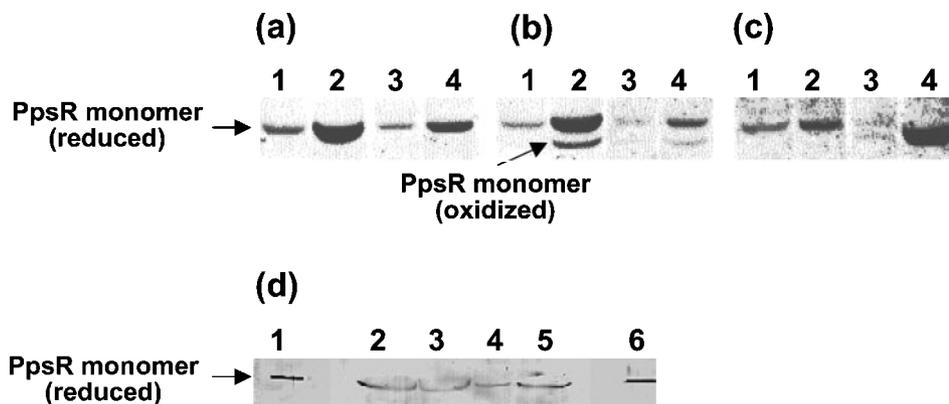
### Determination of *in vivo* redox states of the two cysteines of PpsR in *R. sphaeroides*

We recognized that the formation of intramolecular and intermolecular disulphide bonds in PpsR was correlated with the binding activity of PpsR to the *puc* promoter region. Therefore, to observe whether the growth conditions influence the formation of intramolecular and intermolecular disulphide bonds, we determined the *in vivo* redox states of the two cysteines of PpsR in *R. sphaeroides* by thiol-specific chemical modification with iodoacetamide as an alkylating agent, as shown in Fig. 4(a). PpsR containing a disulphide bond was not observed either in



**Fig. 3.** Analyses of PpsR null mutant (PPS1), wild-type conjugated with pRK415 and conjugant with pRK415::*ppsR*. (a) GMSA of crude extracts (10 µg) of the above strains with the *puc* promoter region. Lanes: 1, purified PpsR (35 ng); 2 and 5, wild-type(pRK415); 3 and 6, wild-type(pRK415::*ppsR*); 4 and 7, PPS1. (b) Western blot (33.3 µg protein) of crude extracts with anti-PpsR antibody. Lanes: 1, molecular mass markers; 2 and 5, wild-type(pRK415); 3 and 6, wild-type(pRK415::*ppsR*); 4 and 7, PPS1. In lane 1, reducing SDS-sample buffer containing  $\beta$ -mercaptoethanol was used; in lanes 2–7, non-reducing SDS-sample buffer was used. O<sub>2</sub>+ and - denote aerobic and anaerobic-dark growth conditions, respectively.

aerobically (lanes 1 and 2) or in anaerobically grown cells (lanes 3 and 4), as for the DTT-treated aliquots in Fig. 4(c), but PpsR containing an intramolecular disulphide bond was formed in the preparation with no modification (Fig. 4b). As shown in Fig. 4(d), when NEM was used in place of iodoacetamide to avoid deprotonation of thiol groups at high pH, the same results were obtained. Even in H<sub>2</sub>O<sub>2</sub>-treated cells, PpsR containing a disulphide bond was not found (lane 2 of Fig. 4d). These results indicate that each thiol group of the two cysteines in PpsR exists in a reduced state irrespective of the oxygen and light; a disulphide bond may be formed by oxygen or its related reactive species during the processes of cell disruption, storage of crude extracts, etc. The results in Fig. 4(a–c) suggest that PpsR levels seem to be regulated by oxygen in wild-type cells. But our many other experiments, including the data in Figs 3(b), 4(d) and Fig. 5(b), showed that PpsR levels in



