Role of PufX Protein in Photosynthetic Growth of *Rhodobacter sphaeroides*. 1. PufX Is Required for Efficient Light-Driven Electron Transfer and Photophosphorylation under Anaerobic Conditions†

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ABSTRACT: The *pufX* gene is essential for photoheterotrophic growth of the purple bacterium *Rhodobacter sphaeroides*. In order to analyze the molecular function of the PufX membrane protein, we constructed a chromosomal *pufX* deletion mutant and phenotypically compared it to a *pufX*$^+$ control strain and to two suppressor mutants which are able to grow photosynthetically in the absence of *pufX*. Using this genetic background, we confirmed that PufX is required for photoheterotrophic growth under anaerobic conditions, although all components of the photosynthetic apparatus were present in similar amounts in all strains investigated. We show that the deletion of PufX is not lethal for illuminated *pufX*$^-$ cells, suggesting that PufX is required for photosynthetic cell division. Since chromatophores isolated from the *pufX*$^-$ mutant were found to be unsealed vesicles, the role of PufX in photosynthetic energy transduction was studied *in vivo*. We show that PufX is essential for light-induced ATP synthesis (photophosphorylation) in anaerobically incubated cells. Measurements of absorption changes induced by a single turnover flash demonstrated that PufX is not required for electron flow through the reaction center and the cytochrome bc_1 complex under anaerobic conditions. During prolonged illumination, however, PufX is essential for the generation of a sufficiently large membrane potential to allow photosynthetic growth. These *in vivo* results demonstrate that under anaerobic conditions PufX plays an essential role in facilitating effective interaction of the components of the photosynthetic apparatus.

Photosynthetic organisms have the ability to convert light to chemical energy by the cooperation of membrane-bound pigment−protein complexes. *Rhodobacter sphaeroides* is a facultative phototrophic, purple non-sulfur bacterium and produces an extensive system of intracytoplasmic membranes (ICM)[1] when grown either anaerobically in the light (photosynthetically) or aerobically in the dark under low oxygen partial pressure (semiaerobically) [reviewed in Kiley and Kaplan (1988)]. The ICM is synthesized from invaginations of the cytoplasmic membrane (Cohen-Bazire et al., 1957) and is functionally differentiated to capture light and catalyze photosynthetic energy conversion.

Two light-harvesting complexes, LH1 and LH2, absorb the light energy and transfer it to the reaction center (RC) [reviewed in Zuber and Brunisholz (1991)]. Each of these membrane complexes consists of two complex-specific polypeptides, α and β, that noncovalently bind bacteriochlorophyll (bchl) and carotenoid molecules. The RC molecules are surrounded and interconnected by a fixed number of LH1 complexes (stoichiometry about 15:1; Monger & Parson, 1977; van Grondelle, 1985). The levels of LH2 antennae, which are arranged peripherally around the RC−LH1 complexes, vary with irradiance and oxygen tension during growth (Aagaard & Sistrom, 1972).

Two membrane-bound complexes form a light-driven, cyclic electron transfer pathway in the ICM: the cytochrome (cyt) bc_1 complex and the RC where the primary photochemistry occurs. The structure of the RC was determined at atomic resolution in *Rhodopseudomonas viridis* [reviewed in Deisenhofer and Michel (1989)] and *R. sphaeroides* [reviewed in (Rees et al. (1989)]. Photooxidation of the primary electron donor P, a bchl dimer, results in charge separation across the membrane (Parson, 1987; Feher et al., 1989). Two subsequent turnovers of the RC lead to the reduction of Q_B, a ubiquinone (Q) molecule bound to the RC. A ubiquinole (QH_2) molecule leaves the RC and enters a pool of Q/QH_2 molecules in the hydrophobic part of the membrane. Cyclic electron flow is completed by the cyt bc_1 membrane complex utilizing QH_2 as an electron donor.

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[1] Abbreviations: bchl, bacteriochlorophyll *a*; CFU, colony-forming unit; cyt, cytochrome; E_h, redox potential; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; Q, ubiquinone; QH_2, ubiquinol; RC, reaction center; WT, wild type.

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The electrons are transferred to cyt c_2, which is located in the periplasm and rereduces the photooxidized bchl dimer of the RC. Transmembrane electron transfer is coupled to the generation of an electrochemical proton gradient across the membrane, thus allowing the formation of ATP by a proton-driven ATP synthase.

The genes for the antenna, the RC, and the cyt bc_1 complex have been cloned and sequenced [reviewed in Kiley and Kaplan (1988)]. The genes coding for LH1-α and -β (*pufB* and *pufA*) and two of the RC subunits (*pufM* and *pufL*) are located adjacently on the chromosome of *R. capsulatus* (Youvan et al., 1984) and *R. sphaeroides* (Kiley et al., 1987). These genes form the so-called *puf* operon that is transcribed as a polycistronic mRNA transcript (Zhu & Kaplan, 1985). An additional open reading frame downstream of *pufM*, termed *pufX*, was originally identified in *R. capsulatus* (Youvan et al., 1984). In *R. sphaeroides*, a similar open reading frame was found to be transcribed as part of the polycistronic *puf* mRNA (Zhu et al., 1986; Lee et al., 1989). In *R. sphaeroides*, the *pufX* gene is expressed as a 9 kDa membrane protein (termed PufX) that is closely associated with the RC−LH1 complex (Farchaus et al., 1992). Recent studies in *R. sphaeroides* (Farchaus et al., 1992; Westerhuis et al., 1993; McGlynn et al., 1994) and *R. capsulatus* (Lilburn et al., 1992) have shown that the PufX protein is required for anaerobic, photosynthetic growth although PufX is not required for *puf* operon expression or primary charge separation in the RC. The precise role of PufX is unknown, but it has been observed that cyclic electron flow (induced by a train of flashes) is impaired in the absence of PufX (Farchaus et al., 1992; Lilburn et al., 1992).

Spontaneous suppressor mutants of a *pufX* deletion mutant of *R. sphaeroides* have been characterized in detail (Barz & Oesterhelt, 1994). In these strains photosynthetic competence is restored by spontaneous point mutations in the plasmid-borne genes *pufB* or *pufA*. These mutations seemed to change the macromolecular antenna structure around the RC, thus allowing photosynthetic growth in the absence of PufX (Barz & Oesterhelt, 1994).

To date, all work on the PufX protein was performed with chromosomal *puf* deletion strains which were complemented with plasmids carrying *puf* operon constructs. These plasmid-complemented strains are unfavorable to determine the function of PufX at a molecular level because of the following reasons: First, the plasmids used for complementation were derivatives of pRK404 (Ditta et al., 1985) that has a copy number of four to six plasmids per cell (Davis et al., 1988). This results in increased expression of the *puf* operon, thereby impeding studies on the stoichiometry of photosynthetic membrane complexes (Farchaus et al., 1990). Second, the previously observed effect of PufX on the levels of LH1 antennae (Farchaus et al., 1992; Westerhuis et al., 1993) could not be quantitated in plasmid-complemented strains due to the additional presence of chromosomal copies of the LH1 genes *pufB* and *pufA*. The fact that single point mutations in *pufB* or *pufA* phenotypically suppress the deletion of *pufX* (Barz & Oesterhelt, 1994) strengthens the importance of studying the putative regulatory role of PufX on LH1 antenna expression under genetically defined conditions. Third, the photosynthetic growth rate of wild type (WT) was found to be about 30% greater than in a mutant carrying a plasmid-borne *puf* operon containing *pufX* (Davis et al., 1988; Farchaus & Oesterhelt, 1989). This observation suggested that cis-acting elements regulate the expression of the *puf* operon in the WT.

Because of these reasons, we constructed a novel *pufX* deletion mutant that carries one copy of a truncated *puf* operon in the correct chromosomal position. We report the characterization of this novel *pufX*$^-$ mutant, a *pufX*$^+$ control strain, and two photocompetent suppressor mutants. In order to study the molecular role of PufX in energy transduction, light-induced absorption measurements were performed. We provide evidence that PufX is essential for photophosphorylation because it plays a vital role in facilitating effective interaction of photosynthetic redox components under anaerobic conditions.

MATERIALS AND METHODS

Materials. DNA-modifying enzymes were from Boehringer Mannheim (Mannheim, FRG). Nylon membranes (Hybond N) for Southern blots were purchased from Amersham-Buchler (Braunschweig, FRG). All other reagents were from Merck (Darmstadt, FRG) or Sigma Chemical Co. (Munich, FRG).

Bacterial Strains and Growth Conditions. R. sphaeroides cultures were grown semiaerobically in Erlenmeyer flasks filled to 50% of the total volume with Sistrom's minimal medium (Sistrom, 1960). The cultures were incubated in darkness at 30 °C and 100 rpm overnight on a gyratory shaker with a displacement radius of 2.5 cm. Aerobic cell growth at high oxygen tension ($pO_2 \approx 200$ mbar) was performed in flasks that were filled to only 20% of their volume and shaken at 300 rpm. Kanamycin (25 μg/mL) was added to *R. sphaeroides* cultures when appropriate. The oxygen partial pressure of these cultures was measured using a Trioxmatic EO 200 Clark-type oxygen electrode connected to an Oxymatic 2000 oximeter from WTW (Weilheim, FRG).

Photoheterotrophic growth on plates was in thermostated light chambers (Oesterhelt, 1982), equipped with BBL GasPak anaerobic systems (BBL Microbiology Systems, Cockeysville, MD) and illuminated by six 60 W incandescent light bulbs. Photoheterotrophic growth in liquid culture was monitored turbidimetrically at 30 °C using a Klett−Summerson colorimeter (No. 66 filter) as described previously (Farchaus et al., 1992). Illumination was provided by far-red light ($\lambda > 680$ nm) defined by spectral-quality Plexiglas manufactured by Rohm-Haas (Darmstadt, FRG). The incident intensity of this light was monitored with an Opto-Meter model 40X (United Detector Technology, Inc.). The rates of photosynthetic growth were determined by fitting the exponential growth phase with a monoexponential function. Only the first 50 h of illumination were considered for *pufX*$^-$ strains because of the spontaneous appearance of suppressor mutants after longer illumination (Barz & Oesterhelt, 1994).

