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The *Rhodobacter sphaeroides* PufX protein is not required for photosynthetic competence in the absence of a light harvesting system

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Abstract

The effects of deletion of the gene encoding the PufX protein from *Rhodobacter sphaeroides* have been examined using bacterial strains with simplified photosystems. We find that the PufX protein is required for photosynthetic growth in strains which have the LH1 antenna complex, but is not required in a reaction centre-only strain, suggesting that the PufX protein does not directly facilitate cyclic electron transfer between the reaction centre and the cytochrome bc_1 complex. The influence of PufX and carotenoid type on the size of the reaction center/LH1 core complex has also been examined in these strains.

Key words: PufX; Cyclic electron transfer; Core complex; Photosynthetic unit; *Rhodobacter sphaeroides*

1. Introduction

The photosynthetic bacterium *Rhodobacter sphaeroides* has a relatively simple photosystem consisting of a core of reaction centres (RC) plus the light harvesting 1 (LH1) antenna, surrounded by a peripheral light harvesting system (LH2) [1]. In the simple 'lake' model [1], captured light energy is transferred from LH2 to LH1 and finally to the reaction centre, the site at which photochemistry takes place. Photochemical charge separation involves the transfer of an electron across the membrane from the dimeric bacteriochlorophylls of the reaction centre (the primary donor), via a bacteriopheophytin molecule, to the initial ubiquinone acceptor (Q_A) and finally to the secondary ubiquinone (Q_B). After rereduction of the primary donor by cytochrome c_2, further electronic excitation of the primary donor results in the transfer of a second electron along the same pathway to Q_B, where the acquisition of two protons results in the formation of ubiquinol which dissociates from the Q_B site into the lipid phase of the membrane. The cytochrome bc_1 complex utilises ubiquinol and oxidised cytochrome c_2 as reductant and oxidant, respectively, with the net result of this cyclic electron transfer being the vectoral translocation of protons across the membrane, forming a transmembrane proton electrochemical gradient.

The genes encoding the reaction centre, LH1 and LH2 proteins have been cloned and sequenced in *Rb. sphaeroides*. The LH1 α and β apoproteins are encoded by *pufA* and *pufB* genes, the L and M subunits of the reaction centre are encoded by *pufL* and *pufM* [2–4] and the reaction centre H subunit is encoded by *puhA* [5]. The *pufB*, *pufA*, *pufL* and *pufM* genes reside within the same operon and are transcribed in the order *BALM* [6]. There are two additional genes within the *puf* operon: upstream of *pufB* there is *pufQ*, which has been implicated in bacteriochlorophyll synthesis [7,8], and downstream of *pufM* there is *pufX* [9]. The *pufX* gene encodes a protein of 82 amino acids which has one possible membrane-spanning helix [9]. Recent studies have shown that strains of *Rb. sphaeroides* and the closely related species *Rb. capsulatus* which lack the *pufX* gene are non-photosynthetic [10,11]. The precise role of the PufX protein is still unknown but it has been shown in both species that cyclic electron flow is severely impaired in the absence of this protein, despite primary charge separation occuring within the reaction centre [11,12]. Additionally, the LH1 : RC ratio has been observed to increase dramatically in the absence of PufX in these same strains [10,11]. These observations have led to proposals that the PufX protein facilitates, directly or indirectly, the flow of ubiquinone and ubiquinol between the Q_B site of the reaction centre and the Q_Z site of the cytochrome bc_1 complex [11–13].

To date, all work on *pufX* has utilised strains in which the peripheral LH2 antenna is present in addition to LH1 and reaction centres. The *Rb. sphaeroides* strains used also contained genomic copies of the LH1 genes, in addition to copies present in a plasmid-based *puf* operon. The present study aimed to determine if photosynthetic growth of *Rb. sphaeroides* has an absolute requirement

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Abbreviations: Rb., *Rhodobacter*; E., *Escherichia*; RC, reaction centre; LH1, light harvesting antenna 1; LH2, light harvesting antenna 2; Q_A, reaction centre primary acceptor quinone Q_B, reaction centre secondary acceptor quinone; Q_Z, quinol oxidase site of the cytochrome bc_1 complex; B_{LH1}, bacteriochlorophylls associated with the LH1 antenna.