**Fig. 4.** Western blot with anti-PpsR antibody for estimation of *in vivo* redox states of the two cysteines in PpsR. (a) Crude extracts treated with iodoacetamide. (b) No treatment. (c) As a control, crude extracts were reduced with DTT before treatment with iodoacetamide. Lanes: 1 and 2, conjugant with pRK415 and pRK415::p*psR* grown aerobically, respectively; 3 and 4, respectively, the above conjugants grown dark-anaerobically. In all lanes, non-reducing SDS-sample buffer was used. (d) In place of iodoacetamide, NEM was used as alkylating agent. Lanes: 1 and 6, purified PpsR; 2–5, NEM-treated crude extracts of wild-type grown aerobically, aerobically, photosynthetically, and dark-anaerobically, respectively. In lanes 1 and 6, reducing SDS-sample buffer containing  $\beta$ -mercaptoethanol was used; in lanes 2–5, non-reducing SDS-sample buffer was used; in lane 2, cells were treated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 5 min before harvesting.

wild-type were similar to each other irrespective of oxygen and light.

### PpsR in an AppA-null background

The AppA-null mutant (APP11) was impaired in its transition from aerobic to photosynthetic growth because of defects in the production of the photosynthetic apparatus (Gomelsky & Kaplan, 1995b). To investigate whether this phenomenon occurred through PpsR as proposed by Gomelsky & Kaplan (1997), APP11 was analysed using GMSA and Western blotting with anti-PpsR antibody. We expected that the binding activity and the protein level of PpsR would be raised by the disruption of AppA since it was proposed as an antirepressor of PpsR as described above. However, the results (Fig. 5a) showed that the binding activity of PpsR decreased more in APP11 (lanes 3 and 6) than in the wild-type (lanes 2 and 5) under both aerobic and anaerobic-dark conditions, and diminished more under anaerobic-dark conditions than under aerobic conditions (lane 3 versus lane 6). The PpsR protein levels checked in the same samples (Fig. 5b) showed similar differences to those seen for the binding activities of PpsR. The binding activity and protein level of PpsR in APP11 were restored by the presence of pRK415::appA, as shown in lanes 4 and 7 of Fig. 5(a, b).

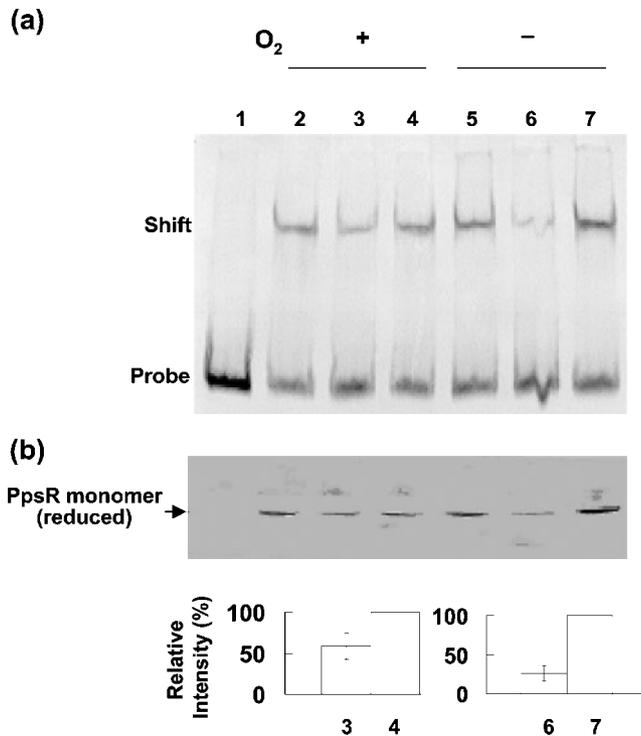
## DISCUSSION

We recognized that the reduction of intramolecular and intermolecular disulphide bonds in PpsR was correlated with the binding activity of PpsR to the *puc* promoter region when using purified PpsR. Therefore, to see whether growth

conditions, especially oxygen, influence the formation of intramolecular and intermolecular disulphide bonds in *R. sphaeroides*, we determined the *in vivo* redox states of the two cysteines in PpsR. The two cysteines of PpsR in living cells had their thiol groups in the reduced state irrespective of growth conditions, which means that PpsR always has the ability to bind to target promoters.