Escherichia coli strains were grown at 37 °C in Luria broth medium (Sambrook et al., 1989). Ampicillin (100 μg/mL) or tetracyclin (10 μg/mL) were used when appropriate. Conjugative transfer of mob$^+$ plasmids into *R. sphaeroides* was carried out using *E. coli* S17-1 (Simon et al., 1983) [*recA pro*$^-$ *res*$^-$ *mod*$^+$ Tp^r Sm^r-pRP4-2-Tc::Mu−Km::Tn7] and the diparental filter-mating procedure described previously (Davis et al., 1988). Exconjugants were selected chemoheterotrophically using kanamycin (25 μg/mL) and tetracyclin (2 μg/mL).

Selection of Green Carotenoid Mutants. To obtain green *R. sphaeroides* strains (Ga type) with blue-shifted carotenoid absorption characteristics, ΔQ−X cells (Barz & Oesterhelt, 1994) were incubated semiaerobically on an agar plate. A clone with a stable green pigmentation (reversion frequency $< 10^{-8}$) was isolated and designated ΔQ−X/g. Integration of the suicide plasmids pSUPtetΔX and pSUPtetC (see Results) into ΔQ−X/g resulted in the green strains PUFΔX/g and PUFC/g, respectively. Southern blot analysis confirmed the expected genetic structure of the *puf* operon region in these mutants.

Recombinant DNA Techniques. Standard molecular biological techniques such as DNA preparation, restriction enzyme digestion, and *E. coli* transformation were performed as described previously (Sambrook et al., 1989). Southern blots were performed using a "DIG Luminescent Detection Kit" following the instructions of the manufacturer (Boehringer Mannheim, Mannheim, FRG).

The suicide plasmid pSUPtetΔX (lacking *pufX*) was constructed in the following manner: After digestion of pRKXmut2T (Farchaus et al., *1990*) with *Bam*HI and *Dra*I (a *Dra*I restriction site is located 55 bp downstream the *puf*M stop codon of pRKXmut2T), the 3.8 kb *puf* operon construct was isolated and ligated to the 7.3 kb *Bam*HI−*Hin*dIII fragment of the suicide vector pSUP202 (Simon et al., 1983). The resulting plasmid was designated pSUPΔX. In order to allow the selection of single-crossover events between the suicide plasmid and the chromosome of *R. sphaeroides*, a tetracyclin resistance gene (Tc^R) was cloned into pSUPΔX. To this end, the 6.5 kb *Eco*RI fragment of pSUPΔX was ligated with the 4.3 kb *Eco*RI−*Sal*I vector of pSUP202, a 1.4 kb *Eco*RI−*Ava*I−Tc^R cartridge (Pharmacia, Piscataway, NJ), and an *Ava*I−*Sal*I linker fragment (constructed from the oligonucleotides 5′-TCGGGCAGCGTG-3′ and 5′-TCGACACGCTGC-3′). The resulting plasmid was named pSUPtetΔX and analyzed by restriction analysis. For the construction of the control strain PUFC (carrying *pufX*), the suicide plasmid pSUPtetC was constructed in the same way by using the 4.1 kb *Bam*HI−*Dra*I fragment that was obtained by digesting pRKXmut2XT (Farchaus et al., 1992) with *Bam*HI and *Dra*I (in this plasmid, a *Dra*I restriction site is located 128 bp downstream the *pufX* stop codon).

Membrane Isolation and Analysis. Chromatophores were isolated from semiaerobically grown cells that were harvested at a defined cell density of 100 ± 5 Klett units. Chromatophores were prepared using a French press cell as described previously (Bowyer et al., 1979). Bchl was evaluated according to Clayton (1966). The total membrane Q content was determined by exhaustive extraction and reversed-phase HPLC analysis, as described previously (Venturoli et al., 1986).

Photophosphorylation Assay in Vivo. Light-induced ATP synthesis was determined in intact cells using a modification of a previously described procedure (Welsch & Smith, 1969). Cell cultures (100 mL) were grown semiaerobically to a cell density of 150 Klett units, harvested by centrifugation, and washed with 20 mM sodium phosphate buffer (pH 7.4). To reduce the high intracellular ATP levels that were observed in recently harvested cells, the cells were resuspended in 100 mL of the same buffer and incubated under semiaeriobic conditions in the dark (24 h, 30 °C). This starvation time was found to reduce the endogenous ATP concentrations to about 25% of the initial value (data not shown). Following starvation, the cells were centrifuged, resuspended in 25 mL sodium phosphate buffer (pH 7.4), and transferred into a thermostated cuvette (30 °C). These steps were performed in complete darkness. Under anaerobic conditions (argon), the cells were stirred for 2 h in the dark before they were illuminated with intense red light defined by a OG590 filter (Schott, Mainz, FRG). For cell lysis, 100 μL aliquots of the cell suspension were rapidly ($t < 5$ s) transferred into 400 μL of 35% cold $HClO_4$ (0 °C) and strongly vortexed for 2 min. The aliquots were neutralized to pH 7.5 ± 0.1 by adding an appropiate volume (670 μL in a typical experiment) of the following solution: 22.5 mL of saturated KOH, 17.5 mL of 1 M KCl, 60 mL of 2 M Tris/HCl (pH 7.5). After the samples were mixed, they were stored at 4 °C and centrifuged immediately before ATP determination. The bioluminescence of the supernatant was measured at 30 °C with a 3M-Biocounter M2010 luminometer, used as described by the manufacturer (Abimed, Düsseldorf, FRG). Before use, the firefly extract FLE-50 (Sigma, München, FRG) was stored overnight in the dark (4 °C) to reduced the amount of endogenous ATP. Calibration curves were obtained with known amounts of ATP that were treated in exactly the same way as the cell aliquots. To normalize the observed ATP concentrations to the number of living cells (CFU), a diluted aliquot of the illuminated cell suspension was spread to an agar plate and incubated at 30 °C.

Reaction Center Photobleaching. To determine the concentration of photoactive RC in chromatophores or intact cells, flash-induced photobleaching of the RC primary donor P865 was measured. Samples were suspended in 100 mM KCl, 50 mM MOPS (pH 7.0), supplemented with 15 μM valinomycin and 2 μM gramicidin, and incubated in the dark for several hours. Flash-induced absorption changes in the Q_y region (680−1000 nm) were recorded using a photodiode array spectrophotometer (Uhl et al., 1985). The intensity of the measuring beam was reduced with a 1% transmittance neutral-density filter and an RG 665 filter (Schott, Mainz, FRG). The sample was excited at 595 nm using an excimer laser (EMG 53 MSC) pumped dye laser (FL3001, Lambda Physics, Göttingen, FRG) with Rhodamine 6B as the dye. The flash had a pulse length of approximately 10 ns. Light-minus-dark absorption changes were recorded as the difference between two spectra recorded 3 μs before and 3 μs after the laser flash, respectively. Four bleaching spectra were averaged for each sample, the dark time between the excitation flashes was 30 s. The RC concentration was calculated using the extinction coefficient $\epsilon_{865} = 113\ mM^{-1}\ cm^{-1}$; Straley et al., 1973).

Determination of Photosynthetic Unit Sizes. Low-temperature (77 K) spectra of chromatophores were recorded as described previously (Barz & Oesterhelt, 1994). Using the extinction coefficients $\epsilon_{800} = 226\ mM^{-1}\ cm^{-1}$ and $\epsilon_{875} = 117\ mM^{-1}\ cm^{-1}$ (Sturgis et al., 1988), the ratio of the bchl molecules bound to LH1 and LH2 ($bchl_{LH1}/bchl_{LH2}$) was calculated from the amplitudes of the absorption peaks at 800 nm (LH2) and 885 nm (LH1). This calculation was based on the assumption that the ratio $\epsilon_{800}/\epsilon_{875}$ (determined at room temperature) did not change significantly upon cooling to 77 K. Total bchl concentration ($bchl_T$) was determined according to Clayton (1966). The stoichiometry of the photosynthetic unit was calculated as $bchl_{LH1}/RC = (bchl_T/RC) \times (bchl_{LH1}/bchl_{LH2})/(1 + bchl_{LH1}/bchl_{LH2})$.

Measurement of Flash-Induced Absorption Changes in Vitro. Kinetic spectrophotometric measurements in chromatophores were performed in an N_2 atmosphere under controlled redox conditions as described previously (Venturoli et al., 1986). All measurements were performed in 100 mM KCl, 50 mM MOPS (pH 7.0) containing the following redox mediators: 1 μM each of phenazine methosulfate, phenazine ethosulfate, and 1-hydroxy-*N*-methylphenazine ethosulfate (pyocyanin); 2 μM of 2,3,5,6-tetramethyl-*p*-phenylenediamine; 10 μM each of *p*-benzoquinone, duroquinone, 1,2-naphthoquinone, and 1,4-naphthoquinone. When flash-induced redox reactions were studied in uncoupled membranes, 10 μM valinomycin and 7 μM nigericin were also added. The electrochromic carotenoid band shift was measured as flash-induced absorption changes at 503 nm (Wraight et al., 1978). The photooxidation of total cyt *c* (both cyt c_1 and cyt c_2) was determined as the difference of absorption changes at 551 and 542 nm (Bowyer et al., 1979).

A xenon flash lamp (EG&G FX201, discharging a 3 μF capacitor charged to 1.5 kV) was used for flash excitation, with a pulse duration at half-maximal intensity of 4 μs. The flash intensity was found to be approximately 90% saturating. Absorbance changes were measured by a single-beam spectrophotometer equipped with a double-grating monochromator (bandwidth 1.5 nm). The photomultiplier was protected by a Corning glass 4/96 filter. A triggered shutter was used to gate the measuring beam (exposure of the sample to the measuring light was no longer than 2 s prior to excitation). Data were acquired by a LeCroy 9410 digital oscilloscope interfaced to an Olivetti M240 personal computer.

