Shortcut of the photosynthetic electron transfer in a mutant lacking the reaction center-bound cytochrome subunit by gene disruption in a purple bacterium, *Rubrivivax gelatinosus*

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Abstract A mutant lacking the reaction center-bound cytochrome subunit was constructed in a purple photosynthetic bacterium, *Rubrivivax gelatinosus* IL144, by inactivation of the cytochrome gene. Photosynthetic growth of the C244 mutant strain occurred at approximately half the rate of the wild-type strain. Although mutagenesis resulted in a greatly reduced amount of membrane-bound cytochromes *c*, illumination induced cyclic electron transfer and the generation of membrane potential in the mutant as observed in the wild-type strain. These findings are consistent with previous observations that the cytochrome subunit is absent in the reaction center complex in some species of purple bacteria and that the biochemical removal of the subunit did not significantly affect the in vitro electron transfer from the soluble cytochrome *c* to the photosynthetic reaction center. These results suggest that the cytochrome subunit in purple bacteria is not essential for photosynthetic electron transfer and growth, even in those species generally containing the subunit.

Key words: Cytochrome subunit; Reaction center; Mutant; Photosynthetic bacteria; Electron transfer; *Rubrivivax gelatinosus*

1. Introduction

The photosynthetic reaction center complex of purple bacteria typically consists of the L, M, H, and cytochrome subunits. The L and M subunits form a heterodimer in the membrane and contain a special pair of bacteriochlorophylls, monomeric bacteriochlorophylls, bacteriopheophytins, and quinones [1]. Electrons are sequentially transferred through the chromophores, from the photo-oxidized special pair at the periplasmic side to the quinones at the cytoplasmic side. The H subunit is located on the cytoplasmic side of the L and M subunits and does not contain any cofactors. The cytochrome subunit protrudes into the periplasmic space and contains four *c*-type hemes. These hemes serve as the immediate electron donors to the photo-oxidized special pair. The oxidized hemes can be re-reduced by a soluble cytochrome *c*.

The electron transfer function and characterization of hemes in the cytochrome subunit has been widely studied in various purple bacteria (e.g. *R. viridis* [2-12], *Rhodospirillum molischianum* [13], *Rubrivivax gelatinosus* [14-16], *Rhodoferax fermentans* [17], *Chromatium vinosum* [18], and *Rhodopseudomonas acidophila* [19]). These studies have presented that the four hemes in the cytochrome subunit differ in redox midpoint potentials (E_m) and peak wavelengths of the α-absorption bands, as shown in a recent review by Nitschke and Dracheva [20]. Two of the hemes show high E_m values and the other two hemes show low E_m values. The four hemes are arranged sequentially, alternating between high and low in the cytochrome subunit perpendicularly to the membrane plane. Typically, one of the high potential hemes is closest to the special pair and serves as the direct electron donor. The significance of this four heme motif and the physiological role of the cytochrome subunit are unknown.

The soluble cytochrome c_2 has been identified as a reductant of the photo-oxidized cytochrome subunit and no other electron transfer pathway had been recognized in purple non-sulfur bacteria. Recently, it was shown that the high potential iron-sulfur protein (HiPIP) is able to reduce the hemes in the cytochrome subunit in two purple non-sulfur bacteria, *R. gelatinosus* [21] and *R. fermentans* [22]. This observation verifies that the soluble cytochrome c_2 is not the sole reductant of the reaction center-bound cytochrome subunit, suggesting the existence of other electron transfer pathways.

Some species of purple bacteria (e.g. *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, and *Rhodopseudomonas palustris*) do not contain the cytochrome subunit. In these species, the photo-oxidized special pair is directly reduced by the soluble cytochrome *c*. Phylogenetic analysis has shown that the absence of the cytochrome subunit occurs independently in purple bacteria, showing that the common ancestor of purple bacteria contained the cytochrome subunit [23,24]. Presumably, the natural deletion of the cytochrome subunit has occurred independently in different lineages as a result of evolution.

Previously, we have shown that removal of the cytochrome subunit through detergent treatment from the purified reaction center complex of *R. gelatinosus* results in direct re-reduction of the special pair by the soluble cytochrome *c* following flash-induced photo-oxidation. The rate of oxidation of the soluble cytochrome *c* by this modified reaction center was comparable to that by the native form of the reaction center that has the tetra-heme cytochrome [14,15]. This observation suggests that the cytochrome subunit does not have an indispensable role in the photosynthetic electron transfer pathways.

