Biochimica et Biophysica Acta, 1100 (1992) 160–170

BBABIO 43596

Pleiotropic effects of *pufX* gene deletion on the structure and function of the photosynthetic apparatus of *Rhodobacter capsulatus*

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(Received 22 July 1991)
(Revised manuscript received 12 January 1992)

Key words: *pufX* gene; Photosynthetic unit; (*R. capsulatus*)

By deletion of the *pufX* gene of *Rhodobacter capsulatus* from a plasmid carrying the *puf* operon and complementation of a chromosomal *puf* operon deletion, we created *pufX* mutants and used them to characterize possible functions of the *pufX* gene product. The *pufX* mutants were incapable of photosynthetic growth in a minimal medium, or in a rich medium at low light intensities, although second-site mutations suppressed this phenotype. Measurements made in vitro with intact and solubilized chromatophore preparations indicated that the individual complexes of the photosynthetic unit seemed to function normally, but electron transfer from the reaction center to the cytochrome b/c_1 complex was impaired. The structures of the photosynthetic apparatus of pseudo-wild type and mutant strains were evaluated using absorption spectroscopy and electron microscopy. The *pufX* mutants had intracytoplasmic membrane invaginations about 50% larger in diameter than those of the pseudo-wild type and higher levels of B870 light-harvesting complex. It is concluded that the PufX protein plays an important role in the structure of the functional photosynthetic unit, and its absence results in loss of efficient electron transfer from the Q_B site of the reaction center to the Q_z site of the cytochrome b/c_1 complex.

Introduction

The light-driven formation of a proton gradient in the facultative photosynthetic bacterium *Rhodobacter capsulatus* normally involves three general types of polypeptide complexes: the light-harvesting antenna complexes (B870 and B800–850), the reaction center and the ubiquinol : cytochrome c_1 oxidoreductase (cytochrome b/c_1) complex. Collectively, these integral membrane complexes comprise what may be called the photosynthetic unit. The structures and functions of many of the polypeptides known to make up the photosynthetic unit are well understood. The genes encoding all known protein components have been sequenced, models have been proposed for their positions in the membrane and the three dimensional structures of reaction centers of closely related bacteria have been determined by X-ray crystallography [1].

Photosynthetic energy transduction is usually thought to begin with the capture of light energy by pigment molecules in the antenna complexes. In general, energy passes from pigment molecules that absorb relatively higher energy (shorter wavelength) light to pigment molecules that absorb relatively lower energy (longer wavelength) light and finally reaches a reaction center. The light energy is trapped efficiently at the reaction center, where it is captured in the form of a charge separation across the cytoplasmic membrane. The energy transferred to the reaction center from the antenna complex causes the oxidation of the bacteriochlorophyll (bchl) *a* special pair. The electron lost by the special pair resides transiently on a bacteriopheophytin molecule and passes sequentially to two quinones (Q_A and Q_B). Meanwhile, the special pair is rapidly reduced by electrons originating from the cytochrome b/c_1 complex. After a second photooxidation event the Q_B quinone is fully reduced to the

Abbreviations: bchl, bacteriochlorophyll; BSA, bovine serum albumin; DBH_2, 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinol; SDS, sodium dodecyl sulfate.

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corresponding quinol, with the requisite two protons having come from the cytoplasm, and leaves the reaction center [2]. The cytochrome b/c_1 complex mediates the formation of a proton gradient as it oxidizes quinols and participates in the reactions leading to transfer of electrons back to the reaction center. The net result of the activation of two reaction centers is the translocation of four protons from the cytoplasm to the periplasm, thus forming a gradient of both electrical charge ($\Delta\Psi$) and proton concentration (ΔpH) [2,3]. If light energy is not transferred to or trapped at the reaction center, it is released primarily in the form of fluorescence at longer wavelengths [4].

There is evidence that the three types of complexes in the photosynthetic unit are not randomly arranged in the inner membrane [5] and that disruption of this arrangement can cause loss of the ability to grow photosynthetically [6,7].

Among the genes encoding the protein components of the photosynthetic unit are those of the *puf* operon, which includes six genes including genes encoding the α and β subunits of the B870 antenna complex, and the L and M subunits of the reaction center. Downstream of these genes is an open reading frame that has been designated the *pufX* gene. Although it has been known for some time that the *pufX* gene is part of polycistronic photosynthesis gene messages, no specific function has yet been ascribed to the putative PufX protein [8–12].

We herein present the results of experiments which show that deletion of the *R. capsulatus pufX* gene impairs the ability of cells to grow photosynthetically,

