THE ROLE OF THE *PUFX* GENE PRODUCT OF *RHODOBACTER CAPSULATUS*

By

TIMOTHY GEORGE LILBURN

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Department of Microbiology

The University of British Columbia
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ABSTRACT

The 2.7 kilobase transcript of the *puf* operon of the photosynthetic bacterium *Rhodobacter capsulatus* has five open reading frames. The gene products of four of these open reading frames (*pufB, A, L,*and *M*) are well characterized as structural polypeptides of the reaction center (*pufL* and *M*) and the B870 light-harvesting antenna complex (*pufB* and *A*). The role of the *pufX* gene product has been unknown. By deleting the *pufX* gene from a plasmid carrying the *puf* operon and using this plasmid to reconstitute a strain of *R. capsulatus* which had the *puf* operon deleted, it was possible to characterize the *pufX* gene product. It was found that the *pufX*$^-$ mutant was unable to grow photosynthetically until a secondary suppressor mutation had occurred. It appeared that either more than one type of suppressor mutation could occur or that one suppressor mutation could be accompanied by further mutations. To determine the nature of the lesion caused by the deletion of *pufX*, the structure of the photosynthetic unit and the ability of the subunits of the photosynthetic unit to accomplish energy and electron transfer of the mutant were compared to a pseudo-wild type. Spectrophotometric techniques, including fluorescence detection, reduced *minus* oxidized spectra, flash-induced absorbance change spectra, and ground state absorption spectra were used for these comparisons as well as biochemical assays. The biochemical assays measured the ability of chromatophores to transfer electrons from a quinone analog to horse-heart cytochrome *c* and to pump protons in response to light irradiation. The results of these comparisons indicated that the individual components of the photosynthetic unit functioned normally but that electron transfer between these components, specifically between the reaction center and the cytochrome b/c_1 complex, was impaired. It thus seemed likely that there was some structural defect in the photosynthetic unit. The structure of the photosynthetic units of pseudo-wild type and mutant strains was probed using sodium dodecyl sulfate-

polyacrylamide gels, absorption spectroscopy, and electron microscopy. It was determined that the mutant had chromatophore vesicles that were about 50% larger than those of the pseudo-wild type and contained higher levels of reaction center and B870 light-harvesting antenna polypeptides. The suppressor mutants also had altered levels of polypeptides and showed differences in the way the expression of their B800-850 polypeptides was regulated. It was concluded that the *pufX* gene product plays a role in the correct assembly of the photosynthetic unit as a structural component of the unit and/or as a regulator of its assembly.

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INTRODUCTION

Rhodobacter capsulatus is a purple non-sulphur bacterium capable of growth by a variety of metabolic modes, including anaerobic photosynthesis and respiration in the dark (46, 23). Its metabolic diversity makes it a good organism for studying photosynthesis since mutations affecting genes whose products are essential for photosynthetic growth are not lethal, as they would be, for example, in plants. Additionally, these bacteria are much more easily manipulated experimentally than plants, the structure of their reaction centre (see below) is very well understood, and many of the genes encoding components of the photosynthetic unit have been cloned. Although the photosynthetic process in these bacteria is simpler than that found in plants, it is similar enough to shed light on this process as it occurs in all photosynthetic organisms. *R. capsulatus* is also a good organism for the study of the regulation of gene expression since different sets of genes are expressed under different growth conditions. Growth conditions are easily manipulated in the laboratory so the expression of a given set of genes can be induced or repressed and this regulation studied.

A switch from aerobic growth to photosynthetic growth in *R. capsulatus* results in the elaboration of extensive invaginations of the inner membrane (51). These invaginations contain the components of the photosynthetic apparatus. These are, principally, the light-harvesting antenna complexes, the reaction centre (RC), and the ubiquinol:cytochrome c_1 oxidoreductase complex (the cytochrome b/c_1 complex). These components include a chain of readily oxidizable and reducible cofactors that mediate the electron transfer pathway of photosynthesis. The light energy captured in photosynthesis is stored or harnessed as a proton gradient.

In photosynthesis, the capture of incident light energy is initially effected by pigment molecules. *R. capsulatus* has two main types of pigments: bacteriochlorophyll, specifically bacteriochlorophyll *a* (bchl *a*), and carotenoids. Free bchl *a* has an absorption maximum of about 770 nm. *In vivo*, the absorption maximum varies according to the environment that the molecule is found in. Thus, the wavelength of light absorbed by this pigment can be "tuned" through association with different polypeptides. There are more than one type of carotenoid in *R. capsulatus*, but in anaerobic cultures of *R. capsulatus* spheroidene is the predominant type whereas in aerobic cultures spheroidenone predominates (26). In addition to their light-harvesting function, carotenoids also protect cells against photooxidative damage from the formation of singlet-state oxygen in the presence of light and bacteriochlorophyll (40). Both types of pigment are found in the RC and in the antenna complexes, with the bulk of the pigments occurring in the antenna complexes.

There are two types of antenna complex: the B870 complex and the B800-850 complex (19). The names of the complexes refer to their major bchl *a* absorption peaks in the near-infrared region of the spectrum. The B870 complex is made up of two pigment-binding polypeptides: the α subunit and the β subunit that have apparent M_r s of 12 and 7 kilodaltons (kDa) respectively. This complex occurs in a fixed stoichiometry relative to the RC with about ten to twenty B870-associated bchl *a* molecules per RC (19). The B800-850 complex in *R. capsulatus* consists of three polypeptides: the α, β, and γ subunits. The apparent M_r s of these subunits are 10, 8, and 14 kDa respectively. The number of B800-850 complexes in the membrane relative to the RC is variable, being inversely proportional to the external light intensity and pO_2. The pigments are bound non-covalently to the α and β subunits of both types of complex. The γ subunit of the B800-850 complex does not bind pigment molecules.

laterally through the membrane from the RC to the cytochrome b/c_1 complex (10).

The cytochrome b/c_1 complex in *R. capsulatus* is made up of four polypeptides. Three of these polypeptides are redox proteins. These are a Rieske iron-sulphur protein (M_r 22.5 kDa), a *b*-type cytochrome (M_r 42.1 kDa) and a cytochrome c_1 (M_r 31.2 kDa) (37). The *b*-type cytochrome apoprotein carries two hemes known as cytochromes b_h, a high potential cytochrome, and b_l, a low potential cytochrome. The fourth protein (M_r 19.8 kDa) has no redox-involved component and no defined role (37). Quinone molecules are also associated with the cytochrome b/c_1 complex [although whether they are tightly bound to the cytochrome b/c_1 complex or exchange rapidly with the cellular pool of quinones is controversial (10, 48)] at two sites. One site, known as the quinol oxidase, or Q_z, site, is responsible for the reduction of the Reiske iron-sulphur cluster and cytochrome b_l. The second site, called the Q_c site, is responsible for oxidation of cytochrome b_h (37).

The cytochrome b/c_1 complex mediates the formation of the proton gradient by which the captured light energy is stored. Formation of the quinol at the primary acceptor site of the RC can be thought of as simultaneously resulting in the oxidation of the iron-sulphur cluster and cytochrome c_1 and reduction of cytochrome b_h of the cytochrome b/c_1 complex. When the quinol from the RC binds to the Q_z site it is presented with two oxidants: the iron-sulphur cluster and cytochrome b_l (see Fig. 1). The electrons are thought to follow the Q cycle proposed by P. Mitchell (42) within the cytochrome b/c_1 complex. One electron is immediately taken up by the iron-sulphur cluster and the second moves at the same time to cytochrome b_l. The two electrons now carried by the *b*-type cytochrome reduce a quinone at the Q_c site, with concomitant uptake of up two protons

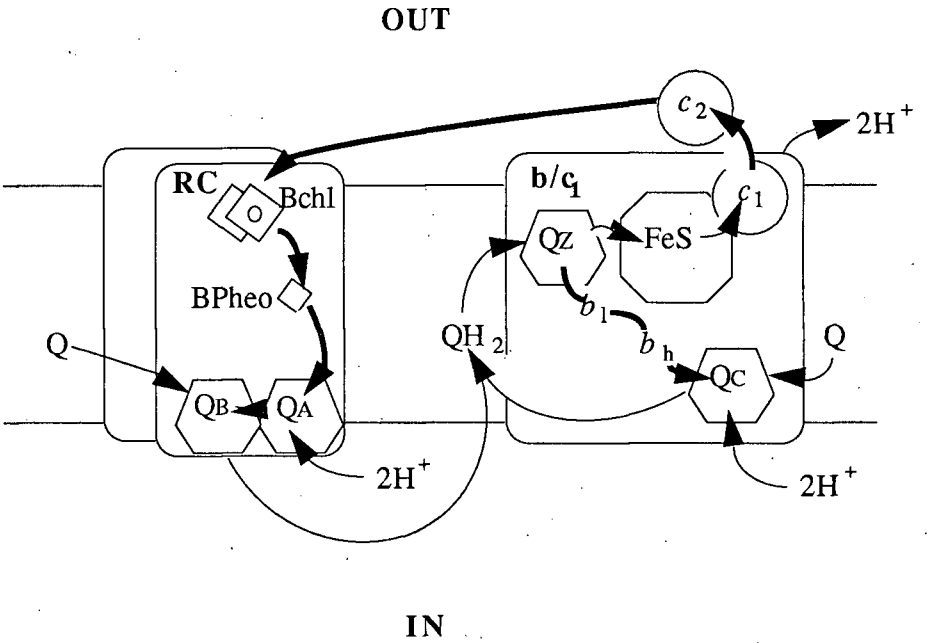


Figure 1. A simplified schematic diagram of electron and proton movement through the reaction centre and cytochrome b/c_1 complex. The heavier lines represent the electrogenic movements of electrons responsible for the carotenoid bandshift. Abbreviations: Bchl, the special pair of the reaction centre; BPheo, bacteriopheophytin; Q, ubiquinone; QH_2, ubiquinol; Q_A and Q_B, the primary electron acceptor; Qz, ubiquinol oxidase site; Qc, ubiquinone reductase site; FeS, Rieske iron-sulphur complex; c_1, cytochrome c_1 (membrane bound); c_2, cytochrome c_2 (diffusible in the periplasm); b_l, the low potential cytochrome b ; b_h, the high potential cytochrome b. For a more detailed explanation see the text.

from the cytoplasm. Now it is the turn of this quinol to move to the Q_z site, and the cytochrome b/c_1 complex is ready to turn over again. The net result, after two RC turnovers, is that the electrons removed from the bchl *a* special pair in the RC are returned to the RC via the cytochrome b/c_1 complex and cytochrome c_2, and 4 protons removed from quinols are translocated to the periplasm. This forms a gradient of both electrical charge ($\Delta\psi$) and proton concentration (ΔpH) (10, 48). Once captured in the form of this proton gradient, energy is used for the phosphorylation of ADP to ATP, for the reduction of NAD^+, for the transportation of ionic species across the membrane, and for motility (47).

An interesting and useful side effect of the movement of electrons and protons across the membrane is a shift in absorption spectra of the carotenoid pigments associated with the B800-850 antenna complexes. This electrochromic red shift in absorbency, called the carotenoid bandshift, is linear with respect to the membrane potential and thus serves as a means of evaluating the ability of a given strain of bacteria to form the transmembrane potential necessary for growth (28).