In the present study, the gene encoding the cytochrome subunit of *R. gelatinosus* was inactivated mutationally to clarify the physiological role of the cytochrome subunit. Growth and electron transfer function of the mutant were analyzed.

2. Materials and methods

2.1. Plasmid construction for deletion of the cytochrome subunit

The genes encoding for the reaction center proteins containing the cytochrome subunit of *R. gelatinosus* IL144 have been cloned and

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sequenced [25,26]. The pGI7 plasmid was derived from the pUC plasmid (Amp^r) and contains the genes for the M and cytochrome subunits (Fig. 1). The pGI7 plasmid was digested at a unique *Bgl*II site within the cytochrome gene. A kanamycin-resistance cartridge was excised from the pUCKM1 plasmid [27] by *Bam*HI digestion and was inserted into pG17 (Fig. 1). The constructed plasmid was electroporated into *R. gelatinosus* cells.

2.2. Electroporation and selection of mutants

R. gelatinosus IL144 was photosynthetically grown at 30°C in 500 ml of PYS medium (0.5% polypeptone, 0.1% yeast extract, 0.5% sodium succinate and minerals, pH 7.0). During the logarithmic growth phase (OD_{660} = 0.5) the cells were stirred on ice for 20 min, then centrifuged in a Hitachi RP8 rotor for 20 min at 6000 rpm and 4°C. The pellet was suspended in 50 ml of ice-cold sterile distilled water and centrifuged briefly. The pellet was resuspended in 10 ml of 10% glycerol/ice-cold sterile distilled water and centrifuged. The pellet was resuspended in 1–2 ml of the 10% glycerol/water, resulting in an approximate cell density of 10^{11}/ml. 40 μl of the cell suspension and 1 μg of the plasmid DNA were mixed, then transferred to an electroporation cuvette (2 mm gap).

Electroporation was carried out using the ECM 600 system (BTX, San Diego, CA) and the same conditions as those established for *R. viridis* [28]. The resistance was set at 246 Ω and the field strength at 12.5 kV/cm, generating approximately 12 ms of pulse length. The cells were immediately transferred to 1 ml of 1% glucose PYS medium and stored on ice for 30 min. The cell suspension was added to 50 ml of PYS medium in an L-tube and grown aerobically at 30°C overnight. A 1 ml aliquot of the culture was added to PYS medium containing 50 μg/ml kanamycin and grown aerobically at 30°C. The cells were transferred and grown on agar plates containing 50 μg/ml kanamycin. The kanamycin-resistant colonies were transferred to plates containing 50 μg/ml ampicillin, and ampicillin-sensitive clones were isolated.

2.3. Preparation of the membrane fraction and measurements

Chromatophores were prepared according to the previously described method [13]. The wild-type and mutant *R. gelatinosus* strains were photosynthetically grown in PYS medium and harvested by centrifugation. The cells were disrupted by passage through a French press. The chromatophores were harvested by differential centrifugation and suspended in buffer containing 50 mM sodium phosphate and 50 mM sodium chloride, pH 7.0. Spectrophotometric results were recorded at UV-160A (Shimadzu, Japan). Xenon-flash-induced absorbance changes were measured with a single-beam spectrophotometer assembled in our laboratory [19].

2.4. DNA purification and Southern blotting

Cells were lysed in buffer containing 40 mM Tris, 0.2 mM EDTA, 1% Tween 20, and 0.2% Nonidet P-40 for 20 min at 60°C. The lysate was mixed with an equal volume of phenol and centrifuged. The aqueous fraction was recovered and mixed with ethanol. The precipitated DNA was recovered with a glass rod and washed once with 70% ethanol. Hybridization was carried out according to a manual on DNA manipulation [29]. 1 μg of DNA was blotted on a Hybond-N nylon membrane (Amersham, UK), and hybridized with a ^{32}P-labeled kanamycin-resistance cartridge, a pUC118, or a part of the gene coding for the M subunit of the reaction center complex, at 65°C overnight.

