

Exchange and Complementation of Genes Coding for Photosynthetic Reaction Center Core Subunits among Purple Bacteria

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Abstract A mutant of the phototrophic species belonging to the β -proteobacteria, *Rubrivivax gelatinosus*, lacking the photosynthetic growth ability was constructed by the removal of genes coding for the L, M, and cytochrome subunits of the photosynthetic reaction center complex. The L, M, and cytochrome genes derived from five other species of proteobacteria, *Acidiphilium rubrum*, *Allochro-matium vinosum*, *Blastochloris viridis*, *Pheospirillum molis-chianum*, and *Roseateles depolymerans*, and the L and M subunits from two other species, *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris*, respectively, have been introduced into this mutant. Introduction of the genes from three of these seven species, *Rte. depolymerans*, *Ach. vinosum*, and *Psp. molis-chianum*, restored the photosynthetic growth ability of the mutant of *Rvi. gelatinosus*,

although the growth rates were 1.5, 9.4, and 10.7 times slower, respectively, than that of the parent strain. Flash-induced kinetic measurements for the intact cells of these three mutants showed that the photo-oxidized cytochrome *c* bound to the introduced reaction center complex could be rereduced by electron donor proteins of *Rvi. gelatinosus* with a $t_{1/2}$ of less than 10 ms. The reaction center core subunits of photosynthetic proteobacteria appear to be exchangeable if the sequence identities of the LM core subunits between donor and acceptor species are high enough, i.e., 70 % or more.

Keywords Photosynthetic reaction center complex · Proteobacteria (Purple bacteria) · *puf* operon · Gene exchange

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Introduction

Photosynthesis was developed early in the evolution of bacteria (Woese 1987) and has been improved with the differentiation of organisms and environmental changes in their habitats. In proteobacteria (purple bacteria), bacteriochlorophyll (BChl) *a* or *b* is used as the main photosynthetic pigment, which can absorb both infrared and visible lights. Light energy captured by the BChls bound to two hydrophobic small peptides, α and β , of the light-harvesting complex is funneled to the photochemical reaction center complex (Gabrielsen et al. 2009). The reaction center complex of purple bacteria consists of, at least, three subunits called L, M, and H (Jones 2009). The L and M subunits are both membrane-bound peptides showing approximately 20 % amino acid sequence identities to each other and form a heterodimeric core of the reaction center complex (Michel et al. 1986). The amino

acid sequences of the L and M subunits are related to those of the D1 and D2 subunits, respectively, of the photosystem 2 reaction center of cyanobacteria and plants, signifying a common origin. The L and M core subunits bind four BChls, two bacteriopheophytins, and two quinones, which are arranged with a two-fold symmetry for transferring electrons across the membrane. Two of the four BChls form a dimer, called the special pair, in the vicinity of the periplasmic surface, which releases an electron when excited by light. In many species, a cytochrome subunit, which contains three or four *c*-type hemes, is bound to the LM core at the periplasmic side and works as the electron donor to the photo-oxidized special pair. The electron from the special pair is transferred through a bacteriopheophytin to the primary electron acceptor, a quinone molecule named Q_A . The electron is then transferred to the second quinone molecule, Q_B , loosely bound to the cytoplasmic side of the LM core. After receiving two electrons and two protons, the reduced Q_B diffuses out of the RC and donates its electrons to the cytochrome bc_1 complex (Okamura et al. 2000), although some of these are used for reduction of NAD^+ via a reverse electron flow mediated by the complex I, according to the redox state in the membrane (Verméglio and Joliot 2014). One of the electrons from the quinol to the bc_1 complex is transferred to the water-soluble electron carrier proteins, such as cytochrome c_2 and HiPIP, and then to the oxidized *c*-type hemes in the RC-bound cytochrome subunit (Lavergne et al. 2009). Some species, such as *Rba. sphaeroides* and *Rps. palustris*, do not have the cytochrome subunit. In this case, the electron is directly transferred from the cytochrome c_2 to the photo-oxidized special pair in the RC (Bullough et al. 2009). This completes a light-induced cyclic electron transfer found in all purple photosynthetic bacteria.