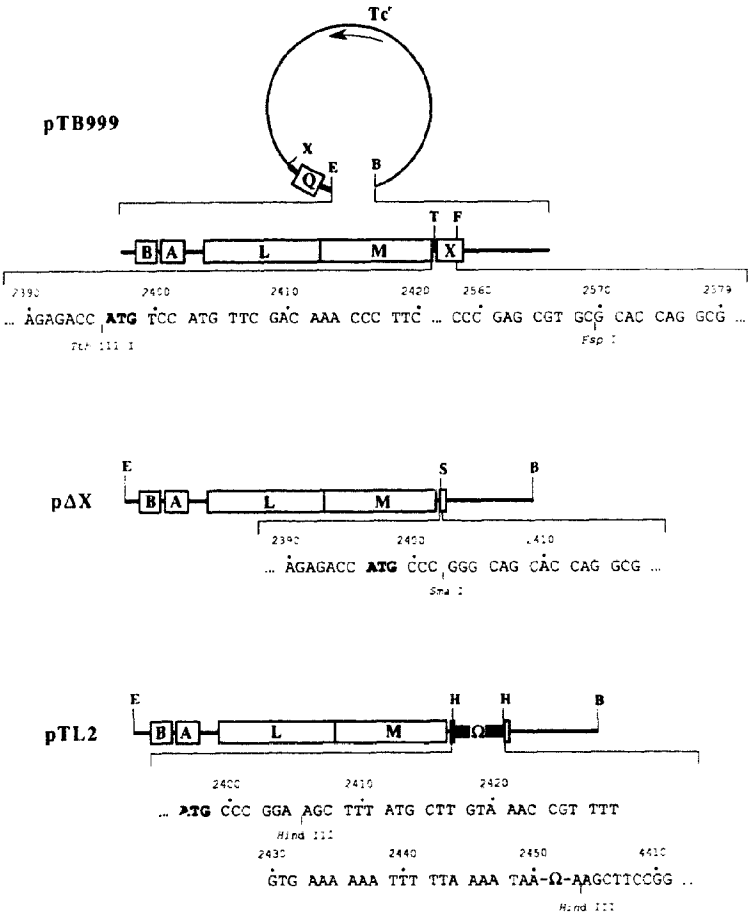

Fig. 1. Representations of plasmids pTB999, pΔX and pTL2. The latter two plasmids were made by deletion and modification of sequences between the *Tth*111I and *Fsp*I sites shown for pTB999. The boxes represent structural genes and the Ω fragment is represented in pTL2 as a heavy black line. The sequences shown 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 *puf* operon *Eco*RI site. Abbreviations: B, *Bam*HI; E, *Eco*RI; F, *Fsp*I; H, *Hin*dIII; S, *Sma*I; T, *Tth*111I; X, *Xho*II.

alters the characteristic stoichiometry of B870 light harvesting to reaction center complexes, and changes the average size of the membrane invaginations that contain the photosynthetic apparatus. Other experiments on electron transport indicate that the *pufX* gene product is essential for efficient transfer of electrons from the reaction center Q_B site to the Q_Z site of the cytochrome b/c_1 complex. We conclude that the PufX protein is necessary for electron transfer within the photosynthetic unit, and is also required for correct assembly of the unit as a structural component and/or as a regulator of its assembly.

Materials and Methods

Bacterial strains and plasmids. The wild type strain B10 of *Rhodobacter capsulatus* has been described [13,14]. Strain ΔRC6 is a *puf*$^-$ derivative of B10 that has a 3316 bp chromosomal DNA deletion. The deletion extends from the *Sal*I site located 40 bp downstream of the start of the *pufQ* gene to the *Xho*II site 488 bp downstream of the 5′ end of the *pufX* gene, with the neomycin phosphotransferase structural gene interposed between these two sites [9].

Strain ΔRC6 *crtD* is identical to *R. capsulatus* ΔRC6 except that it carries a mutation in the *crtD* gene encoding a carotenoid biosynthetic enzyme, introduced by gene transfer agent transduction with strain DE442 as donor [15]. 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 evaluation of the carotenoid bandshift and cytochrome absorbencies much easier.

Plasmid pTB999 (Fig. 1) carries the *pufQ*, *B*, *A*, *L*, *M* and *X* genes on a DNA fragment that extends from the *Xho*II site located 348 bp upstream of the *puf* operon promoter to the *Nru*I site 620 bp downstream of the end of the *pufX* gene. Deletion of the DNA between the filled-in *Tth*111I and the *Fsp*I sites in the *pufX* gene, followed by insertion of a *Sma*I linker at the site of the deletion, created a 174 bp translationally in-frame deletion in *pufX*. Substitution of this deleted gene for the wild type gene carried on pTB999 created the plasmid pΔX. Therefore, plasmid pΔX is identical to pTB999 except for this deletion in the *pufX* gene (Fig. 1).

Plasmid pTL2 (Fig. 1) is identical to pΔX except that it has an Omega (Ω) fragment (Amersham) inserted at the deletion site, accomplished by insertion of a *Hin*dIII linker in the *Sma*I site of pΔX followed by insertion of an Ω fragment in this *Hin*dIII site. The Ω fragment carries transcription and translation terminators at both ends and the truncated *pufX* polypeptide encoded by pTL2 is only 17 amino acids in length, of which only the N-terminal methionine is a *pufX* residue (Fig. 1). Cells of *R. capsulatus* ΔRC6 reconstituted with pΔX or pTL2 were phenotypically identical in all ways tested, but we herein present the results obtained with pTL2.

Growth conditions. All *R. capsulatus* strains were grown in RCV minimal medium [14], sometimes supplemented as noted in the text, at 34°C. High oxygen (aerobic) cultures were grown in flasks filled to 10% of their nominal capacities and shaken at 300 rpm in a rotary shaking water bath. Oxygen-limited cultures were grown in flasks filled to 80% of their nominal volumes and shaken at 150 rpm. Photosynthetic cultures were grown in screw-cap tubes filled to capacity and held in a glass-sided water bath in front of tungsten filament light sources of varying intensity. All cultures used in growth experiments were inoculated to a turbidity of 20 Klett units (approx. $8 \cdot 10^7$ cfu/ml) and growth was followed by measuring the turbidity of cultures using a Klett-Summerson Colorimeter equipped with filter No. 66. Photosynthetic cultures were inoculated from oxygen-limited cultures in stationary phase. Dark anaerobic cultures were grown in RCV medium supplemented with 20 mM fructose and 30 mM dimethyl sulfoxide [16]. Plate cultures were grown on RCV medium supplemented with 15 g/l agar, and media for plasmid-carrying strains were supplemented with 0.5 μg of tetracycline per ml. Photosynthetically grown plate cultures were grown in anaerobic jars (BBL).

Isolation of chromatophores. Chromatophores (inner-membrane vesicles containing the photosynthetic apparatus) were prepared as described [17] from cells grown under reduced aeration (see above) until exponential growth had stopped due to oxygen limitation. Cell densities were typically $4.5 \cdot 10^8$ to $5.5 \cdot 10^8$ cfu/ml (120 to 150 Klett units; see Fig. 2A). Chromatophores used for the cytochrome b/c_1 assays were solubilized using 1.5 g of *n*-dodecylmaltoside (Boehringer-Mannheim) per g of protein as described [18].