2.5. Polymerase chain reaction (PCR) and sequencing

Amplification of the DNA fragment containing *pufC* was performed using polymerase chain reaction (PCR) method as described in [30]. One of the primers had a sequence, 5′-GCCCAGCTGTTCTGGGTCGG-3′, the motif of which is seen at the 3′ portion of *pufM* (at position −233 from *pufC*). The other primer has a sequence, 5′-CGGCTTGTAGGCACCCTGGTGGCA-3′, seen at the 3′ portion of *pufC* (at position 956). The reaction was performed using Zymoreacter (ATTO, Japan) following the protocol supplied by the manufacturer. Sequencing was performed using a Dye Primer Cycle Sequencing Kit and a Model 373A DNA Sequencer (Applied Biosystems Japan). The primer for the sequencing had a sequence, 5′-GTGACCTGCTACTACACCTGCCA-3′, which is seen at position 444 in *pufC* (43 nt upstream from the *Bgl*II site).

3. Results and discussion

Deletion of the cytochrome subunit in *R. gelatinosus* was accomplished through double cross-over recombination by insertion of a kanamycin-resistance cassette into the gene coding for this subunit. The C2 strain was selected in an initial screening using agar plates containing kanamycin. Although wild-type *R. gelatinosus* is ampicillin-sensitive, the C2 strain was ampicillin-resistant, demonstrating that the C2 strain genome carries all characteristics of the pGI7 plasmid. This observation indicates that single cross-over recombination occurred. Several non-selective transfers of the C2 strain resulted in the cultivation of a kanamycin-resistant and ampicillin-sensitive strain that was named C244. Figure 2 shows the results of the Southern blot analysis of genomic DNA from the wild-type, C2, and C244 strains of *R. gelatinosus*. In this analysis, C244 exhibited a positive signal against the kanamycin-resistance gene, but not against the pUC gene. These results suggest that the gene for the cytochrome subunit of strain C244 was inactivated by insertion of the kanamycin-resistance gene during double cross-over recombination. This hypothesis was confirmed by polymerase chain reaction (PCR) using primers annealing to the 3′ regions of *pufM* and *pufC* and by the DNA sequencing. As shown in Fig. 3, a 1.1 kb band was produced by the wild type and a 2.7 kb band by the C244 strain. The nucleotide sequence of a part of the 2.7 kb DNA fragment showed that in the C244 strain, the kanamycin-resistance cartridge is inserted within *pufC* at the unique *Bgl*II site.

The C244 strain was grown under both aerobic respiratory and anaerobic photosynthetic conditions. Absorption spectra

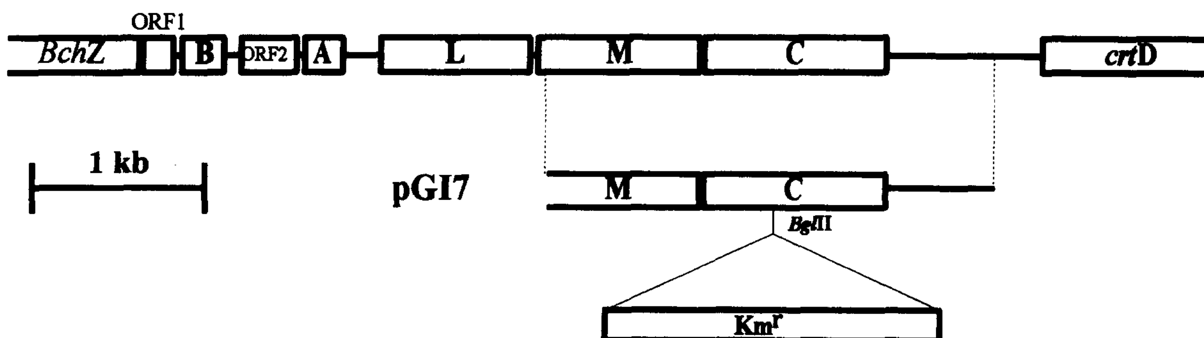

Fig. 1. Restriction map of photosynthetic genes (*puf* operon) of *R. gelatinosus* and construction of a plasmid for inactivation of cytochrome subunit gene. A kanamycin resistant cartridge was inserted at a unique *Bgl*II site in the pGI7.

