Photosynthetic Deficiency of a *pufX* Deletion Mutant of *Rhodobacter sphaeroides* Is Suppressed by Point Mutations in the Light-Harvesting Complex Genes *pufB* or *pufA*†

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ABSTRACT: The *pufX* gene of the facultative phototroph *Rhodobacter sphaeroides* encodes a membrane protein that is required for photoheterotrophic growth. Deletion of *pufX* impairs the photosynthetic generation of a transmembrane potential, suggesting a role for the PufX protein in light-driven cyclic electron transfer [Farchaus, J. W., et al. (1992) *EMBO J. 11*, 2779–2788]. Here we describe the isolation and characterization of 65 spontaneous suppressor mutants in which photosynthetic competence was restored by secondary mutations. Genetic analysis revealed the occurrence of single point mutations altering highly conserved residues within the light-harvesting complex, B875. One of three tryptophan codons was changed to stop or arginine codons in 89% of these suppressor mutants. Spectral characterization and Western blot analysis were used to examine the B875 assembly and the stable expression of the altered light-harvesting polypeptides. Three different groups of suppressor mutants were found: (1) No stable expression of altered B875 polypeptides was detected for the α43W→* and β44W→* mutants. (2) There was expression of the mutated B875-β chain, but no stable B875 assembly in the β47W→R mutants. (3) Intact B875 complexes were found for the α47S→F or β20H→R mutants. These results provide evidence that the differently altered B875 polypeptides do not substitute directly for the PufX protein but lead to structural rearrangements in the macromolecular membrane organization, thus restoring a sufficiently high capacity for light-driven cyclic electron transfer.

Photosynthetic organisms have the ability to convert light to chemical energy by the cooperation of membrane-bound pigment–protein complexes. *Rhodobacter* (*R.*)[1] *sphaeroides* is a facultative, phototrophic, purple non-sulfur bacterium and produces an extensive system of intracytoplasmic membranes (ICMs) when grown either anaerobically in the light (photosynthetically) or chemotrophically in the dark under low oxygen partial pressure (semi-aerobically) (Kiley & 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 membrane-bound complexes form a light-driven, cyclic electron transfer pathway in the ICM: the cytochrome (cyt) bc_1 complex and the reaction center (RC) where the primary photochemistry occurs. The structure of the RC was determined at atomic resolution in *Rhodopseudomonas viridis* [review: Deisenhofer and Michel (1989)] and *R. sphaeroides* [review: Rees et al. (1989)]. Photooxidation of a bacteriochlorophyll (bchl) dimer in the RC 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 quinone molecule bound to the RC. A quinole molecule leaves the RC and enters a pool of quinone/quinole molecules in the hydrophobic part of the membrane. Cyclic electron flow is completed by the cyt bc_1 membrane complex utilizing quinole as an electron donor. The electrons are transferred to soluble 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.

Two light-harvesting complexes, B875 and B800-850, serve as antennae that capture and transfer light energy to the RC [review: Zuber and Brunisholz (1991)]. B875 is characterized by its room-temperature near-IR absorption maximum at 875 nm (888 nm at 77 K), while B800-850 has two maxima at 800 and 850 nm. Each of these membrane complexes contains equimolar amounts of two complex-specific polypeptide subunits, designated α and β, which exist in the ICM as aggregates of α/β-heterodimers. The B875-α/β dimer binds two bchl and two carotenoid molecules (Broglie et al., 1980), while each B800-850-α/β heterodimer contains three bchl molecules and one carotenoid (Zuber, 1985). The RC molecules are surrounded and interconnected by a fixed number of B875 complexes (stoichiometry about 15:1; Monger & Parson, 1977; van Grondelle, 1985). The levels of B800-850 antennae vary with irradiance and oxygen tension during growth (Aagaard & Sistrom, 1972) and are arranged peripherally around the RC–B875 core complexes.

The genes for the RC, the light-harvesting antennae, and the cyt bc_1 complex have been cloned and sequenced [review: Kiley and Kaplan (1988)]. The genes coding for B875-β and -α (*pufB* and *pufA*) and two of the RC subunits (*pufL* and *pufM*) are located adjacently on the chromosomes of *R. capsulatus* (Youvan et al., 1984) and *R. sphaeroides* (Kiley et al., 1987). These genes are transcribed as a polycistronic

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[1] Abbreviations: bchl, bacteriochlorophyll a; CFU, colony-forming unit; cyt, cytochrome; ICM, intracytoplasmic membrane; PS+, photosynthetic; PS−, non-photosynthetic; *R.*, *Rhodobacter*; RC, reaction center; WT, wild type.

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Table 1: Strains and Plasmids Used in This Study[a]

strain or plasmid	relevant characteristics	reference
R. sphaeroides		
PUFΔLMX21	genomic deletion of *pufLMX*, insertion of Km^R gene	Farchaus & Oesterhelt, 1989
ΔQ–X	genomic deletion of *pufQBALMX*, insertion of Km^R gene	Barz et al., unpublished
Sup	spontaneous, photocompetent suppressor mutants of PUFΔLMX21 (p2T)	this study
plasmids		
pMXmut1	pMa/c (Stanssens et al., 1989) with 5.3-kb *Bam*HI–*Hin*dIII *puf* operon insert	Farchaus et al., 1990
pRKXmut2T (abbrev p2T)	pRK404 (Ditta et al., 1985) with 3.8-kb *Bam*HI–*Hin*dIII *pufQBALM* insert and a 60-bp transcription terminator sequence inserted after *pufM*	Farchaus et al., 1990
pRKXmut2XT (abbrev p2XT)	p2T with *pufX* inserted between *pufM* and transcription terminator sequence	Farchaus et al., 1992
pXsup	p2T derivatives isolated from spontaneous, photocompetent suppressor mutants of PUFΔLMX21 (p2T)	this study

[a] The suppressor mutants Sup and the corresponding plasmids pXsup were numbered with three-figure codes (i.e., Sup101 or pXsup101) and are described in detail in Table 2.

mRNA transcript, indicating that they are members of a single operon (termed the *puf* operon for photosynthetic unit, fixed) (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 this species, a 9 kDa membrane protein is expressed from *pufX* under photosynthetic or semi-aerobic conditions of growth (Farchaus et al., 1992). This gene product copurified with detergent-solubilized RC-B875 complexes, indicating a close association of the PufX protein with these core complexes (Farchaus et al., 1992). Deletion of *pufX* caused a non-photosynthetic (PS^-) phenotype, despite the fact that *puf* operon expression and RC photoactivity were unaffected in the absence of *pufX* (Farchaus et al., 1990). The impairment of light-driven cyclic electron transfer was observed in a mutant lacking *pufX*, suggesting that PufX was required for the generation of a membrane proton gradient upon illumination (Farchaus et al., 1992). The precise role of the PufX gene product in cyclic electron transfer has not yet been determined. Spontaneous suppressor mutants of a *pufX* deletion mutant have been observed in *R. sphaeroides* (Farchaus et al., 1992) and *R. capsulatus* (Lilburn & Beatty, 1992). In these strains, photosynthetic competence seemed to be restored by secondary mutations. To further understand the role in *pufX* for cyclic electron transfer, we investigated the molecular basis of suppression by studying which secondary genes can substitute for *pufX* after they have undergone a genetic alteration.

In this study, we report the isolation and the characterization of 65 spontaneous suppressor mutants from the *R. sphaeroides* *pufX*-minus strain PUFΔLMX21 (p2T) (Farchaus et al., 1990; see Table 1 for the abbreviation of the plasmid name). Detailed genetic analysis revealed the occurrence of single point mutations in *pufA* or *pufB*, leading to the alteration of highly conserved tryptophan residues in most of the suppressor mutants. We show that these tryptophan mutations cause severe alterations in the structure and function of the B875 antennae. Evidence is provided that suppression is caused by rearrangement of the macromolecular complex organization around the RC.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *R. sphaeroides* cultures were grown semi-aerobically 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. 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 with a Klett-Summerson colorimeter (No. 66 filter) as described previously (Farchaus et al., 1992). 1 Klett unit corresponds to approximately 10^7 CFU/mL. Kanamycin (25 μg/mL) was added to *R. sphaeroides* cultures as was tetracycline (2 μg/mL) when a strain harbored a derivative of pRK404 (Ditta et al., 1985). However, tetracycline was not used for photosynthetic growth to avoid the generation of growth-inhibitory products (Martin et al., 1987).

Escherichia (*E.*) *coli* strains were grown in LB medium (Sambrook et al., 1989) at 37 °C. Ampicillin (100 μg/mL) or tetracycline (10 μg/mL) was 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).