Spectrophotometric analyses. Absorption spectra were obtained of aerobically and photosynthetically grown intact cells (about $1.8 \cdot 10^9$cells suspended in 1 ml of 22.5% bovine serum albumin (BSA) in RCV medium) using a Hitachi U-2000 double-beam spectrophotometer (bandwidth of 2 nm).

Reduced minus oxidized differential absorption spectra and flash spectroscopy were carried out as described [19,20].

Thin-section electron microscopy. Cells from cultures grown under oxygen-limited conditions were fixed according to the method of Ryter and Kellenberger [21] and stained with lead salts and uranyl acetate. Silver sections were examined and photographed using a Zeiss EM C10 electron microscope. The mean diameter of chromatophore invaginations in electron micrographs was determined by averaging 35 direct measurements made on each strain.

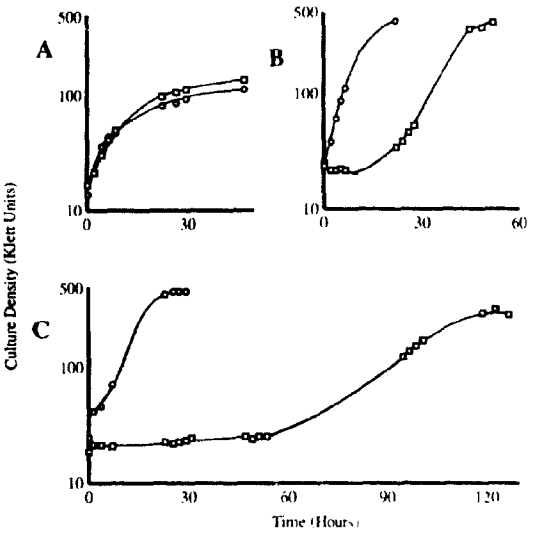

Fig. 2. Comparison of growth of *R. capsulatus* strains ΔRC6(pTB999) (○) and ΔRC6(pTL2) (□) under three different conditions of growth: A, grown under low aeration; B, photosynthetic growth conditions, incident light intensity = 1000 μE m^2 per s; C, as in B but light intensity = 120 μE m^2 per s.

Other assays. Light intensity was measured using a model LI-185B photometer equipped with a LI-190SB quantum sensor (Li-Cor).

Protein measurements used the Lowry assay as modified by Peterson [22] with bovine serum albumin as the standard.

The quinol-dependent cytochrome b/c_1 complex activity was assayed as described [23] except that the concentration of DBH_2 was 60 μM, the concentration of horse heart cytochrome *c* was 50 μM, and malonate was omitted. The rates of reduction of horse heart cytochrome *c* were calculated with an extinction coefficient of 21.1 mM^{-1} cm^{-1} [24]. DBH_2 was synthesized as described [25] except that flash column chromatography was substituted for preparative thin-layer chromatography. DBH_2 was reduced prior to use as described [26], except the initial reductant was sodium thiosulfate rather than sodium hydrosulfite and the reduced substrate was finally dissolved in dimethyl sulfoxide rather than ethanol.

The relative concentration of quinones was determined as described [27].

Results

Growth capabilities and kinetics of strains containing wild type and pufX deletion genes

Although *puf* operon mRNA levels were similar in *R. capsulatus* ΔRC6(pTB999) and ΔRC6(pTL2) (T.G. Lilburn, M.Sc. thesis, University of British Columbia, 1990), there were significant differences in photosynthetic growth, as detailed below.

Fig. 2A shows growth curves for ΔRC6(pTB999) and ΔRC6(pTL2) grown aerobically in the absence of illumination, in which it can be seen that ΔRC6(pTL2) grew at approximately the same rate and to the same density as ΔRC6(pTB999). Under conditions of dark anaerobic growth with dimethyl sulfoxide as the terminal electron acceptor, the mutant again grew similarly to strain ΔRC6(pTB999) (T.G. Lilburn, M.Sc. thesis, University of British Columbia, 1990).

Shown in Fig. 2B and C are growth curves obtained for these two strains with cultures grown photosynthetically under two light intensities. Strain ΔRC6(pTB999) showed little or no lag time before initiation of exponential growth under either light condition, whereas ΔRC6(pTL2) always underwent a long lag period before growth was detectable. The length of this lag period was always longer at the reduced light intensity and it was extremely variable; for cultures grown at 120 μE/m^2 per s it ranged from approx. 36 to 111 h. Furthermore, the rates of photosynthetic exponential growth obtained with ΔRC6(pTB999) cultures were fairly reproducible (±17%), whereas the growth rates of ΔRC6(pTL2) cultures, once initiated, were more variable (±36%). Photosynthetic subculture of ΔRC6(pTL2) resulted in reduction or loss of the lag.

These results led us to suspect that secondary (suppressor) mutants which had regained the ability to grow photosynthetically had arisen within the ΔRC6(pTL2) cultures. Indeed, plates that were streaked with cells from a stationary phase, photosynthetically grown ΔRC6(pTL2) culture were found to give rise to colonies displaying a variety of pigmentation types that could be distinguished with the naked eye. When plates were spread with cells from aerobically grown cultures of ΔRC6(pTL2) and incubated photosynthetically it was found that none of the cells from the aerobic culture were initially capable of photosynthetic growth. Eventually, a few colonies appeared, 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 ΔRC6(pTL2) cultures, by spreading known numbers of cells on solid media followed by incubation under anaerobic, illuminated growth conditions, gave a value of approx. 10^{-5}.