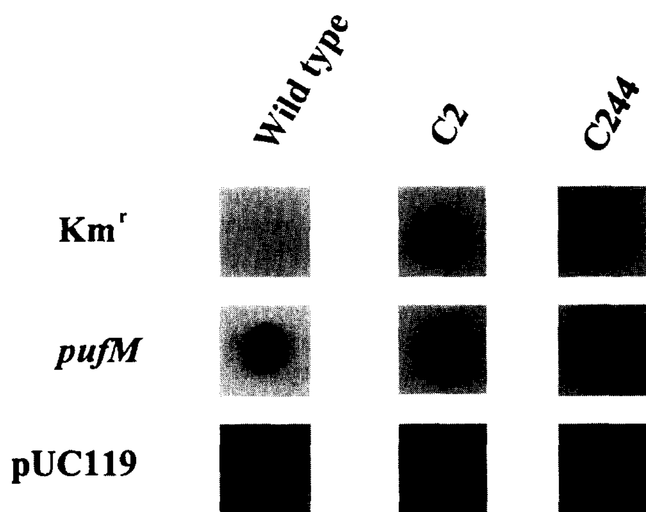

Fig. 2. DNA analysis of mutated strains of *R. gelatinosus* by Southern blotting. Each dot contained 1 μg of genomic DNA from a strain shown in the upper area in the figure. The genes used as the probes are represented in the left side. Hybridization was performed at 65°C overnight.

of the wild-type and C244 membranes, which were grown under photosynthetic conditions, showed no significant differences, indicating an insignificant mutagenic change in the contents of photosynthetic pigment-protein complexes. Figure 4A shows the deletion effect on the C244 cytochrome subunit by highlighting differences between the redox spectra of membranes isolated from the photosynthetically grown mutant and wild-type strains. Most soluble cytochromes are lost in the membrane preparations. The height of the α-absorption band of *c*-type hemes in C244 was approximately 10% of that in the wild type. Because the reaction center-bound subunit was the major *c*-type cytochrome in the membrane, this variance shows that synthesis of the cytochrome subunit in C244 does not occur. Figure 4B shows the flash-induced kinetics of the re-reduction of the photo-oxidized reaction centers in the wild-type and C244 membranes. In the wild type, the photo-oxidized special pair is quickly re-reduced within 1 ms by a heme in the cytochrome subunit. Re-reduction of the photo-oxidized special pair is significantly slower in the C244 strain, and no absorbance changes derived from the cytochrome *c* are observed. The kinetic trace of the special pair in C244 seems to also contain a rapid re-reduction phase, but the reductant should be other than cytochromes *c*.

Figure 5 shows the kinetics of the changes of the membrane potential in the wild-type and C244 membrane fractions, which were measured using the carotenoid band shift. The induction of continuous light resulted in the nearly equivalent membrane potential of both C244 and wild-type membranes, demonstrating that mutagenesis had no effect on function of

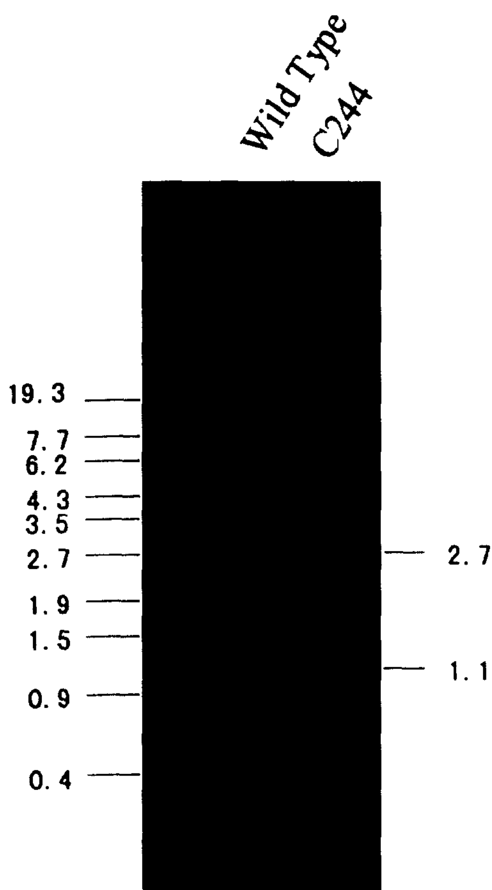

Fig. 3. DNA fragments containing *pufC* amplified by polymerase chain reaction (PCR). Two primers for the PCR had sequences homologous to the 3′ region of *pufL* and 3′ region of *pufC*, respectively. The *Eco*T14I digest of λ DNA was employed as a DNA molecular weight marker. The numbers indicate the lengths of the DNA fragments in kilobase(s).