DNA Sequence Analysis and Mutagenesis. Standard molecular biological techniques such as DNA preparation, restriction enzyme digestion, and *E. coli* transformation were performed as described previously (Sambrook et al., 1989). Oligonucleotides were synthesized by the phosphoramidite method on an Applied Biosystems Model 381A DNA synthesizer. DNA dideoxy sequencing was performed using an Applied Biosystems Model 373A DNA sequencer and a Taq DyeDeoxy Terminator Cycle Sequencing Kit as suggested by the manufacturer (Applied Biosystems Inc., Weiterstadt, FRG). For sequence analysis of *pufA* and *pufB*, 1000-bp fragments were amplified from each pXsup plasmid by PCR. These templates (300 ng) were used for thermocyclic sequencing with three forward and two backward primers, respectively.

Plasmids containing suppressor mutations identical to those of pXsup101, -102, or -106 were detected by colony hybridization (Grunstein & Hogness, 1975) of the various *E. coli* S17-1 (pXsup) strains. The oligonucleotides 5′-CTACAACTGACTGGAAATC-3′, 5′-GGCGTCCGCGGTTCTGA-3′, 5′-CTACAACTAGCTGGAAATC-3′ (complementary to the

mutated regions in pXsup101, -102, and -106), 5′-CTACAACTGGCTGGAAATC-3′, and 5′-GGCGTCCGTGGTTCTGA-3′ (complementary to the corresponding sequences in WT) were 5′-labeled with ^{32}P (Sambrook et al., 1989) and used for colony hybridization as described before (Farchaus et al., 1993). The filters were washed under increasingly stringent conditions by raising the temperature. A temperature of 47 °C allowed hybridization of the oligonucleotides with perfect complementary sequences, but not with sequences containing a single base-pair mismatch.

Site-specific mutagenesis was performed by using recombinant PCR (Higuchi et al., 1988). In the first step of this procedure, a mismatch primer led to the introduction of a specific mutation close to one end of a PCR product. In a second PCR reaction, two separate PCR fragments that overlapped with their mutated ends were combined and reamplified, yielding one longer product that then contained an internal point mutation. After each PCR reaction, excess primers were removed by Centricon 100 (Amicon, Witten, FRG) ultrafiltration as described by Higuchi et al. (1988). To prevent unwanted mutagenesis through misincorporations by the Taq DNA polymerase, we used Vent DNA polymerase (New England Biolabs, Beverly, MA). This thermostable DNA polymerase possesses a 3′→5′ proofreading activity, resulting in a higher fidelity of base incorporation during PCR (Mattila et al., 1991). The following mismatch primers were used: 5′-GAGATTTCCAGTCAGTTGTAGCTGGGGG-3′, 5′-CCCCCAGCTACAACTGACTGGAAATCTC-3′, 5′-CTCCTCAGAACCGCGGACGCCAGA-3′, 5′-TCTGGCGTCCGCGGTTCTGAGGAG-3′, 5′-CTCCTCAGAACCCCGGACGCCAGA-3′, and 5′-TCTGGCGTCCGGGGTTCTGAGGAG-3′. The PCR-generated fragments were digested using *Ase*I and *Pvu*II. The resulting 839-bp inserts were cloned into pMXmut1 (Farchaus et al., 1990; see Table 1), a derivative of the phasmid pMa/c (Stanssens et al., 1989). Subcloning of the resulting modified *puf* operon fragments into p2T (see Table 1) using *Bam*HI and *Hin*dIII yielded pRKα43*, pRKβ47R, and pRKβ47G.

For the construction of the *puf* deletion strain ΔQ–X, an *Nru*I restriction site was introduced by two base-pair substitutions in the first two codons of the *pufQ* gene (ATGAGC→ATTCGC). Since the stop codon of the *bchA* gene immediately upstream of *pufQ* (Hunter et al., 1991) was destructed by these mutations, an additional CAA→TAA mutation was introduced in the terminal glutamine codon of *bchA*. A recombinant PCR product (1.3 kb) obtained with the primers 5′-TCGAAGACGAAAGGGCA-3′ and 5′-GCATGATCGCGAATTAGTTCTCTCCCTTCC-3′ was cloned into the *Bam*HI–*Nru*I fragment (4.6 kb) of pMXmut1 (Farchaus et al., 1990; see Table 1). The resulting plasmid was designated pMa2.0. A kanamycin resistance (Km^R) gene was isolated from the transposon Tn5 (Jorgensen et al., 1979) with *Bam*HI and *Hin*dIII. The restriction ends of the resulting 1.9-kb fragment were converted to blunt ends using the Klenow fragment of DNA polymerase I (Sambrook et al., 1989). Cloning of this Km^R gene into the recombinant *Nru*I site of pMa2.0 yielded a plasmid in which the Km^R gene was flanked by the sequences originally located upstream and downstream of the *puf* operon. *Bam*HI and *Hin*dIII were used to subclone this interposon construct into the suicide vector pSUP202 (Simon et al., 1983). After conjugation of the resulting plasmid into *R. sphaeroides* WT cells, exconjugants with a stable Km^RTc^S PS^- phenotype were selected as described previously (Farchaus & Oesterhelt, 1989). Southern blot analysis confirmed that the Km^R interposon had replaced the *puf* operon genes by a double-crossover event. The *puf* deletion strain was designated ΔQ–X.

Membrane Isolation and Analysis. For the isolation of ICM, *R. sphaeroides* was grown semi-aerobically in 1-L cultures and harvested at 100–110 Klett units by centrifugation (30 min, 4 °C, 9000*g*). The cell pellet was resuspended in 5 mL of 20 mM Tris (pH 7.5). The subsequent French press and centrifugation steps were performed as described by Bowyer et al. (1979). For Western blot analysis of lysed *R. sphaeroides* cells, the French press lysate was centrifuged (30 min, 4 °C, 12000*g*) to remove cell debris and unbroken cells. The bchl contents of ICM preparations were determined after extraction into acetone–methanol (7:2, v/v) (Clayton, 1966). Protein concentration was measured with the bicoinic acid method. For the determination of ICM proteins, an aliquot of ICM was extracted with acetone–methanol (7:2, v/v). After centrifugation, the protein pellet was dissolved in 0.1 M NaOH–1% SDS, and the protein content was determined using BCA Protein Assay Reagent, as described by the manufacturerer (Pierce, Rockford, IL). Bovine serum albumin was used as a standard.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using the Tricine buffer system for small polypeptides as described by Schägger and von Jagow (1987). We used a 15.5% acrylamide–1% bisacrylamide gel containing 13% (w/v) glycerol. Before electrophoresis, the protein samples were denatured at 40 °C in 2% SDS, 50 mM dithioerythritol, 50 mM $NaCO_3$, and 12% sucrose for 30 min. Low molecular weight markers were used (Sigma Chemical Co., Munich, FRG).

Protein transfer to nitrocellulose membranes for Western blot analyses was carried out in 0.2 M glycine, 25 mM Tris (pH 7.5), 0.02% (w/v) SDS, and 20% (v/v) methanol at 1 mA/cm^2 using a semidry blot apparatus (LKB, Piscataway, NJ).

Immunodetection was performed using an ECL Western blotting system as suggested by the manufacturerer (Amersham, Braunschweig, FRG). The anti-B875 primary antibody (kindly provided by J. Takemoto; Takemoto et al., 1987) was used at a final dilution of 1:2000. Goat anti-rabbit antibody coupled with alkaline phosphatase (Sigma Chemical Co., Munich, FRG) was used as the secondary antibody in a 1:1500 dilution.

Spectroscopy. Low-temperature (77 K) spectra of ICMs were recorded using an Aminco DW-2A spectrophotometer equipped with a liquid nitrogen Dewar flask and a Hamamadzu IR photomultiplier (Model 473). ICMs were suspended in a 50 mM MOPS–100 mM KCl buffer (pH 7.5) to a final bchl concentration of 20 μM. Glycerol was added to 45% (v/v) prior to freezing of the samples in a low-temperature cuvette (d = 0.2 cm) by immersion in liquid nitrogen. For recording the 77 K spectra with intact cells, cultures of known cell density were harvested by centrifugation. The cells were resuspended in fresh medium to a number of 10^{10} cells/mL and frozen in liquid nitrogen after adding glycerol to 50% (v/v).

Materials. DNA-modifying enzymes were obtained from Boehringer Mannheim (Mannheim, FRG) and used according to the instructions of the manufacturer. Nitrocellulose membranes (0.2 μm) for Western blots were from Schleicher and Schuell (Dassel, FRG). [α-^{32}P]dATP (800 Ci/mmol) and [γ-^{32}P]dATP (5000 Ci/mmol) were obtained from Amersham (Braunschweig, FRG). Antibiotics and all other chemicals were of analytical grade or better and purchased from local distributors.