While this paper was being prepared, a report describing the redox property and DNA-binding activity of PpsR was published (Masuda & Bauer, 2002). That report presented two different results from ours: first, all of PpsR is oxidized to form an intramolecular disulphide bond under aerobic conditions; second, only PpsR containing intramolecular disulphide bond (oxidized monomer) was shown on SDS-PAGE. Unlike the trend of increasing DNA-binding activity by the reduction of the disulphide bond in our data, Masuda & Bauer (2002) found that DNA-binding activity to the *puc* promoter region was decreased 2.2-fold by the reduction of the intramolecular disulphide bond in PpsR. However, we could not obtain a PpsR pool containing only intramolecular disulphide bond and its own binding activity to the target promoter could not be determined, so the comparison in DNA-binding activity might not be meaningful.

In *E. coli*, cytosolic redox potential was reported to lie between approximately  $-260$  and  $-280$  mV under aerobic and anaerobic conditions (Gilbert, 1990). So even if cells grow under aerobic conditions, the cytoplasm maintains a reduced state, and the cysteines in most proteins exist in their reduced state. When cysteines are oxidized to form a disulphide bond or their thiol groups are converted to



**Fig. 5.** Analyses of wild-type conjugated with pRK415, APP11 with pRK415, and APP11 with pRK415::appA. (a) EMSA of crude extracts (16 µg) with the *puc* promoter region. (b) Western blot of crude extracts (40 µg) with anti-PpsR. Lanes: 1, no protein; 2 and 5, wild-type(pRK415); 3 and 6, APP11(pRK415); 4 and 7 APP11(pRK415::appA). The protein levels in APP11(pRK415) and APP11(pRK415::appA) were quantified in three independent experiments, and the values were converted into percentages and then averaged. O<sub>2</sub> + and - denote aerobic and anaerobic-dark growth conditions, respectively.

sulfenic acid by oxidative stress or certain stimuli, these are reconverted into thiol groups by cellular reductants like reduced glutathione or thioredoxin or the glutaredoxin system (Åslund *et al.*, 1999; Claiborne *et al.*, 1999; Lee *et al.*, 2002). In the case of extracellular proteins containing several cysteine residues, disulphide bonds are made by the periplasmic Dsb system in prokaryotes, or the oxidative protein folding system residing in the endoplasmic reticulum in eukaryotes (Kadokura *et al.*, 2003; Fassio & Sitia, 2002). Therefore, since PpsR is not an extracellular protein, it seems unlikely that all of the oxidized PpsR can withstand the tendency to reduction due to the presence of so many reductants under reduced cellular conditions, even though the cells are under aerobic conditions.

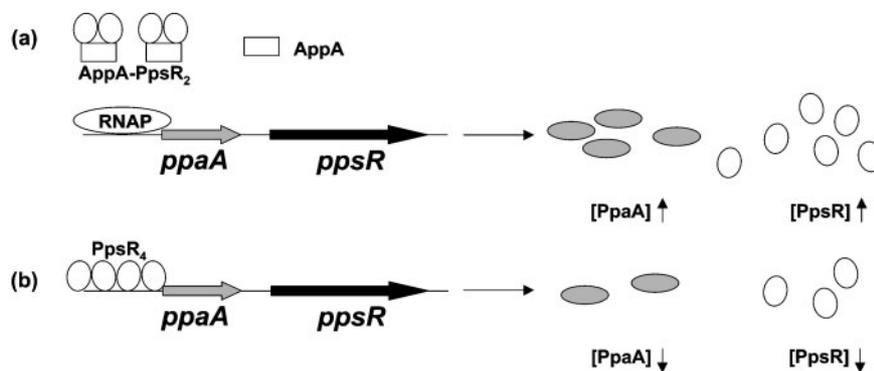
A protein containing an intramolecular disulphide bond usually migrates more quickly during SDS-PAGE than when it is fully reduced, because of a decrease in chain flexibility and hydrodynamic volume (Loferer *et al.*, 1995; Kang *et al.*, 1999; Lee *et al.*, 2002). Furthermore, since

the cysteines in PpsR are separated by 173 amino acid residues in the primary amino acid sequence, the structural difference between oxidized and reduced monomers may be increased. Therefore, as expected, the two forms were easily discriminated without any modifying reagent when subjected to SDS-PAGE.