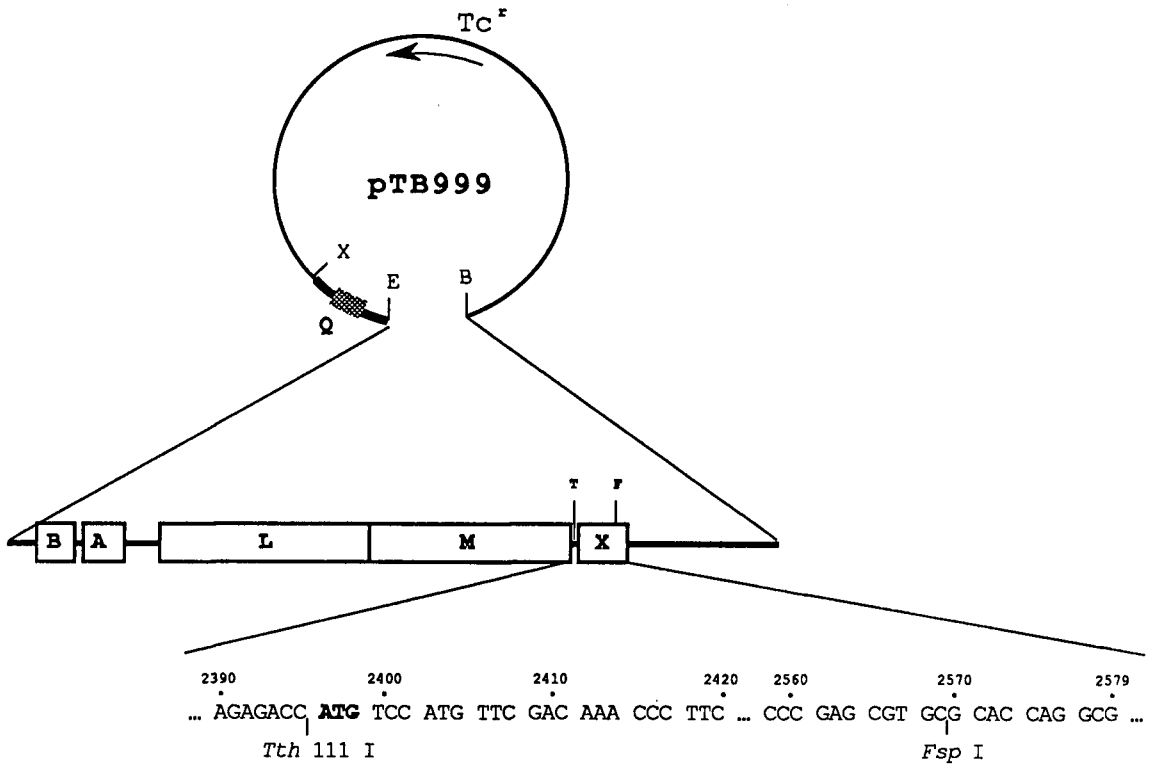
The expression of photosynthesis genes has been shown to be regulated both by light intensity (24, 69) and oxygen concentration (pO_2) (8). Regulation of expression of photosynthesis genes has been shown to occur at the level of transcription (8, 35), post-transcriptionally (5, 69), and post-translationally (16, 17, 34). It also has been speculated that expression of some pigment-binding polypeptides is regulated at the level of translation (34). How synthesis of the pigment and peptide components of the photosynthetic apparatus is coordinated and how conditions outside the cell are reported to postulated regulatory proteins are both open questions at present. It appears, however, that ex-

pression of polypeptide and pigment biosynthesis genes may be interdependent. For example, if bchl *a* synthesis in *R. capsulatus* is blocked, whether by transposon mutagenesis of a gene encoding a bchl *a* biosynthetic enzyme (68) or by chemical inhibition of bchl *a* biosynthetic enzymes (34), cells are not able to grow photosynthetically because, in addition to the absence of bchl *a*, the RC and antenna apo-peptides do not accumulate in the cell. A second example is the loss of expression of the *pucA, B,* and *E* genes when the *crtI* gene is mutated by insertion of a transposon (68).

The genes encoding photosynthetic apparatus components in *R. capsulatus* that have been sequenced include the genes for cytochrome c_2 (*cycA*) (11), for the apoproteins of the three characterized components of the cytochrome b/c_1 complex (*petA, B,* and *C*) (12), for the L, M, and H subunits of the RC (*pufL* and *M* and *puhH*) (63), for the β and α subunits of the B870 complex (*pufB* and *A*) (63), for the β, α, and γ subunits of the B800-850 complex (*pucB, A,* and *E*) (64, 54), for a bacteriochlorophyll synthesis enzyme (*bchC*) (61), and for eight carotenoid biosynthesis enzymes (*crtA, B, C, D, E, F, I,* and *K*) (2). The *puf* operon (Fig. 2) includes an open reading frame called C2397 or *pufX*. (63). No function has been ascribed to the putative protein encoded by this open reading frame, although the gene is known to be transcribed as part of a polycistronic mRNA molecule that encodes the other components of the *puf* operon (7).

I report here that a deletion mutation of the *pufX* gene affects the ability of cells to grow photosynthetically and that pleiotropic secondary mutations arise that suppress the effect of the *pufX* mutation. Although these secondary mutations restore the ability of cells to grow photosynthetically, they also cause changes in the expression of carotenoid genes and B800-850 genes. I hypothesize that the *pufX* protein is necessary for the correct assembly and/or structural configuration of components of the photosynthetic apparatus.

Figure 2. Plasmids pTB999, pΔX, and pTL2. The latter two plasmids were made by deleting the *Eco*R I to *Bam*H I fragment of pTB999 and inserting the designated *Eco*R I to *Bam*H I fragments. The cross-hatched and open boxes represent structural genes and the Ω fragment is represented in pTL2 as a heavy black line. The sequences shown all include the start codon of the *pufX* open reading frame, shown in bold type. The numbers above the sequence refer to the number of base-pairs downstream of the *Eco*R I site. Abbreviations: B, *Bam*H I; E, *Eco*R I; F, *Fsp* I; H, *Hin*d III; S, *Sma* I; T, *Tth*111 I; X, *Xho* II.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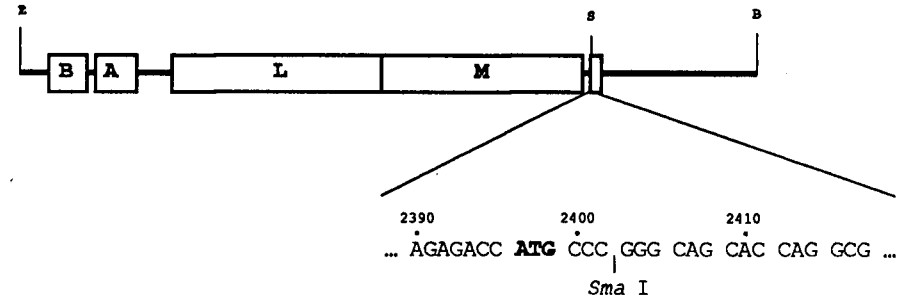
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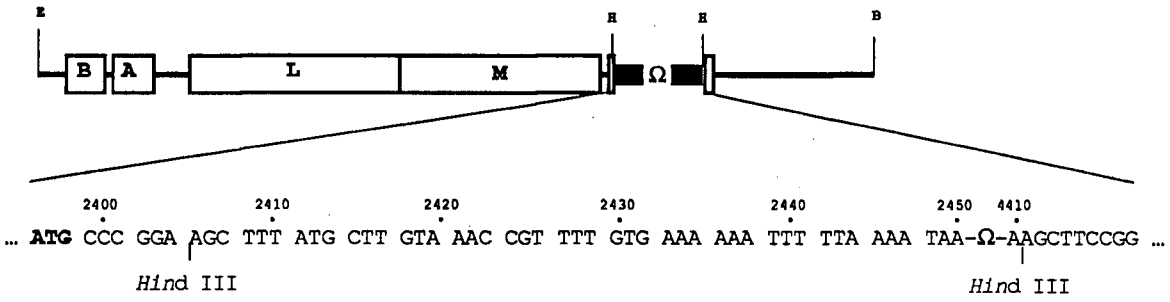
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MATERIALS AND METHODS

1. Bacterial strains and plasmids. *Rhodobacter capsulatus* B10 is a wild type strain (39). *R. capsulatus* U43 is a *puf*$^-$ *puc*$^-$ derivative of *R. capsulatus* MW442 (*puc*$^-$) created by deleting 2,778 bp of the *puf* operon carrying the *pufB, A, L, M,* and *X* genes and part of the *pufQ* gene (65). *R. capsulatus* ΔRC6 is a *puf*$^-$ strain that has a 3,316 bp chromosomal DNA deletion. The deletion includes the entire *puf* operon downstream of a *Sal*I site found 40 bp downstream of the start of the *pufQ* gene and extends to an *Xho* II site 488 bp downstream of the 5' end of the *pufX* gene. This deletion was, in essence, first created on a pUC13 derivative pJAJ21 (30) by ligating a 1.6 kb fragment of transposon Tn5 DNA carrying the neomycin phosphotransferase structural gene (4) between a 0.75 kb fragment of *R. capsulatus* DNA corresponding to the chromosomal sequence upstream of the *Sal* I site marking the 3' end of the deletion, and a 1.73 kb fragment of *R. capsulatus* DNA corresponding to the chromosomal DNA downstream of the *Xho*II site marking the 5' end of the deletion (7,1). The *R. capsulatus*::Tn5 DNA was excised from the resulting plasmid as a *Hin*dIII-*Bam*HI fragment and ligated into the plasmid pRK404, which is able to replicate in *R. capsulatus*, but is somewhat unstable and is lost in the absence of selection (30). This pRK404 derivative was named pJAJ105.

Plasmid pJAJ105 was conjugated into *R. capsulatus* B10 to allow reciprocal exchange of the plasmid-borne *puf* deletion/ *neo* insertion with the chromosomal copy of the *puf* operon, followed by plasmid loss. Recombinants were identified by their resistance to kanamycin and their photosynthetically deficient phenotype. The replacement of the *puf* operon was confirmed by Southern blotting.

R. capsulatus ΔRC6 *crtD* was made from *R. capsulatus* B10 as follows. First B10 was mixed with the supernatant liquid from a culture of *R. capsulatus* DE442. The latter strain is an over-producer of the *R. capsulatus* gene transfer agent and carries a mutation in the *crt*D gene encoding a carotenoid biosynthetic enzyme (62, B. Marrs, personal communication). The cells were plated and colonies were screened for green pigmentation (since *crt*D mutants produce only the carotenoid neurosporene, they are green in color). The same *Hin*dIII-*Bam*HI fragment that was used to create ΔRC6 (see above) was conjugated into B10 *crtD* but in this case the vector was the suicide plasmid pSUP201(53). ΔRC6 *crtD* was isolated by selecting for kanamycin resistant recombinants, and screening for photosynthetically deficient colonies. Since the final product of the carotenoid biosynthetic pathway in the *crtD* mutant is neurosporene, the 500-600 nm region of the spectrum is simplified making examination of the carotenoid bandshift much easier.

Plasmid pTB999 (Fig. 2) carries the *pufQ, B, A, L, M,* and *X* genes along with about 0.7 kb of the region upstream of the *pufQ* gene. Deletion of the DNA between the filled-in *Tth*111I site and the *Fsp*I site in the *pufX* gene, followed by insertion of a *Sma*I linker at the site of the deletion, created a 174 bp in-frame deletion in *pufX*. Substitution of this deleted gene for the wild type gene carried on pTB999 created the plasmid pΔX. Plasmid pΔX is identical to pTB999 except for this deletion in the *pufX* gene (Fig. 2).

Plasmid pTL2 (Fig. 2) is identical to pΔX except that it has an Omega (Ω) fragment (Amersham) inserted at the deletion site. The reading frame of *pufX* was destroyed by insertion of a *Hind*III linker in the *Sma*I site followed by insertion of an Ω fragment in this *Hind*III site. The Ω fragment carries transcriptional and translational terminators at

both ends and the truncated *pufX* polypeptide carried on pTL2 is only 17 amino acids in length, of which only the N-terminal methionine is a *pufX* residue. Plasmid pTL2 was used in complementation studies in the strains ΔRC6 and ΔRC6 *crtD* to determine the effects of the loss of the *pufX* gene product.

2. Growth conditions. All *R. capsulatus* strains were grown in RCV medium at 34°C (60). High oxygen cultures were grown in flasks filled to 10% of their nominal volume and shaken at 300 RPM in a rotary shaking water bath. Oxygen-limited cultures were grown in flasks filled to 80% of their nominal volume and shaken at 150 RPM. Photosynthetically grown cultures were grown in screw-cap tubes filled to capacity and held in a glass-sided water bath in front of light sources of varying intensity. All cultures used in growth experiments were inoculated to an optical density of 20 Klett units (*ca.* 8 x 10^7 cfu / ml) and growth was followed by measuring the optical density of the culture using a Klett-Summerson Colorimeter. Photosynthetic cultures were inoculated from oxygen-limited cultures in stationary phase. Dark anaerobic cultures were grown in RCV medium supplemented with 20mM fructose and 30mM dimethylsulphoxide (DMSO). DMSO served as an electron acceptor for anaerobic respiration (23). Plate cultures were grown on RCV medium supplemented with 15 g / l agar. Media for plasmid-carrying strains were supplemented with 0.5 μg of tetracycline per ml., and 0.1% yeast extract was added to plates used for fluorescence experiments. Photosynthetically grown plate cultures were grown in anaerobic jars at 34° C.