The genes coding for the subunits of the RC together with those of the light-harvesting 1 (LH1) complex form an operon called *puf*. The arrangement of genes in this operon is well conserved among the purple photosynthetic bacteria in the order of *pufB*, *-A*, *-L*, and *-M*, coding for the β and α subunits of the LH1 and the L and M subunits of the RC, respectively. In species having the RC-bound cytochrome subunit, the *pufC* gene coding for this subunit is located at the end of this operon as *pufBALMC*. The gene coding for the H subunit of the RC, *puhA*, is not included in the *puf* operon and forms an operon called *puh* together with the genes encoding proteins, possibly functioning in the formation process or stabilization of the RC-LH1 complex (Aklujkar et al. 2005, 2006). In *Rhodobacter capsulatus* and *Rba. sphaeroides*, the genes coding for the enzymes required for synthesis of BChls and carotenoids are flanked by the *puf* and *puh* operons (Coomber et al. 1990; Bauer et al. 1991). Such a gene island is called the photosynthesis gene cluster (PGC). Other species of purple photosynthetic

bacteria also have the PGC, although the arrangement of operons in this gene cluster is not always conserved (Igarashi et al. 2001; Nagashima and Nagashima 2013). In photosynthetic organisms other than purple bacteria and Heliobacteria, the genes for photosynthesis show scattered locations.

The purple photosynthetic bacteria group is not monophyletic. Phylogenetic studies based on comparison of the nucleotide sequences of 16S rRNA have shown that purple bacteria can be divided into five groups (classes): α , β , γ , δ , and ϵ (Woese 1987). Photosynthetic species, including aerobic anoxygenic phototrophic species, which can synthesize BChls but are incapable of photosynthetic growth under anaerobic conditions (Shimada 1995; Yurkov and Csotonyi 2009), are found only in the α , β , and γ groups with mixed distributions in many non-photosynthetic species. Such scattered distribution of photosynthetic species has been interpreted as the result of independent losses of the photosynthetic phenotype in many lineages in the course of adaptation to different environments without any light, from a common ancestor possessing photosynthetic ability (Woese 1987). This interpretation seems to be reasonable in the view of multiple metabolic pathways for energy conversion other than photosynthesis. In addition, horizontal gene transfer may also have contributed to the scattered distribution of photosynthetic species in purple bacteria. When the amino acid sequences of the L and M subunits of the RC are used for phylogenetic tree construction, species belonging to the β and γ groups are positioned among members of the α group while retaining the branching topology in each of the groups. Such positioning, inconsistent with that in the phylogenetic tree based on 16S rRNA sequences, has been interpreted as the result of the horizontal transfer of photosynthesis genes between ancestral species of the β and γ groups and those of the α group (Igarashi et al. 2001; Nagashima et al. 1997a).

Horizontal transfer of photosynthesis genes has not been fully verified by laboratory experiments. The PGC from the *Rhodobacter* species has been cloned in *Escherichia coli* (Marrs 1981), but no photosynthetic phenotypes and no BChl syntheses have been reported so far. Among photosynthetic species, however, it has been shown that a deficiency in the process of BChl synthesis can be restored by introducing genes from distantly related species (Xiong et al. 1998; 2000). The *puf* operon deletion mutant of *Rba. capsulatus* recovered the ability of photosynthetic growth by the expression of the *puf* operon from *Rba. sphaeroides* (Zinsel et al. 1989) but not by the introduction of the *puf* operon from the aerobic anoxygenic phototrophic bacterium *Roseobacter denitrificans*, although a functional reaction center is heterologously assembled (Kortlüke et al. 1997).

In the present study, the genes coding for the subunits of the reaction center complex were exchanged between a β -purple bacterium, *Rvi. gelatinosus*, and seven additional species belonging to various lineages of purple bacteria in order to estimate how much difference in the core of the reaction center complex is acceptable for restoring the photosynthetic phenotype. This should be the first step in providing experimental evidence of the horizontal transfer of photosynthesis genes and will contribute to studies on artificial photosynthesis using biosystems.