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for PufX, or whether the non-photosynthetic phenotype of *pufX*-deficient strains is related to changes in the properties of the antenna complexes which result from the absence of the PufX protein. To this end, reaction centre-only strains of *Rb. sphaeroides* have been constructed both with and without the *pufX* gene to determine whether PufX is essential for physiologically relevant rates of light-driven electron transfer. In addition, RC/LH1 strains which lack the *pufX* gene have been constructed in order to determine the effect of *pufX* on the size of the RC/LH1 core complex in the absence of genomic copies of the LH1 and LH2 structural genes. These strains have allowed for the first time a direct estimate to be made of RC/LH1 core sizes in the presence and absence of PufX.

2. Materials and methods

2.1. Media, strains, plasmids and growth conditions

All bacterial strains and plasmids were as described in [14,15] except plasmids pRKEH10X$^-$ and pRKEH10DX$^-$ which are described in section 3.

Escherichia coli strains were grown in Luria-Bertani medium at 37°C supplemented with tetracycline at 10 $\mu g \cdot ml^{-1}$.

Rb. sphaeroides strains were grown at 34°C in M22+ medium, supplemented with 0.1% casamino acids. Antibiotics were added where appropriate at the following concentrations ($\mu g \cdot ml^{-1}$): tetracycline (1), streptomycin (5), kanamycin (20). Dark, semiaerobic growth was achieved in 30 ml universal bottles containing 10 ml of medium, or in 500 ml conical flasks containing 400 ml of medium, in an orbital incubator at 150 rpm. Photoheterotrophic growth was obtained by inoculating 30 ml universal bottles filled with medium with cells from a semiaerobic culture harvested in early stationary phase, to give a final absorbance at 680 nm of 0.1–0.2. Growth then took place at 34°C and at an illumination of 150 $W \cdot m^{-2}$, and was monitored by measuring the absorbance of aliquots at 680 nm.

2.2. Genetic procedures

Plasmids to be introduced into *Rb. sphaeroides* were initially transformed into *E. coli* strain S17-1. Conjugative crosses were then performed as described in [16].

2.3. Preparation of membranes

Intracytoplasmic membranes were prepared using a French pressure cell, as described in [17], from two independent isolates of each strain.

2.4. Spectroscopy

Absorbance spectroscopy on membrane preparations was performed using a Guided Wave Model 260 fibre optic spectrophotometer (Guided Wave Inc., El Dorado Hills, CA, USA). Absorbance of bacteriochlorophyll in acetone/methanol extracts of membrane preparations was measured using a Beckman DU 640 spectrophotometer (Beckman Instruments UK, High Wycombe, UK).

2.5. Calculation of reaction centre concentration

To calculate the reaction centre concentration from room temperature absorbance spectra the extinction coefficient of 312 $mM^{-1} \cdot cm^{-1}$ at 805 nm for photooxidised reaction centres was used. This coefficient was calculated using the value of 288 $mM^{-1} \cdot cm^{-1}$ at 802 nm for detergent-purified reaction centres in the ground state [18], by comparing the amplitude of the peak in oxidised and reduced reaction centres in membranes from the antenna-deficient strain RCO2/DD13. (On photooxidation of the reaction centre the peak at 802 nm shows a red-shift of 3 nm and a 10% increase in intensity). The absorbance at 805 nm in RC/LH1 membranes was corrected for the contribution from the LH1 antenna by measuring the ratio of the absorbances at 805 nm and 875 nm for membranes prepared from strains which contain only the LH1 complex (described in [14]). The contribution of LH1 to the absorbance at 805 nm in the RC/LH1 membranes was then calculated by multiplying the measured absorbance at 875 nm by this ratio. It should be noted that because of the configuration of the optics in the Guided Wave spectrophotometer the sample is irradiated with white light, resulting in the full bleaching of the low energy exciton component of the Qy transition of the reaction centre primary donor at 870 nm, as observed in reaction centre-only membranes (data not shown), and therefore the absorbance at 875 nm was due solely to the LH1 antenna bacteriochlorophylls. The contribution of LH1 to the absorbance at 805 nm was subtracted from the measured absorbance at 805 nm to give the true absorbance of the reaction centres, and this value was then used to calculate the concentration of reaction centres. This calculation was performed on three dilutions of each membrane preparation.