In order to determine the stoichiometry of the photosynthetic redox components, trains of closely spaced flashes were used to photoactivate chromatophores inhibited with 10 μM valinomycin, 7 μM nigericin, and 10 μM antimycin A. Following a train of eight flashes (20 ms apart), the total concentration of photooxidizable RC was measured at E_h = 160 mV from the absorption changes at 542 nm (using the extinction coefficient ϵ = 10.3 mM^{-1} cm^{-1}; Dutton et al., 1975; Bowyer et al., 1981). Total photooxidizable cyt (c_1 + c_2) was measured spectroscopically following an analogous procedure and using an extinction coefficient $\epsilon_{551-542}$ = 19.5 mM^{-1} cm^{-1} (Dutton et al., 1975; Bowyer et al., 1979 and 1981). Cyt b_{561} redox changes were measured similarly using $\epsilon_{561-569}$ = 19.5 mM^{-1} cm^{-1} (Bowyer et al., 1981; Crofts et al., 1983). Measurements performed in the presence of antimycin and myxothiazol demonstrated that any photoinduced signal due to RC and cyt (c_1 + c_2) redox changes was eliminated at the wavelength pair 561−569 nm, also in chromatophores isolated from PUFΔX/g, Sup101/g, and Sup102/g.

Light-Induced Absorption Difference Spectroscopy in Vivo. Light-induced absorption changes in intact cells were determined as described in detail in the following article (Barz et al., 1995).

RESULTS

Construction of a Chromosomal pufX Deletion Strain. Complementation of a *pufLMX* deletion strain with plasmids carrying different *puf* operon constructs demonstrated that the *pufX* gene product (termed PufX) is strictly required for photoheterotrophic but not for chemoheterotrophic growth of *R. sphaeroides* (Farchaus et al., 1990, 1992).

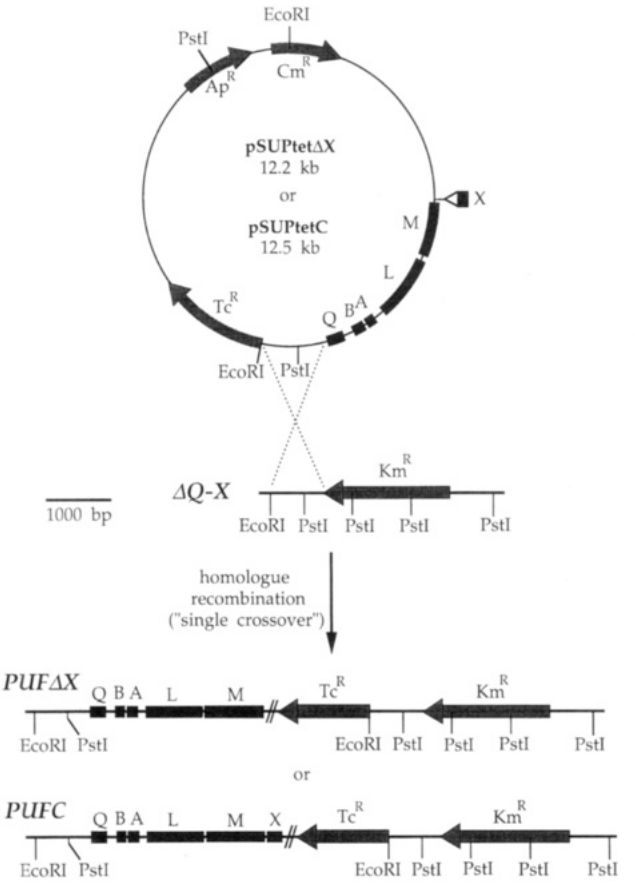

FIGURE 1: Construction of the mutant strains PUFΔX and PUFC by integration of suicide plasmids lacking or carrying *pufX*. The 0.8 kb sequence upstream the *puf* operon is identical in the suicide plasmid and in the genome of ΔQ−X so that it is the preferred site for a single-crossover event (indicated by the dashed cross). "//" indicates the 7.3 kb fragment of the suicide plasmids located between the end of the *puf* operon and the Tc^R gene.

As tools for the determination of the molecular function of the PufX protein, these plasmid-complemented mutants were unfavorable for several reasons (see introduction). Therefore, we constructed a novel *pufX* deletion mutant that carries one copy of each of the genes *pufQBALM* in the correct chromosomal position. As described under Materials and Methods, a truncated *puf* operon construct was cloned into the suicide vector pSUP202 (Simon et al., 1983). The resulting plasmid pSUPtetΔX (Figure 1) was conjugated into ΔQ−X, a deletion strain in which the complete *puf* operon had been replaced with a kanamycin resistance (Km^R) gene (Barz & Oesterhelt, 1994). Since the suicide plasmid was unstable in *R. sphaeroides*, the tetracyclin resistance (Tc^R) gene was only expressed if a single crossover event had integrated the suicide plasmid into the genome of ΔQ−X (Figure 1). The 0.8 kb DNA fragment on the 5′ end of the Km^R gene was the expected insertion site of pSUPtetΔX because this sequence was identical in the ΔQ−X genome and in the suicide plasmid.

As shown in Figure 1, integration of pSUPtetΔX into ΔQ−X resulted in a *pufX* deletion mutant, designated PUFΔX, that carried the genes *pufQBALM* in their correct chromosomal position. To distinguish the *pufX* phenotype from phenotypes possibly caused by the insertion of vector sequences (including the Tc^R gene), we constructed a control

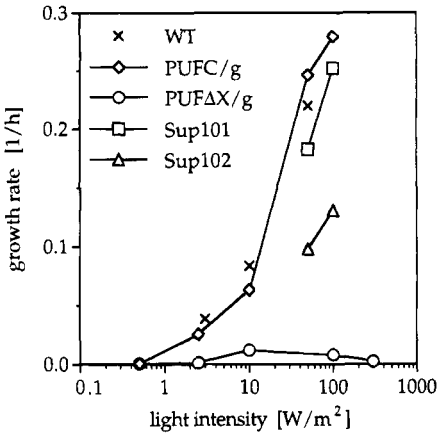

FIGURE 2: Dependence of photoheterotrophic growth rates on the incident light intensity. Anaerobic cultures were inoculated with semiaerobically grown precultures and exposed to incandescent far-red light of the intensities indicated. Growth rates were determined by fitting the exponential phase of photosynthetic growth curves with a monoexponential function.

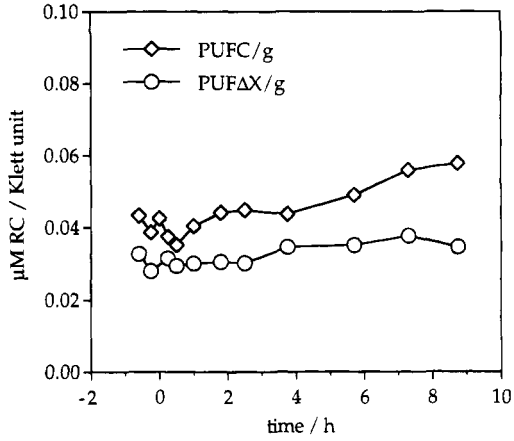

FIGURE 3: Lack of RC degradation during prolonged illumination. Anaerobic cultures were inoculated with semiaerobically grown precultures and exposed to incandescent white light (100 W/m²) at 30 °C. At the times indicated, aliquots were taken and used to determine the RC concentration by flash-induced photobleaching (see Materials and Methods). Cellular RC concentrations are given relative to the number of Klett units determined for each culture.

strain PUFC (see Figure 1: insertion of pSUPtetC into ΔQ−X). Since PUFC was genetically identical to PUFΔX except for the presence of *pufX*, these strains were well suited for determining the role of *pufX*.

For each mutant, one clone with a stable Km^R Tc^R phenotype and WT pigmentation was used for further characterization. Southern blot analysis (using a 0.6 kb DNA fragment carrying *pufBA* and a 1.8 kb probe specific to Km^R) was performed to confirm that the suicide plasmids had integrated correctly into the chromosome of ΔQ−X (data not shown). To obtain pigmentation mutants with blue-shifted carotenoid absorption characteristics (Ga type, Griffiths & Stanier, 1956), green mutants of PUFΔX and PUFC were isolated (see Materials and Methods) and designated PUFΔX/g and PUFC/g, respectively. Absorption spectra of these strains showed the same carotenoid peaks (at 431, 459, and 491nm) as a *R. sphaeroides* mutant known to accumulate neurosporene (Farchaus & Oesterhelt, 1989). Since spontaneous mutations in the *crtD* gene occur frequently in *R. sphaeroides* (Garí et al., 1992) and lead to the accumulation of neurosporene, the green pigmentation of PUFΔX/g and PUFC/g is likely to be caused by such a *crtD* mutation.

Phenotypic Characterization of the Strains. To confirm the requirement of *pufX* for photosynthetic growth in a genetic background based on chromosomal *puf* genes, the growth phenotypes of the newly generated strains were studied under defined conditions. As observed before for plasmid-complemented strains (Farchaus et al., 1990, 1992), *pufX* was not required for chemoheterotrophic growth at high ($pO_2 \approx 200$ mbar) or low ($pO_2 = 0.5-0.8$ mbar) oxygen tensions (data not shown). Under photoheterotrophic conditions, however, *pufX* was strictly required for growth. As shown in Figure 2, the photosynthetic phenotypes of WT and PUFC/g were indistinguishable whereas PUFΔX/g did not grow even at very high irradiance (500 W/m²). Thus, *pufX* is also essential for photosynthetic growth when a single copy of the *puf* genes is located on the chromosome.

To determine whether the lack of *pufX* was lethal for illuminated *R. sphaeroides* cells or whether it only impaired the ability of photosynthetic growth (i.e., cell division), a $pufX^-$ culture was illuminated for 50 h under anaerobic conditions while another $pufX^-$ culture was incubated anaerobically in the dark. Aliquots were taken from both cultures, diluted, spread on agar plates, and incubated aerobically. Similar numbers of colonies were obtained from the differently incubated cultures (data not shown) giving evidence that the photosynthetic incompetence of PUFΔX/g was not caused by light-induced death of the $pufX^-$ cells. Instead, photosynthetic growth (cell division) was impaired in the absence of *pufX*.

A possible reason for the impairment of photosynthetic growth of PUFΔX/g could be the light-induced degradation of the RC. To study a potential role of PufX in protecting anaerobic cells against photodamage, the stability of the RC was determined during prolonged illumination of PUFC/g and PUFΔX/g cells under anaerobic conditions. Figure 3 shows that the concentration of photobleachable RC was similar in cells containing or lacking *pufX* for an illumination period of about 10 h. Since the levels of RC per cell did not decrease in the absence of PufX, light-induced degradation of the RC could be excluded.