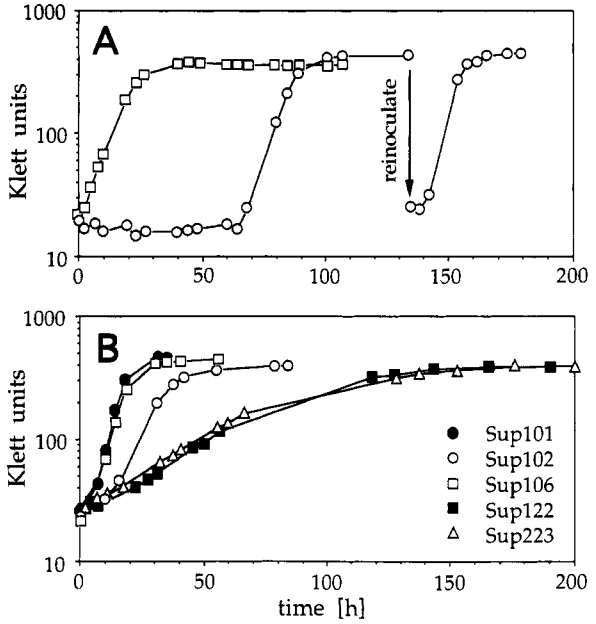

FIGURE 1: Photoheterotrophic growth curves determined for anaerobic liquid cultures inoculated from semi-aerobically grown cultures and exposed to a far-red irradiance of 100 W/m^2: (A) growth curves of PUFΔLMX21 (p2XT) (□) and PUFΔLMX21 (p2T) (○); (B) growth curves of five spontaneous mutants suppressing the photosynthetic deficiency of PUFΔLMX21 (p2T).

RESULTS

Selection of Spontaneous Suppressor Mutants. The *R. sphaeroides pufLMX* deletion strain PUFΔLMX21 (Farchaus & Oesterhelt, 1989) was complemented with p2T, a *puf* operon carrying plasmid lacking *pufX* (see Table 1). This genetic transfer was not sufficient to restore photoheterotrophic competence at low or high irradiance (Farchaus et al., 1990). The same experiment carried out with plasmid p2XT (see Table 1), which was generated by reinsertion of *pufX* into p2T, allowed photosynthetic growth, thus clearly demonstrating that *pufX* is essential (Farchaus et al., 1992).

As shown in Figure 1A, the lack of photosynthetic growth of an anaerobic liquid culture of PUFΔLMX21 (p2T) persisted for only 60–80 h. After this period, sudden growth was reproducibly observed. When these photosynthetically grown cultures were used as an inoculum, an anaerobic liquid culture exhibited no significant lag prior to the onset of growth (right half of Figure 1A), indicating that the growth under photosynthetic selection pressure was caused by cells that are genetically distinct from the initial inoculum. The alternative explanation is that the semi-aerobically grown inoculum was unable to adapt to photosynthetic conditions, which made an additional experiment necessary. Serial dilutions of a semi-aerobically grown PUFΔLMX21 (p2T) culture were spread on agar plates and incubated for 5 days under aerobic or photosynthetic (20 W/m^2) conditions, respectively. Photocompetent colonies of different sizes arose with a frequency of 10^{-6}–10^{-5}, indicating the occurrence of individual, phenotypically distinct mutants. In contrast, all cells of the photosynthetically grown up culture (end of the growth curve in Figure 1A) had a PS$^+$ phenotype when illuminated anaerobically on agar plates. These observations suggest that photosynthetic conditions select for spontaneous mutants that are able to grow photosynthetically in the absence of *pufX*.

To further examine the function of *pufX* in *R. sphaeroides*, we investigated the mutagenic events that compensate for the lack of *pufX*. Aliquots from 20 independent cultures of PUFΔLMX21 (p2T) were spread on agar plates and illuminated anaerobically for 5 days. Photocompetent colonies were obtained with a frequency of about 10^{-5}. From each plate, 3–4 colonies of different sizes were picked and streaked to purity in two steps. First, the cells were incubated aerobically in the dark, giving colonies of normal size for all mutants. After a second round of streak-purification under photosynthetic conditions, 65 of 70 mutants were found to have a PS$^+$ phenotype. Under semi-aerobic conditions, growth of these mutants was not appreciably affected when compared to PUFΔLMX21 complemented with p2T or p2XT (data not shown). Next, a colony hybridization with a 350-bp *pufX* DNA probe was performed with all 65 mutants to exclude the possibility that *pufX*-carrying contaminations had been isolated. Since *pufX* was not detected in any of the mutants (data not shown), contaminations (as well as reversions of the *pufX* genotype) could be excluded. Therefore, the photocompetent mutants carry the original *pufX* deletion and suppress the photosynthetic deficiency by additional mutations. These suppressor mutants were named Sup and numbered with a three-figure code (i.e., Sup101).

To avoid selective pressure by light, cultures of suppressor mutants were always grown semi-aerobically in the dark (with photosynthetic growth tests being the only exception). For five suppressor mutants, the photosynthetic growth rates of anaerobic cultures were determined at 100 W/m^2 after inoculation with semi-aerobically grown precultures. Figure 1B shows three distinct growth profiles, indicating that the different suppressor mutants correspond to at least three different genotypes.

Genetic Characterization of Second-Site Suppressor Mutants. The frequency of occurrence of photocompetent suppressor mutants on agar plates was 10^{-6}–10^{-5}, suggesting that spontaneous mutations might have been selected. To understand the genetic basis of suppression, we localized the mutated region in the suppressor mutants. The different suppressor mutants contained p2T-derived plasmids, called pXsup (i.e., pXsup101 in Sup101), which complement the *pufLMX* deletion strain PUFΔLMX21 (see Figure 2). To decide whether the PS$^+$ phenotype was due to a plasmid-borne mutation or to a genomic alteration (or both), all 65 suppressor plasmids were isolated, individually transformed into *E. coli* S17-1 (Simon et al., 1983), and conjugated into PUFΔLMX21, which had not been subjected to photosynthetic conditions before. The resulting PUFΔLMX21 (pXsup) strains were purified and tested for photosynthetic competence. A total of 63 of 65 pXsup plasmids (97%) was able to complement PUFΔLMX21 to a PS$^+$ phenotype, showing that mutations on these plasmids were sufficient to compensate for the lack of *pufX*. For each of the 63 complemented strains, the photosynthetic phenotype was identical to that of the original suppressor mutant (data not shown). This excluded the possibility of additional chromosomal mutations being relevant for suppression. For 2 of the 65 suppressors (3%), the p2T-derived plasmid did not confer photosynthetic activity on PUFΔLMX21, indicating that something other than plasmid-encoded mutations contributed to the recovery of photosynthetic competence in these mutants (see Discussion).

To further localize the mutations on the suppressor plasmids, the *pufBALM* insert of pXsup101 was digested into three fragments of similar size by using the restriction sites *Bam*HI, *Pst*I, *Pvu*II, and *Hin*dIII (see Figure 2B). Each of these fragments was exchanged separately with the corresponding fragment of p2T by appropriate digestions and religation. Thus, each of the three resulting plasmids contains a 1–1.5-

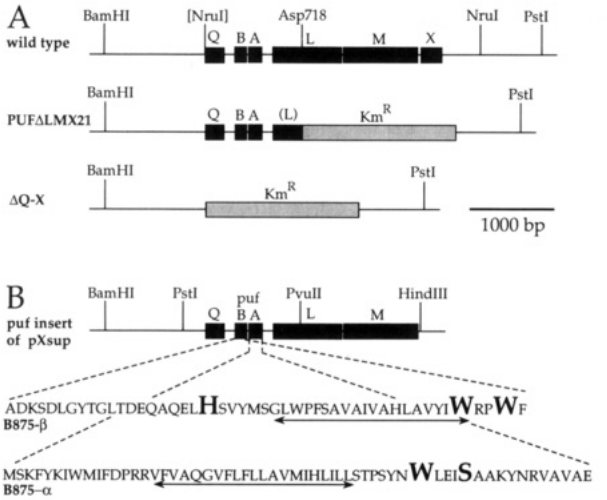

FIGURE 2: Schematic presentation of the *puf* operon of the strains used in this study. (A) Partial restriction map of the *puf* operon in the chromosomal DNA of wild type, PUFΔLMX21, and ΔQ–X. The Km^R interposon has been used to replace parts of the *puf* operon. ΔQ–X was constructed by using a recombinant *Nru*I restriction site (shown in brackets) and the *Nru*I site 3′ of the *puf* operon (see Materials and Methods). (B) Restriction map of the *puf* operon insert of the pXsup suppressor plasmids and protein sequences of the polypeptides B875-α and B875-β encoded by *pufB* and *pufA*, respectively. Arrows indicate the hydrophobic membrane-spanning domain. Single residues, which are altered in mutants suppressing the photosynthetic deficiency of a *pufX* deletion strain, are emphasized.

kb fragment originating from pXsup101, with the rest of the plasmid being identical to p2T. Control ligations without pXsup101-derived fragments did not result in plasmids that could be transformed into *E. coli*, thus confirming the exchanges of the three fragments between pXsup101 and p2T. After these plasmids were transferred to PUFΔLMX21, the photosynthetic phenotypes were tested. The 1.4-kb *Pst*I–*Pvu*II fragment carrying the genes *pufQ*, *pufB*, and *pufA* of pXsup101 was found to contain the genetic region suppressing photosynthetic deficiency. Complete sequencing of this 1.4-kb fragment revealed a single base-pair substitution in *pufA* as the only difference between pXsup101 and p2T (see Table 2). Thus, this point mutation in *pufA* was responsible for suppression. When the *pufBA* genes of pXsup102, -103, -104, -105, and -106 were sequenced, single base-pair mutations were also found. The mutation found in pXsup103, -104, and -105 was identical to the one in pXsup102; pXsup106 carried a mutation in the same codon as pXsup101 (Table 2).