2.6. Calculation of the number of LH1 bacteriochlorophylls per reaction centre

Membranes from RC/LH1 strains were extracted with acetone:methanol (7:2) and the absorbance at 747 nm and 771 nm was measured. The concentrations of total bacteriochlorophyll were calculated using the equations given in [19]. For each dilution of each membrane preparation the estimated reaction centre concentration (see above) was used to calculate the concentration of reaction centre bacteriochlorophyll; this was then subtracted from the total measured bacteriochlorophyll concentration for each dilution to give the concentration of bacteriochlorophyll associated with the LH1 antenna. The number of LH1 bacteriochlorophylls per reaction centre was calculated from this figure and the associated reaction centre concentration, and the mean determined for each strain.

3. Results

3.1. Construction of pufX *deletion strains*

In previous studies a plasmid-based copy of the *puf* operon was engineered so as to contain several useful restriction sites, yielding a plasmid named pRKEH10 [15] (Fig. 1). The majority of the *pufB* gene and all of the *pufA* gene were then deleted from this to give plasmid pRKEH10D, enabling the genes of the RC L and M subunits to be expressed in the absence of the LH1 structural genes [15]. In order to make versions of these two plasmids in which *pufX* was deleted, a *Sma*I partial digestion was followed by restriction of the unique, engineered, *Bam*HI site immediately upstream of *pufX*. The *Bam*HI site was then filled in and, after ligation, clones were selected in which the *Bam*HI–*Sma*I fragment containing the *pufX* gene had been deleted. These were identified initially by restriction analysis, and the absence of *pufX* was then confirmed by DNA sequencing. In the resulting constructs, the transcription terminator downstream of *pufX* [9] was left intact. Thus two new plasmids were obtained which lacked *pufX* but encoded reaction centres and LH1 (pRKEH10X$^-$) or reaction centres only (pRKEH10DX$^-$) (Fig. 1).

All four plasmids in Fig. 1 were transferred into *Rb. sphaeroides* strain DD13 in which the *pucBA* genes, encoding the LH2 antenna complex, and the *pufBALMX* operon have been replaced by antibiotic resistance cassettes [14]. The resulting transconjugant strains RCLH12/DD13 and RCLH12X$^-$/DD13 contained reaction centres and LH1 with and without *pufX*, respectively, whereas strains RCO2/DD13 and

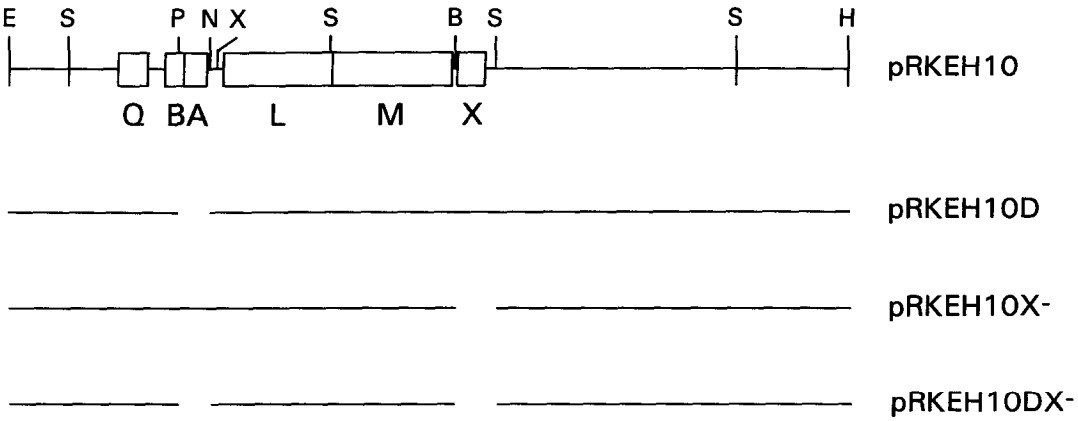

Fig. 1. Restriction map of pRKEH10 [15] and the regions which have been deleted to form pRKEH10D [15], pRKEH10X⁻ and pRKEH10DX⁻. E, *Eco*RI; S, *Sma*I; P, *Pvu*I; N, *Nae*I; X, *Xba*I; B, *Bam*HI; H, *Hin*dIII. Note that the *Xba*I and *Bam*HI sites were introduced by site-directed mutagenesis [15].