It was known that the repressor activity of PpsR was active even under anaerobic conditions (Gomelsky & Kaplan, 1997). In apparent agreement with that result, we have shown that binding activity of PpsR to target promoters was maintained even under anaerobic conditions. Even in the results of Masuda & Bauer (2002), not all of the repressor activity of PpsR disappeared under anaerobic conditions, since the DNA-binding activity of the reduced PpsR decreased about 2.2-fold. However, in *R. capsulatus*, a reporter gene assay showed that transcriptional activity of photosystem genes (*puc*, *crt* and *bch*) was higher in DB469 (a CrtJ-null mutant) than in the wild-type under aerobic conditions but those in the two strains were almost the same under anaerobic-light conditions (Ponnampalam *et al.*, 1995). The DNA-binding activity of the reduced form is about 4.5-fold lower than that of the oxidized one. Unlike PpsR, CrtJ does not seem to work well under anaerobic conditions, which is consistent with the data of Masuda *et al.* (2002).

It would not make sense to maintain repressor activity under anaerobic conditions, since it has long been known that photosystem genes are expressed in large quantities under anaerobic conditions (Cohen-Bazire *et al.*, 1957; Aagaard & Sistrom, 1972). Since PpsR-binding sites overlap the -10 and -35 promoter regions of *puc*, *crt* and *bch* with RNA polymerase binding sites, it is difficult for these phenomena to happen simultaneously. On the other hand, under anaerobic conditions, several transcriptional activators are activated by the reduction of oxygen: e.g. PrrA, phosphorylation by PrrB; FnrL, reassembly of cofactor (Eraso & Kaplan, 1995; Zeilstra-Ryalls & Kaplan, 1998). Also, there was a report that RegA, a PrrA homologue in *R. capsulatus*, competed with CrtJ to bind to the *bchC* promoter region (Bowman *et al.*, 1999). Therefore, it is proposed that an anaerobic activator regulates photosystem gene expression under these conditions.

As mentioned above, photosystem gene expression was severely impaired in the AppA-null mutant APP11; this might be due to PpsR, since AppA was proposed to function as an antirepressor of PpsR (Gomelsky & Kaplan, 1995b; Gomelsky & Kaplan, 1997). And recently antirepressor (PpsR) action of AppA was verified by *in vitro* complex formation of the two proteins (Masuda & Bauer, 2002). We also investigated the DNA-binding activity to target the promoter, and the protein level of PpsR, in an AppA null background to validate the relationship between the two proteins. Although the DNA-binding activity to the target promoter and the protein level of PpsR were expected to increase in an AppA-null background, both of them in fact decreased when compared with those in



**Fig. 6.** A model combining the model of Masuda & Bauer (2002), the data of Gomelsky *et al.* (2003), and our data for controlling *ppsR* expression in wild-type (a) and AppA-null mutant (b) under anaerobic-dark conditions.