To avoid DNA sequencing of those remaining plasmids that might be identical to pXsup101, -102, or -106, colony hybridizations were performed with oligonucleotides of sequences corresponding to the mutated region in pXsup101, -102, and -106. Oligonucleotides with WT sequences of the mutation sites were used as controls. Very stringent hybridization conditions were used to allow the detection of single point mutations (see Materials and Methods). A total of 53 of the 63 pXsup plasmids (84%) proved to have a mutation identical to that of either pXsup101, -102, or -106 (see Table 2). The *pufBA* genes of the remaining 10 pXsup plasmids were then sequenced. An additional seven pXsup plasmids were shown to have a single point mutation in *pufBA*, but at positions different from the ones already identified.

The suppressor mutants were picked from only 20 independent agar plates. Therefore, not all of them were expected to be genetically independent from each other. The colony hybridization revealed that in 17 cases, two colonies that had been picked from one agar plate were identical. These 17 mutants were probably duplicates of already existing suppressor mutants, thereby reducing the number of truly independent suppressor mutants to 48 (including 46 strains carrying plasmid-borne mutations).

Table 2 summarizes the mutations found in *pufBA* and lists the corresponding changes in the B875 polypeptides α and β. In 89% of the independent, plasmid-borne suppressor mutants, one of the tryptophan residues α43W, β44W, or β47W was exchanged. The aromatic residues in these positions are highly conserved in all purple bacterial B875 complexes (Zuber & Brunisholz, 1991). The codons for α43 and β44 were altered in the mutants, generally to stop codons (*) and tryptophan β47 was always changed to an arginine. Mutations of histidine β20 (to arginine) or serine α47 (to phenylalanine) were observed in only two cases and allowed only poor photosynthetic growth. The generation time could not be determined quantitatively for these two mutants (>50 h in Table 2) because further, more effective suppressor mutations occured after 60–80 h of anaerobic illumination. In three of the 63 pXsup plasmids no mutation could be identified in the *pufBA* region (including 200 bp upstream of *pufB*), showing that a mutation in another plasmid-borne gene can also cause suppression (see Discussion). Except for B875-β20H, all mutated residues are located on the carboxyl-terminal side of the B875-α and -β proteins (see Figure 2B).

It should be emphasized that only for pXsup101 was the 1.4-kb fragment carrying the suppressor mutation completely sequenced. All other pXsup plasmids had only been sequenced in the *pufBA* region. To further demonstrate that the identified mutations are sufficient for the suppression of photosynthetic deficiency, the most frequently found point mutations in *pufA* (α43*) and *pufB* (β47R) were constructed by site-specific mutagenesis (see Materials and Methods). After verification of the desired sequence in the mutagenized region, the resulting plasmids pRKα43* and pRKβ47R were introduced into PUFΔLMX21. The mutants containing the α43* or β47R mutations had the same photosynthetic phenotypes as the original suppressors Sup101 and Sup102 (data not shown). From these observations, we conclude that the point mutations analyzed were the only relevant mutations in Sup101 and Sup102 and were sufficient to compensate for the lack of *pufX*.

Transfer of Suppressor Plasmids into a Mutant Deleted for All puf Genes. The various suppressor plasmids restore the photosynthetic competence of the deletion strain PUFΔLMX21, which is deleted for *pufLMX* but still contains chromosomal copies of *pufBA* upstream of the kanamycin resistance cartridge (see Figure 2A). The merodiploid situation with plasmid-derived and chromosomal copies of *pufBA* could lead to the expression of both mutated and WT B875 polypeptides. To examine whether the presence of the mutated *pufBA* alone or the additional presence of WT *pufBA* was required for suppression, the various pXsup plasmids were tested for their ability to complement a strain deleted for the *puf* genes, including *pufBA*. We constructed ΔQ–X by replacing all of the *puf* operon genes of *R. sphaeroides* with a kanamycin resistance gene (see Figure 2A and Materials and Methods). Complementation of ΔQ–X with p2T (lacking *pufX*) and p2XT (containing *pufX*) confirmed the requirement of *pufX* for photosynthetic competence also in this genetic background.

The various pXsup plasmids were conjugated into ΔQ–X and tested for their ability to restore the PS^+ phenotype. As shown in the left half of Table 3, plasmids with missense mutations (like pXsup102) complement ΔQ–X to photosyn-

Table 2: Genetic Characterization of the pXsup Plasmids Isolated from the Suppressor Mutants[a]

suppressor strain	generation time at 100 W/m² (h)	suppressor plasmid	mutated gene	mutated amino acid of B875	mutation	percentage of occurrence
Sup101	4.0	pXsup101	*pufA*	α43	TGG→TGA W→*	33
Sup102	7.2	pXsup102	*pufB*	β47	TGG→CGG W→R	35
Sup106	4.4	pXsup106	*pufA*	α43	TGG→TAG W→*	11
Sup122	23	pXsup122	*pufB*	β44	TGG→TGA W→*	8.7
Sup133	>50	pXsup133	*pufB*	β20	CAC→CGC H→R	2.2
Sup141	>50	pXsup141	*pufA*	α47	TCT→TTT S→F	2.2
Sup223	25	pXsup223	*pufB*	β44	TGG→TAG W→*	2.2
Sup113		pXsup113				
Sup175	>50	pXsup175		no mutation in *pufBA*		6.5
Sup292		pXsup292				

[a] The generation times of the suppressor mutants (carrying chromosomal *pufBA* genes) were determined under photoheterotrophic growth conditions using an irradiance of 100 W/m². The control strain PUFΔLMX21 (p2XT) had a generation time of 4.2 h. Generation times labeled >50 h could not be determined quantitatively, but were at least 50 h. The occurrence of identical mutations among the 46 independent plasmid-borne suppressor mutations is shown on the right.

Table 3: Biochemical Characterization of the Various ΔQ–X (pXsup) Mutants[a]

pXsup	mutation	phenotype	λ_{max} of B875 (nm)	expression of B875-α	expression of B875-β
101/106	α43*	PS⁻	–	–	traces
102	β47R	PS⁺	–	traces	+
122/223	β44*	PS⁻	–	traces	–
133	β20R	PS⁺	883	+	+
141	α47F	PS⁺	880	+	+
pRKβ47G	β47G	PS⁺	879	+	+

[a] The B875 absorption spectra were measured at 77 K. At this temperature, membranes of ΔQ–X (p2T) or ΔQ–X (p2XT) have a B875 absorption peak of λ_{max} = 888 nm. The photosynthetic phenotypes were tested at low (10 W/m²) or high (100 W/m²) irradiance. The expression of B875-α/β was determined by SDS–PAGE and Western blotting using an anti-B875 antibody.

thetic competence, whereas plasmids carrying nonsense mutations do not allow photosynthetic growth of ΔQ–X at high or low irradiance. However, ΔQ–X (pXsup101) was found to contain normal amounts of photobleachable RC (data not shown), excluding the lack of functional RC as the reason for photosynthetic deficiency. Apparently, in the α43* and β44* mutants, the presence of both the WT and mutated *pufBA* copies is required for suppression.