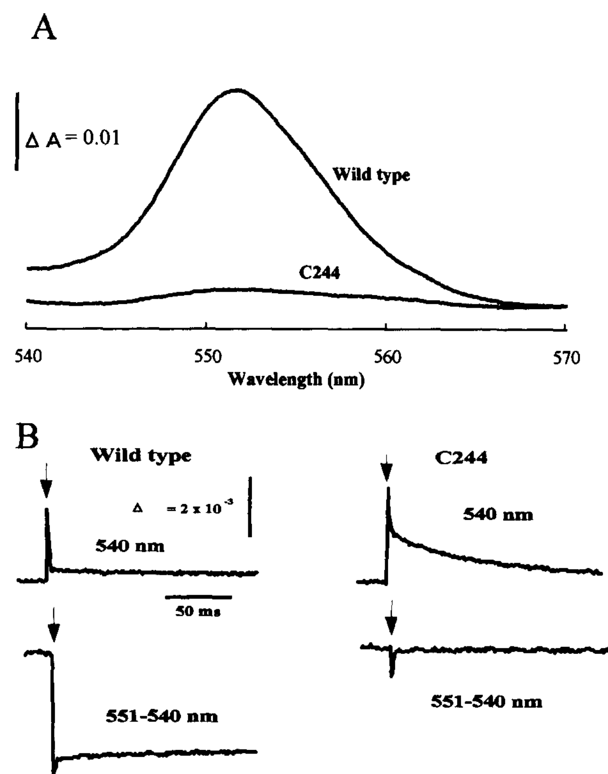

Fig. 4. Reduced-minus-oxidized difference spectra (A) and kinetics of re-reduction of photo-oxidized reaction centers (B) in the membranes of the wild type and C244 of *R. gelatinosus*. Membranes were suspended in the buffer containing 50 mM sodium phosphate and 50 mM sodium chloride, pH 7.6. The concentration was adjusted to absorbance of 3.0 at 860 nm. (A) Reduced minus oxidized difference spectra were obtained by subtracting spectra of the samples added 3.3 mM potassium ferricyanide from those added 1 mM sodium dithionite. (B) Membranes were suspended in the buffer containing 1 mM sodium ascorbate and 1 μM valinomycin.

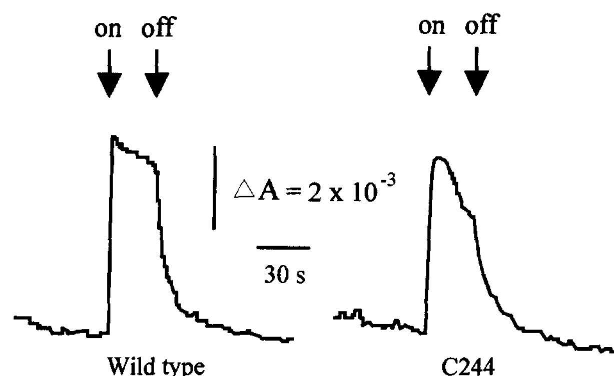

Fig. 5. Membrane potential changes in the chromatophore membranes of the wild type and C244 of *R. gelatinosus* measured using the carotenoid band shift. Membranes were suspended in the buffer containing 5 mM sodium phosphate, 5 mM sodium chloride, 1 mM sodium ascorbate and 10 μM 2,3,4,6-tetramethyl-phenylene diamine, pH 7.6. The concentration was adjusted to absorbance of 2.0 at 860 nm. The measurements were performed by recording the time course of the difference absorbance change at 495 minus 511 nm.

the photosynthetic reaction center complex, except to inhibit synthesis of the cytochrome subunit. These results indicate that deletion of the cytochrome subunit does not significantly affect construction of other photosynthetic proteins and that the cytochrome subunit is not indispensable in *R. gelatinosus*. This finding is consistent with our previous observation that removal of the cytochrome subunit by detergent treatment results in an electron transfer rate comparable to that of the native reaction center complex in vitro [14,15]. The photosynthetic growth of the mutant strain suggests that the shortcut electron transfer also occurs in the growing cells.

Most purple bacteria contain the cytochrome subunit. Several species of divergent lineages among the purple bacteria, however, do not contain this subunit. It has been previously suggested through observation of at least four independent phylogenetic lines that the cytochrome subunit has become less necessary in some species and subsequently lost during the course of evolution [23,24]. The present results support this theory.