RCO2X⁻/DD13 both lacked LH1 but contained reaction centres with and without *pufX*, respectively. The same four plasmids were also conjugated into a spontaneous green-coloured derivative of DD13, DD13/G2 [14] (which has neurosporene as the main carotenoid) so that any effects of carotenoid composition could be investigated.

3.2. Growth of *pufX*⁺ and *pufX*⁻ strains under photoheterotrophic conditions

The complemented DD13 strains described above were grown under dark semiaerobic conditions until early stationary phase, by which time induction of the photosynthetic apparatus had occurred. Photosynthetic cultures were then inoculated using these oxygen-limited cells and the growth monitored at 680 nm. At the end of the growth experiment the presence or absence of the *pufX* gene was confirmed by rescuing the plasmid DNA from the cells in the culture followed by restriction analysis and DNA sequencing.

From Fig. 2 it can be seen that strain RCLH12/DD13 grew photosynthetically after a lag phase of approximately 35 h, with a mean doubling time of 5.5 h during the logarithmic growth phase. In contrast, the growth of RCLH12X⁻/DD13 was unpredictable and the cultures which did grow photosynthetically did so only after a prolonged lag phase of between 100 and 150 h; the doubling times during photosynthetic growth, if achieved, also varied. These results demonstrated that PufX is required for photoheterotrophic growth in strains which have both reaction centres and LH1 antenna complexes, confirming previous results obtained with antenna-containing PufX-deficient strains (Farchaus et al. [10]). The unpredictable growth observed for RCLH12X⁻/DD13 has been observed for similar *Rb. sphaeroides* strains and has been attributed to the occurrence of suppressor mutations which allow photosynthetic growth in the absence of PufX [10].

The growth of strains RCO2/DD13 and RCO2X⁻/DD13 was also monitored (Fig. 2). Both strains had a very similar pattern of photoheterotrophic growth with no lag phase and mean doubling times of approximately 11 h (RCO2/DD13) and 10 h (RCO2X⁻/DD13). In a typical experiment an absorbance at 680 nm of between 0.6 and 0.8 was reached for both strains, with no statistically significant difference between the two strains in terms of the final absorbance reached. This level of growth contrasts with the final absorbance at 680 nm of strain RCLH12/DD13 of between 2.0 and 2.5, and can be accounted for by the lack of light harvesting complexes in the RCO2/DD13 and RCO2X⁻/DD13 strains which leads to inhibition of photosynthetic growth by self-shading as the cultures thicken. This self-shading was also manifested in the relatively short period of true logarithmic growth exhibited by both RC-only strains (Fig. 2).

3.3. Estimation of the size of the RC/LH1 core in *pufX*⁺ and *pufX*⁻ RC/LH1 strains

Intracytoplasmic membranes were prepared from two independent isolates of each of the RCLH12/DD13 and RCLH12X⁻/DD13 strains. Room temperature absorption spectra of these membranes were normalised according to the reaction centre concentration, estimated as described in section 2. Comparison of the RCLH12/DD13 and RCLH12X⁻/DD13 spectra (Fig. 3A) indicated that the core size had increased slightly in the absence of PufX. The absolute core sizes were then estimated by measuring the number of LH1 bacteriochlorophylls per reaction centre (see section 2). From Table 1 it can be seen that there was a 1.16-fold increase in the core size on removal of PufX.

Transconjugant strains were also generated using the neurosporene-containing deletion strain DD13/G2, and membrane spectra were obtained for these strains (denoted as RCLH12/DD13/G2 and RCLH12X⁻/DD13/

G2) (Fig. 3B). In this carotenoid background the absence of PufX led to a 1.5-fold increase in core size (Table 1), revealing that the relative effect of PufX upon core size was dependent upon the carotenoid content of the photosystem. Comparing the core size arising from the same plasmid in the DD13 and DD13/G2 backgrounds it can be seen that pRKEH10 produced a larger core size in the DD13 background than in DD13/G2, whereas the core sizes produced by the *pufX*-deficient plasmid pRKEH10X$^-$ were equal in the two backgrounds.