This phenomenon was demonstrated directly by introducing two plasmids, p2T and pXsup101, into the *pufBA*-less deletion strain ΔQ–X. For this purpose, we conjugated pXsup101 into ΔQ–X (p2T). Since the recipient in this conjugation experiment was already resistant to tetracycline (due to p2T), photosynthetic conditions were used for the selection of ΔQ–X (p2T, pXsup101) cells. Photocompetent suppressor mutants can also arise from ΔQ–X (p2T) (see Discussion), and therefore, the extent of conversion to a PS⁺ phenotype had to be compared to the extent of suppression in a control experiment. Conjugation of p2XT (carrying *pufX*) into ΔQ–X (p2T) gave rise to 1.9×10^{-3} photocompetent colonies (positive control), about 100 times more than a conjugation control run in the absence of donor cells (negative control), revealing the suppression rate of 1.8×10^{-5}. The ΔQ–X (p2T, pXsup101) mutant gave rise to 1.0×10^{-3} photosynthetic colonies, demonstrating a PS⁺ phenotype in the presence of both p2T and pXsup101. Sequencing of PCR-amplified *pufA* fragments from four colonies of ΔQ–X (p2T, pXsup101) revealed a mixture of α43W and α43* codons, confirming the presence of both plasmids. This experiment clearly proves that the presence of both mutated and WT *pufA* genes is required for suppression in the α43* mutant. Similar results were obtained when p2T was conjugated into ΔQ–X (pXsup101) (data not shown). The conjugation of the *pufX*-carrying plasmid p2XT into ΔQ–X (pXsup101) also gave rise to photocompetent colonies with a frequency of about 10^{-3}, showing that suppression was not disturbed by the reinsertion of *pufX*.

Biochemical Characterization of the Suppressor Mutants. To understand the mechanism of suppression by certain point mutations in *pufBA*, we first examined the expression of B875 complexes in the suppressor mutants. Absorption spectra were measured with ICMs isolated from semi-aerobically grown cultures of five different suppressor and two control strains (Figure 3). The cultures were harvested at identical cell densities of 100 Klett units (1 Klett unit corresponds to approximately 10^7 CFU/mL) because low amplitudes of B800-850 were advantageous, allowing a clear investigation of the B875 expression in the mutants carrying *pufBA* point mutations. The amount of B800-850 is known to increase with the increasing cell density of a culture growing under oxygen-limiting conditions (Aagaard & Sistrom, 1972; Farchaus et al., 1990). Absorption spectra were measured at 77 K for better resolution of the overlapping B875/B800-850 peaks. As shown in Figure 3, B875 and B800-850 were detected in all suppressor mutants. The absorption maxima of B875 were shifted to 883 nm in Sup133 and Sup141 (compared to 888 nm in PUFΔLMX21 (p2T)).

It has been established that deletion of *pufX* changes the stoichiometries within the photosynthetic apparatus by increasing the amount of B875 and decreasing the level of B800-850 (Farchaus et al., 1990, 1992). The *pufX*⁺ and *pufX*⁻ control spectra shown in Figure 3 confirm the increase in B875 relative to B800-850 after deletion of *pufX*. In the five suppressor mutants, the stoichiometry of B875 to B800-850 was reduced to values lower than those in PUFΔLMX21 (p2XT) (see inset of Figure 3).

Since the absorption spectra in Figure 3 were normalized to total bchl content, the absolute amounts of B875 could not be identified from them. To estimate the total B875 content,

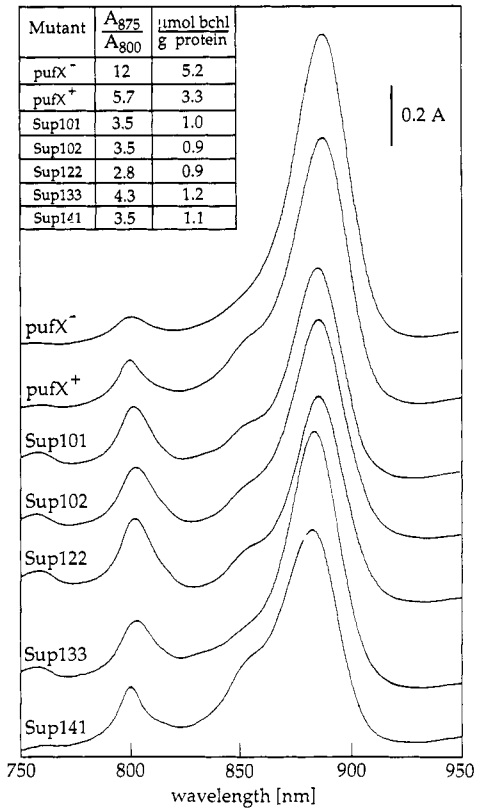

FIGURE 3: Low-temperature (77 K) absorption spectra of ICMs isolated from semi-aerobically grown cells, which were harvested at a cell density of 100–110 Klett units. ICMs were suspended to a final bchl concentration of 20 μM. Glycerol was added to 45% (v/v) prior to freezing in liquid nitrogen. The spectra were vertically displaced from each other for clarity. The inset shows the amplitudes of the various spectra at 800 and 875 nm. PUFΔLMX21 (p2T) is abbreviated *pufX*⁻; *pufX*⁺ indicates the PUFΔLMX21 (p2XT) control.

the bchl/protein stoichiometry was determined for all of the preparations (inset of Figure 3). Compared to the control membranes, the levels of bchl per protein were drastically reduced in the suppressor mutant membranes. Therefore, the amounts of B875 per membrane protein were lowered approximately 3–4-fold by the suppressor mutations. A similar reduction in the amount of B875 was observed when absorption spectra were recorded from intact cells of Sup101, Sup102, and both control strains (data not shown).

The presence of B875 in the suppressor mutants might be solely due to the chromosomal, unmutated *pufBA* genes. In order to clarify whether a functional B875 antenna could be expressed from the mutated *pufBA* genes alone, absorption spectra were measured with ICM isolated from ΔQ–X carrying the various suppressor plasmids. The ΔQ–X (pXsup) cultures were harvested at 100 Klett units, a cell density at which B875 should be clearly detectable because of low B800-850 levels (see above). As shown in Figure 4, little if any B875 could be found in those mutants that carry a mutation in one of the conserved tryptophan codons of *pufBA*. The weak absorption signal at approximately 880 nm might be caused by the reaction center special pair P865. Second-derivative analysis revealed no further absorption signals overlapping with one of the B800-850 peaks, so that a large blue shift of the B875 signal could be excluded. The suppressor plasmids pXsup133 and -141, which had been isolated only once from poorly growing colonies, lead to the expression of B875 complexes with slightly blue-shifted absorption peaks (see Table 3).

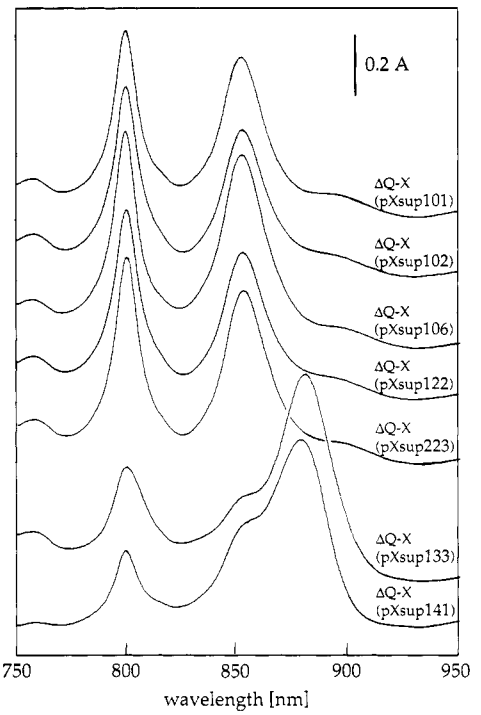

FIGURE 4: Low-temperature (77 K) absorption spectra of ICMs isolated from the various ΔQ–X (pXsup) mutants. The cells were grown semi-aerobically and harvested at a cell density of 100–110 Klett units. Other conditions were the same as for Figure 3. The spectra were vertically displaced from each other for clarity.

The absence of B875 absorption peaks demonstrated that mutations in highly conserved tryptophan codons of *pufBA* prevented the assembly of B875 complexes from their components. Alternatively, the B875 polypeptides were not stably inserted into the membrane. To decide between these alternatives, isolated ICMs were separated by SDS–PAGE. Due to interference with the detection of B875 polypeptides from highly expressed B800-850 polypeptides, the same ICM preparations (isolated from low-density cells) were used as for measuring the absorption spectra. The B875-α and -β proteins could be assigned to protein bands in the 5–10-kDa region by comparison with ΔQ–X lacking any B875 genes (Figure 5A, lane 1).