Stoichiometry of the Components of the Photosynthetic Apparatus. Previously, studies with *R. sphaeroides* $pufX^-$ strains demonstrated that the deletion of *pufX* increases the amount of LH1 and decreases the level of LH2 (Farchaus et al., 1990, 1992; Westerhuis et al., 1993; McGlynn et al., 1994). However, the plasmid-complemented strains used in those studies were unfavorable for determining the role of PufX in regulating the antenna expression because of plasmid copy number effects and, in the case of the earlier studies, because of additional, genomic copies of the LH1 genes. Since the mutants described in this study carry a single copy of the *puf* genes in the correct chromosomal location, they are perfectly suitable to analyze the role of *pufX* in regulating the stoichiometry of the photosynthetic unit. Absorption spectra were recorded using chromatophores isolated from semiaerobically grown cells of PUFC/g and PUFΔX/g. In addition, two neurosporene-accumulating suppressor mutants, Sup101/g and Sup102/g, were used in this study. These strains were obtained by conjugating the plasmids pXsup101 and pXsup102 (Barz & Oesterhelt, 1994) into the neurosporene-accumulating *pufLMX* deletion strain PUFΔLMX21/3 (Farchaus & Oesterhelt, 1989). The plasmids pXsup101 and

Table 1: Size of the Photosynthetic Unit and the Stoichiometries of the Redox Components of the Photosynthetic Electron Transfer Chain (Molar Ratios) in Semiaerobically Grown Chromatophores[a]

mutant	$bchl_{LH1}$/ $bchl_{LH2}$	bchl/RC	$bchl_{LH1}$/ RC	Q/RC	cyt b_{561}/ RC	cyt c_2/ RC
PUFC/g	0.83	90	41	63	0.85	1.6
PUFΔX/g	1.5	80	48	43	1.3	>2.5
Sup101/g	1.3	11	5.9	49	1.0	>3.1
Sup102/g	1.7	8.1	5.1	65	0.72	>2.5

[a] Different chromatophores preparations were used for the left and right halves of this table. Total bchl was evaluated according to Clayton (1966). The concentrations of bchl molecules bound to LH1 ($bchl_{LH1}$) or LH2 ($bchl_{LH2}$) were calculated from 77 K absorption spectra as described under Materials and Methods. For the left half of this table, RC concentrations were determined by photobleaching induced by a laser flash (Materials and Methods). The total ubiquinone (Q) content of the membranes was determined by exhaustive extraction and reversed HPLC analysis, as described previously (Venturoli et al., 1986). The total concentrations of photooxidizable RC (right half of the table), photooxidizable cyt (c_1 + c_2), and of photoreducible cyt b_{561} were measured using a train of eight flashes, as described under Materials and Methods. The amount of photooxidizable cyt c_2 was obtained by subtracting the amount of photoreducible cyt b_{561} from the concentration of total cyt (c_1 + c_2), assuming a stoichiometry of one cyt c_1 per cyt b_{561}. In the case of PUFΔX/g, Sup101/g, and Sup102/g, a lower limit of cyt c_2 stoichiometry is given, since even a train of fifteen closely spaced flashes could not photooxidize the full complement of cyt c_2.

pXsup102 carry genetically well-characterized point mutations in *pufBA* genes that allow photosynthetic growth in the absence of *pufX* (see Figure 2). Absorption spectra were measured at 77 K for better resolution of the overlapping LH1 and LH2 peaks. The cultures were harvested at identical cell densities of 100 ± 5 Klett units. As observed with plasmid-complemented strains (Westerhuis et al., 1993), the LH1 absorption maximum was slightly red-shifted in the absence of *pufX* (885 nm in PUFC/g; 887 nm in PUFΔX/g).

The total concentrations of bchl and RC were measured using chromatophores of PUFC/g, PUFΔX/g, Sup101/g, and Sup102/g. The size of the photosynthetic unit ($bchl_{LH1}$/RC) was calculated using the molar ratios $bchl_{LH1}/bchl_{LH2}$ and bchl/RC (see left half of Table 1). The $bchl_{LH1}$/RC data obtained for PUFC/g and PUFΔX/g were a little larger than the values (25−35) obtained for WT (Aagaard & Sistrom, 1972, Westerhuis et al., 1993), but were significantly lower than in plasmid-complemented mutants (see Discussion). In the chromosomal *pufX*$^-$ mutant, $bchl_{LH1}$/RC was raised by about 17% when compared to the *pufX*$^+$ control (Table 1). Thus, the results obtained in this genetic background clearly confirm that *pufX* has some effect on the expression of LH1. Interestingly, the suppressor mutants Sup101/g and Sup102/g had drastically smaller values of bchl/RC and $bchl_{LH1}$/RC, indicating severe changes in the antenna structure of these photocompetent suppressor mutants (see Discussion).

To further understand the requirement of *pufX* for photosynthetic growth, the molar stoichiometries of the photosynthetic redox components were determined. Chromatophores of PUFC/g, PUFΔX/g, Sup101/g, and Sup102/g were exposed to trains of actinic flashes, and the flash-induced absorption differences were measured at selected wavelengths. We observed that all four strains contain functionally active RC, cyt b_{561} and cyt c_2. The right half of Table 1 shows the stoichiometries of the main components of the cyclic electron transfer chain, expressed as the molar ratios with the RC concentration. The molar ratios of the redox components of PUFC/g are in good agreement with previously published stoichiometries in chromatophores isolated from semiaerobically grown WT cells (Robertson et al., 1986). In the absence of *pufX*, the stoichiometries of the redox components differ slightly from those in WT. While the total Q content in PUFΔX/g was a little lower than in PUFC/g, the levels of photoreducible cyt b_{561} and of photooxidizable cyt c_2 were even increased in *pufX*$^-$ strains.

Characterization of Light-Driven ATP Synthesis in Vivo. The requirement of *pufX* for photosynthetic growth as well as for flash-induced cyclic electron transfer (Farchaus et al., 1992; Lilburn et al., 1992) suggests that the PufX protein is involved in photosynthetic energy transduction. To directly demonstrate the importance of PufX for the conversion of light into chemical energy, light-induced ATP synthesis (photophosphorylation) was investigated in PUFC and PUFΔX. In addition to these strains, the photocompetent suppressor mutants Sup101 and Sup102 (Barz & Oesterhelt, 1994) were also studied. The photophosphorylation experiments were performed with intact cells since chromatophore vesicles of PUFΔX, Sup101, and Sup102 were found to be unsealed (see below). As shown in Figure 4, PufX is clearly required for light-driven ATP synthesis. While a significant light-induced increase of ATP concentration was measured in PUFC at an irradiance of 500 W/m^2, photophosphorylation was not carried out by PUFΔX (Figure 4A,B). In the *pufX*$^-$ strain, a small increase of ATP concentration was observed only during the first illumination period but not during a second illumination (Figure 4B). Even when the irradiance was increased to 2000 W/m^2, no ATP synthesis was observed in PUFΔX (Figure 4C). Similar experiments at high irradiance with the suppressor strains Sup101 and Sup102 revealed that light-driven ATP synthesis is restored in these mutants (Figure 4 D,E). Thus, specific point mutations in the LH1 genes (carried on multicopy plasmids) can suppress the impairment of photophosphorylation caused by the deletion of *pufX*.

Flash-Induced Electrochromic Carotenoid Band Shift in Vitro. To study the previously observed (Farchaus et al., 1992; Lilburn et al., 1992) role of PufX in cyclic electron transfer in this new genetic background, the flash-induced generation of a membrane potential was analyzed spectroscopically. The electrochromic shift in the LH2 carotenoid absorption spectrum was used as an indicator of the transmembrane potential (Jackson & Dutton, 1973; Junge & Jackson, 1982). Chromatophores isolated from semiaerobically grown cells of PUFC/g, PUFΔX/g, Sup101/g and Sup102/g were subjected to single-turnover flashes under defined redox conditions. As shown in Figure 5, the rise kinetics of the carotenoid band shift was biphasic in PUFC/g. The fast phase resulting from charge separation in the RC was not resolved kinetically in this measurement. The slow phase, attributed to the turnover of the cyt bc_1 complex, was found to have a time constant ($t_{1/2}$ ≈ 1.5 ms) in agreement with published values (Jackson & Dutton, 1973; Prince & Dutton, 1977). For PUFC/g chromatophores, the kinetics of the slow phase depended on the ambient redox potential in a manner described previously (Crofts et al., 1983; Prince, 1990). In contrast, no carotenoid band shift was found for PUFΔX/g chromatophores (Figure 5). Similar traces were observed for Sup101/g (data not shown) and Sup102/g (Figure 5). Only a very small ΔA_{503} signal was observed in these three strains despite the presence of normal

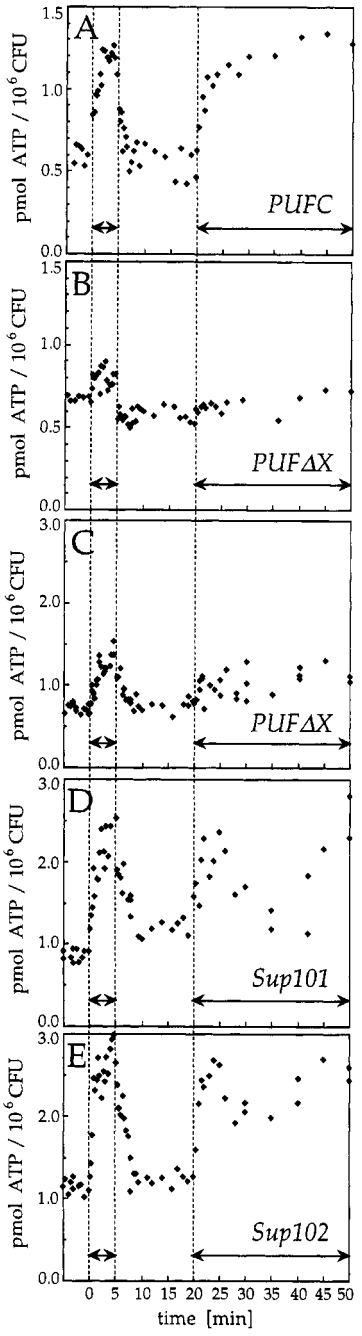

FIGURE 4: Photophosphorylation in intact cells of PUFC (A), PUFΔX (B, C), Sup101 (D) and Sup102 (E). Cell cultures were grown semiaerobically to 150 Klett units, incubated for 24 h in 20 mM sodium phosphate buffer (pH 7.4) to reduced endogenous ATP, and transferred into a anaerobic cuvette that was thermostated to 30 °C and equipped with a magnetic stirrer. After anaerobic (argon) incubation for 2 h in the dark, aliquots were taken from each cell suspension at the times indicated. Cell lysis and quantitative ATP determinations were performed as described under Materials and Methods. Agar plates spread with diluted aliquots of each cell suspension were incubated aerobically to determine the number of colony forming units (CFU). The periods of illumination are indicated by the arrows. Irradiances of 500 W/m^2 (panels A and B) or 2000 W/m^2 (panels C−E) were used.