The growth experiments described above were carried out using RCV minimal medium. When analogous experiments were done using a rich medium, YPS [14], and a light intensity of 120 μE/m^2 per s, it was seen that ΔRC6(pTL2) was able to grow photosynthetically about as well as ΔRC6(pTB999). When the light intensity was reduced to 35 μE/m^2 per s, ΔRC6(pTL2) showed a lag and a slower growth rate relative to

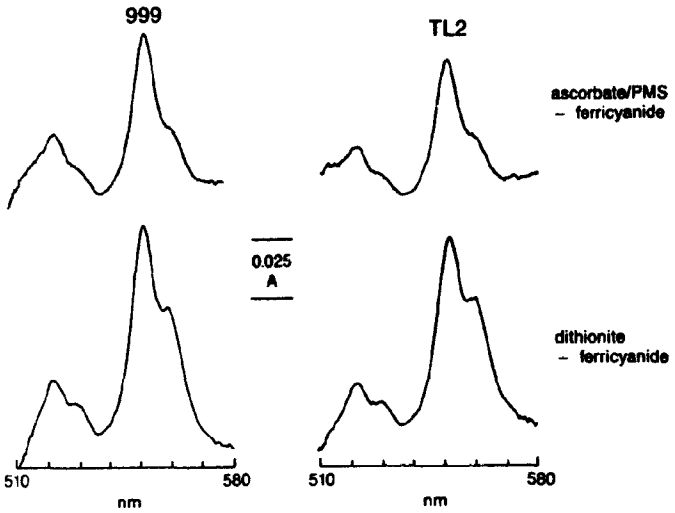

Fig. 3. Reduced minus oxidized absorption spectra of cytochromes in chromatophores from strains ΔRC6*crtD*(pTB999) (left) and ΔRC6*crtD*(pTL2) (right). Chromatophores (113 μM bchl) were suspended in 20 mM *N*-morpholinopropanesulfonate-100 mM KCl (pH 7.0), a few crystals of potassium ferricyanide were added and a baseline spectrum was recorded. A few crystals of sodium ascorbate were then added, the sample was made 2 μM with respect to *N*-methylphenazonium methosulphate (PMS) and the difference spectrum (indicated as ascorbate/PMS-ferricyanide) was recorded. To obtain the second difference spectrum from each sample (indicated as dithionite-ferricyanide) a few crystals of sodium dithionite were added to the sample cuvette and a new difference spectrum recorded.

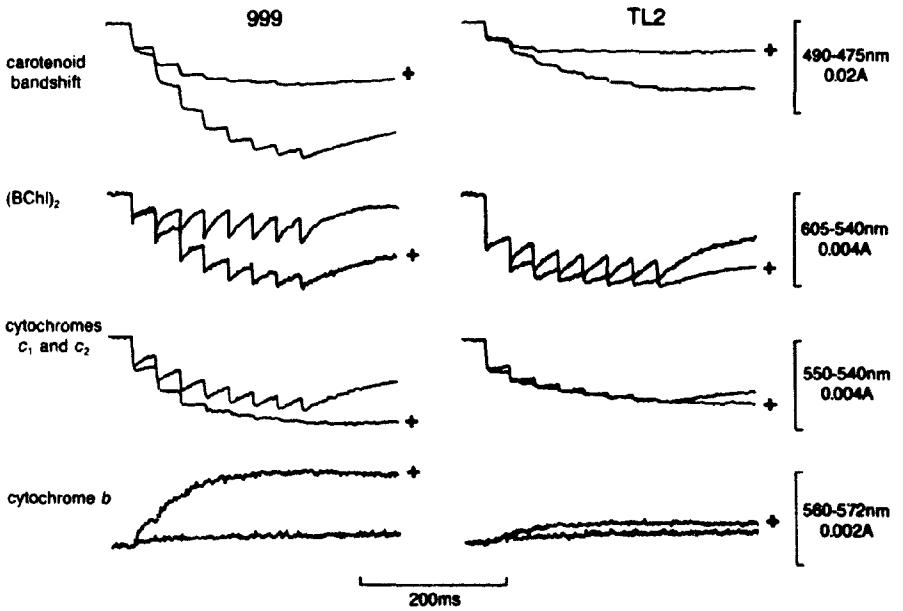

Fig. 4. Responses of the carotenoid bandshift (490–475 nm), reaction center (605–540 nm), *c*-type cytochromes (550–540 nm), and *b*-type cytochromes (560–572 nm) in chromatophores from strains ΔRC6*crtD*(pTB999) (left) and ΔRC6*crtD*(pTL2) (right) to eight actinic flashes. Chromatophores (25 μM bchl) were suspended in the same buffer as in Fig. 3 and the E_h was brought to about 150 mV by the addition of a few crystals of sodium ascorbate. Traces for each strain were recorded separately in the presence (+) and absence of 2 μM antimycin, and overlaid for ease of comparison.

ΔRC6(pTB999), although the growth rates of some of these cultures did increase after 50 to 60 h presumably because of the growth of suppressor mutants (T.G. Lilburn, M.Sc. thesis, University of British Columbia, 1990).

Reduced minus oxidized differential absorption spectra

For these and other oxidation-reduction experiments a *crtD* mutant derivative of ΔRC6 was constructed (see Materials and Methods). Optical difference spectra were used to estimate the relative amounts of *c*- and *b*-type cytochromes in chromatophores prepared from Δ*RC6crtD*(pTB999) and Δ*RC6crtD*(pTL2). Ascorbate, plus the redox mediator *N*-methylphenazonium methosulphate, reduces high potential cytochromes including cytochromes c_1 and c_2 (which have α band absorption maxima at about 550 nm) and some *b*-type cytochromes, which have a band absorption maxima at about 560 nm. Dithionite reduces both these high potential cytochromes and low potential cytochromes, which are mainly *b*-type cytochromes. Fig. 3 shows that, although levels of *c*-type cytochromes in Δ*RC6crtD*(pTL2) were slightly lower than those seen in Δ*RC6crtD*(pTB999), the levels of *b*-type cytochromes were almost identical in the two strains. This implies that the two strains have approximately the same amounts of cytochrome b/c_1 complexes. Because there are *b*- and *c*-type cytochromes in *R. capsulatus* other than those known to be directly involved in photosynthetic electron transfer, these spectra must be regarded as only semi-quantitative indicators of the levels of the cytochrome b/c_1 complex, but they do point to the fact that deletion of the *pufX* gene does not lead to a gross alteration in the concentration of the cytochrome b/c_1 complex.