3. Isolation of chromatophores. Chromatophores (inner-membrane vesicles containing the photosynthetic apparatus) were prepared as described in Zilsel *et al.* (66) from cells grown under reduced aeration and disrupted by passage through a French

pressure cell.

4. Enhanced fluorescence studies. *R. capsulatus* strains ΔRC6(pTB999), ΔRC6(pTL2), and three secondary mutant strains were examined for enhanced fluorescence by irradiating colonies with incident light of from 400 to 600 nm wavelength and scanning them through a video camera fitted with a Wratten 87c filter, which transmits visible and infrared light of greater than 740 nm wavelength. The image was digitized and enhanced using a Kontron (Munich) SEM-IPS image analysis system. This procedure is described fully in Zilsel *et al.* (66).

5. Light-induced *ΔpH* experiments. Experiments were carried out essentially as described by von Stedingk and Baltscheffsky (58) except that the chromatophores were prepared as described in Zilsel *et al.* (66) using an unbuffered salt solution (150 mM NaCl, 100 mM KCl) in place of C buffer. Chromatophores were suspended in three ml of salt solution in a temperature-controlled cuvette at a concentration of about 20 μM bchl *a*. Light of varying intensities was directed onto the suspension and the *pH* change was measured using a Fisher model 320 *pH* meter equipped with a Fisher calomel microcombination electrode, and recorded on a Beckman chart recorder.

6. Spectrophotometric analyses. Absorption spectra of aerobically and photosynthetically grown whole cells (about 1.8×10^9 cells suspended in 22.5% BSA in RCV medium) and chromatophores (diluted to 500 μg of protein per ml in 10mM Tris, 1mM EDTA) were obtained using a Hitachi U2000 spectrophotometer.

Reduced minus oxidized differential absorption spectra and flash spectroscopy were carried out in the laboratory of R. C. Prince at Exxon Research and Engineering Company,

Annandale, New Jersey. Full descriptions of the techniques used are found in Davidson *et al.* (13) and Prince *et al.* (49).

7. Gel electrophoresis of proteins. Gel electrophoresis of proteins was carried out as described in Zilsel *et al.*(66). About 50 μg of protein were loaded per lane. Heme staining was carried out in the dark essentially as described by Goodhew *et al.* (25).

8. Thin-section electron microscopy. Cells from cultures grown under oxygen-limited conditions were fixed according to the method of Ryter and Kellenberger (31) and stained with lead salts and uranyl acetate. Silver sections were examined and photographed using a Zeiss EM C10 electron microscope.

9. RNA blots. Total cellular RNA was harvested as described by von Gabain *et al.* (57), run on a 1% agarose/ formaldehyde gel (38) (8.5 μg RNA per lane), and blotted on to a nylon membrane. The blot was pre-hybridized with salmon sperm DNA (500 μg/ ml in 5x SSC (38), 1% SDS, 50% formamide, and 10 mM EDTA) for two hours at 42° Celsius (3). A probe consisting of *puf* DNA that had been ^{32}P-labelled by the random oligonucleotide primer method (22) was then added to the prehybridization mix and hybridization was performed over night at 42° Celsius (3). The blot was washed twice for ten minutes in 2X SSC + 0.1% SDS at 52° Celsius and once for fifteen minutes in 0.2X SSC + 0.1% SDS at 58° Celsius and used to make an autoradiogram.

10. Sequence analysis. The hydropathic index of the *pufX* polypeptide was plotted using the method of Kyte and Doolittle with a window size of 15 amino acids (36). The prediction of membrane associated helices was done using the methods of Eisenberg *et al.* (20), Rao and Argos (50), and Klein *et al.* (32). Sequence alignments were done

using a method based on that of Miller and Myers (41). The versions of all five programs used were those found on release 6.01 of the software package PC/GENE (IntelliGenetics, Inc.).

11. Other assays. Light intensity was measured using a model LI-190SB quantum photometer (Li-Cor Inc.).

Protein measurements were done using the Lowry assay as modified by Peterson (45) with bovine serum albumin as the standard.

Total cellular bchl *a* was determined by extracting cells with acetone:methanol (7:2) and measuring the absorption of the extracts at 770 nm (9).

The cytochrome b/c_1 complex activity was assayed as described by Berry and Trumpower (6) except that the concentration of 2,3-dimethoxy-5-methyl-6-n-decyl-1,4-benzoquinol (DBH_2), a substrate for this assay, used was 40 μM and the assay was carried out at a pH of 6.8. Reduction of horse heart cytochrome *c* was followed on a Varian DMS 100 spectrophotometer using an extinction coefficient (reduced minus oxidized) equal to 18.5 mM^{-1} cm^{-1}. DBH_2 was synthesized according to the protocol published by Wan *et al.* (59) except that flash column chromatography was substituted for preparative TLC. The synthesis was carried out in the laboratory of E. Piers. The substrate was reduced prior to use using a modification (E. Berry, personal communication) of the method published by Trumpower and Edwards (55) in which the initial reductant was sodium thiosulfate rather than sodium hydrosulfite and the reduced substrate was finally dissolved in DMSO rather than ethanol.

Succinate-cytochrome *c* reductase activity was assayed as described by Trumpower and Edwards (55) except that the potassium cyanide concentration was 5 mM.

The mean diameter of chromatophore invaginations in electron micrographs was determined by direct measurement with 35 measurements made on each strain.

RESULTS

1. Effects of *pufX* gene deletion and interposon mutations on *puf* operon mRNA abundance. RNA blots of an in-frame deletion mutant of the *pufX* gene and an Ω insertion mutant (see Materials and Methods) revealed that the molar ratio of *pufBA* to *pufLM* transcript segments was similar to the ratio obtained with a plasmid-borne wild type copy of the *puf* operon (Fig. 3). Therefore, the resultant phenotypes of these *pufX* mutations would be unlikely to be due to secondary effects stemming from perturbations in the amounts of light-harvesting and reaction center messages, and would most likely be solely due to the absence of the *pufX* gene product. This was an important consideration because a previous study of a *pufX* deletion mutant had utilized a strain that had reduced amounts of the *pufLM* message segment, and reduced absorbency of the reaction center at about 804 nm (24). The possibility that the mutations that we have constructed somehow cause an overall increase in transcription of the *puf* operon has not been directly tested but is unlikely for two reasons. Firstly, the intensities of the bands in Figure 3 from RNA preparations isolated from different strains were approximately the same and, secondly, in a *puc*$^-$ strain background the absorption at 805 nm, which is due to RC bchl *a*, was the same as in the wild type (data not shown).

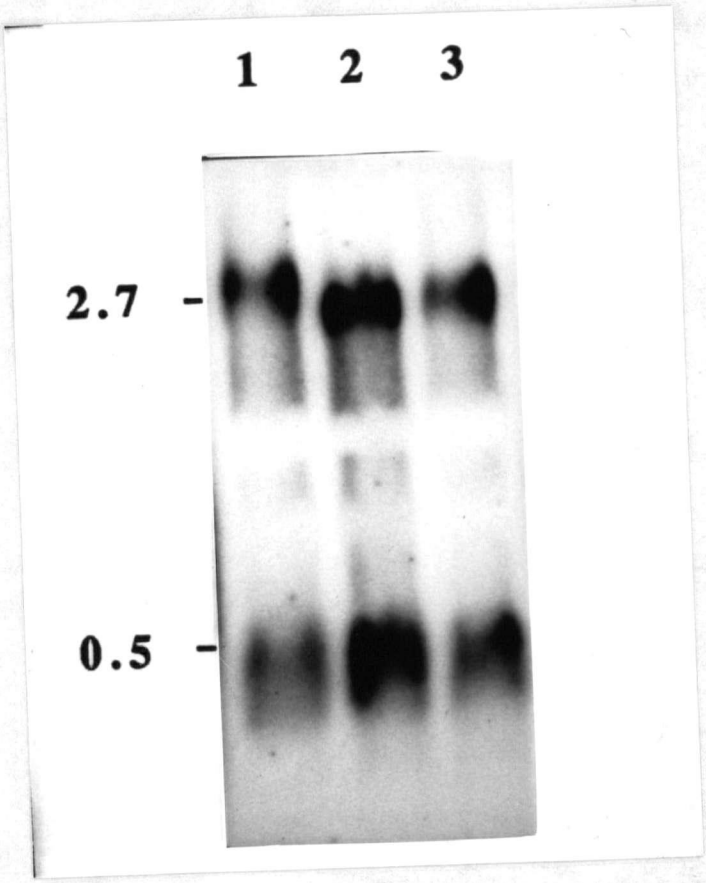


Figure 3. Autoradiogram of a blot of total RNA, 8.5 μg loaded per lane, extracted from: 1, ΔRC6(pTB999) [the pseudo-wild type]; 2, ΔRC6(pTL2) [the deletion-insertion mutant]; 3, ΔRC6(pΔX) [the deletion mutant]. The blot was probed with [α–^{32}P]-labelled *puf* DNA. Numbers on left refer to the approximate size in kilobases of wild type *puf* transcripts.

2. Growth capabilities and kinetics of strains containing wild type and *pufX* deletion genes. Figure 4A shows growth curves for *R. capsulatus* ΔRC6 carrying either wild type or *pufX* mutant genes on a plasmid and grown aerobically in the absence of illumination. The mutant grew at the same rate and to the same density as the pseudo-wild type strain under these conditions. Under conditions of dark anaerobic growth with DMSO as the terminal electron acceptor, again the mutant grew at the same rate and to the same density as the pseudo-wild type.

Also shown in Figure 4B and C are growth curves obtained for these two strains with cultures grown photosynthetically under two light intensities. The pseudo-wild type strain (containing pTB999) showed little or no lag time before initiation of exponential growth under either light condition, whereas the mutant reproducibly underwent a very long lag period before growth was detectable. The length of this lag period was variable, but was always longer at reduced light intensities, and ranged from 36 to 111 hours. Furthermore, the exponential rate of photosynthetic growth obtained with the pseudo-wild type strain was fairly reproducible, whereas the growth rate of the *pufX* mutant strain, once initiated, was more variable as evidenced by the standard deviations shown in Table I. Photosynthetic subculture of the photosynthetically grown *pufX* mutant strain resulted in loss or reduction of the lag. These results led us to suspect that secondary (suppressor) mutants that had regained the ability to grow photosynthetically were arising within the *pufX*$^-$ cultures. Indeed, plates that were streaked with cells from a stationary phase photosynthetically grown *pufX*$^-$ culture were found to give rise to colonies displaying a variety of pigmentation types that could be distinguished with the naked eye. These suspicions were reinforced when plates were spread with cells from aerobically grown cultures of the mutant and incubated photosynthetically. It was seen that none of the cells from the aerobic culture were initially capable of photosynthetic growth. Eventually, a

Figure 4. Comparison of growth of *R. capsulatus* strains ΔRC6(pTB999), ΔRC6(pTL2), ΔRC6(pTL2)-1, ΔRC6(pTL2)-7, and ΔRC6(pTL2)-9 under three different conditions: A, grown under low aeration; B, photosynthetic growth conditions, light intensity ≈ 100 Watts / m^2; C, as in B but light intensity ≈ 12 Watts / m^2.