amounts of carotenoids (data not shown). This small ΔA_{503} signal was insensitive to the ionophores valinomycin and nigericin, indicating that it was not caused by a membrane potential but by RC photooxidation (Bowyer, 1979). Since the primary photochemistry of the RC was not impaired in the absence of PufX (see below), the lack of the carotenoid

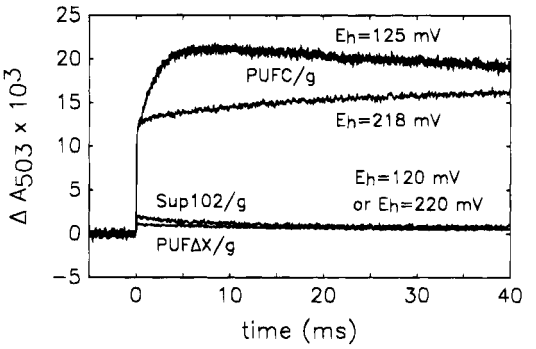

FIGURE 5: Kinetics of the electrochromic carotenoid band shift induced by a single-turnover flash in redox-poised chromatophores of PUFC/g, PUFΔX/g, and Sup102/g. The assay conditions are described under Materials and Methods. After flash excitation (at time zero), absorption changes were measured at 503 nm. Traces are the average of eight measurements and are normalized to the same concentration of total photooxidizable reaction center (0.17 μM). The instrument response time was 50 μs.

band shift suggests that the chromatophores isolated from mutants lacking *pufX* were unsealed vesicles (see Discussion). This conclusion was supported by the results of the following experiment.

Chromatophores Vesicles Isolated from Mutants Lacking PufX Are Not Sealed. Using a train of 20 saturating flashes, the amount of photooxidizable total cyt *c* (cyt c_1 + c_2) was recorded in chromatophores. As shown in Figure 6A (upper trace), the oxidation of cyt *c* in PUFC/g chromatophores is saturated by multiple flashes. To study the permeability of chromatophore vesicles, the photooxidation of cyt *c* was studied after the addition of mammalian cyt *c*. As shown in the lower trace of Figure 6A, the addition of exogenous cyt *c* to PUFC/g chromatophores did not markedly change the extent of flash-induced cyt *c* oxidation (less than 20% stimulation). Thus the majority of RC complexes of this strain could interact only with the cyt c_2 that was trapped inside sealed chromatophores vesicles and not with external cyt *c*. In chromatophores isolated from PUFΔX/g or Sup102/g, the photooxidation of cyt *c* was much slower than in PUFC/g chromatophores (Figure 6B,C, upper traces). In these membrane preparations, the amount of photooxidizable cyt (c_1 + c_2) was significantly larger and saturation was not so evident. The addition of exogenous cyt *c* to these chromatophores resulted in a considerably increased rate and extent of cyt *c* oxidation following the first three or four flashes. Further cyt *c* oxidation persisted after the fifteenth flash (lower traces in panels B and C), when essentially all endogenous cyt *c* molecules had been fully oxidized (upper traces). Very similar data were obtained when either freshly prepared or frozen vesicles were used, thus demonstrating that the membrane integrity is not affected by freezing and thawing of the chromatophores. These data show that most, or all, RC molecules of PUFΔX/g or Sup102/g chromatophores were accessible to cyt *c* that had been added to the external bulk phase. This observation could be interpreted in several alternative ways (see Discussion). As the most convincing interpretation, we conclude that unsealed vesicles were formed in the absence of the PufX protein. For this reason, the role of PufX in generating a membrane potential could not be studied *in vitro*.

Characterization of Single-Turnover Electron Transport in Vivo. Since semiaerobic growth competence in the absence of *pufX* is clearly a proof of coupled membranes *in*

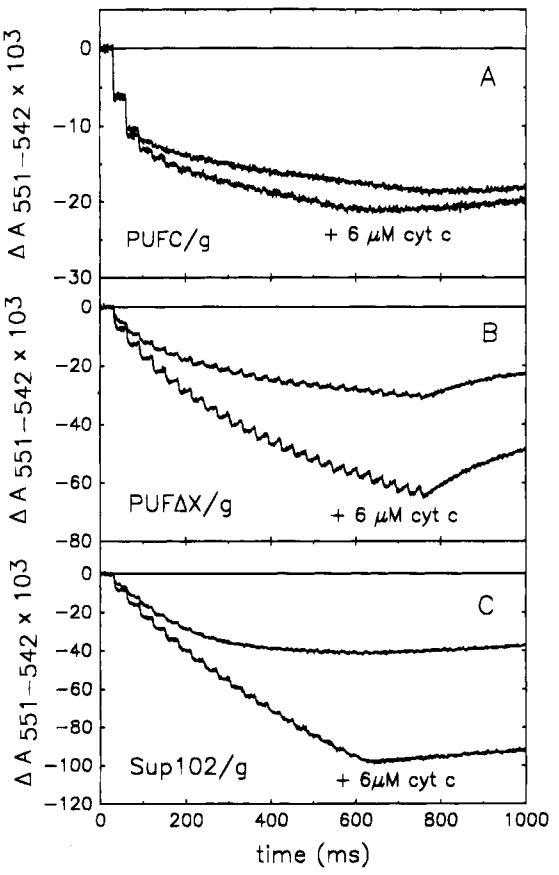

FIGURE 6: Oxidation of exogenous cytochrome *c* by chromatophores isolated from (A) PUFC/g, (B) PUFΔX/g, or (C) Sup102/g. Chromatophores were suspended to a final RC concentration of approximately 0.5 μM in 100 mM KCl, 50 mM MOPS (pH 7.0) containing 10 μM valinomycin, 5 μM antimycin A, 1 mM ascorbate, and 1 mM KCN. The samples were subjected to 20 (A, B) or 25 (C) single-turnover flashes fired 30 ms apart. Flash-induced absorption changes due to the oxidation of cyt *c* were measured at 551 and 542 nm. In each panel, the upper trace shows oxidation of the endogenous cyt ($c_1 + c_2$); the lower traces were obtained after addition of 6 μM horse heart cyt *c*. The traces are averages of four measurements, and the instrument response time was 500 μs.

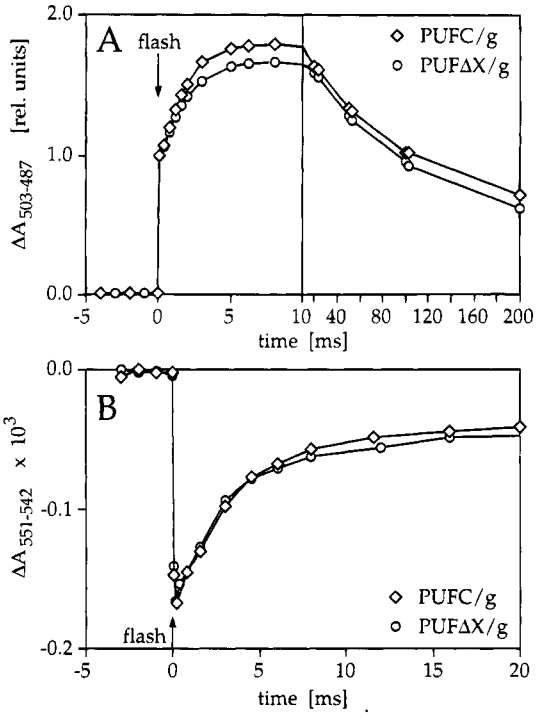

FIGURE 7: Flash-induced absorption changes in anaerobic cells of PUFC/g and PUFΔX/g. (A) The electrochromic carotenoid band shift following a single-turnover flash (indicated by the arrow) was measured at 503−487 nm and normalized to the signal observed 20 μs after the flash excitation. (B) Flash-induced absorption changes due to cyt *c* oxidation were determined at 551−542 nm using cell suspesions of identical cell density.

vivo, semiaerobically grown cells were used to study the role of PufX in cyclic electron transfer. After single turnover flash excitation, the electrochromic carotenoid band shift was detected under anaerobic conditions. Figure 7A shows the rise and decay kinetics of the $\Delta A_{503-487}$ signals obtained with PUFC/g and PUFΔX/g cells. In order to facilitate comparison of the kinetics, the traces in Figure 7A were normalized to the fast phase of the carotenoid band shift that resulted from charge separation in the RC. The rationale for this normalization was that the cellular concentration of photoactive RC was independent of PufX [see Figure 3 and Farchaus et al. (1990)] whereas the levels of LH2 carotenoids (which cause the carotenoid band shift; Holmes et al., 1980) were not.

For PUFC/g, the flash-induced carotenoid signal observed *in vivo* (Figure 7A) was similar to the trace measured *in vitro* at $E_h \approx 125$ mV (Figure 5). Therefore, the anaerobic conditions used to study cyclic electron flow *in vivo* seemed to be characterized by an at least partially reduced quinone pool. In PUFC/g, the time constant of the slow phase ($t_{1/2} \approx 1.1$ ms) was similar to published values (Cotton & Jackson, 1982). In anaerobically incubated PUFΔX/g cells, both the fast and the slow phases ($t_{1/2} \approx 1.2$ ms) of the carotenoid band shift were observed, demonstrating that PufX was not required *in vivo* for turnover of the cyt bc_1 complex following flash-induced charge separation in the RC. The decay rates of the carotenoid band shift ($t_{1/2} = 120 \pm 10$ ms) were also unchanged in the absence of PufX (Figure 7A), excluding the possibility of a general nonspecific leakiness of the membrane in PUFΔX/g. When the flash-induced carotenoid band shift was studied using anaerobically incubated cells of Sup101/g or Sup102/g, similar traces were observed (data not shown).