Figure 6A shows that the growth rate of C244 under anaerobic photosynthetic conditions was slower than that of the wild-type strain. Under photosynthetic conditions, C244 reproduced at a significantly reduced rate of 270 min, almost double that of the wild-type strain at 140 min. In contrast, the aerobic respiratory reproduction rates of the C244 and wild-type strains were identical at 130 min (Figure 6B). These results indicate that the absence of the cytochrome subunit is disadvantageous for photosynthetic growth under the conditions used in the present study. This growth inhibition may be caused by lack of rapid re-reduction of the photo-oxidized special pair by the bound cytochrome subunit, resulting in an increased rate of electron back transfer to the photo-oxidized special pair. Another possibility is the existence of an electron donor other than soluble cytochrome *c* reducing the cytochrome subunit. HiPIP, a high-E_m redox protein, may be one such element since it has been shown that HiPIP serves as an electron donor to the cytochrome subunit in *R. gelatinosus* TG-9 [21] and *R. fermentans* [22].

Figure 6 shows a lag-time in growth of approximately 4 h for the wild-type and 20 h for the C244 strain, subsequent to initial aerobic growth using residual oxygen. This lapse is presumably due to the synthesis of the photosynthetic apparatus, although it is difficult to explain why the growth curve of C244 has such an extensive lag-time between aerobic and photosynthetic growth. The presence of the cytochrome subunit may expand the range of redox conditions suitable for appropriate photosynthetic electron transfer. When the ambient redox potential is low, the cytochrome subunit may facilitate rapid adaptation of the photosynthetic apparatus to the change in ambient redox conditions. Another possibility is that the cytochrome subunit may have an important role in the higher-order assembly of the photosynthetic apparatus in forming the complete electron transfer pathway.

Purple bacterial species lacking the cytochrome subunit (e.g. *R. sphaeroides* and *R. rubrum*) exhibit significant aerobic growth rarely observed in species that contain the cytochrome subunit (e.g. *R. viridis* and *R. molischianum*). In the present study, it was observed that *R. gelatinosus* was able to grow photosynthetically and aerobically with the removal of the cytochrome subunit, and may even be in the midst of an evolutionary process of deleting the cytochrome subunit. Deletion mutants of the cytochrome subunit in purple bacteria, which only grow photosynthetically, merit continued research. Further physiologic and kinetic C244 studies, and additional site-directed mutagenesis will lead to clarification of the roles and evolution of the cytochrome subunit.

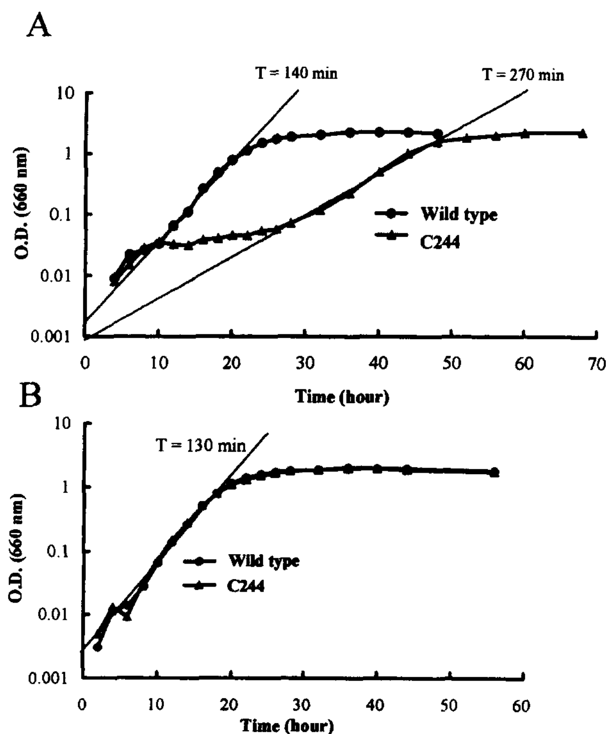

Fig. 6. Growth curves of wild type and C244 of *R. gelatinosus* under photosynthetic (A) and aerobic (B) conditions. The measurements were started by inoculation of 1/1000 volume of late log phase cells grown aerobically in PYS medium. Cultures were grown at 30°C. Photosynthetic cultivation was performed in screw-capped tubes filled with the medium in the light supplemented with 60 W tungsten lamp placed 20 cm apart from the cultures. The absorbance at 660 nm was measured in the tube, the diameter of which was 18 mm. The average of three out of five independent measurements omitting the minimum and the maximum was plotted against the time.

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