4. Discussion

Previous work on *Rb. sphaeroides* strains containing LH2, LH1 and reaction centres has illustrated that the lack of PufX has two major effects: light-driven cyclic electron transfer is impaired [12], and the LH1:RC ratio increases substantially [13]. Very similar results have also been reported for an analogous strain of *Rb. capsulatus* where PufX was reported to facilitate, either directly or indirectly, ubiquinone/ubiquinol exchange between the Q_B site of the reaction centre and the Q_Z site of the cytochrome bc_1 complex [11]. The results reported in this manuscript demonstrate that PufX has no significant effect upon the rate of photosynthetic growth in strains which lack both types of antenna complex, indicating that photosynthetic growth of *Rb. sphaeroides* does not have an absolute requirement for the PufX protein. This supports the hypothesis that the effect of removal of the PufX protein on the exchange of ubiquinone/ubiquinol between the reaction centre and the cytochrome bc_1 complex is indirect, since any direct effect upon cyclic electron transfer should be apparent in the presence or absence of a light harvesting antenna.

Previous work [12] has noted that strains of *Rb. sphaeroides* lacking *pufX* have elevated levels of LH1, and it has been estimated that there was an increase in the number of LH1 bacteriochlorophylls per reaction centre from 76 up to 167 when comparing RC/LH1 strains with and without *pufX*, respectively [13]. This enhanced core size was linked with the hypothesis of limitation of the oligomerization state of LH1 by the pufX protein, the additional LH1 in the absence of pufX contributing to the impairment of cyclic electron flow between the reaction centre and the cytochrome bc_1 complex [13]. However, this enhancement was in addition to a 2.4-fold increase in core size in the *pufX*$^+$ RC/LH1 strain as compared with the wild-type, which gave rise to large, separate antenna domains unconnected to reaction centres [13]. The use of LH2-deficient strains in the present study has allowed a direct estimate to be made for the first time of the effect of removal of *pufX* on the size of the RC/LH1 core complex. The estimated number of LH1 bacteriochlorophylls per reaction centre was between 23 and 35 for all the constructs tested, reflecting previously measured wild-type levels [20]; moreover, the observed increase in core size when comparing strain RCLH12X$^-$/DD13 with RCLH12/DD13 was only 1.16, demonstrating that the impairment in cyclic electron flow in the absence of PufX can be achieved without any large increase in the number of LH1 antenna. The differences in core size between those reported in this manuscript and those in [13] can be explained by the large differences between the respective strains used; the strains used in this study did not contain any LH2 antenna, nor did they contain any genomic copies of the LH1 genes in addition to the copies in the plasmid-based *puf* operon expression system. Both of these factors have the potential to affect the LH1:RC stoichiometry.

In contrast to the DD13 carotenoid background, core sizes in the neurosporene-containing strains showed a 1.5-fold increase when strain RCLH12X$^-$/DD13/G2 was compared with strain RCLH12/DD13/G2 (Table 1). Why the absence of PufX should lead to a larger increase in the core size in this carotenoid background is not known, but the larger core size in strain RCLH12/DD13 as compared with strain RCLH12/DD13/G2 (Table 1) suggests that LH1 complexes may be inherently less stable in the presence of neurosporene. The fact that in the absence of PufX the size of the RC/LH1 core in the two carotenoid systems is equal could be due to enhanced