The observation that single-flash excitation resulted in normal cyclic electron transfer in anaerobically incubated *pufX*$^-$ cells was confirmed when the photooxidation and rereduction of cyt *c* was studied *in vivo* (Figure 7B). Flash-induced redox reactions of total cyt *c* (cyt $c_1 + c_2$) were recorded by measuring absorption changes at 551 and 542 nm (Prince & Dutton, 1977, 1978). As shown in Figure 7B, the amplitude of cyt *c* oxidation (normalized to cell density) was very similar for PUFC/g and PUFΔX/g cultures, demonstrating that PufX is not required for single-turnover oxidation of cyt *c in vivo* (data not shown). Similar results were obtained by monitoring the rereduction of photooxidized RC *in vivo* at 542 nm (data not shown). Figure 7B also shows that the rereduction kinetics of cyt *c* were similar in PUFC/g and PUFΔX/g, confirming the interpretation that the turnover of the cyt bc_1 complex was normal under the anaerobic conditions used here. In conclusion, the results shown in Figure 7 demonstrated that PufX was not required for a single turnover of the RC and the cyt bc_1 complex under anaerobic conditions.

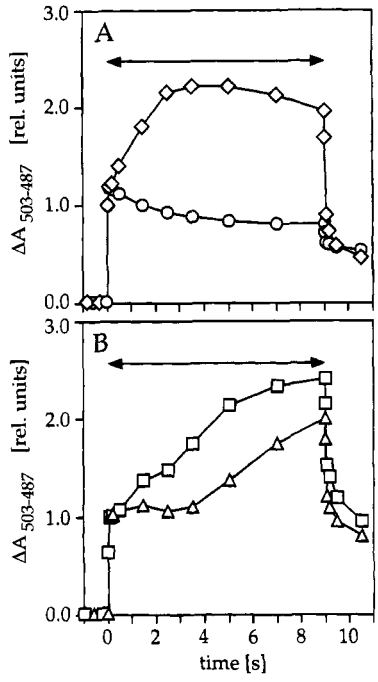

FIGURE 8: Electrochromic carotenoid band shift induced *in vivo* by continuous illumination (indicated by the double-headed arrows). Saturating light (50 W/m^2) was used to excite anaerobic cells of PUFC/g (◇), PUFΔX/g (○), Sup101/g (□) and Sup102/g (△). Light-induced absorption changes were measured at 503−487 nm and are normalized as in Figure 7A.

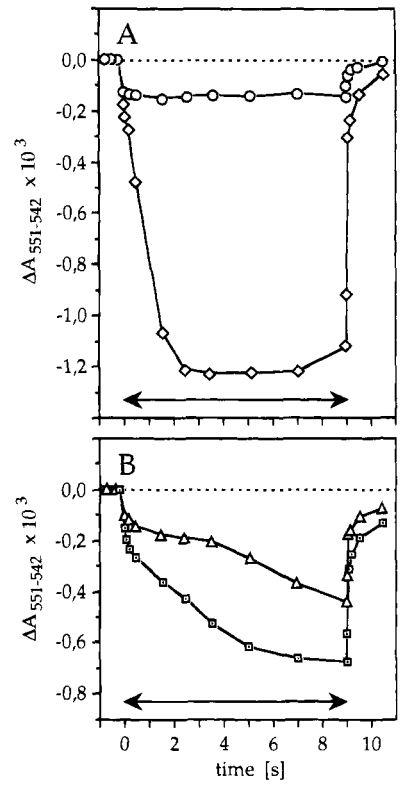

FIGURE 9: Light-induced oxidation of cyt *c* in anaerobic cells of PUFC/g (◇), PUFΔX/g (○), Sup101/g (⊡), and Sup102/g (△). Saturating light (50 W/m^2) was used for cell excitation (indicated by the double-headed arrows). Absorption changes were determined at 551−542 nm.

Cyclic Electron Transfer Induced by Continuous Illumination in Vivo. *In vivo* excitation with one single turnover flash did not reveal an essential role of PufX in cyclic electron transfer. However, flash excitation does not correspond to the continuous illumination used to study photosynthetic growth or photophosphorylation. To determine the efficiency of cyclic electron transfer under physiological conditions, the light-induced generation of a membrane potential was measured using continuous light. Figure 8 shows the electrochromic carotenoid band shift induced *in vivo* by a period of saturating illumination. In PUFC/g cells, there was a fast rise of the $\Delta A_{503-487}$ signal (first data point 20 ms after the onset of illumination) followed by a slower increase for about 2 s (Figure 8A).

A drastically different time course was observed during continuous illumination of cells lacking *pufX* (Figure 8A). After a fast rise of the $\Delta A_{503-487}$ signal (t < 20 ms), no further increase of the carotenoid band shift of PUFΔX/g was found at any light intensity used. Interestingly, this signal closely resembled the trace observed during very weak illumination (0.5 W/m^2) of PUFC/g (data not shown). This very low irradiance does not allow photosynthetic growth of PUFC/g (see Figure 2), demonstrating that the light-induced membrane potential observed during strong illumination of PUFΔX/g is not sufficient to sustain photosynthetic growth. In the suppressor mutants Sup101/g and Sup102/g, the light-induced generation of the membrane potential was partially restored (Figure 8B). Interestingly, the rate of the slow increase of the $\Delta A_{503-487}$ signal correlated with the photosynthetic growth rates of the four strains investigated (see Figure 2).

Similar results were obtained when the light-induced oxidation of cyt *c* was studied during prolonged illumination. Figure 9A demonstrates that the level of steady-state photooxidation (e.g., the ratio of light-induced oxidation and rereduction) of cyt *c* was drastically reduced in the absence of PufX. Contrary to PUFC/g, only a small $\Delta A_{551-542}$ signal was observed in PUFΔX/g cells (induced within 20 ms). In the *pufX*$^-$ strain, the steady-state level of cyt *c* photooxidation was not further increased during prolonged illumination, whereas in PUFC/g cells a much larger signal was built in the second time range. As observed for the carotenoid band shift, the steady-state levels of cyt *c* photooxidation were partially restored in the suppressor strains Sup101/g and Sup102/g (Figure 9B).

A possible explanation for the low levels of cyt *c* photooxidation in the absence of *pufX* could be that cyt c_2 is already oxidized in dark-adapted PUFΔX/g cells. To estimate the amounts and redox state of cyt *c*, intact cells of PUFC/g, PUFΔX/g, Sup101/g, and Sup102/g were treated with ferricyanide or dithionite. Optical difference spectra revealed that all four strains contained similar amounts of cyt *c* (λ_{max} = 551 nm) and cyt *b* (λ_{max} = 561 nm). Furthermore, the addition of dithionite to untreated cells did not change the intensity of the cyt *c* absorption band of each strain, thus excluding the possibility that cyt c_2 was preoxidized in dark-adapted PUFΔX/g cells.

In conclusion, these results show that PufX is involved in the light-driven generation of a membrane potential under anaerobic conditions. Although PufX is not required for flash-induced electron transport, this membrane protein is essential for efficient multiple turnover of the cyclic electron transfer chain in continuous light, consistent with the observation of a requirement of PufX for photophosphorylation.

DISCUSSION

To understand the essential role of *pufX* for photoheterotrophic growth of *R. sphaeroides*, we characterized photophosphorylation and light-driven electron flow in two genetically well-defined strains which differ only in the presence or absence of *pufX*. In addition, two photocompetent suppressor strains were studied to learn how the requirement of PufX can be suppressed by specific point mutations in *pufBA* (Barz & Oesterhelt, 1994).

The elevated levels of LH1 antennae in the absence of PufX (Farchaus et al., 1992; Westerhuis et al., 1993; McGlynn et al., 1994) and the association of PufX with solubilized RC−LH1 complexes (Farchaus et al., 1992), as well as the fact that single point mutations in *pufBA* suppress the requirement of PufX (Barz & Oesterhelt, 1994), suggested a functional correlation between PufX and the LH1 antenna complexes. In the plasmid-complemented *pufX* mutants constructed previously, copy number effects and the presence of both chromosomal and plasmid-borne copies of *pufBA* prevented a quantitative analysis of the *pufX* function (Farchaus et al., 1990, 1992; Westerhuis et al., 1993). To study the molecular role of PufX in a genetically well-defined background, we constructed two strains PUFC and PUFΔX by integrating suicide plasmids into the chromosome of the *puf* deletion strain ΔQ−X (Figure 1). Since PUFC and PUFΔX carry a single *puf* operon construct (containing or lacking *pufX*) at the correct chromosomal position, normal expression of *puf* operon genes was expected for these strains. Indeed, the photosynthetic growth rates observed for PUFC (or the green derivative PUFC/g) were identical to WT at all light intensities tested (Figure 2), whereas mutants carrying a plasmid-borne *puf* operon (containing *pufX*) have photosynthetic growth rates that are about 30% smaller (Davis et al., 1988; Farchaus & Oesterhelt, 1989). This observation demonstrates that cis-acting elements regulate the expression of the *puf* operon in *R. sphaeroides*. This should be taken into consideration when assessing growth studies in which plasmid-borne *puf* genes were used (Farchaus et al., 1992; McGlynn et al., 1994).

Like a plasmid-complemented strain lacking *pufX* (Farchaus et al., 1990), the chromosomal *pufX*$^-$ mutants PUFΔX and PUFΔX/g did not grow photosynthetically at any light intensity tested (Figure 2). This requirement of PufX for photosynthetic growth of *R. sphaeroides* was observed in succinate or malate minimal medium as well as in rich medium (data not shown). In contrast, a *pufX*$^-$ mutant of *R. capsulatus* was found to grow photosynthetically in a rich medium at high light intensity (Lilburn et al., 1992). In our study, the requirement of *pufX* for photosynthetic growth was also observed in the absence of kanamycin (data not shown), contrary to a recent report stating that *pufX* was only required in the presence of kanamycin (Kaplan & Donohue, 1993). Since the viability of *pufX*$^-$ cells is not decreasing more rapidly during illumination than in the dark, the absence of *pufX* is not lethal for illuminated cells. This observation demonstrates that PufX is not required for protecting anaerobic cells against photodamage but for photosynthetic cell division (growth). This interpretation was confirmed directly by showing that, both in the presence or absence of *pufX*, the cellular levels of photobleachable RC are constant in cells exposed to continuous illumination (Figure 3).