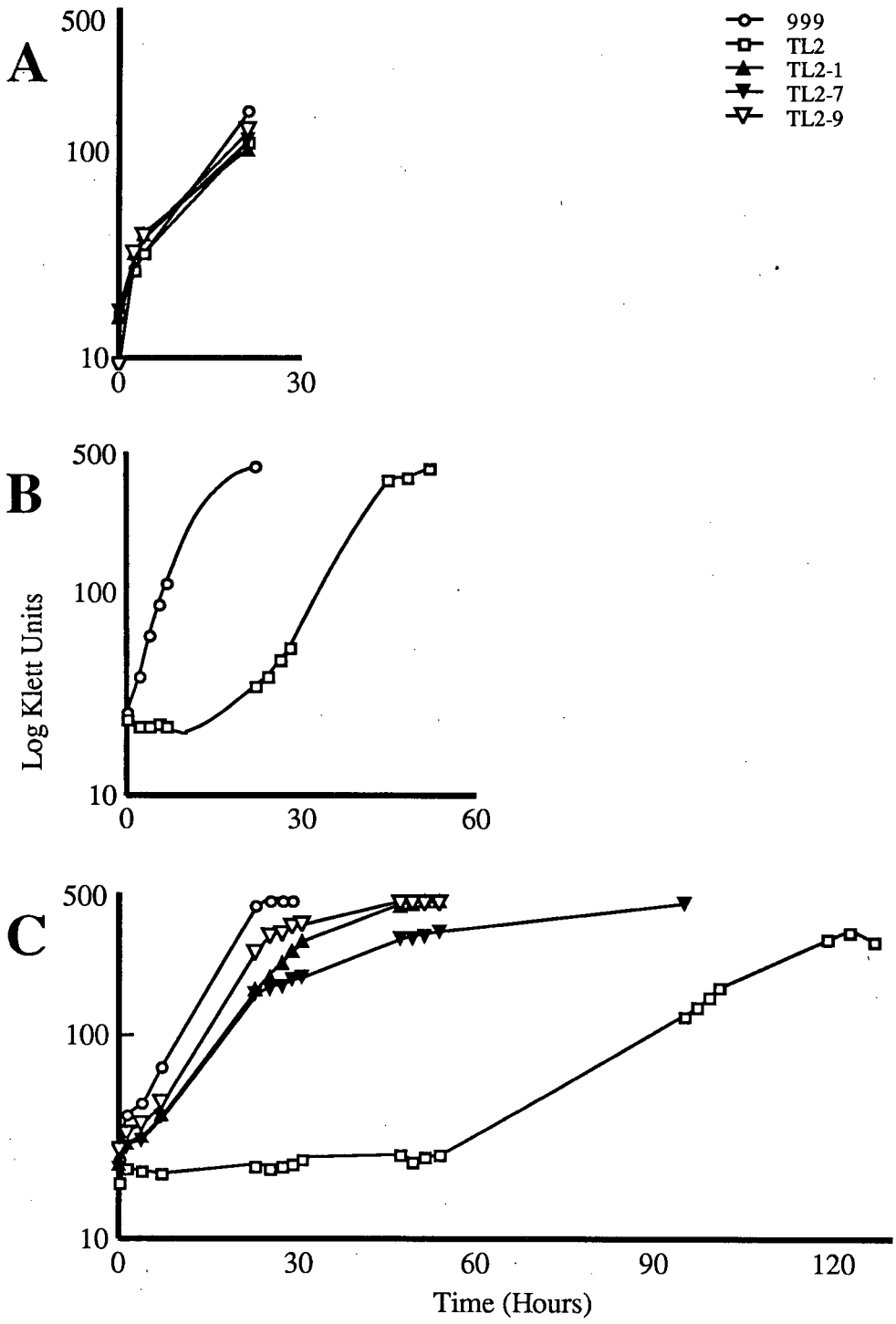

A
500
100
10
0
30
999
TL2
TL2-1
TL2-7
TL2-9
B
500
100
10
0
30
60
Log Klett Units
C
500
100
10
0
30
60
90
120
Time (Hours)

Table I: Photosynthetic growth rates and lag times of *R. capsulatus* strains ΔRC6(pTB999), ΔRC6(pTL2), ΔRC6(pTL2)-1, ΔRC6(pTL2)-7, and ΔRC6(pTL2)-9. Grown at 12 Watts per square meter.

Strain	Growth Rate (hrs.)	Lag Time (hrs.)
ΔRC6(pTB999)	4.8 ± 0.8	0
ΔRC6(pTL2)	8.9 ± 3.2	62.4± 25.4
ΔRC6(pTL2)-1	4.0	3.2
ΔRC6(pTL2)-7	8.2	10.3
ΔRC6(pTL2)-9	6.2	16.8

few colonies began to grow and these colonies showed a diversity of pigmentation similar to that seen on the plates spread from the photosynthetically grown liquid cultures. Subsequent estimation of the frequency of such mutants in populations of aerobically grown *pufX*$^-$ cultures by spreading known numbers of cells on solid media, followed by incubation under anaerobic, illuminated growth conditions, gave a value of approximately 10^{-5}.

Three of these suspected secondary mutants were purified from photosynthetically grown *pufX*$^-$ cultures and designated ΔRC6(pTL2)-1, ΔRC6(pTL2)-7, and ΔRC6(pTL2)–9. As Figure 4A shows, these secondary mutants displayed the same growth characteristics under aerobic growth conditions as the pseudo-wild type and the primary mutant, ΔRC6(pTL2). When placed under photosynthetic growth conditions, it was evident that the secondary mutants had regained the ability to grow photosynthetically (Fig. 4C). A lag was still evident for these strains but it was reduced by 73% in ΔRC6(pTL2)-9, by 84% in ΔRC6(pTL2)-7, and by 96% in ΔRC6(pTL2)-1 as compared to ΔRC6(pTL2) (see Table I). The growth rates of two of the secondary mutants, ΔRC6(pTL2)-7 and ΔRC6(pTL2)-9, were slower than that of the pseudo-wild type but ΔRC6(pTL2)-1 had an essentially wild type growth rate.

3. **Light-induced Δ*pH* Experiments** - At light intensities of 100 W/m^2, chromatophores made from *pufX*$^-$ mutants in both the ΔRC6 and the U43 backgrounds were able to form *pH* gradients at a rate and of a magnitude comparable to the pseudo-wild type chromatophores. In the ΔRC6 background this ability fell off with decreasing light intensities and at a light intensity of 0.1 W/m^2 the rate and the magnitude were 60% of pseudo-wild type levels. In the U43 background this ability was maintained at light intensities as low as 0.05 W/m^2 (Table II).

Table II: Light-induced proton translocation in chromatophore preparations from *R. capsulatus* strains ΔRC6(pTB999), ΔRC6(pTL2), U43(pTB999), and U43(pTL2) at a light intensity of 0.1Watts per square meter.

Chromatophores From:	Magnitude (nmoles protons translocated per: nmole Bchla)	Magnitude (nmoles protons translocated per: mg protein)	Rate (nmoles protons translocated per minute per: nmole Bchla)	Rate (nmoles protons translocated per minute per: mg protein)
ΔRC6(pTB999)	0.5 ± 0.07	21.2 ± 2.6	0.5 ± 0.14	22.8 ± 5.8
ΔRC6(pTL2)	0.3 ± 0.03	12.0 ± 1.2	0.3 ± 0.07	13.4 ± 2.6
U43(pTB999)	0.9 ± 0.05	19.7 ± 1.0	1.4 ± 0.11	28.3 ± 2.18
U43(pTL2)	0.9 ± 0.21	20.5 ± 4.7	1.5 ± 0.26	34.3 ± 6.0

4. Absorption spectroscopy

(a) Scans. Figures 5 and 6 show typical scans of whole cells of *R. capsulatus* strains ΔRC6(pTB999), ΔRC6(pTL2), ΔRC6(pTL2)-1, ΔRC6(pTL2)-7, and ΔRC6(pTL2)-9 grown under photosynthetic and oxygen limiting conditions. The RC, B870 and B800-850 complexes can be approximately quantified on the basis of their absorption. It can be seen that cells of the *pufX* mutant ΔRC6(pTL2) grown under low oxygen conditions [Fig. 5(i)] showed a strong B870 shoulder compared with the pseudo-wild type strain ΔRC6(pTB999) [Fig. 5(ii)]. The magnitude of the B870 peak in ΔRC6(pTL2) was such that the longest wavelength absorption maximum.(normally at 857 nm) was shifted to 858 nm. Aerobically grown secondary mutants also showed this increase in B870 absorption and the resulting red-shift was more exaggerated with redshifts of 4 nm in ΔRC6(pTL2)-9 and 16 nm in ΔRC6(pTL2)-7 [Fig. 6A (ii) and (iii)]. In contrast with ΔRC6(pTL2), however, the apparent increase in B870 absorption in the secondary mutants grown under low oxygen conditions was at least partly due to decreases in the absolute amount of B800-850 complexes. When scans of ΔRC6(pTL2)-7 and ΔRC6(pTL2)-9 cells were compared to scans of ΔRC6(pTB999) cells, all grown under photosynthetic conditions, it was observed that the red-shift seen in the mutants was only about 1 nm, and that overall absorption in the near infrared region was about 20% higher. There are two reasons for these phenomena: 1) the levels of B870 complexes have increased and are higher than the levels seen in the pseudo-wild type as evidenced by the size of the shoulder on the B850 absorption peaks and 2) the levels of B800-850 complexes in these two secondary mutants relative to B870 complex levels are higher under photosynthetic growth conditions compared with cultures grown under low oxygen tension (see Table III). Therefore, the red shift was less in photosynthetically grown cultures than in cultures grown under low oxygen conditions because of the relative increase in B800-

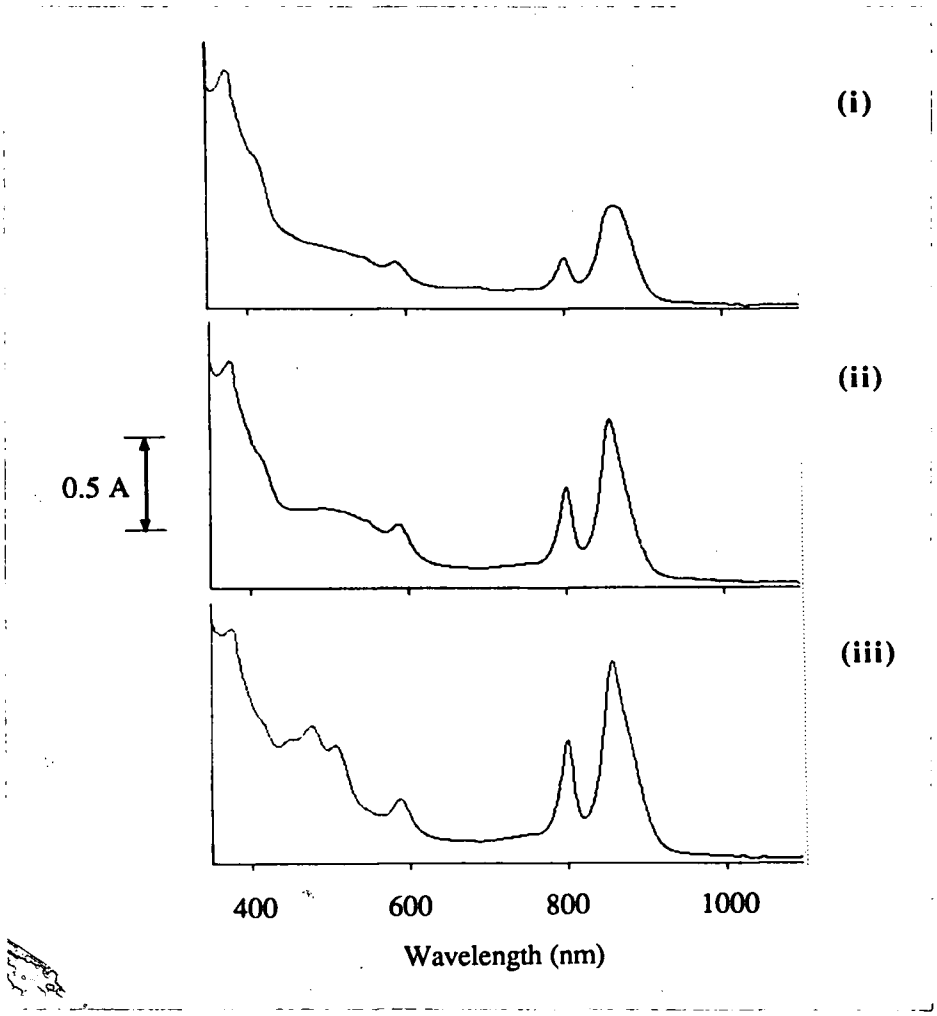


Figure 5. Absorption spectra of intact cells of *R. capsulatus* strains (i) ΔRC6(pTL2), grown under low aeration; (ii) ΔRC6(pTB999), grown under low aeration; (iii) ΔRC6(pTB999) grown under photosynthetic growth conditions. ΔRC6(pTL2) does not grow under photosynthetic growth conditions.

Figure 6. Absorption spectra of intact cells of *R. capsulatus* strains (i) ΔRC6(pTL2)–1, (ii) ΔRC6(pTL2)-7, and (iii) ΔRC6(pTL2)-9. A, cells grown under low aeration; B, cells grown under photosynthetic growth conditions.