wild-type and complemented cells (Fig. 5a, b). It is difficult to understand this result from the viewpoint of the antirepressor activity of AppA. Nevertheless, we present a plausible model to rationalize our data and the others (Fig. 6). APP11, impaired in the photosystem, contains fewer PpsR than wild-type and the difference was more obvious under anaerobic-dark conditions than under aerobic conditions. Anti-repressor (PpsR) action of AppA was verified by *in vitro* complex formation of the two proteins under anaerobic-dark conditions (Masuda & Bauer, 2002). Recently PpaA was reported to activate photosystem gene expression and PpsR mediates the repression of *ppaA* gene expression (Gomelsky *et al.*, 2003). The *ppaA* and *ppsR* genes are located close to each other and have the same transcriptional direction. Therefore, if these two genes are an operon, AppA could indirectly activate transcription of the genes by binding PpsR under anaerobic-dark conditions (Fig. 6a), but in the AppA-null mutant, the two genes are downregulated by PpsR and photosystem genes are also downregulated by decreased PpaA and derepressed PpsR even though the protein level of PpsR is low (Fig. 6b). Under aerobic conditions, a similar explanation is possible and the decrease of the differences between wild-type and AppA-null mutant may be ascribed to the weak interaction of oxidized AppA and PpsR. In our experiment, the DNA-binding activity of PpsR increased in proportion to the protein level in the wild-type compared with the AppA-null mutant. It might be due to the dissociation of the complex (AppA and PpsR) by light or oxidized AppA in the experiment according to the model of Masuda & Bauer (2002). PpsR had a redox-sensitive property but remained in the reduced state in the cell, and its amount was reduced by disruption of AppA.

## ACKNOWLEDGEMENTS

We thank Drs Jeong Kook Lee, Samuel Kaplan and Mark Gomelsky for help with donation of several experimental materials, and give special thanks to Dr Lee for help with discussions. This work was supported by a Research Fellowship of the BK21 project.

## REFERENCES

- Aagaard, J. & Siström, W. R. (1972). Control of synthesis of reaction center bacteriochlorophyll in *Rhodospseudomonas sphaeroides*. *Photochem Photobiol* **15**, 209–225.
- Åslund, F., Zheng, M., Beckwith, J. & Storz, G. (1999). Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status. *Proc Natl Acad Sci U S A* **96**, 6161–6165.
- Bowman, W. C., Du, S., Bauer, C. E. & Kranz, R. G. (1999). *In vitro* activation and repression of photosynthesis gene transcription in *Rhodobacter capsulatus*. *Mol Microbiol* **33**, 429–437.
- Braatsch, S., Gomelsky, M., Kuphal, S. & Klug, G. (2002). A single flavoprotein, AppA, integrates both redox and light signals in *Rhodobacter sphaeroides*. *Mol Microbiol* **45**, 827–836.
- Claiborne, A., Yeh, J. I., Mallett, T. C., Luba, J., Crane, E. J. 3rd, Charrier, V. & Parsonage, D. (1999). Protein-sulfenic acids: diverse roles for an unlikely player in enzyme catalysis and redox regulation. *Biochemistry* **38**, 15407–15416.
- Cohen-Bazire, G., Siström, W. R. & Stanier, R. Y. (1957). Kinetic studies of pigment synthesis by non-sulfur purple bacteria. *J Cell Comp Physiol* **49**, 25–68.
- Davis, J., Donohue, T. J. & Kaplan, S. (1988). Construction, characterization, and complementation of a Puf<sup>-</sup> mutant of *Rhodobacter sphaeroides*. *J Bacteriol* **170**, 320–329.
- Eraso, J. M. & Kaplan, S. (1995). Oxygen-insensitive synthesis of the photosynthetic membranes of *Rhodobacter sphaeroides*: a mutant histidine kinase. *J Bacteriol* **177**, 2695–2706.
- Fassio, A. & Sitia, R. (2002). Formation, isomerisation and reduction of disulphide bonds during protein quality control in the endoplasmic reticulum. *Histochem Cell Biol* **117**, 151–157.
- Gilbert, H. F. (1990). Molecular and cellular aspects of thiol-disulfide exchange. *Adv Enzymol Relat Areas Mol Biol* **63**, 69–172.
- Gomelsky, M. & Kaplan, S. (1995a). Genetic evidence that PpsR from *Rhodobacter sphaeroides* 2.4.1 functions as a repressor of *puc* and *bchF* expression. *J Bacteriol* **177**, 1634–1637.
- Gomelsky, M. & Kaplan, S. (1995b). *appA*, a novel gene encoding a trans-acting factor involved in the regulation of photosynthesis gene expression in *Rhodobacter sphaeroides* 2.4.1. *J Bacteriol* **177**, 4609–4618.
- Gomelsky, M. & Kaplan, S. (1997). Molecular genetic analysis suggesting interaction between AppA and PpsR in regulation of photosynthesis gene expression in *Rhodobacter sphaeroides* 2.4.1. *J Bacteriol* **179**, 128–134.