The lack of photosynthetic growth even at very high irradiance excludes the requirement of PufX for the capture or transfer of light energy to the RC because, under high light conditions, *R. sphaeroides* is able to grow photosynthetically in the absence of all antenna complexes (Meinhardt et al., 1985; Jones et al., 1992). Recently, a spectroscopic study confirmed that the efficiency of energy transfer to the RC was independent of PufX (Westerhuis et al., 1993). In addition to this functional connection between antennae and RC, the LH1 complexes are structurally connected to the RC, since solubilized RC−LH1 complexes could be isolated in the absence of PufX (see accompanying article). Thus, PufX is not required for the stable association of LH1 antennae complexes with the RC.

The use of the new chromosomal *pufX* deletion strain in the present study has allowed for the first time to precisely determine the effect of deletion of *pufX* on the size of the RC−LH1 complex. When the stoichiometry of the various components of the photosynthetic apparatus was determined in the new mutants (Table 1), we observed a slight increase of $bchl_{LH1}$/RC (about 17%) upon deletion of *pufX*. This small increase in the size of the photosynthetic unit is drastically different from the large increase (120%) observed in plasmid-complemented strains, where $bchl_{LH1}$/RC values of 76 and 167 were observed (Westerhuis et al., 1993). The fact that deletion of *pufX* increases the size of the photosynthetic core complex much less than the presence of plasmid-borne *puf* genes (Westerhuis et al., 1993) suggests that this slightly increased core size is not the direct cause of the photosynthetic deficiency. Similarly, the molar stoichiometries of the main photosynthetic redox components (RC, Q, cyt b_{561}, cyt c_2) differed slightly in the presence or absence of PufX (Table 1), but did not explain the requirement of PufX for photosynthetic growth. Most of these stoichiometries were in good agreement with previous results that had been obtained using chromatophores from semiaerobically grown cells of *R. capsulatus* (Robertson et al., 1986). Only the molar ratio cyt b_{561}/RC was smaller in our study (0.7−1.3) than the value of 2.7 determined before (Robertson et al., 1986). This difference could possibly be explained with the high sensitivity of the expression of cyt bc_1 genes to the oxygen partial pressure and therefore to actual growth conditions.

In a plasmid-complemented *pufX*$^-$ strain, spontaneous mutations in the plasmid-borne *pufBA* genes restore the ability to grow photosynthetically in the absence of PufX (Barz & Oesterhelt, 1994). Interestingly, such photocompetent suppressor mutants could also be isolated from PUFΔX, a strain carrying no plasmid. Therefore, mutations other than plasmid-borne mutations are able to suppress the requirement of PufX for photosynthetic growth. Like in the plasmid-borne suppressor strains, photosynthetic competence might be restored in these new suppressors by spontaneous point mutations in the LH1 antenna genes. Since the molecular basis of these chromosomal mutations is not known, these suppressor strains were not further used in this study. Instead, two genetically well-defined suppressor mutants, Sup101 and Sup102, were included into the biochemical and spectroscopic analysis of the PufX function. As described before (Barz & Oesterhelt, 1994), these strains contain a mixture of chromosomal WT and plasmid-borne mutant *pufBA* genes. The plasmid pXsup101 carries a nonsense mutation in the codon of tryptophan 43 of the LH1

α-subunit, whereas a missense mutation in pXsup102 causes a tryptophan to arginine change in codon 47 of LH1-β (Barz & Oesterhelt, 1994). Although these strains carry a plasmid-borne *puf* operon (causing copy number effects and altered expression rates), they were useful tools to determine if single LH1 mutations suppress the requirement of PufX for cyclic electron transfer.

Interestingly, Sup101/g and Sup102/g have much smaller values of $bchl_{LH1}$/RC than PUFC/g and PUFΔX/g (see Table 1), indicating that the size of the photosynthetic unit is drastically reduced in these suppressor mutants. This result is in good agreement with an earlier analysis of the LH1 levels of these strains (Barz & Oesterhelt, 1994). We suggest that the decrease in the LH1 levels causes severe changes in the antenna structure of the suppressor mutants thus restoring sufficient cyclic electron transfer to allow photosynthetic growth. This interpretation is consistent with a recent study showing that PufX has no significant effect on the photosynthetic growth rates of *R. sphaeroides* strains lacking both types of antenna complexes (McGlynn et al., 1994). Apparently, PufX is only required for photosynthetic growth when normal antenna levels are present in the membrane (see accompanying article).

Photophosphorylation experiments showed that PufX is strictly required for light-driven ATP synthesis at high irradiance (Figure 4). However, in the absence of PufX, a small increase in the intracellular ATP levels was observed during the first few minutes of illumination, indicating that some initial photophosphorylation occurs before energy transduction is impaired. In the suppressor mutants Sup101 and Sup102, light-driven ATP synthesis was restored to high levels. Following a fast generation of ATP after the onset of illumination, however, a transient decrease of intracellular ATP levels was observed (Figure 4 D,E), possibly indicating an oscillatory behavior of the cellular ATP pool in these mutants.

Photophosphorylation could not be studied in fresh cells because a long starvation period was required to reduce large intracellular energy storages. In addition, high light intensities had to be used to reveal light-induced ATP synthesis because the method of acidic cell lysis and neutralization (Welsch & Smith, 1969) caused small pH fluctuations that led to some scattering of the bioluminescence data. These experimental problems could not be avoided by measuring photophosphorylation *in vitro* because *pufX*$^-$ chromatophores were deficient in generating a flash-induced membrane potential (Figure 5). The fact that exogenous cyt *c* can access the photooxidized special pair of *pufX*$^-$ chromatophores (Figure 6) suggests that membrane vesicles are unsealed in the absence of *pufX*.

In principle, an alternative explanation for these findings could be that chromatophores lacking PufX are sealed but inverted (periplasmic side facing the outside). However, such "inverted chromatophores" would generate a normal membrane potential upon flash excitation. Since essentially no carotenoid band shift was observed in the chromatophores lacking PufX (Figure 5), this interpretation can be excluded. Moreover, no carotenoid band shift could be induced in valinomycin-treated PUFΔX/g chromatophores by K^+ diffusion potentials (Junge & Jackson, 1982), in contrast to PUFC/g chromatophores (data not shown). Another possible explanation for the observed access of exogenous cyt *c* to the special pair might be that individual *pufX*$^-$ vesicles contain a mixed population of RC complexes (having different orientations with respect to the membrane). However, such a change of RC orientation would have to occur during chromatophore preparation because the RCs are normally oriented in intact *pufX*$^-$ cells. Such rearrangements in integral membrane proteins have never been demonstrated, so this interpretation seems very unlikely. We conclude that the deletion of *pufX* changes the membrane structure of *R. sphaeroides* in a way that isolated chromatophores are no longer sealed vesicles. Consistent with this interpretation, one washing step at high ionic strength (400 mM KCl) removed a much larger fraction of cyt c_2 from chromatophores of PUFΔX/g than of PUFC/g (leaving a cyt c_2/RC ratio of 0.58 for PUFΔX/g and of 0.95 for PUFC/g).

In contrast to *R. sphaeroides*, a flash-induced membrane potential could be detected in *pufX*$^-$ chromatophores from *R. capsulatus* (Lilburn et al., 1992), indicating that PufX is not required for vesicle integrity in this species. In a very recent study, it was observed that second-site suppressors (carrying nonsense mutations in the second codon of *pufA*) partially loose their ability to generate a membrane potential during preparation of chromatophores, indicating a destabilization of the photosynthetic unit in the absence of PufX (Lilburn et al., 1995). This conclusion is consistent with our findings of the role of PufX on conferring membrane integrity in *R. sphaeroides*.

Due to the fact that chromatophores lacking PufX were unsealed, informations on cyclic electron transfer were obtained *in vivo*. Following a single-turnover flash, the kinetics of electron flow through the RC and the cyt bc_1 complex were indistinguishable in the presence or absence of PufX, showing that these complexes are active under the anaerobic conditions used in this study. Similar conclusions were drawn from measurements in *R. capsulatus* chromatophores, where the individual complexes of the photosynthetic apparatus were found to function normally in the absence of PufX (Lilburn et al., 1992).

Since single-turnover studies could not reveal any role of PufX in energy transduction, multiple-turnover experiments were performed. In anaerobically incubated cells, PufX was found to be required for the efficient generation of a photosynthetic membrane potential during prolonged illumination (Figure 8). Interestingly, the absence of PufX did not change the carotenoid band shift obtained 20 ms after the onset of illumination, but prevented the generation of a large potential on a seconds time scale. The molecular nature of this slow increase of membrane potential is not known. However, this slow component of the carotenoid band shift was found to be only transient when a 60 s period of saturating light was used to excite PUFC/g cells: After rising for several seconds, the $\Delta A_{503-487}$ signal decreased to its initial value (observed 20 ms after the onset of illumination) in less than 20 s (data not shown). When the light intensity was reduced, both the extent and the generation rate of this transient membrane potential decreased (data not shown). Such a transient membrane potential has been observed previously using intact cells of *R. capsulatus* (Cotton & Jackson, 1984). In that study, the decay of carotenoid band shift after several seconds of illumination could be blocked by venturicidin, demonstrating that it was caused by increased proton flow through the F_0 subunit of the ATP synthase. As shown in another study, the membrane potential must reach a threshold to allow proton flow through

the ATP synthase and catalytic ATP synthesis (Taylor & Jackson, 1985). Thus, the slow generation of a membrane potential apparently reflects multiple-turnover cyclic electron transfer that causes the activation of ATP synthesis when the threshold of membrane potential becomes exceeded. This interpretation is confirmed by the observation that the increase in potential (above the initial value observed after 20 ms) is strictly required for photosynthetic growth competence of PUFC/g cells. At very low irradiance (0.5 W/m^2), where PUFC/g cannot grow photosynthetically (Figure 2), such a transient membrane potential is no longer observed, clearly correlating this signal to the photosynthetic growth ability.