Figure 5A shows that the various ΔQ–X (pXsup) mutants express *pufBA* in different amounts. B875 polypeptides with nonsense mutations (predicted size for α43*, 5.0 kDa; for β44*, 4.7 kDa) could not be detected in the membrane (lanes 4, 6, 7, and 10). On the contrary, B875-β47R was clearly expressed in pXsup102 (lane 5) as a protein migrating slower in SDS–PAGE than B875-β47W (lanes 2 and 3). Interestingly, the second unmutated B875 polypeptide was not detectable in significant amounts in any of the tryptophan mutants. On the other hand, both B875 proteins were expressed from pXsup133 and -141 (lanes 8 and 9), although in reduced amounts compared to WT B875.

To further examine the expression of B875 proteins that were undetectable after SDS–PAGE and Coomassie (or silver) staining, Western blot analysis was performed with polyclonal anti-B875 antibodies (Takemoto et al., 1987). As shown in Figure 5B, these antibodies only weakly cross-react with B800-850 polypeptides (lane 1). Compared to membranes of ΔQ–X (p2T) or ΔQ–X (p2XT), which gave rise to intense B875-α/β bands (lanes 2 and 3), the original suppressors Sup101, -102, and -122 (containing chromosomal *puf*BA) contained smaller amounts of B875 proteins (lanes 4–6). B875-β was recognized by the anti-B875 antibodies to a smaller extent than B875-α

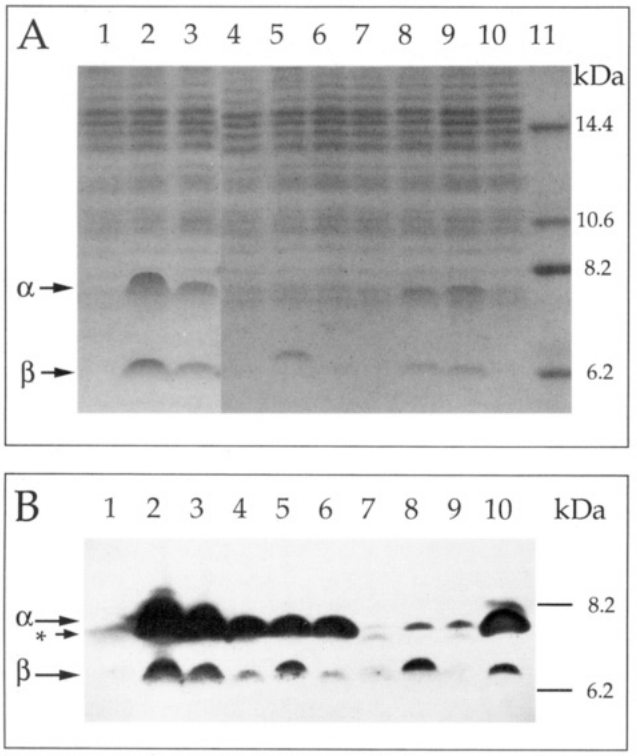

FIGURE 5: (A) Coomassie-stained SDS–PAGE of ICMs isolated from ΔQ–X (lane 1) and from ΔQ–X complemented with p2T (lane 2), p2XT (lane 3), pXsup101 (lane 4), pXsup 102 (lane 5), pXsup106 (lane 6), pXsup122 (lane 7), pXsup133 (lane 8), pXsup141 (lane 9), or pXsup223 (lane 10). Molecular weight markers were loaded in lane 11. The ICMs were from the same preparations as those used for Figure 4. For each sample, a total of 5 μg of protein was separated on an acrylamide gel using the tricine buffer system described by Schägger and von Jagow (1987). The positions of unmodified B875-α and -β polypeptides are indicated on the left. (B) Detection of B875-α and -β polypeptides in ICMs using Western blotting with anti-B875 antibodies. ICMs were isolated from ΔQ–X (lane 1), ΔQ–X (p2T) (lane 2), ΔQ–X (p2XT) (lane 3), Sup101 (lane 4), Sup102 (lane 5), Sup122 (lane 6), ΔQ–X (pXsup101) (lane 7), ΔQ–X (pXsup102) (lane 8), ΔQ–X (pXsup122) (lane 9), and ΔQ–X (pRKβ47G) (lane 10). A total of 3 μg of protein was used for each sample. The positions of unmodified B875-α and -β polypeptides are indicated on the left. The asterisk shows the position of the B800-850-α protein, which weakly cross-reacts with the anti-B875 antibodies.

(lanes 2 and 3; Takemoto et al., 1987) and could barely be detected in the suppressor mutants Sup101 or Sup122. While β47R clearly was detected in Sup102 membranes (migrating slower than β47W), no truncated polypeptides α43* or β44* could be found in ICM isolated from Sup101 or Sup122, respectively (see Discussion).

In membranes isolated from ΔQ–X (pXsup101, -102, or -122) (lanes 7–9), the weak cross-reactivity of anti-B875 antibodies with B800-850-α could be revealed. However, no B875-α43* or -β44* chains were detectable in the membrane (lane 7 and 9), showing that trunction of the carboxyl terminus impaired the membrane expression of B875-α/β. On the other hand, β47R was detected as a membrane protein (lane 8). Interestingly, unmutated B875-α was found only in trace amounts in ICM preparations (lanes 8 and 9), whereas unmutated B875-β was not detectable (lane 7). When larger amounts of membranes (6 μg of total protein) were examined by anti-B875 Western blotting, no B875-α43* or -β44* polypeptides could be detected (data not shown). However, unmutated B875-β was found in trace amounts in ΔQ–X (pXsup101). To exclude the presence of truncated α43* or β44* polypeptides in cell compartments other than ICM, anti-B875 Western blotting was also performed with the lysed cells of the corresponding mutants. Again no B875-α43* or -β44* bands could be found (data not shown).

The bchl-binding ability and membrane expression of mutated B875 polypeptides are summarized in Table 3. Three different sets of clones were obtained as suppressors of photosynthetic deficiency. In the nonsense mutants α43* and β44*, the truncated B875 proteins were not stably expressed in the membrane; as a result, no B875 absorption could be detected. β47R was stably inserted into the membrane as a mutated protein that did not bind bchl. Finally, the β20R and α47F mutants expressed B875 complexes with slightly blue-shifted absorption peaks. These observations suggest that suppression may be mechanistically different in these three suppressor classes. The very dissimilar growth rates of the α43* and β44* mutants (see Figure 1B) indicate two differently efficient subgroups of the nonsense suppressor class (see Discussion).

Specificity of the B875 Mutation Necessary for Suppression. Genetic investigation of 48 independent suppressor mutants revealed that the distribution of mutation sites in *pufBA* was highly nonrandom, with many W→* or W→R changes occuring in three tryptophan codons. Alteration of these highly conserved tryptophan residues prevents the assembly of bchl-containing B875 complexes in the membrane.

To understand the nature of *pufX*-independent photosynthetic growth, we examined whether the observed nonrandom distribution of B875 mutations was necessary for effective suppression. Alternatively, the observed specificity of mutations could be the mechanistic result of spontaneous mutagenesis events. Hypermutability of certain DNA regions, so-called "hot spots" (Benzer, 1961), in *pufBA* might exchange certain bases more frequently than others. Site-specific mutagenesis was performed to clarify which alterations of tryptophan β47 allow suppression. This residue was mutated to arginine in 35% of 46 independent suppressor plasmids (pXsup102), but not to any other amino acid that could be achieved by a single base mutation of a TGG codon (Gly, Leu, Ser, Cys, stop). The plasmid pRKβ47G was constructed by recombinant PCR (see Materials and Methods) to yield a B875-β protein with glycine at position 47 (mutation TGG→GGG). After pRKβ47G was transferred into PUFΔLMX21 and ΔQ–X, the resulting clones were found to have a PS^+ phenotype, showing that for functional suppression β47W could be altered to an amino acid other than arginine. The generation time of PUFΔLMX21 (pRKβ47G) was determined to be 8.1 h at an irradiance of 100 W/m^2.

To examine the expression of β47G, ICMs were isolated from ΔQ–X (pRKβ47G) and analyzed by Western blotting (Figure 5B, lane 10). B875-α and -β were detected in slightly lower amounts than the control ΔQ–X (p2XT) (lane 3). As shown in Figure 6, the absorption spectrum of ΔQ–X (pRKβ47G) revealed a B875 absorption peak, which was shifted to λ_{max} = 879 nm (Table 3), whereas λ_{max} = 888 nm was observed with unaltered B875 at 77 K (Farchaus et al., 1990; Westerhuis et al., 1993). Hence, the suppression of photosynthetic deficiency by mutations of highly conserved B875 tryptophans did not require the loss of bchl binding observed in α43*, β47R, and β44*. Apparently, the β47G mutant is similar to the β20R and α47F mutants, which are expressed as membrane proteins and bind bchl. Thus, the frequent and independent occurrence of β47R mutants (Sup102) must be the result of the mechanism of spontaneous mutagenesis.