- Gomelsky, M., Horne, I. M., Lee, H.-J., Pemberton, J. M., McEwan, A. G. & Kaplan, S. (2000). Domain structure, oligomeric state, and mutational analysis of PpsR, the *Rhodobacter sphaeroides* repressor of photosystem gene expression. *J Bacteriol* **182**, 2253–2261.
- Gomelsky, L., Sram, J., Moskvina, O. V., Horne, I. M., Dodd, H. N., Pemberton, J. M., McEwan, A. G., Kaplan, S. & Gomelsky, M. (2003). Identification and in vivo characterization of PpaA, a regulator of photosystem formation in *Rhodobacter sphaeroides*. *Microbiology* **149**, 377–388.
- Gregor, J. & Klug, G. (1999). Regulation of bacterial photosynthesis genes by oxygen and light. *FEMS Microbiol Lett* **179**, 1–9.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- Kadokura, H., Katzen, F. & Beckwith, J. (2003). Protein disulfide bond formation in prokaryotes. *Annu Rev Biochem* **72**, 111–135.
- Kang, J. G., Paget, M. S. B., Seok, Y. J., Hahn, M. Y., Bae, J. B., Hahn, J. S., Kleanthous, C., Buttner, M. J. & Roe, J. H. (1999). RsrA, an anti-sigma factor regulated by redox change. *EMBO J* **18**, 4292–4298.
- Keen, N. T., Tamaki, S., Kobayashi, D. & Trollinger, D. (1988). Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. *Gene* **70**, 191–197.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lee, S. R., Yang, K. S., Kwon, J., Lee, C., Jeong, W. & Rhee, S. G. (2002). Reversible inactivation of the tumor suppressor PTEN by H<sub>2</sub>O<sub>2</sub>. *J Biol Chem* **277**, 20336–20342.
- Loferer, H., Wunderlich, M., Hennecke, H. & Glockshuber, R. (1995). A bacterial thioredoxin-like protein that is exposed to the periplasm has redox properties comparable with those of cytoplasmic thioredoxins. *J Biol Chem* **270**, 26178–26183.
- Masuda, S. & Bauer, C. E. (2002). AppA is a blue light photoreceptor that antirepresses photosynthesis gene expression in *Rhodobacter sphaeroides*. *Cell* **110**, 613–623.
- Masuda, S., Dong, C., Swem, D., Setterdahl, A. T., Knaff, D. B. & Bauer, C. E. (2002). Repression of photosynthesis gene expression by formation of a disulfide bond in CrtJ. *Proc Natl Acad Sci U S A* **99**, 7078–7083.
- Penfold, R. J. & Pemberton, J. M. (1994). Sequencing, chromosomal inactivation, and functional expression in *Escherichia coli* of *ppsR*, a gene which represses carotenoid and bacteriochlorophyll synthesis in *Rhodobacter sphaeroides*. *J Bacteriol* **176**, 2869–2876.
- Ponnampalam, S. N. & Bauer, C. E. (1997). DNA binding characteristics of CrtJ. *J Biol Chem* **272**, 18391–18396.
- Ponnampalam, S. N., Buggy, J. J. & Bauer, C. E. (1995). Characterization of an aerobic repressor that coordinately regulates bacteriochlorophyll, carotenoid, and light harvesting-II expression in *Rhodobacter capsulatus*. *J Bacteriol* **177**, 2990–2997.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Simon, R., Priefer, U. & Puhler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. *Bio/Technology* **1**, 784–791.
- Taylor, B. L. & Zhulin, I. B. (1999). PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol Mol Biol Rev* **63**, 479–506.
- Zeilstra-Ryalls, J. H. & Kaplan, S. (1998). Role of the *fnrL* gene in photosystem gene expression and photosynthetic growth of *Rhodobacter sphaeroides* 2.4.1. *J Bacteriol* **180**, 1496–1503.