When PUFΔX/g cells were illuminated at very high irradiance, the membrane potential did not exceed the small value that was reached within 20 ms (Figure 8). Apparently, the light-induced membrane potential is too small in these cells to activate ATP synthesis, thereby explaining the photosynthetic incompetence of strains lacking PufX. The membrane potential that was generated during prolonged illumination of Sup101/g and Sup102/g confirmed the functional correlation between the slow increase in membrane potential and the photosynthetic phenotype. These suppressor mutants partially restore both the generation rates of membrane potential and the photosynthetic growth rate; in doing so Sup101/g is much more efficient than Sup102/g (Figures 2 and 8). Similar results were obtained when the steady-state levels of cyt *c* photooxidation were studied during continuous illumination (Figure 9). Although dark-adapted *pufX*$^-$ cells contain normal amounts of reduced cyt c_2, the rate of photooxidation of cyt c_2 (compared to the rate of rereduction) is drastically lower in these cells than in the presence of *pufX*. While the deletion of *pufX* does not affect cyt *c* redox kinetics following single-flash excitation (Figure 7B), *pufX* is required for the effective photooxidation of cyt *c* during prolonged illumination.

In conclusion, these results clearly confirm our previous result that *pufX* is essential for the efficient generation of a photosynthetic membrane potential under anaerobic conditions (Farchaus et al., 1992). Although *pufX* is not required for electron transport after single-flash excitation, this membrane protein enhances the rate of multiple-turnover electron flow over relatively long time scales, as required for photosynthetic growth and photophosphorylation. However, specific mutations in the antenna structure suppress the requirement of *pufX* for photosynthetic growth of *R. sphaeroides* by somehow restoring efficient electron transfer (see accompanying paper).

The *in vivo* experiments described in this paper were performed under anaerobic conditions, where the Q pool is known to be prevalently reduced (Jones et al., 1990). In order to localize the role of *pufX* for cyclic electron transfer more precisely, we studied the redox dependence of electron flow in the absence of *pufX*. This work is described in the accompanying article.

REFERENCES

Aagaard, J., & Sistrom, W. R. (1972) *Photochem. Photobiol. 15*, 209−225.

Barz, W. P., & Oesterhelt, D. (1994) *Biochemistry 33*, 9741−9752.

Barz, W. P., Verméglio, A., Francia, F., Venturoli, G., Melandri, B. A., & Oesterhelt, D. (1995) *Biochemistry 34*, 15248−15258.

Bowyer, J. R. (1979) Ph.D. Thesis, University of Bristol, U.K.

Bowyer, J. R., Tierney, G. V., & Crofts, A. R. (1979) *FEBS Lett. 101*, 201−206.

Bowyer, J. R., Meinhardt, S. W., Tierney, G. V., & Crofts, A. R. (1981) *Biochim. Biophys. Acta 635*, 167−186.

Clayton, R. K. (1966), *Photochem. Photobiol. 5*, 669−677.

Cohen-Bazire, G., Sistrom, W. R., & Stanier, R. Y. (1957) *J. Cell. Comp. Physiol. 49*, 25−68.

Cotton, N. P. J., & Jackson, J. B. (1982) *Biochim. Biophys. Acta 679*, 138−145.

Cotton, N. P. J., & Jackson, J. B. (1984) *Biochim. Biophys. Acta 767*, 618−626.

Crofts, A. R., Meinhardt, S. W., Jones K. R., & Snozzi, M. (1983) *Biochim. Biophys. Acta 723*, 202−218.

Davis, J., Donohue, T. J., & Kaplan, S. (1988) *J. Bacteriol. 170*, 320−329.

Deisenhofer, J., & Michel, H. (1989) *EMBO J. 8*, 2149−2170.

Ditta, G., Schmidhauser, T., Yakobson, E., Lu, P., Liang, X. W., Finlay, D. R., Guiney, D., & Helinski, D. R. (1985) *Plasmid 13*, 149−153.

Dutton, P. L., Petty, K. M., Bonner, K. S., & Morse, S. D. (1975) *Biochim. Biophys. Acta 387*, 536−556.

Farchaus, J. W., & Oesterhelt, D. (1989) *EMBO J. 8*, 47−54.

Farchaus, J. W., Grünberg, H., & Oesterhelt, D. (1990) *J. Bacteriol. 172*, 977−985.

Farchaus, J. W., Barz, W. P., Grünberg, H., & Oesterhelt, D. (1992) *EMBO J. 11*, 2779−2788.

Feher, G., Allen, J. P., Okamura, M. Y., & Rees, D. C. (1989) *Nature 339*, 111−116.

Garí, E., Gibert, I., & Barbé, H. (1992) *Mol. Gen. Genet. 232*, 74−80.

Griffiths, M., & Stanier, R. Y. (1956) *J. Gen. Microbiol. 14*, 599−715.

Holmes, N. C., Hunter, C. N., Niederman, R. A., & Crofts, A. R. (1980) *FEBS Lett. 115*, 43−48.

Jackson, J. B., & Dutton, P. L. (1973) *Biochem. Biophys. Acta 325*, 102−113.

Jones, M. R., Richardson, D. J., McEvan, A. G., Ferguson, S. J., & Jackson, J. B. (1990) *Biochim. Biophys. Acta 1017*, 209−216.

Jones, M. R., Fowler, G. J. S., Gibson, L. C. D., Grief, G. G., Olsen, J. D., Crielaard, W., & Hunter, C. N. (1992) *Mol. Microbiol. 6*, 1173−1184.

Junge, W., & Jackson, J. B. (1982) in *Energy Conservation by Plants and Bacteria* (Govindjee, Ed.) Vol. 1, pp 589−646, Academic Press, New York.

Kaplan, S., & Donohue, T. J. (1993) in *The Photosynthetic Reaction Center* (Deisenhofer, J., & Norris, J. R., Eds.) Vol. 1, pp 101−132, Academic Press, San Diego.

Kiley, P. J., & Kaplan, S. (1988) *Microbiol. Rev. 52*, 50−69.

Kiley, P. J., Donohue, T. J., Havelka, W. A., & Kaplan, S. (1987) *J. Bacteriol. 169*, 742−750.

Lee, J. K., Dehoff, B. S., Donohue, T. J., Gumport, R. I., & Kaplan, S. (1989) *J. Biol. Chem. 264*, 19354−19365.

Lilburn, T. G., Haith, C. E., Prince, R. C., & Beatty, J. T. (1992) *Biochim. Biophys. Acta 1100*, 160−170.

Lilburn, T. G., Prince, R. C., & Beatty, J. T. (1995) *J. Bacteriol. 177*, 4593−4600.

McGlynn, P., Hunter, C. N., & Jones, M. R. (1994) *FEBS Lett. 349*, 349−353.

Meinhardt, S. W., Kiley, P. J., Kaplan, S., Crofts, A. R., & Harayama, S. (1985) *Arch. Biochem. Biophys. 236*, 130−139.

Monger, T. G., & Parson, W. W. (1977) *Biochim. Biophys. Acta 460*, 393−407.

Oesterhelt, D. (1982) *Methods Enzymol. 88*, 417−420.

Parson, W. (1987) in *Photosynthesis* (Amesz, J., Ed.) pp 43−61, Elsevier Science Publisher B.V., Amsterdam.

Prince, R. C. (1990) in *The Bacteria* (Krulwich, T., Ed.) Vol. 12, pp 111−149, Academic Press, Orlando, FL.

Prince, R. C., & Dutton, P. L. (1977) *Biochim. Biophys. Acta 462*, 731−747.

Prince, R. C., & Dutton, P. L. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.), pp 439−453, Plenum Press, New York.

Rees, D. C., Komiya, H., Yeates, T. O., Allen, J. P., & Feher, G. (1989) *Annu. Rev. Biochem. 58*, 607−633.

Robertson, D. E., Davidson, E., Prince, R. C., van der Berg, W. H., Marrs, B. L., & Dutton, P. L. (1986) *J. Biol. Chem. 261*, 584−591.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) in *Molecular cloning: A laboratory manual*, Cold Spring Harbor Laboratory Press, Plainview, New York.
Simon, R., Priefer, U., & Pühler, A. (1983) *Bio/Technology 1*, 784−791.
Sistrom, W. R. (1960) *J. Gen. Microbiol. 22*, 778−785.
Straley, S. C., Parson, W. W., Mauzerall, D. C, & Clayton, R. K. (1973), *Biochim. Biophys. Acta 305*, 597−609.
Sturgis, J. N., Hunter, C. N., & Niederman, R. A. (1988) *Photochem. Photobiol. 48*, 243−247.
Taylor, M. A., & Jackson, J. B. (1985) *FEBS Lett. 192*, 199−203.
Uhl, R., Meyer, B., & Desel, H. (1985) *J. Biochem. Biophys. Methods 10*, 35−48.
van Grondelle, R. (1985) *Biochim. Biophys. Acta 811*, 147−195.
Venturoli, G., Fernández-Velasco, J. G., Crofts, A. R., & Melandri, B. A. (1986) *Biochim. Biophys. Acta 851*, 340−352.
Welsch, F., & Smith, L. (1969) *Biochemistry 8*, 3403−3408.
Westerhuis, W. H. J., Farchaus, J. W., & Niederman, R. A. (1993) *Photochem. Photobiol. 58*, 460−463.
Wraight, C. A., Cogdell, R. J., & Chance, B. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) pp 471−511, Plenum Press, New York.
Youvan, D. C., Bylina, E. J., Alberti, M., Begusch, H., & Hearst, J. E. (1984) *Cell 37*, 609−619.
Zhu, Y. S., & Kaplan, S. (1985) *J. Bacteriol. 162*, 925−932.
Zhu, Y. S., Kiley, P. J., Donohue, T. J., & Kaplan, S. (1986) *J. Biol. Chem. 261*, 10366−10374.
Zuber, H., & Brunisholz, R. A. (1991) in *The Chlorophylls* (Scheer, H., Ed.) pp 627−703, CRC Press, Boca Raton, FL.

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