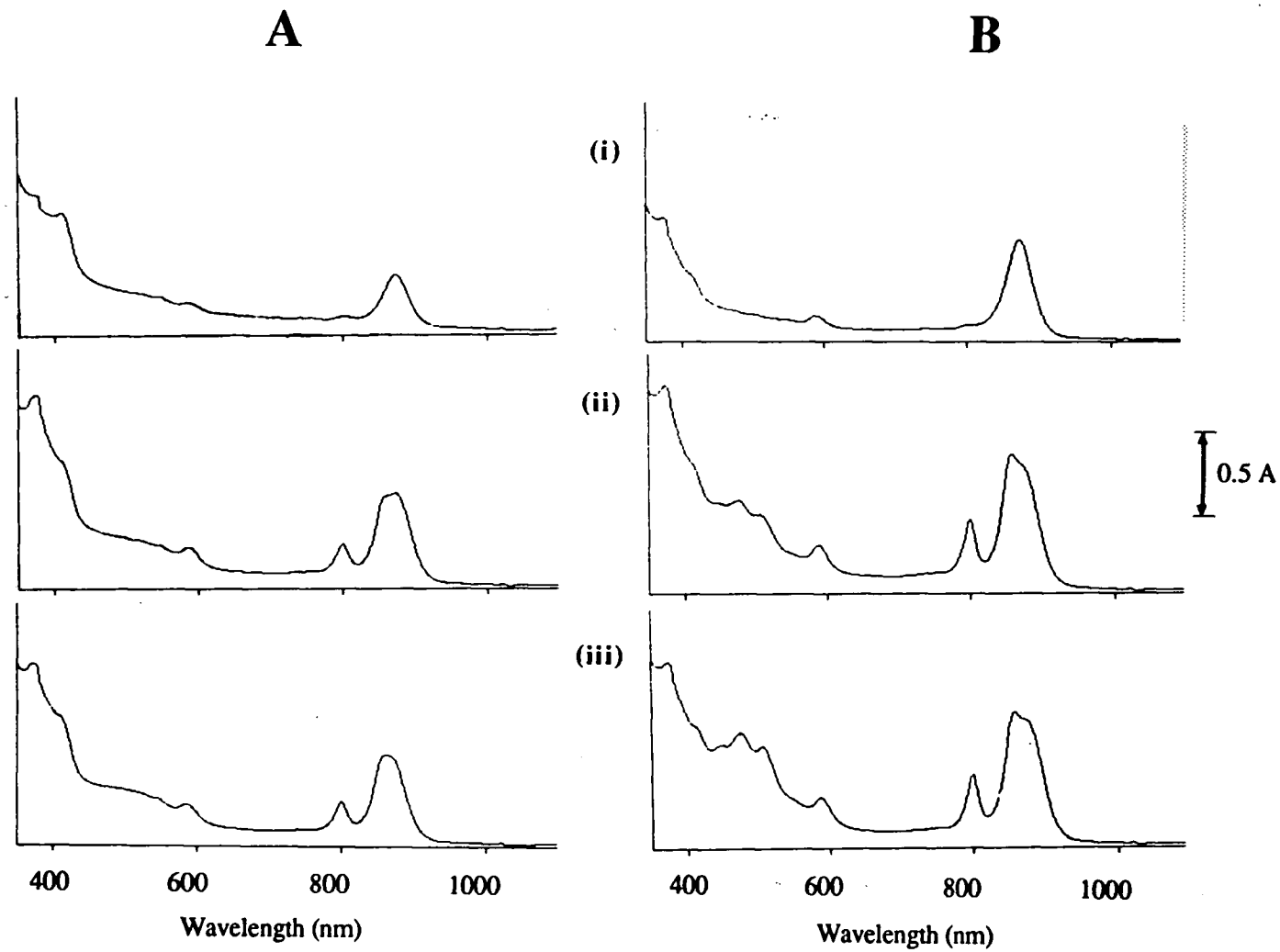

A
B
(i)
(ii)
(iii)
0.5 A
400
600
800
1000
Wavelength (nm)
400
600
800
1000
Wavelength (nm)

Table III: Magnitude of absorption of mutant strains of *R. capsulatus*. relative to ΔRC6(pTB999)[a].

Strain	Relative Absorption At:	
	802nm	857nm
Low Oxygen Grown		
ΔRC6(pTB999)	1.0	1.0
ΔRC6(pTL2)	1.0	1.1
ΔRC6(pTL2)-1[b]	0.4	—
ΔRC6(pTL2)-7	0.6	0.8
ΔRC6(pTL2)-9	0.6	0.8
Photosynthetically Grown		
ΔRC6(pTB999)	1.0	1.0
ΔRC6(pTL2)[c]	—	—
ΔRC6(pTL2)-1	0.3	—
ΔRC6(pTL2)-7	1.0	1.2
ΔRC6(pTL2)-9	1.0	1.2

[a] The peak intensity was calculated by dividing the absorption at the given wavelength by the absorption at 650 nm.

[b] This strain does not make B800-850 antenna complexes.

[c] This strain does not grow photosynthetically.

850 absorption. The secondary mutant ΔRC6(pTL2)-1 does not have B800-850 complexes nor does it contain any carotenoids. Its furthest-red absorption peak, occurring at 874 nm, is due solely to B870 and RC absorption.

(b) Reduced *minus* oxidized differential absorption spectra. Optical difference spectra were used to determine the relative amounts of *c* and *b*-type cytochromes in chromatophores prepared from the pseudo-wild type and mutant strains. Ascorbate, plus a trace of the redox mediator N-methyl-phenazonium methosulphate, reduces high potential cytochromes including cytochromes c_1 and c_2, which have absorption maxima at about 550 nm, and some *b*-type cytochromes, which have absorption maxima at about 560 nm. Dithionite additionally reduces low potential cytochromes, which are mainly *b*-type cytochromes. Figure 7 shows that, although levels of *c*-type cytochromes in ΔRC6(pTL2) were slightly lower than those seen in ΔRC6(pTB999), levels of *b*-type cytochromes were almost identical in the two strains. This implies that the two strains have approximately the same amount of cytochrome b/c_1 complexes. Because there are lesser amounts of *b*- and *c*-type cytochromes in *R. capsulatus* other than those directly involved in photosynthetic electron transfer, these spectra must be regarded as qualitative, or semi-quantitative indicators of the levels of cytochrome b/c_1 complexes.

(c) Flash spectroscopy. Cyclic electron flow in the pseudo-wild type and mutant strains was assessed using the technique of flash spectroscopy. Chromatophores were exposed to a series of eight saturating actinic flashes and the difference spectra at selected wavelengths were measured on a dual wavelength, double beam spectrophotometer. Depending on the wavelengths monitored, it was possible to to measure the carotenoid bandshift (by monitoring at 490 *minus* 475 nm) or to reveal the oxidation and reduction of

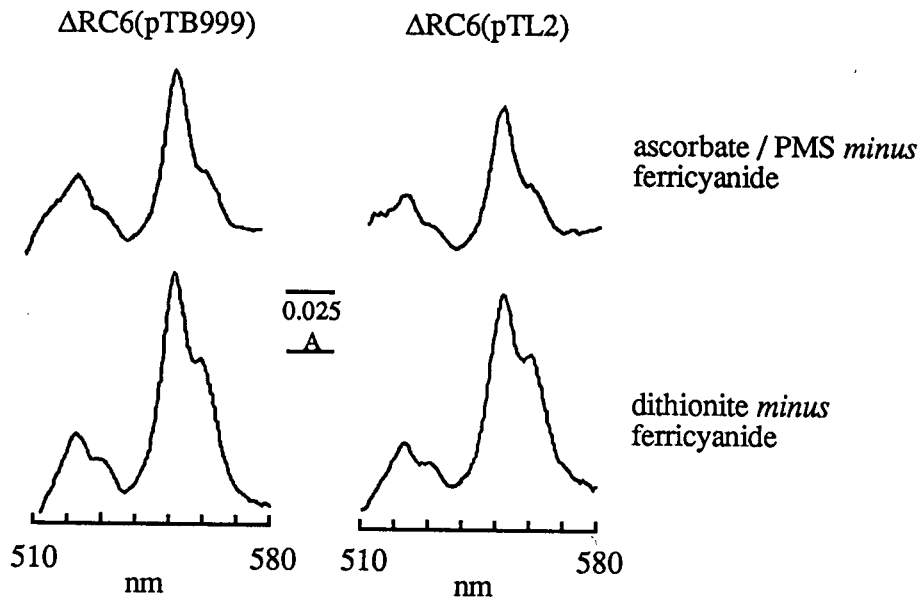


Figure 7. Absorption spectra of cytochromes in chromatophores from the pseudo-wild type [ΔRC6(pTB999)] and mutant [ΔRC6(pTL2)] strains.

individual components of the cyclic electron pathway. Thus, oxidation and rereduction of the bchl *a* special pair in the RC could be seen as differences in absorption at 605 *minus* 540 nm. Similarly, oxidation and reduction of *c*-type cytochromes were followed at 550 *minus* 540 nm, and reduction of cytochromes b_l and b_h of the cytochrome b/c_1 complex was followed at 560 *minus* 572 nm. Figure 8 shows the results of these experiments. Spectra of both strains were measured at each wavelength pair in the presence and absence of antimycin. Antimycin is an inhibitor of cytochrome b/c_1 complex function in chromatophores that prevents electron transfer from cytochrome b_h to the quinone in the Q_c site (see Fig. 1). This means that, in the presence of antimycin, after several flashes the high and low potential *b*-type cytochromes will be fully reduced while the RC special pair and cytochromes c_1 and c_2 will be oxidized since cyclic electron flow has been halted. These effects are clearly seen in the data for ΔRC6(pTB999) in Figure 8. In the absence of antimycin, the RC special pair is quickly reduced by cytochrome c_2 after each flash and cytochromes c_1 and c_2 are in turn reduced by the Rieske iron-sulphur complex. In contrast, after about four flashes in the presence of antimycin all light-induced movement of electrons and protons across the chromatophore membrane ceases, as is evidenced by the carotenoid bandshift data, and the *b*-type cytochromes are fully reduced.

Turning to the data for ΔRC6(pTL2), it is apparent that this strain has some defect in its cyclic electron pathway. The carotenoid bandshift indicates that the *pufX*$^-$ mutant is still able to generate a transmembrane potential, but the magnitude of the potential is only about one third of the potential generated by the pseudo-wild type. The RC special pair is reduced, but only to an extent that is marginally greater than the reduction in the presence of antimycin, and the same is true of reduction of cytochromes c_1 and c_2. It should be noted that after eight flashes some reduction of the special pair is still occurring. The