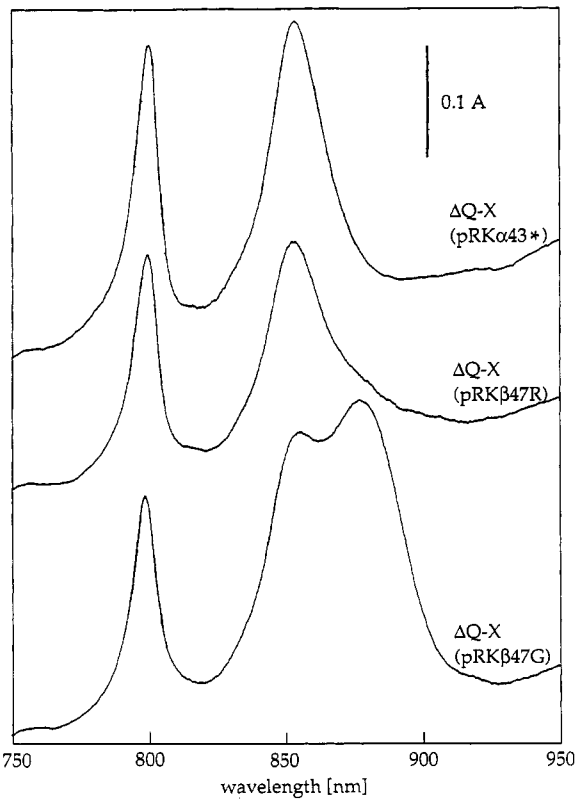

FIGURE 6: Low-temperature (77 K) absorption spectra of intact cells of the site-specific mutants ΔQ–X (pRKα43*), ΔQ–X (pRKβ47R), and ΔQ–X (pRKβ47G). Cells were grown semi-aerobically to a cell density of 110–120 Klett units, harvested by centrifugation, and resuspended to a final density of 5×10^9 cells/mL. Glycerol was added to 50% (v/v) prior to freezing in liquid nitrogen. The spectra were vertically displaced from each other for clarity.

DISCUSSION

To understand the role of *pufX* in the photoheterotrophic growth of *R. sphaeroides*, we analyzed intergenic suppressor mutations that compensate for the lack of *pufX*. The molecular basis for suppression was studied to learn which secondary genes can substitute for *pufX* after they have undergone a suitable mutation.

A large number of suppressor mutants was isolated and characterized to determine all of the mutations that allow efficient suppression. To avoid sequence analysis of identical mutants that descended from one early mutagenic event in the preculture, care was taken to isolate genetically independent suppressors. For that reason, cells originating from 20 independent clones of PUFΔLMX21 (p2T) were spread on agar plates. A total of 65 photocompetent colonies of different sizes was purified after illumination of these plates. A large number of these suppressors was expected to be genetically independent from each other. However, in 17 cases, two of the Sup101, -102, or -106 colonies that had been picked from one plate were found to be identical. Therefore, 17 of the mutants isolated might be duplicates, thereby reducing the number of truly independent clones to 48.

Most of the pXsup plasmids (97%) were found to suppress the PS^- phenotype of PUFΔLMX21. The equivalence of photosynthetic phenotypes between the original and the complemented strains showed that only plasmid-borne mutations were relevant for suppression. Subcloning, sequencing, and oligonucleotide hybridization revealed that certain mutations occurred repetitively (89%) in three tryptophan codons of *pufA* and *pufB*. The fact that only a few genotypes were found among the 48 analyzed independent phenotypes guaranteed that the suppressor mutants observed were major representatives. This observation strengthens the significance of the suppressor events in understanding the function of *pufX*. By site-specific mutagenesis, the α43* and β47R mutations identified in pXsup101 and pXsup102 (68% of 46 independent mutant plasmids) were shown to be the only mutations necessary for suppression. For the remaining pXsup plasmids, mutations other than the ones identified could not be excluded. However, the fact that a single point mutation caused suppression in 68% of the mutants made a second mutation quite unlikely (especially for Sup122, for which four independent colonies were found). The plasmids pXsup113, -175, and -292 contained unidentified mutations outside the *pufBA* region. This observation demonstrated that other plasmid-borne mutations, possibly in the RC genes *pufLM*, could compensate for the lack of *pufX*.

The tryptophan residues α43W, β44W, and β47W are located at the periplasmic end of the membrane, spanning helices of B875-α and -β, and are highly conserved among all purple bacterial B875 complexes (Zuber & Brunisholz, 1991). Interestingly, no B875 absorption signal could be detected in the mutants carrying alterations in these residues, pointing to functional significance of the conserved tryptophans for B875 assembly. Several highly conserved aromatic amino acids (α41Y, α43W, β42Y, β44W, and β47W) in the transmembrane helices of B875-α and -β are located in the vicinity of the intramembrane histidine, which most probably coordinates the bchl molecules (Zuber & Brunisholz, 1991). Therefore, these aromatic residues have been suggested to interact with the bchl molecules, thus influencing the spectral properties of the antenna complexes (Brunisholz et al., 1984; Zuber, 1993).

The suppressor mutants, as isolated in this study, have a merodiploid *pufBA* genotype because of the presence of a genomic copy of *pufBA*. For that reason, the ability of the mutated *pufBA* genes to express the α or β chains and to assemble B875 was investigated after transfer of the pXsup plasmids into ΔQ–X, a deletion strain lacking the entire *puf* operon. Absorption spectroscopy and Western blot analysis revealed three entirely different classes of suppressor mutations (see Table 3).

(1) The α43* and β44* mutants do not assemble B875 complexes in the absence of chromosomal *pufBA* genes. The truncated α or β proteins could not be detected in the ICMs or lysed cells of these mutants. It cannot be excluded that traces of α43* were expressed and migrated together with B875-β. On the other hand, β44* definitely was not expressed stably. Interestingly, the second, unmutated B875 polypeptide was found only in trace amounts in these mutants, probably because they are unable to insert into the membrane. It has been observed before that B875-α and -β proteins are not integrated independently, but interact with each other during membrane insertion (Richter & Drews, 1991).

Site-directed mutagenesis of α43W in *R. capsulatus* (Babst et al., 1991) and *R. sphaeroides* (J. D. Olsen and C. N. Hunter, unpublished data) revealed a blue shift of the B875 peak by 8–18 nm when this residue was altered to other hydrophobic amino acids. A hydrogen bond between α43W and the exiton-coupled B875 bchl dimer was found by resonance Raman spectroscopy (J. D. Olsen et al., unpublished data), directly proving a structural role for α43W in binding bchl. However, the α43 stop codon does not alter a single amino acid, but leads to the truncation of 16 carboxyl-terminal residues from B875-α. Similarly, five carboxyl-terminal residues of B875-β

were deleted by the β44W→* mutation. The lack of B875 complexes in these mutants demonstrates an important role of the missing carboxyl termini for stable membrane insertion or B875 assembly.

(2) The β47W→R missense mutant expressed a mutated β chain with reduced migration in SDS gels. Although SDS gels separate proteins mainly due to their molecular weights, the additional positive charge of β47R might be a possible explanation for the slower gel mobility. Compared to WT, the unaltered B875-α polypeptide was expressed only in trace amounts, showing that stable membrane integration of B875-α was impaired by β47R (but not by β47G; see below). As a consequence, no B875 absorption peak could be found.

(3) The suppressor mutations β20H→R and α47S→F, which allow only poor photosynthetic growth, led to the expression of B875-α and -β in smaller amounts than in WT. Histidine β20 is conserved among all purple bacterial antenna β chains, indicating the functional importance of this residue (Zuber & Brunisholz, 1991). On the other hand, α47S is the only nonconserved B875 residue that was altered in a suppressor mutant. In the absence of chromosomal *pufBA* genes, both of these mutants assemble B875 with slightly blue-shifted (5–8 nm) absorption peaks, indicating that β20H and α47S influence the spectral properties of B875. A very similar situation was found in ΔQ–X (pRKβ47G), a β47G mutant that was made by site-directed mutagenesis. In contrast to ΔQ–X (pXsup102) (carrying the β47R mutation), WT amounts of B875 polypeptides were expressed in ΔQ–X (pRKβ47G). The B875 complex was assembled and had a blue-shifted (9 nm) absorption signal, suggesting that β47W also modulates the spectral properties of B875.