Light-dependent electron flow through the reaction center and cytochrome b/c_1 complexes

Cyclic electron flow in Δ*RC6crtD*(pTB999) and Δ*RC6crtD*(pTL2) was assessed using flash spectroscopy. Chromatophores were exposed to a train of eight saturating actinic flashes and the differences in absorption at selected wavelengths were measured, in the presence and absence of antimycin. (Antimycin is an inhibitor of electron transfer from cytochrome b_h to the quinone in the Q_c site of the cytochrome b/c_1 complex.) In these experiments the ambient potential (E_h) was determined by the presence of sodium ascorbate. Under these conditions the cytochromes c_1 and c_2 and the Rieske cluster are essentially fully reduced, while Q_Z and the *b* cytochromes are essentially fully oxidized. The results obtained with Δ*RC6crtD*(pTB999) are shown on the left of Fig. 4. In the absence of antimycin the majority of the reaction centers ($(Bchl)_2$; monitored at 605–540 nm) were rapidly reduced after each flash, and the oxidation of the cytochromes c_1 and c_2 responsible for this reduction was partially masked by their rapid reduction by the Rieske iron sulfur cluster, which was in turn reduced by cyclic electron flow initiated by the light flashes. The concomitant generation of a transmembrane potential was evinced by the carotenoid bandshift. The addition of antimycin interrupted the cyclic electron flow, so oxidized cytochromes c_1 and c_2 were no longer reduced after the first flash and were essentially completely oxidized by the later flashes. At the same time the *b* cytochromes were stranded in the reduced form, and cyclic electron flow with its associated generation of the transmembrane electrochemical gradient ceased.

Turning to the data for Δ*RC6crtD*(pTL2), shown on the right of Fig. 4, it is apparent that this strain has a significant defect in its light-initiated electron transfer. Although the extent of the carotenoid bandshift after the first flash was substantial, there was relatively little additional change after subsequent flashes. Even after eight flashes the magnitude of the bandshift was only about one third of that generated in Δ*RC6crtD*(pTB999). Although the special pair and cytochromes *c* were capable of photobleaching, these components were not rereduced as rapidly as in the preparation from Δ*RC6crtD*(pTB999). The addition of antimycin had only a small effect on the kinetics of cyclic electron transfer. We attribute these differences to be due to a reduction in electron flow to, or through, the cytochrome b/c_1 complex of Δ*RC6crtD*(pTL2).

Single flash kinetics of the carotenoid bandshift

Fig. 4 shows that cyclic electron transfer was essentially nonexistent in Δ*RC6crtD*(pTL2) under the conditions of that experiment, suggesting that although the reaction center was working normally, the electrons coming out of the reaction center did not reach the cytochrome b/c_1 complex. This might be have been due to interruption of electron flow out of the reaction center, or into the cytochrome b/c_1 complex. One way to assess which one of these alternatives might be more important would be to repeat the experiment of Fig. 4 under conditions where Q_Z is chemically reduced before the flash. Under such conditions, which are optimal for cyclic electron flow and associated phosphorylation of ADP [2], electrons are available within the cytochrome b/c_1 complex to reduce the Rieske iron sulfur cluster and the cytochromes *b* as soon as the Rieske cluster is oxidized by cytochrome c_1, so the kinetics of cyclic electron flow, and of the concomitant carotenoid bandshift, are no longer limited by the rate of arrival of electrons from Q_B (see Ref. 2). This experiment was done and is described below.

The upper portion of Fig. 5 compares the carotenoid bandshift in Δ*RC6crtD*(pTB999) with Q_Z either oxidized or reduced before the flash. Phases I and II were not resolved kinetically on this time scale, but together

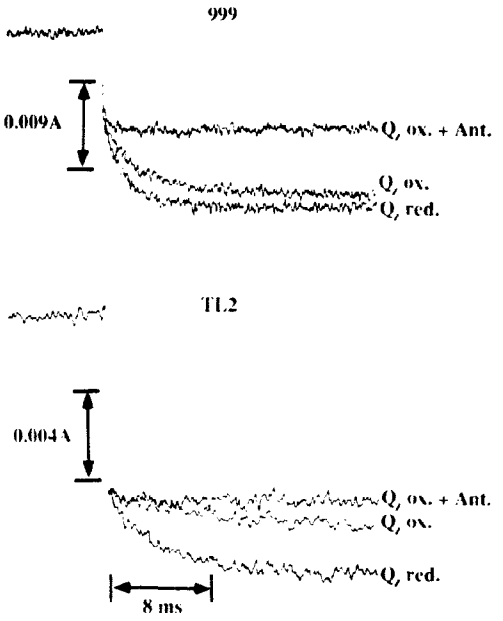

Fig. 5. Single flash carotenoid bandshift kinetics in chromatophores from *R. capsulatus* strains ΔRC6*crtD*(pTB999), designated 999, and ΔRC6*crtD*(pTL2), designated TL2. Chromatophores were suspended in 20 mM 4-morpholinepropanesulfonic acid, 100 mM KCl, 1 mM $MgCl_2$ (pH 7) to a final bchl concentration of 25 μM. 5 μM diaminodurene-2,3,5,6-tetramethylphenylenediamine, *N*-methylphenazonium methosulfate and *N*-ethylphenazonium ethosulfate were added as redox mediators. The redox midpoint potential was poised at $E_h \approx 90$ mV (Q_z red.) or at $E_h \approx 225$ mV (Q_z ox.), and antimycin added to 2 μM where indicated. A single flash was delivered and the kinetics of the optical change at the wavelength pair 490–475 nm were monitored.

they gave rise to the bandshift seen in the presence of antimycin. As expected (see Ref. 2), starting with Q_z reduced stimulated the rate of the third phase of the bandshift, although it had only a minor effect on the magnitude of phase III, perhaps because in the Q_{ox} preparation the quinone pool was not completely oxidized.