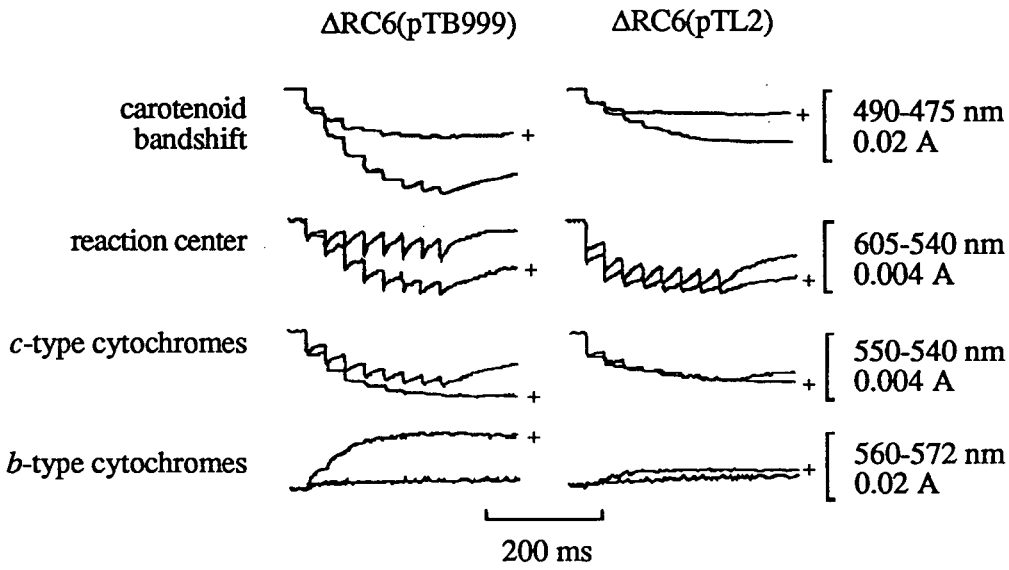


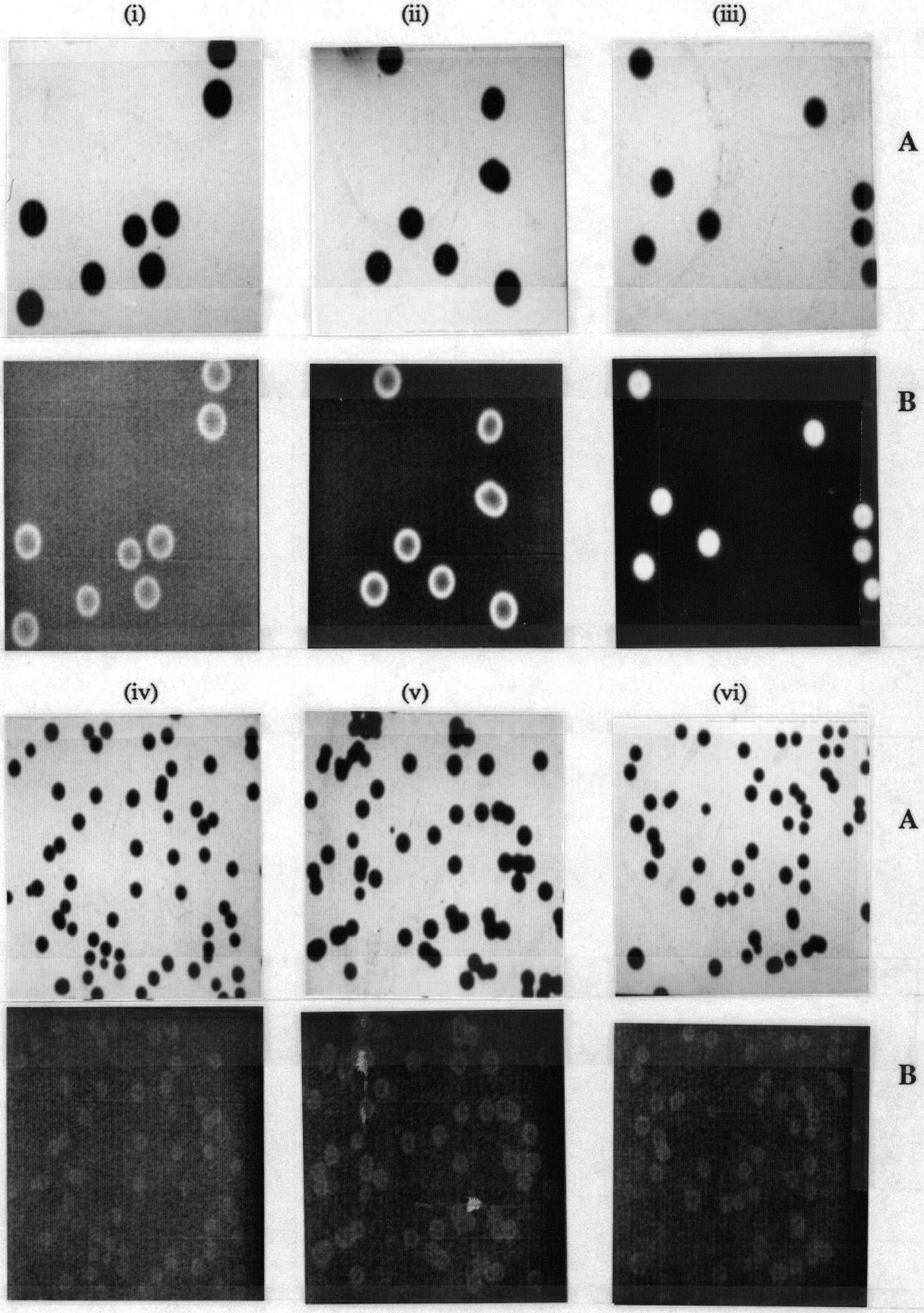
Figure 8. Absorption changes in the carotenoids (490-475 nm), reaction center (605-540 nm), *c*-type cytochromes (550-540 nm), and *b*-type cytochromes (560-572 nm) of chromatophores in response to eight actinic flashes. Traces for each strain were recorded separately in the presence (+) and absence of 2 μM antimycin and overlaid for ease of comparison.

cytochromes b_l and b_h are only slightly reduced after four flashes in ΔRC6(pTL2) in the presence of antimycin, but the kinetics of reduction seem comparable to those seen in the pseudo-wild type.

5. Fluorescence emission. Results of the fluorescence studies are shown in Figure 9. In principle, if the *pufX*$^-$ mutant is deficient in its ability to transfer light energy from the light-harvesting complex to the RC it will show higher levels of fluorescence than the pseudo-wild type under identical conditions (16). Fluorescence in the primary and secondary mutants was at approximately the same level as in the pseudo-wild type strain.

6. SDS-PAGE analysis of chromatophore membrane proteins. Figure 10(A) shows the results of SDS-PAGE analysis of chromatophores from *R. capsulatus* strains grown under oxygen-limiting conditions. When the pseudo-wild type ΔRC6(pTB999) and the primary mutant ΔRC6(pTL2) were compared, some differences in their polypeptide profiles were seen. Specifically, ΔRC6(pTL2) had higher levels of the B870 polypeptides (seen most easily with the 12 kDa α subunit), the RC polypeptides and an unidentified polypeptide of M_r 32 kDa. The *pufX*$^-$ suppressor mutants gave diverse banding patterns on the gel. Like ΔRC6(pTL2), these mutants had elevated levels of the two B870 antenna complex polypeptides. The levels of B800-850 polypeptides in ΔRC6(pTL2)-7 and ΔRC6(pTL2)-9 were about as high as the levels seen in the pseudo-wild type strain and ΔRC6(pTL2), but ΔRC6(pTL2)-1 had no B800-850-associated polypeptides. Also, the amount of the 32 kD polypeptide present in two of the secondary mutants, ΔRC6(pTL2)-7 and ΔRC6(pTL2)-9, was much lower than the amount seen in the other three strains. ΔRC6(pTL2)-1 had a higher level of this polypeptide than all the other strains . A heme stain of the same gel [Fig. 9(B)], which selectively stained *c* type cyto

Figure 9. Relative fluorescence of colonies of *R. capsulatus* strains (i) ΔRC6(pTB999), [pseudo-wild type, a negative control], (ii), ΔRC6(pTL2), [the deletion insertion mutant], (iii) ΔRC6(pΔ4), [complementing plasmid carries only *pufQ*, a positive control], (iv) ΔRC6(pTL2)-1, (v) ΔRC6(pTL2)-7, (vi) ΔRC6(pTL2)-9. A, viewed under visible light; B, viewed through an infrared filter.


(i)
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A
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(iv)
(v)
(vi)
A
B

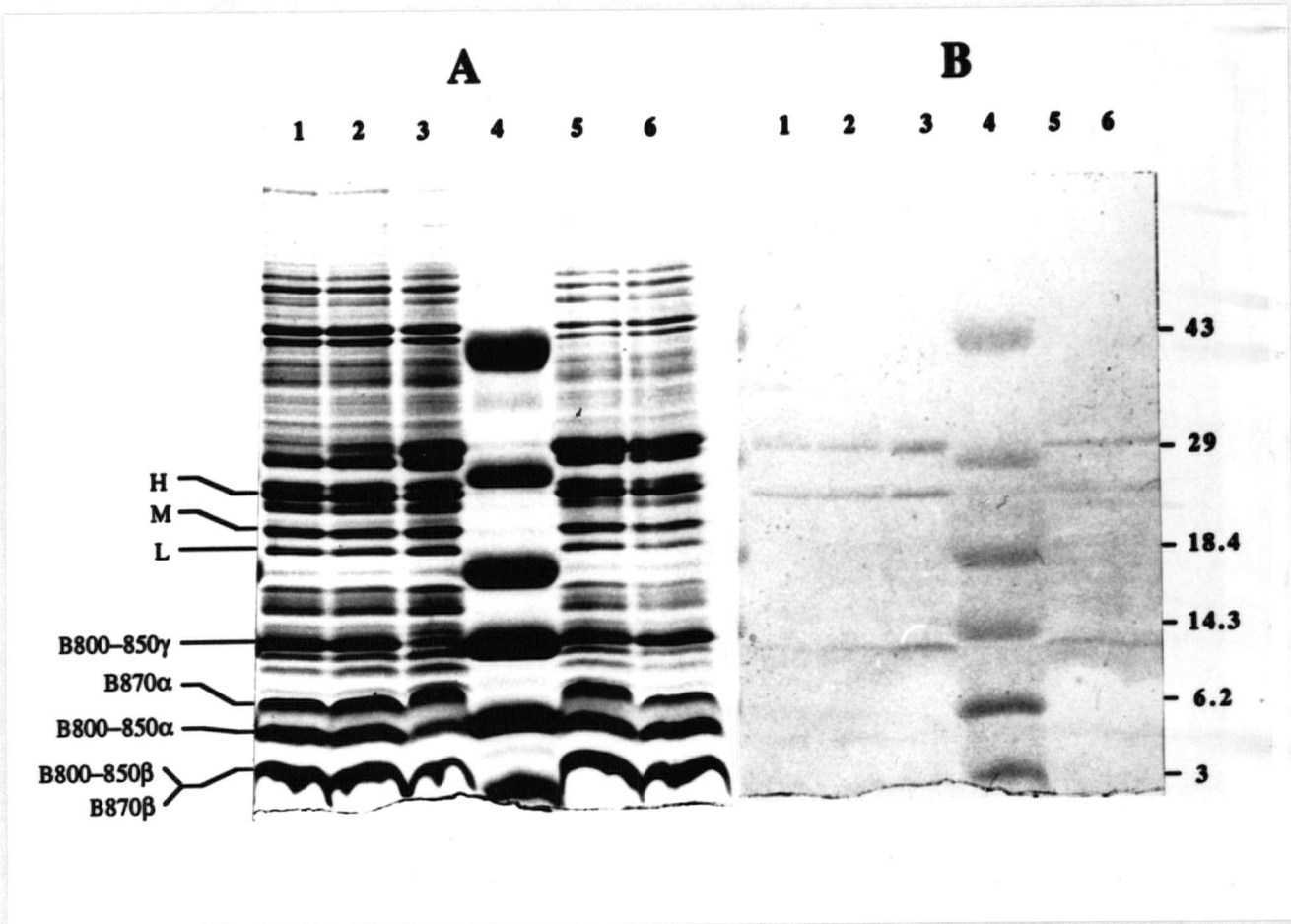


Figure 10. SDS-polyacrylamide gel electrophoresis of chromatophore vesicles from *R. capsulatus* strains: 1, ΔRC6(pTB999); 2, ΔRC6(pTL2), the deletion-insertion mutant; 3, ΔRC6(pTL2)-1, a secondary mutant that lacks functional B800-850 antenna complexes; 4, Molecular weight markers; 5, ΔRC6(pTL2)-7; 6, ΔRC6(pTL2)-9. The bands corresponding to the RC subunits H, M, and L are indicated on the right, as are the subunits of the B870 and B800-850 light-harvesting complexes. The positions of the molecular mass markers (in kDa) are shown on the left. A: Coomassie stain, B: Heme stain of the same gel.

chromes, revealed three bands with M_rs of 31, 26, and 13 kDa. Two of these bands can be reliably identified as the cytochrome c_1 (31 kDa) and a mixture of cytochromes c_2 and c' (13 kDa). The identity of the 26 kDa band is not known. ΔRC6(pTL2) had less staining of the 26 kDa cytochrome than the pseudo-wild type. If it is assumed that the intensity of staining reflects the amount of cytochrome present, then strain ΔRC6(pTL2)-1 had higher levels of all three of these cytochromes than all the other strains. Strains ΔRC6(pTL2)-7 and ΔRC6(pTL2)-9 both had low levels of cytochromes c_1 and c' relative to ΔRC6(pTB999), ΔRC6(pTL2), and ΔRC6(pTL2)-1.

7. Electron microscopy. Representative transmission electron micrographs of thin sections of cells from cultures of ΔRC6(pTB999) and ΔRC6(pTL2) grown under oxygen-reduced conditions are shown in Figure 11. The inner membrane invaginations of ΔRC6(pTL2) had a mean diameter about 50% greater than those of ΔRC6(pTB999).

8. Characterization of cytochrome b/c_1 complexes. Electron flow through cytochrome b/c_1 complexes in whole chromatophores was tested using inner membrane preparations from cells grown under oxygen-limiting conditions (see Table IV). Comparisons of electron flow from the succinate dehydrogenase-ubiquinol reductase complex to cytochrome c_2 were also made. Here again electron flow in preparations from the mutant seemed unimpaired when compared to the pseudo-wild type. However, these experiments cannot be interpreted reliably (see Discussion).