In ΔQ–X (pRKβ47G), the PS^+ phenotype was restored in the presence of a B875 complex. Therefore, the lack of B875 observed in ΔQ–X (pXsup102) is not required for suppression. Suppression by both mutations, β47W→G and β47W→R, makes a direct, structural role for the β47R protein in substituting for the PufX protein unlikely. Instead, the photosynthetic competence seems to be restored by removing the conserved β47W residue. This raised the following question: why was β47W altered to β47R in 35% of the independent suppressor mutants, but not to any other amino acid that could be achieved by a single one-base mutation of a TGG codon (Gly, Leu, Ser, Cys, stop)? Since the photosynthetic growth rates of the β47R and β47G mutants were found to be comparable in a PUFΔLMX 21 background, β47G mutants should not have been lost upon spontaneous occurrence. In addition, only CGG was found as the codon at position β47, although AGG also would have resulted in β47R. Therefore, the T→C transition seems to be the preferential mutagenic event at the first base of codon β47, giving rise to the exclusive isolation of arginine mutants in this position.

A direct structural substitution for *pufX* by the various suppressor mutations is very unlikely, even for those mutants that stably express an altered B875 polypeptide. The very differently mutated α or β proteins cannot be assumed to take over the precise structural role of the PufX protein. Severe changes in the spectral properties of B875 by the observed mutations in highly conserved tryptophans point to the requirement of damaging B875. On the basis of this interpretation, one might be tempted to assume a role for *pufX* in protecting cells from photoinhibition. However, no light-induced damage of the RC was observed in cells lacking *pufX* (Barz et al., unpublished observation), excluding such a possibility.

The p2T-derived plasmids of two suppressor mutants, Sup123 and Sup254, were not sufficient to restore the photosynthetic competence of PUFΔLMX21. Spectral analysis demonstrated the lack of B800-850 complexes in Sup123 (data not shown), indicating that a genetic alteration impaired the expression of B800-850 in this mutant. We did not reveal whether the suppression in Sup123 and Sup254 was based on a single chromosomal mutation or on a more complex situation. Preliminary results indicated the occurrence of more than one mutation in Sup254.

It was surprising that 90% of all independent suppressor mutants carried a plasmid-borne *pufBA* mutation, since a similar mutation in the chromosomal *pufBA* genes should also allow suppression. However, no such mutation could be isolated (with the possible exception of Sup254). This observation can partially be explained by the copy number of the pRK404-derived plasmid (4–6 per cell; Davis et al., 1988). Another explanation might be an increased proportion of spontaneous mutations on the plasmid compared to the chromosome. However, the alternative possibility that the observed specificity of plasmid-borne *pufBA* mutations was functionally required for suppression could not be excluded.

In *R. capsulatus*, *pufX* is also required for photoheterotrophic growth (Lilburn et al., 1992). Recently, three suppressor mutants of a *pufX* deletion mutant of *R. capsulatus* were isolated and characterized (Lilburn & Beatty, 1992). Two of them include mutations in the *pufBALM*-carrying plasmid, leading to altered stoichiometries of the light-harvesting complexes. Although no sequence analysis of these mutants was presented, their high levels of B875 expression (in the absence of chromosomal *pufBA* genes) indicate that the genetic alterations in these plasmids are different from the tryptophan mutations found in *R. sphaeroides*. In a third *R. capsulatus* suppressor mutant, the plasmid did not confer photosynthetic growth on the *puf* deletion strain, suggesting a chromosomal mutation. No B800-850 antenna could be detected in this mutant, although B800-850-α and -β polypeptides were present in normal amounts. Except for its much higher photosynthetic growth rate, this mutant appeared to be similar to Sup123 in our study.

The α43* and β44* mutants are photosynthetically incompetent in the absence of WT *pufBA* genes (see Table 3), despite the presence of normal levels of photobleachable RC. Even very high irradiance did not allow photosynthetic growth of these mutants. Since the strains still contain B800-850, which is able to directly transfer excitation energy to the RC (Hess et al., 1993), energy transfer to the RC cannot be limiting in these mutants. The requirement of both WT and mutated *pufBA* genes for suppression was proven directly by introducing two plasmids, coding for α43W and α43*, into ΔQ–X. Obviously, the α43* mutation is necessary, but not sufficient, for suppression. Additional WT B875 is needed for function and can be encoded by plasmid-borne as well as by chromosomal *pufBA* genes.

Therefore, one would expect to observe α43* mutations in suppressors isolated from ΔQ–X (p2T) (lacking chromosomal *pufBA* genes). Under photosynthetic selection, a suitable mutation would lead to a mixture of plasmids coding for α43W and α43*. This was indeed observed when 30 new suppressor mutants were isolated from ΔQ–X (p2T). In the majority of the new pXsup plasmids, α43*, β47R, or β44* mutations identical to those in pXsup101, -102, or -106 were found by oligonucleotide hybridization (data not shown). While the plasmids coding for β47R restored the photosynthetic competence of ΔQ–X, introduction of plasmids isolated from an

α43* mutant into ΔQ–X did not result in a PS$^+$ phenotype, as was expected for a mixture of α43W- and α43*-encoding plasmids. Since the double-complementation experiment had shown that such a mixture of plasmids is sufficient for suppression in ΔQ–X, additional mutations acting in concert with the α43* mutation could be excluded.

No α43* or β44* polypeptides could be detected in Sup101 or Sup122, although the presence of the corresponding genes was functionally required for suppression. This suggested a similar mechanism of suppression for these nonsense mutants. However, the different growth rates observed for Sup101 and -122 have clearly shown that the α43* mutation allows a more efficient suppression than the β44* mutation. Therefore, the nonsense suppressor class can be divided into two subgroups of different efficiencies.

What is the molecular basis for suppression in the mutants carrying nonsense mutations? The lack of detectable α43* or β44* polypeptides and the additional requirement of WT B875 cause an enigmatic interpretation: One possible explanation would be that trace amounts of α43* or β44* (too little to be detected by Western blot analysis) might be expressed and exert a catalytic function in the presence of WT B875 (i.e., by interaction with B875). The B875 complex might also stabilize α43*/β44* in the membrane, so that the truncated chains are not stably inserted in the absence of WT B875. Alternatively, the expression of α43* or β44* might be undetected by Western blot analysis due to some detection artifact (i.e., weak detection of B875-β by the anti-B875 antibodies or no detection of polypeptides missing their carboxyl termini).

As another suggestion, the nonsense mutations might prevent some negative effect of WT B875 that is caused by the deletion of *pufX*. Instead of stably expressing a protein with an active role in suppression, the α43* or β44* mutants might restore photosynthetic competence by reducing the levels of B875, thereby changing its oligomeric structure. This possibility is strongly supported by the low amounts of B875 observed in the suppressor mutants. On the basis of the amount of membrane protein, the suppressors express only 20–35% of the levels of B875 found in unmutated control strains (see Figures 3 and 5). This model is supported by spectroscopic analysis of PUFΔLMX21 (p2T), which recently demonstrated a role for *pufX* in regulating both the levels and the supramolecular organization of the B875 complex (Westerhuis et al., 1993). That study suggested that the PufX protein limits the aggregation state of B875, assuring the proper functional arrangement of the RC–B875 core complex. This suggestion is in accordance with the localization of the PufX protein in the RC–B875 core complex (Farchaus et al., 1992).

The requirement of WT B875 in the α43*/β44* mutants cannot be seen in the light-harvesting function, because *R. sphaeroides* cells lacking the B875 antenna are photosynthetically viable at the irradiance used in this study (Jones et al., 1992; Hess et al., 1993). Instead, the presence of both altered and unaltered *pufBA* genes may be necessary to cooperatively change the macromolecular membrane structure around the RC in a way that restores photosynthetic competence.

What conclusion concerning the function of *pufX* can be drawn from the suppressor analysis? The absence of *pufX* causes an impairment in light-driven cyclic electron transfer, although all protein–pigment complexes required for photosynthesis are functionally assembled (Farchaus et al., 1992). A recent study on the role of *pufX* in *R. capsulatus* also found a lesion in cyclic electron transfer in the absence of *pufX* (Lilburn et al., 1992). Those data suggested an impairment of electron transfer from the RC–Q_b site to the cyt bc_1–Q_z site. A similar observation was made in *R. sphaeroides*. Preliminary measurements have shown a clear correlation between the generation of membrane potential in continuous light and the photosynthetic growth rates of WT, Sup101, Sup102, and a mutant carrying a chromosomal *pufX* deletion (Barz et al., unpublished observations). Apparently, an altered organization of the RC environment in the absence of *pufX* results in defects in the lateral, intramembrane ubiquinole diffusion.

The suppressor analysis presented here points to a central role for *pufX* in organizing the macromolecular structure of the ICM. We suggest that the various suppressor mutants structurally rearrange the RC surroundings, thus restoring a sufficiently high capacity for light-driven electron transfer. Our knowledge of all data currently available is not sufficient to explain the precise function of *pufX* in light-driven cyclic electron transfer. All results, however, support the idea that *pufX* is required for the RC–cyt bc_1 interaction by facilitating the macromolecular organization of the photosynthetic unit for efficient quinole exchange.

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