The lower portion of Fig. 5 shows the same experiment with ΔRC6*crtD*(pTL2). Concordant with Fig. 4, there was little phase III of the bandshift when Q_z was oxidized prior to the flash, although the extent of phases I plus II was similar to that seen in ΔRC6*crtD* (pTB999) (note the different scales). In this case, however, there was a dramatic effect on both the kinetics and the apparent extent of phase III when Q_z was reduced prior to the flash. It is thus clear that the Q_z site of the cytochrome b/c_1 complex of ΔRC6*crtD* (pTL2) behaves almost normally if quinones are chemically reduced prior to a flash. Note, however, that the extent of phase III in this strain under these conditions was not equal to the sum of phases I plus II, as it was

TABLE I

Cytochrome b/c_1 complex specific activities found in chromatophore fractions solubilized with n-dodecylmaltoside

Strain	Cytochrome b/c_1 complex specific activity [a]		
	crude chromatophores plus DM	non-solubilized chromatophores (pellet) [b]	solubilized chromatophores (supernatant liquid) [b]
ΔRC6(pTL2)	107	20	192
ΔRC6(pTB999)	121	26	173

[a] μmol cytochrome *c* reduced per min per mg protein.
[b] Solubilized chromatophores were centrifuged at 100000 × *g* for 90 min.

in ΔRC6*crtD*(pTB999), but only approximately half this in magnitude. This indicates that even when Q_z is chemically reduced prior to the flash, and is able to donate electrons to the Rieske cluster and cytochromes *b* during cyclic electron flow, there is still an impair-

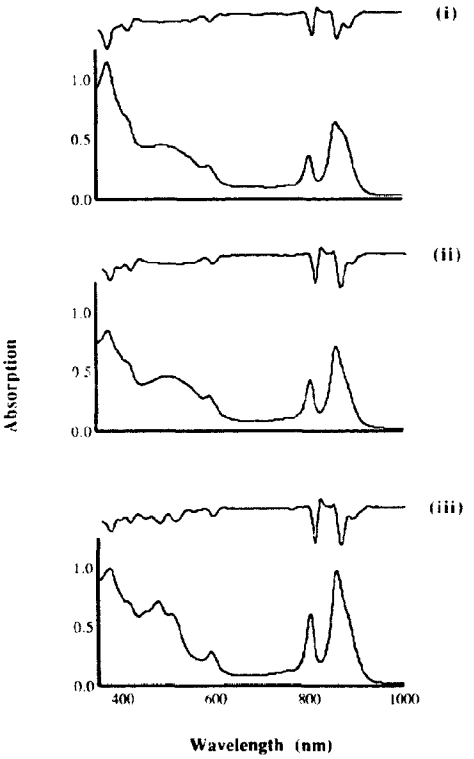

Fig. 6. Absorption spectra of equal numbers of intact cells of *R. capsulatus* strains: (i) ΔRC6(pTL2), grown under low aeration; (ii) ΔRC6(pTB999), grown under low aeration; (iii) ΔRC6(pTB999) grown photosynthetically. The fourth derivatives of the spectra (upper traces) are also shown.

ment in the rate of re-reduction of Q_Z by the reaction center.

Relative concentrations of quinones

Light-dependent electron flow from the RC to the cytochrome b/c_1 complex could in principle be restricted by low levels of quinones in chromatophore invaginations of ΔRC6(pTL2) cells. Comparison of relative concentrations of quinones in chromatophores from ΔRC6(pTL2) and ΔRC6(pTB999) showed that, at protein concentrations of 10 mg/ml, chromatophores from ΔRC6(pTB999) contained quinones at a concentration of 1.8 μM whereas the concentration in ΔRC6(pTL2) was 1.7 μM.

Quinol-dependent electron flow through the cytochrome b/c_1 complex

Because light-dependent electron flow to, or through, the cytochrome b/c_1 complex was greatly impaired in ΔRC6*crtD*(pTL2), yet the amounts of *b*- and *c*-type cytochromes and quinones seemed similar to ΔRC6*crtD*(pTB999), we assayed electron flow through the solubilized cytochrome b/c_1 complex in chromatophores with an excess of DBH_2 as electron donor (see Table I). This was done with chromatophore preparations from cells grown under oxygen-limiting conditions. The rates obtained (173 nmol cytochrome *c* reduced/min per mg protein for RC6 (pTB999) and 192 nmol cytochrome *c* reduced/min