9. Sequence analysis. A hydropathic plot of the amino acid sequence encoded by *pufX* [Fig. 12(A)] indicates that the *pufX* gene product could be a transmembrane protein. The hydrophobic portion of the sequence extends from amino acid (aa) 19 to aa 52. The most

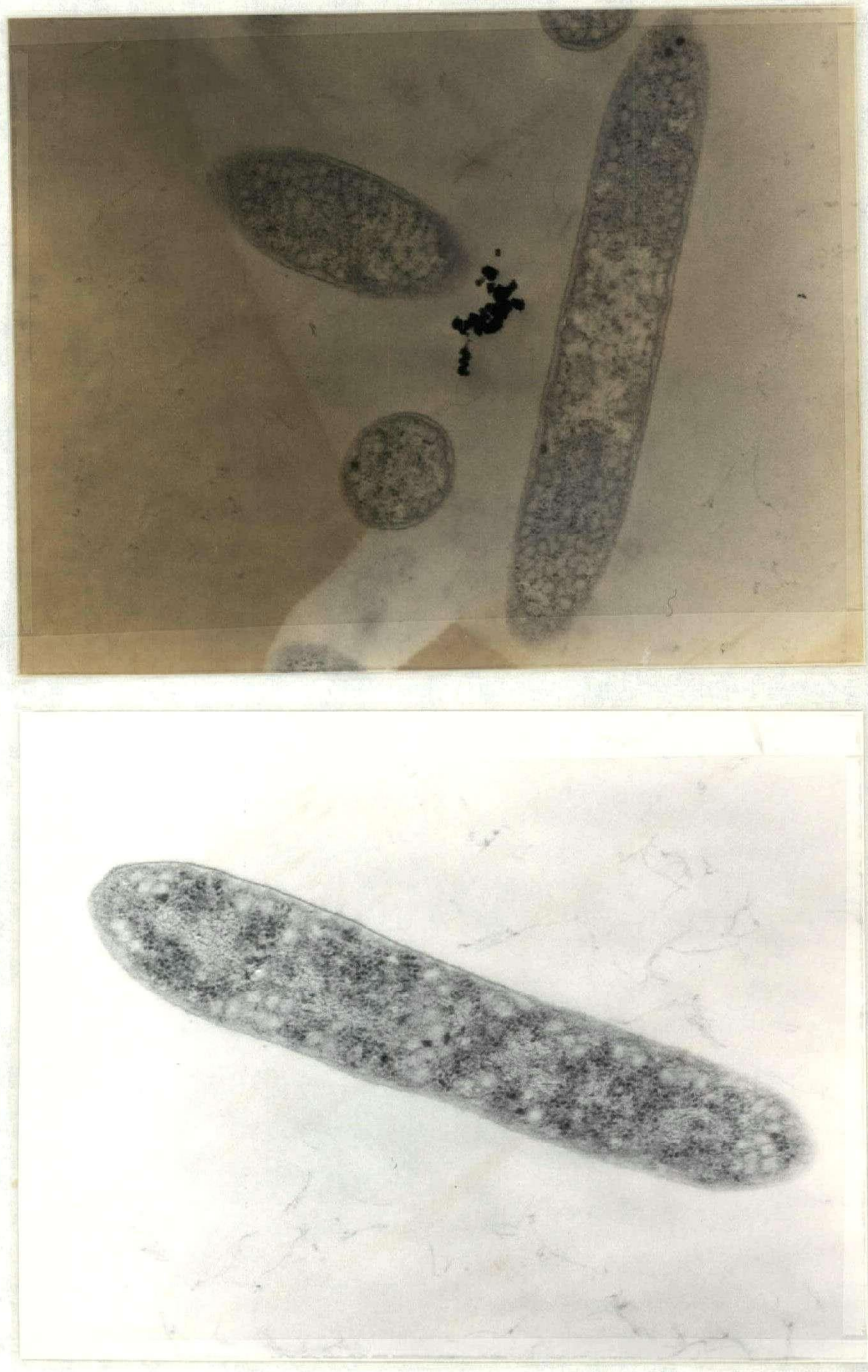
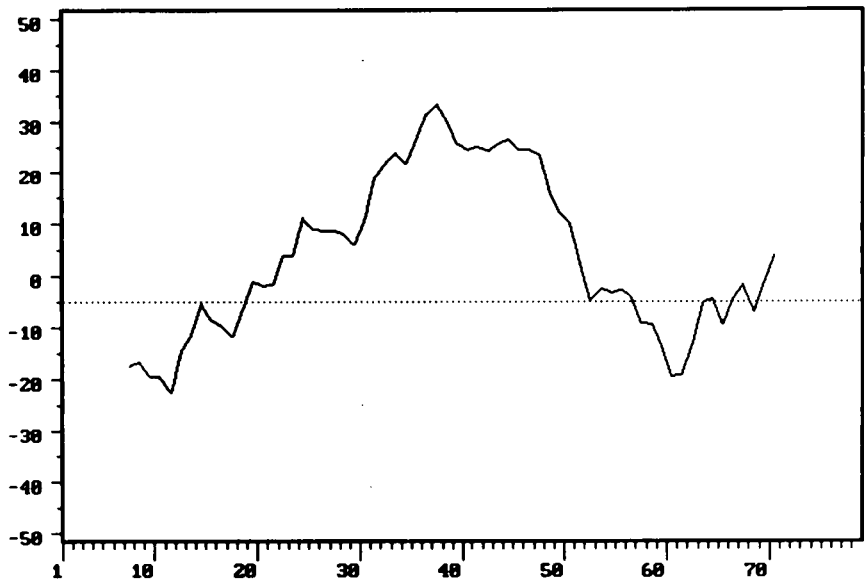


Figure 11. Transmission electron micrographs of thin sections of: A ΔRC6(pTB999) and B ΔRC6(pTL2).

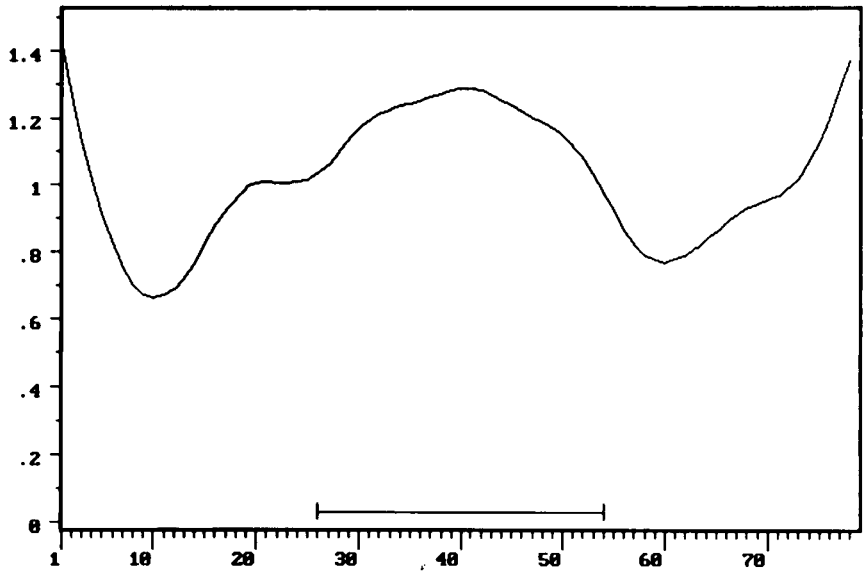
Table IV: Ubiquinol:cytochrome b/c_1 oxidoreductase complex activity in chromatophores from *R. capsulatus* strains ΔRC6(pTB999) and ΔRC6(pTL2)

Strain	Specific Activity (μmoles cytochrome *c* reduced per minute per mg protein)
ΔRC6(pTB999)	38
ΔRC6(pTL2)	38



A

Hydropathic index of PUFX$RCAPS from amino acid 1 to amino acid 78.
Computed using an interval of 15 amino acids. (GRAVY = 2.5).



B

Smoothed curve of PUFX$RCAPS from amino acid 1 to amino acid 78.
The y axis value represents the buried helix parameter.

Figure 12. (A) Hydropathy plot and (B) prediction of trans-membrane helix based on the amino acid sequence of *pufX*.

hydrophobic portion is the sequence stretching from aa 32 to aa 48, and when the entire *pufX* amino acid sequence is analysed using three different secondary structure prediction programs the sequence from aa 27 to aa 46 is consistently picked as a transmembrane α-helix. The results from application of the program RAOARGOS (33b) are shown in Figure 12(B). The results from hydropathy plots and similar analytical programs do not constitute proof of a protein's secondary structure, but they have successfully predicted transmembrane helices in the RC of a species related to *R. capsulatus, Rhodopseudomonas viridis*. The existence of these helices in *R. viridis* has been confirmed by X-ray crystallography (27).

The amino acid sequence of *pufX* was compared with those of light-harvesting polypeptides from *R. capsulatus, Rhodobacter sphaeroides, Rhodospirillum rubrum, Rhodopseudomonas viridis,* and *Chloroflexus aurantiacus* using a method based on that of Needleman and Wunsch (43). The greatest degree of similarity was with the B870 α subunit of *R. capsulatus*; with 5 gaps permitted, 27.6 % of the residues were identical and 15.5 % were similar when the program of Myers and Miller (41) was used (Fig. 13). This degree of similarity just falls short of Doolittle's criteria for relatedness(18), so it may be more realistic to say that the similarity found indicates similarity of structure rather than a common ancestry for the two proteins.

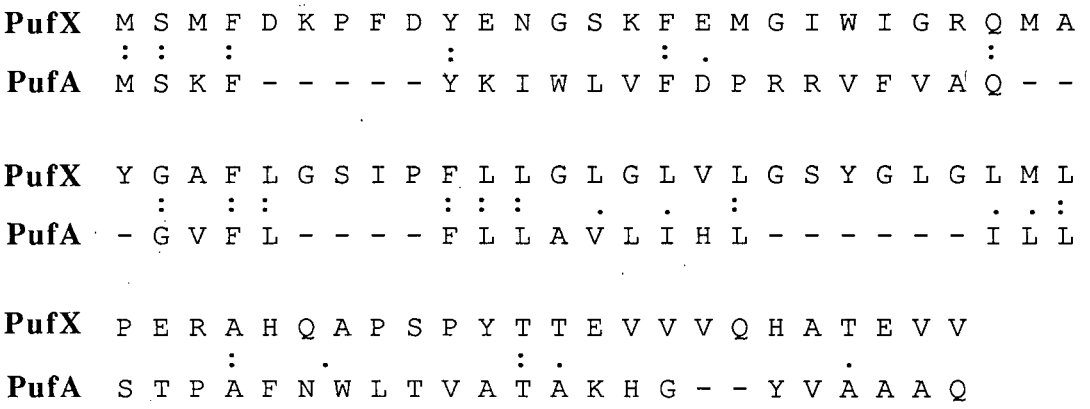


Figure 13. A comparison of the amino acid sequences encoded by the genes *pufA* (PufA) and *pufX* (PufX). Five gaps are inserted in the *pufA* amino acid sequence. Using this alignment, sixteen of the residues are identical (shown by :) and nine are similar (shown by .).

DISCUSSION

1. The primary mutant ΔRC6(pTL2)

Rhodobacter capsulatus ΔRC6(pTL2) grows normally under both low oxygen and high oxygen conditions; therefore, no deficiencies in respiratory ATP generation exist. This strain is unable to grow under photosynthetic growth conditions. The *pufX* gene product must, therefore, be involved in the process of photosynthetic growth. It is noteworthy that it is part of an operon which encodes five other polypeptides that, collectively, are essential for photosynthetic growth but are otherwise superfluous.

The localization and characterization of the defect in the photosynthetic unit caused by the deletion of the *pufX* gene were the goals of the experiments done after the growth experiments. Incident light energy is first captured by the antenna complexes, and the fluorescence emission experiments should have revealed any defect in the *pufX*$^-$ mutant's ability to capture light energy or to pass this energy on to the RC. Since fluorescence emission from the mutant was about the same as that seen in the pseudo-wild type, it was concluded that the defect must lie elsewhere.