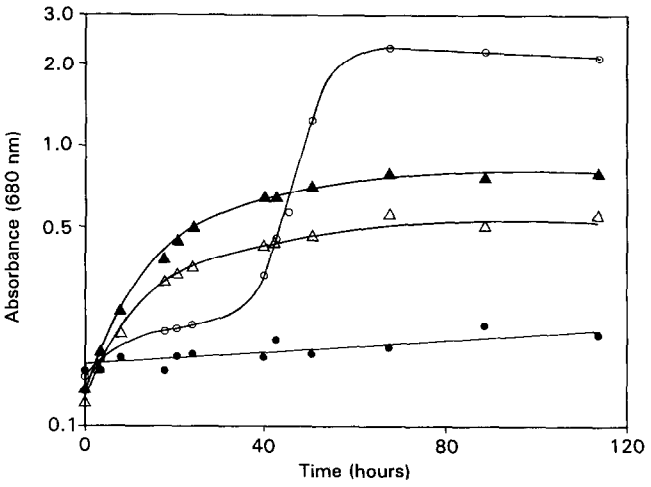

Fig. 2. Growth of strains RCLH12/DD13 (○), RCLH12X$^-$/DD13 (●), RCO2/DD13 (D) and RCO2X$^-$/DD13 (▲) under photoheterotrophic conditions.

Table 1
Estimation of the number of LH1 bacteriochlorophylls per reaction centre in *pufX*$^+$ and *pufX*$^-$ strains (see section 2)

Strain	$\frac{[B_{LH1}]}{[RC]}$	$\frac{RCLH12X^-}{RCLH12}$
RCLH12/DD13	30.8 ± 1.1	1.16
RCLH12X$^-$/DD13	34.9 ± 2.8	
RCLH12/DD13/G2	23.6 ± 1.8	1.49
RCLH12X$^-$/DD13/G2	34.9 ± 1.8	

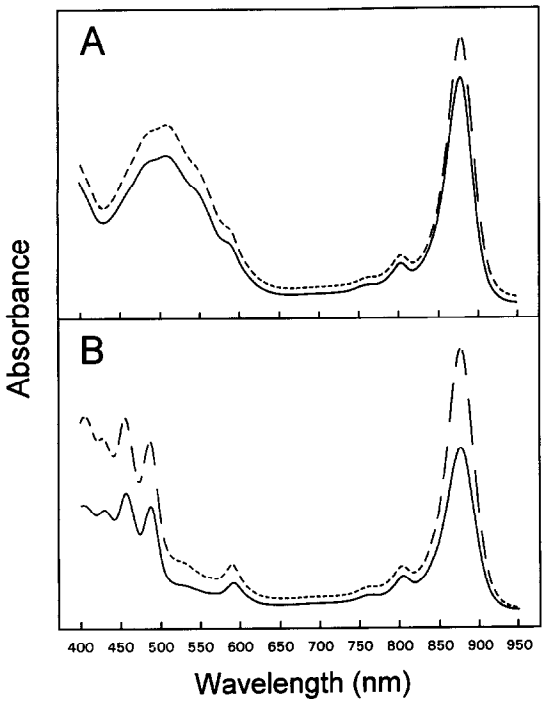

Fig. 3. Room temperature absorbance spectra of (A) RCLH12/DD13 and RCLH12X⁻/DD13 (dashed line) and (B) RCLH12/DD13/G2 and RCLH12X⁻/DD13/G2 (dashed line). All spectra were corrected to a reaction centre concentration of 0.1 mM, estimated as described in section 2.

stability or increased synthesis of LH1 in the PufX-deficient neurosporene strain. The data also suggests that PufX may be linked with the carotenoid binding site of LH1. The influence of both PufX and carotenoid type on LH1 stability is currently being studied in LH1-only strains of *Rb. sphaeroides* in order to examine this point further.

The mechanism by which removal of the PufX protein disrupts ubiquinone/ubiquinol exchange between the reaction centre and the cytochrome bc_1 complex is still not known. Despite there being no large increase in the number of LH1 bacteriochlorophylls per reaction centre in the DD13 strains described in the present study, the suggestion [13] that PufX affects the supramolecular organisation of LH1 may still be valid. Since there is evidence that PufX is an integral membrane protein [12] it is feasible that PufX interacts with the reaction centre complex and acts directly to prevent LH1 from physically blocking the Q_B site, thus allowing quinol-mediated electron flow between the Q_B site of the reaction centre and the Q_Z site of the cytochrome bc_1 complex. Future analysis of the photosynthetically competent *pufX*⁻ suppressor mutants observed by ourselves and others, together with the use of LH1-only strains, may help in understanding the molecular basis of the effects of PufX, which may involve a specific molecular interaction with the reaction centre and/or the LH1 complex.

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