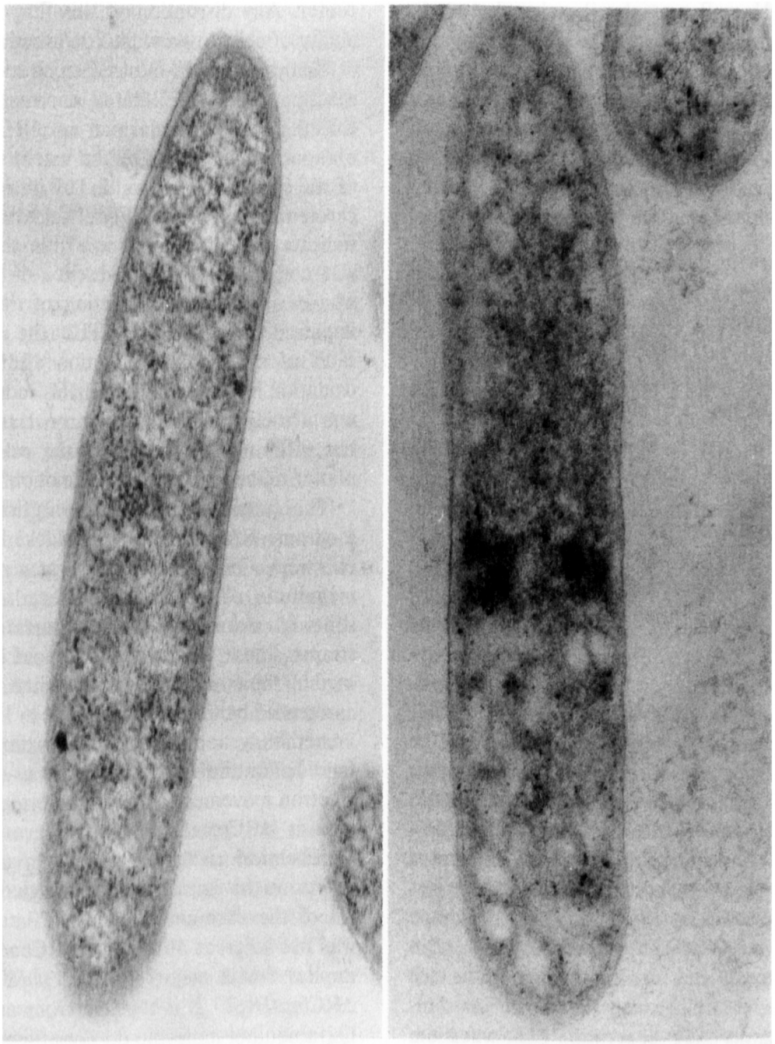

Fig. 7. Transmission electron micrographs of thin sections of cells grown with low aeration. A: ΔRC6(pTB999) and B: ΔRC6(pTL2).

per mg protein for RC6(pTL2)) indicated that there was no great difference in the specific activities of solubilized cytochrome b/c_1 complexes from the two strains.

Absorption spectroscopy of intact cells

Fig. 6 shows representative absorption spectra from single scans of intact cells of ΔRC6(pTB999) and ΔRC6(pTL2) grown under photosynthetic or oxygen limiting conditions, and the fourth derivatives of these spectra. The amounts of B870 and B800–850 complexes can be estimated on the basis of their absorption peaks. Cells of ΔRC6(pTL2) grown under low oxygen conditions (Fig. 6(i)) reproducibly showed an increased B870 shoulder compared with ΔRC6(pTB999) (Fig. 6(ii)), whereas the magnitudes of the B800–850 absorption peaks were more variable. These differences were reproducible in many experiments with intact cells and chromatophores, and show that elevated levels of the B870 complex resulted from the *pufX* mutation. These data were supported by the results of SDS-PAGE of chromatophore proteins, which showed increased amounts of the B870 complex peptides in chromatophores from cells of ΔRC6(pTL2) (T.G. Lilburn, M.Sc. thesis, University of British Columbia, 1990).

Electron microscopy of thin sections of ΔRC6 cells containing either pTB999 or pTL2

Fig. 7 shows representative electron micrographs of thin sections of ΔRC6(pTB999) and ΔRC6(pTL2) cells grown under oxygen-limited conditions. Direct measurements of the diameters of chromatophore invaginations in these and similar micrographs showed that chromatophores of ΔRC6(pTL2) had a mean diameter approx. 50% greater than that of ΔRC6(pTB999). However, chromatophores from ΔRC6*crtD*(pTL2) did not clearly separate from ΔRC6*crtD*(pTB999) chromatophores during rate zonal centrifugation in 5–35% sucrose density gradients (unpublished data).

Discussion

In order to investigate possible functions of the *pufX* gene product, the experiments described above were done on a pseudo-wild type control strain (*R. capsulatus* ΔRC6(pTB999)) and a *pufX* insertion/deletion mutant (*R. capsulatus* ΔRC6(pTL2)), and a variety of changes in phenotype attributable to the loss of the PufX protein was observed.

R. capsulatus ΔRC6(pTL2) grew at normal rates under both low oxygen and high oxygen conditions (see Fig. 2); therefore, it would seem that no major deficiencies in respiratory ATP generation exist when the *pufX* gene is deleted. On the other hand, strain ΔRC6(pTL2) was unable to grow in minimal RCV medium under photosynthetic growth conditions (see Fig. 2). We attribute the growth that eventually was obtained to the presence of second-site suppressor mutants present (at a level of about 10^{-5}) in *pufX* mutant cultures. The lack of photosynthetic growth, except in the rich YPS medium at higher light intensities (≥ 120 μE m^{-2} per s), implies that the *pufX* mutant was greatly impaired, although not absolutely lacking, in some photosynthetic process. This is consistent with the increased length of lag times with decreased light intensity. It is noteworthy that the *pufX* gene is part of an operon which encodes five other polypeptides that, collectively, are essential for normal photosynthetic growth [7,8].

Photosynthetic energy transduction depends on the flow of electrons through the reaction center, to the cytochrome b/c_1 complex via a quinol, through the cytochrome b/c_1 complex and back to the reaction center. Any disruption of this flow would impair the ability of cells to grow photosynthetically.

Analysis of light-induced electron flow through the reaction center in isolated chromatophores revealed that the bchl *a* special pair in ΔRC6*crtD*(pTL2) was photoactive, as shown by the extent of photobleaching of the special pair as well as by phases I and II of the carotenoid bandshift (Figs. 4 and 5). These results indicate that electron flow within the reaction center was not affected by the absence of the PufX protein, whereas subsequent reduction of the bchl dimer was impaired. In ΔRC6*crtD*(pTL2) the initial extents and rates of cytochrome reduction after the first photooxidation indicate that under this redox condition there was a small amount of electron transfer (see Fig. 4), but with subsequent flashes the extents and rates of photooxidized cytochrome reduction were much lower.

The possibility that electron flow within the cytochrome b/c_1 complex was blocked was evaluated in two ways. One method used was a comparison of the magnitude of single flash-induced carotenoid bandshifts in redox-poised chromatophores from the two strains. Phase III of the carotenoid bandshift is mediated by the b/c_1 complex, and when this portion of the carotenoid bandshift is examined in Fig. 5 two facts are immediately apparent: (i) when the ubiquinone pool (and hence the Q_Z ubiquinone) was poised oxidized, electron movement through the cytochrome b/c_1 complex in ΔRC6*crtD*(pTL2) was severely impaired, and (ii) chemical reduction of the Q_Z ubiquinone largely overcame this impairment, although the extent of phase III of the carotenoid bandshift in ΔRC6*crtD*(pTL2) was not as great as seen in ΔRC6*crtD*(pTB999). This implies that a major effect of *pufX* gene deletion in ΔRC6*crtD*(pTL2) is that electrons associated with the Q_B ubiquinol molecule do not efficiently reach the Q_Z site of the cytochrome b/c_1 complex.