The capture of light energy at the RC depends on the cyclic flow of electrons through the RC, to the cytochrome b/c_1 complex via a quinol, through the cytochrome b/c_1 complex, and back to the RC via cytochrome c_2. Any disruption of this flow will disrupt the cell's ability to grow photosynthetically. Preliminary kinetic spectrophotometric analysis of light-induced electron flow through the RC in isolated chromatophores (Fig. 7) showed that the bchl *a* special pair in the mutant is oxidized as quickly as the special pair in the pseudo-wild type

(taking into account the time scale used) but that re-reduction occurred more slowly. This would indicate that electron flow through the RC is not impaired and the extent of cytochrome c_1 and c_2 oxidation after the initial flash supports this assertion. Hence, it seems likely that a charge gradient is formed within the RC, that is, that quinones are being reduced.

To test the possibility that electron flow through the cytochrome b/c_1 complex was blocked, the ability of cytochrome b/c_1 complexes in crude chromatophore preparations to reduce the ubiquinone analogue DBH_2 and oxidize horse heart cytochrome *c* was measured. Electron flow through the cytochrome b/c_1 complex of the *pufX*$^-$ mutant seemed normal. A problem with this experiment is that the chromatophores prepared by French pressure cell disruption are predominantly (~80-90%) "inside-out" (52), that is, the vesicles enclose periplasmic material. Therefore, the horse heart cytochrome *c* added to the chromatophore suspension was on the "wrong" side of the membrane; to mimic the normal electron acceptor, cytochrome c_2, it should be inside the chromatophore vesicles. To improve the experiment, the chromatophore vesicles could be solubilized or, better yet, the cytochrome b/c_1 complexes could be purified. However, light-driven electron flow in chromatophores does not suffer from this drawback. When electron flow through the cytochrome b/c_1 complex was evaluated spectrophotometrically, it was evident that this flow was slower than that seen in the wild type. However, it was not clear that the retarded rate was due to a defect in the cytochrome b/c_1 complex *per se*; in view of the fact that reduction of the special pair was still occurring after eight flashes and that the cytochromes b_l and b_h are being reduced at a rate comparable to the rate seen in the pseudo-wild type, it appeared that electron flow was impaired by a deficiency of electron transfer between the RC and the cytochrome b/c_1 complex rather than by any defect in intra-cytochrome b/c_1 complex electron transfer.

It seemed possible that, because light-dependent cyclic electron flow in the *pufX*

mutant was impaired, this mutant might lack the ability to transfer protons across the inner membrane via the cytochrome b/c_1 complex. The light-induced *ΔpH* experiments were done to test this hypothesis. In these experiments a 40% difference was seen in the rate at which protons were pumped across the membrane between the *pufX*$^-$ mutant and the pseudo-wild type, but only when the experiments were done in a strain, ΔRC6, which had B800-850 complexes. In the U43 strain background the light-stimulated rate of change in *pH* was the same in the mutant and the pseudo-wild type. This result alone would lead one to conclude that the presence of B800-850 complexes is implicated in the *pufX*$^-$ mutant's inability to grow photosynthetically; this possibility is further discussed below. Although in growth experiments using the U43 strain background the *pufX*$^-$ mutant showed the same phenotype as it did in the ΔRC6 strain background (which seemed to imply that the presence or absence of B800-850 light-harvesting complexes was irrelevant), the nature of the *puc* mutation in U43 is not well characterized. This strain is believed to have suffered a point mutation in one of the *puc* genes (65). The result of such a mutation could be to prevent B800-850 complex formation while simultaneously affecting the *pufX*$^-$ phenotype so that it differed slightly from the phenotype in the ΔRC6 (*puc*$^+$) background. Because of uncertainties about the exact nature and effects of the *puc* mutation in U43, it was decided to focus on the ΔRC6 host strain (which is wild type apart from the *puf* operon deletion).

Since it seemed possible that photosynthetic deficiency arose from an inability to transfer energy or electrons from one component of the photosynthetic unit to another, it was decided to investigate the possibility of a fault in the way these components were assembled. Did the absence of the *pufX* gene product cause some disabling alteration in the structure and/ or stoichiometry of the photosynthetic unit? The structure and stoichiometry of the photosynthetic unit were examined in three different ways: 1) sodium dodecyl sulphate-

polyacrylamide gel electrophoresis (SDS-PAGE) of chromatophores, 2) absorption spectroscopy of whole cells and chromatophores, and 3) electron microscopy of thin sections of cells grown under reduced oxygen conditions.

These three techniques revealed differences between the *pufX*$^-$ mutant and the pseudo-wild type in both stoichiometry and structure. The *pufX*$^-$ mutant had higher levels of RC and B870 polypeptides than the pseudo-wild type and also had larger chromatophore vesicles. How these differences caused the *pufX*$^-$ mutant's inability to grow photosynthetically could not be precisely determined, but it is certainly credible that a disruption in the structure of the photosynthetic unit should affect the function of the photosynthetic unit. The secondary structure data indicating the *pufX* gene product may be a transmembrane protein, with sequence similarity to the B870 α polypeptide, are also suggestive of a structural role for the *pufX* polypeptide.

Since the stoichiometry of RC and B870 polypeptides is thought to be controlled via the stoichiometry of short and long *puf* operon mRNA transcripts, and since mRNA stoichiometry has been shown to be affected by deletions in the *puf* operon it was first necessary to show that deletion of the *pufX* gene was not effecting a change in polypeptide stoichiometry by causing a change in mRNA stoichiometry. RNA blots showed that the deletion of the *pufX* gene did not affect the stoichiometry of short and long *puf* operon mRNA's. Therefore, changes seen in the make-up of the photosynthetic unit must be due to the absence of the *pufX* gene product, and these changes are manifested post-transcriptionally.

2. The secondary mutants

By isolating and characterizing some of the secondary mutants arising from attempts to grow ΔRC6(pTL2) photosynthetically, it was hoped to shed some light on the nature of the lesion caused by the absence of *pufX*.

Typically, after a long lag photosynthetically grown cultures of the primary mutant grew and plates spread from stationary phase cultures had colonies with a variety of phenotypes. Some of these colonies arose from the cells used to inoculate the culture and are made up of cells genetically identical to ΔRC6(pTL2). Most of the colonies, however, are made up of secondary mutants. Looking only at growth rates of the secondary mutants isolated, two phenotypes are seen: 1) cells that are now able to grow photosynthetically at the same rate as the pseudo-wild type, and 2) cells that grow photosynthetically at about half the rate of the pseudo-wild type. Similarily, if the absorption spectra are examined two phenotypes are seen: 1) a spectrum that shows a complete lack of carotenoids and B800-850 complexes, and 2) a spectrum that under low oxygen conditions shows levels of B800-850 complexes depressed by 20-25% such that the farthest red absorption peak is red-shifted from 1 to 17 nm, and under photosynthetic growth conditions shows levels of B800-850 complexes elevated by 20-25% relative to the pseudo-wild type. The different absorption spectrum of ΔRC6(pTL2)-1 corresponded with its SDS-PAGE profile in that the B800-850 polypeptides were missing from the gel and there was no absorption due to this antenna complex. On the other hand, the SDS-PAGE profiles of ΔRC6(pTL2)-7 and ΔRC6(pTL2)-9 did not correspond to the spectra of these two strains; both strains showed diminished absorption due to B800-850 antenna complexes relative to the pseudo-wild type, but the gel showed that the level of B800-850 antenna complex-associated polypeptides was equal to or, perhaps, higher than the levels seen in the pseudo-wild type. Photosynthetically grown cultures of ΔRC6(pTL2)-7 and

ΔRC6(pTL2)-9 have increased B800-850 absorption relative to ΔRC6(pTB999), so it is possible that expression of the associated polypeptides is equally high under both growth conditions, but that the degree of incorporation of the polypeptides into functional complexes is different under the two growth conditions. Note that a given growth rate phenotype is not necessarily associated with a specific absorption spectrum; the three secondary mutants discussed here are typical of the thirty secondary mutants seen, but preliminary characterization of these thirty secondary mutants showed that at least two of them that lacked B800-850 complexes had a doubling time twice as long as the pseudo-wild type. The spectrophotometric and SDS-PAGE data on the secondary mutants is probably more illuminating than the growth rate data. All the secondary mutants had altered polypeptide stoichiometry in their photosynthetic units compared to both the pseudo-wild type and the primary mutant. Additionally, those secondary mutants that still had B800-850 complexes showed altered regulation of expression of those complexes. The levels of these complexes under low oxygen conditions were lower than the levels of expression seen in the pseudo-wild type even though expression of the *puc* genes encoding the B800-850 polypeptides is thought to be fully induced under these conditions. The levels of these complexes were also disproportionately high under photosynthetic growth conditions.

CONCLUSION

The results show that the loss of the *pufX* gene product deprives *R. capsulatus* cells of their ability to grow photosynthetically. This stands in contrast to the results of Klug and Cohen (23) who found that deletion of *pufX* did not result in loss of the ability to grow photosynthetically, although it did result in a decrease in the growth rate and a change in the absorp-

tion spectrum. The phenotype of the *pufX* mutant observed by these authors is typical of some of the secondary mutants isolated from photosynthetically grown cultures inoculated with ΔRC6(pTL2).

In principle, *pufX* could be involved in photosynthesis in three ways. First, the *pufX* gene product could be directly involved as a pigment-binding polypeptide. Since the main pigment-binding polypeptides of *R. capsulatus* have been well characterized this possibility seems remote. Such a polypeptide would be expected to occur in the same stoichiometry as the *pufL* and *M* polypeptides, but it cannot be identified when the SDS-PAGE profiles of the mutant and pseudo-wild type are compared. A second possible role of the *pufX* gene product is that of an "accessory" structural polypeptide analogous to the B800-850 γ polypeptide. Perhaps a component (or components) of the photosynthetic apparatus is incorrectly assembled in the absence of the *pufX* gene product, resulting in inefficient photosynthetic unit function. An example of this was reported by Jackson *et al.* (21). In *R. capsulatus* PBS108, a photosynthetically deficient mutant that lacks B870 antenna complexes but that has functional RC, B800-850, and cytochrome b/c_1 complexes, they showed that the RC was incorrectly assembled in the membrane. Again, if this were the case one would expect to see the *pufX* gene product occurring in the membrane in amounts at least as great as the RC polypeptides, although it could be a peripheral membrane protein lost during chromatophore purification. A third possible role for the *pufX* gene product is that of a regulatory protein. In principle, regulation could occur at one of three levels: 1) at the level of mRNA synthesis (*i.e.* transcriptional regulation), 2) at the level of protein synthesis (*i.e.* translational regulation), and 3) at the level of assembly of the photosynthetic unit (*i.e.* post-translational regulation). Transcriptional regulation does not seem likely since RNA blots of the *pufX*$^-$ mutants show that levels of *puf* operon transcripts, both full-length and processed, do not differ significantly from those seen in the pseudo-wild type. This leaves the two possibilities of translational and

post-translational regulation. Translational regulation seems the less likely choice. SDS-PAGE shows that all the known polypeptides necessary for photosynthesis are present in aerobically grown ΔRC6(pTL2) cells as well as in the secondary mutants. Absorption spectra of these cells shows that the ratio of B870 to B800-850 complexes in ΔRC6(pTL2) is about 50% higher than in ΔRC6(pTB999) and lower than in the two secondary mutants that still contain B800-850 complexes. This indicates that the *pufX*$^-$ mutation somehow changes the configuration of the photosynthetic unit and that the suppressor mutations overcome this defect by modifying the structure of the photosynthetic unit even further. This conclusion is also supported by the results of the thin-section electron microscopy which show a size difference between pseudo-wild type and mutant inner membrane invaginations. Thus, it would seem to be the regulation of assembly of the photosynthetic unit that is faulty in the mutant. Failure to maintain the correct structure of the photosynthetic unit due to a breakdown in regulation would be phenotypically indistinguishable from the absence of *pufX* as an accessory polypeptide. Therefore, it seems safe to conclude that the ΔRC6(pTL2) gene product is somehow involved in the assembly of functional photosynthetic units either as a structural part of the photosynthetic unit or as a regulator of its assembly. These two possibilities are not mutually exclusive.

The characterization of the *pufX* mutant *R. capsulatus* ΔRC6(pTL2) has made it clear that the *pufX* gene product is essential for the interactions between the RC and the cytochrome b/c_1 complex that are needed for photosynthetic growth. The precise role of the *pufX* gene product remains unclear, but the nature of the secondary suppressor mutations indicates that it interacts with more than one component of the photosynthetic apparatus. Further characterization of the secondary mutants should shed more light on the role of the *pufX* gene product in photosynthesis and, indeed, on the overall process of photosynthesis.

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