We also tested the ability of solubilized cytochrome b/c_1 complexes to catalyze reduction of cytochromes c when provided with an excess of the ubiquinone analogue DBH_2 (see Table I). Using this criterion, electron flow through the b/c_1 complexes of the two strains were comparable. When the concentration of DBH_2 was titrated, the rates obtained showed non-Michaelis-Menten kinetics, so we could not assess the affinity of the Q_Z site for quinol in this way.

Since it seemed likely that the photosynthetic deficiency in ΔRC6(pTL2) arose from an inability to transfer electrons from one component of the photosynthetic unit to another, the possibility of a fault in the way these components were assembled was investigated. The structure and stoichiometry of the photosynthetic unit were examined in two ways presented here: (1) absorption spectroscopy of whole cells and chromatophores, (2) electron microscopy of chromatophore invaginations in thin sections of cells grown under reduced oxygen conditions.

The absorption scans of intact cells of ΔRC6(pTL2) (see Fig. 6) revealed an increase in the amount of B870 absorption in comparison to ΔRC6(pTB999), and this change was also apparent from the B870α and B870β protein band intensities in SDS-PAGE of chromatophore preparations (T.G. Lilburn, M.Sc. thesis, University of British Columbia, 1990).

Transmission electron micrographs of thin sections of cells from cultures of ΔRC6(pTB999) and ΔRC6 (pTL2) grown under oxygen-reduced conditions, shown in Fig. 7, revealed that the inner membrane invaginations of ΔRC6(pTL2) had a mean diameter about 50% greater than those of ΔRC6(pTB999).

How these differences relate to the inability of strain ΔRC6(pTL2) to grow photosynthetically is unclear, but it is certainly credible that a disruption in the protein composition of the photosynthetic unit could affect chromatophore size. The increase in chromatophore size may be a secondary effect, although our data do not allow us to distinguish between possible alternative cause and effect relationships between intracytoplasmic membrane area and amounts of photosynthetic membrane proteins. However, preliminary fluorescence experiments indicate that energy transfer from the B870 light-harvesting complex to the reaction center does not seem to be impaired in the *pufX* mutant (N. Woodbury, personal communication).

The results of the electron microscopy and absorption spectroscopy experiments, taken together, indicate that the *pufX* mutation changes the structure of the photosynthetic unit. Thus, it would seem that assembly of the photosynthetic unit is faulty in ΔRC6(pTL2), perhaps because the PufX polypeptide interacts both with bchl-protein complexes and the cytochrome b/c_1 complex. It has been proposed that, in vivo, reaction centers form supercomplexes with a subset of cytochrome b/c_1 complexes [5,19,28]. We speculate that the PufX protein may be a structural component of the photosynthetic unit that physically links the reaction center to the cytochrome b/c_1 complex. Indeed, computer programs used to evaluate possible properties of the PufX protein (Refs. 29–31; as found on PC/GENE 6.01 (Intelligenetics)) indicate that the PufX protein is likely to be a transmembrane protein, and displays 26% amino acid identity when aligned with the *R. capsulatus* B870α peptide sequence. However, the possibility that the PufX protein could otherwise facilitate ubiquinol production or transfer cannot be ruled out. For example, it is possible that the binding constant of the semiquinone at the Q_B site of the reaction center is significantly lower in the absence of the PufX protein. This would result in the more frequent release of the semiquinone from the Q_B site, a consequent drop in the number of electrons reaching the Q_Z site of the cytochrome b/c_1 complex and an impairment in photo-induced cyclic electron flow. However, it is also possible that the affinity of the Q_Z site for quinol is reduced. Evidently, the suppressor mutations overcome some of the effects resulting from the absence of the PufX protein by further modifying the structure of the *pufX* photosynthetic unit (unpublished data). Alternatively, the *pufX* gene product may be involved catalytically in regulation of the assembly of the photosynthetic unit, although these two possibilities are not mutually exclusive.

Farchaus et al. have reported the results of experiments on a similar *pufX* mutant of *R. sphaeroides*, in which they found that a deletion strain seemed to be incapable of photosynthetic growth [12]. A *pufX* mutant strain studied by these authors also had increased amounts of the B870 complex homologue (LH1) and suppressor mutants capable of photosynthetic growth arose after prolonged incubation of cultures. A reaction center preparation from this strain showed photobleaching equivalent to a control preparation from a pseudo-wild type strain, although no experiments on electron transfer between components of the photosynthetic apparatus were reported. Nevertheless, these data are indicative of very similar functions for the PufX proteins of these two species.

The results presented in this communication show that the loss of the *pufX* gene product deprives *R. capsulatus* cells of their ability to grow photosynthetically in a minimal medium. Although this apparently contrasts with a previous publication in which it was reported that deletions of the *pufX* gene did not eliminate the ability to grow photosynthetically, this difference may be ascribed to the fact that these authors grew their mutant strain in a complex medium [11]. Photosynthetic growth on rich but not minimal media was also seen in a strain of *R. capsulatus* that was constructed to contain the *R. sphaeroides* cy-

tochrome b/c_1 complex, and which also seemed to lack the requisite interaction between the cytochrome b/c_1 complex and the reaction center [19].

In view of these collective results, it seems that a major function of the PufX protein is to facilitate, directly or indirectly, electron transfer from the Q_B site of the reaction center to the Q_z site of the cytochrome b/c_1 complex.

Acknowledgements

We thank W. Parson for assistance in the early stages of this work, B. Marrs for the *crtD* GTA overproducer strain *R. capsulatus* DE442, E. Piers for help with DBH_2 synthesis and M. Weis for instruction in electron microscopy. T.G.L. thanks the B.C. Medical Services Foundation for a predoctoral scholarship.

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