Materials and Methods

Cultivation of Bacterial Strains and DNA Purification

Rte. depolymerans 61A (DSM 11813^T), *Rvi. gelatinosus* IL144 (NBRC 100245), and its derivatives were grown in a PYS medium (Nagashima et al. 1997a). *Blc. viridis* (DSM133^T), *Rps. palustris* CGA009 (ATCC BAA-98), and *Rba. sphaeroides* IL106 (NBRC 100037) were photosynthetically grown in a malate-basal medium (0.5 % sodium malate, 0.1 % ammonium sulfate, 0.1 % yeast extract, 0.1 % vitamin solution (Nagashima et al. 1997a), and 20 mM potassium phosphate, pH 6.8). *Ach. vinosum* D (DSM180^T) and *Psp. molischianum* (DSM120^T) were grown under photosynthetic conditions according to Bose (1963) and Nagashima et al. (1993), respectively. *Aph. rubrum* (ATCC 35905^T) was aerobically grown according to Nagashima et al. (1997b). The cells grown to exponential growth phase were sedimented by centrifugation and used for DNA purification as described in a previous paper (Nagashima et al. 1996).

Construction of *Rvi. gelatinosus* Mutant Lacking the Reaction Center L, M, and Cytochrome Subunits

A plasmid pGELPUF (Maki et al. 2003), which is a pUC119-based plasmid containing whole *puf* genes of *Rvi. gelatinosus* IL144, was digested by restriction enzymes *Sph*I and *Stu*I. The linearized plasmid was self-ligated by treatments with a Klenow fragment and a DNA ligase using a Blunting Kit (TAKARA BIO INC., Otsu, Japan). These treatments removed the entire lengths of *pufL* and *pufM* and about 4/5 of the length of the N-terminal of *pufC* from the plasmid, as shown in Fig. 1. This manipulated DNA insert was transferred to a suicide vector, pJPCm (Ohmine et al. 2009), after digestion with *Sac*I and *Bam*HI restriction enzymes, and maintained in cells of *E. coli* strain S17-1 λ pir. Then, a DNA fragment containing *sacRB* genes and a kanamycin-resistant cartridge was inserted at the unique *Sac*I restriction site in this plasmid. The resultant plasmid was named pJP Δ gelpufLMC and

introduced into the cells of *Rvi. gelatinosus* IL144RL2 by trans-conjugation from the *E. coli* S17-1 λ pir host cells. The *pufLMC* genes were removed from the *Rvi. gelatinosus* IL144RL2 genome via a two-step homologous recombination, which had been screened by kanamycin resistance for the first and by sucrose resistance for the second, as previously described (Ohmine et al. 2009). The *Rvi. gelatinosus* mutant lacking *pufLMC* without any insertions of antibiotics-resistant cartridges was named Δ pufLMC after confirmations of the mutation by genomic Southern hybridization, PCR, and DNA sequencing experiments.

Introduction of Exogenous *pufLM(C)* Genes into the Cells of *Rvi. gelatinosus* Δ pufLMC

A 1-kb *Rvi. gelatinosus* DNA region immediately upstream of the start codon of *pufL* was amplified by PCR using the plasmid pGELPUF (Maki et al. 2003) as the template and a set of primers, M13F+7 (5'-GTAAACGACGGCCAGTGAATTTCG) and GpufL-23R (5'-CATATTGATTCTCCGACCGACGG). The M13F+7 primer has a cohesive sequence (24-bases) to the 5' side of the M13 multi cloning site in the *lacZ* gene of the plasmid. A DNA region containing whole *pufLMC* of each of *Aph. rubrum*, *Ach. vinosum*, *Blc. viridis*, *Psp. molischianum*, and *Rte. depolymerans* or whole *pufLM* of each of *Rba. sphaeroides* and *Rps. palustris* was also amplified by PCR using primer sets as shown in Table 1. Through these PCR experiments, the forward primers have a cohesive sequence (15-bases) to the direct upstream of *Rvi. gelatinosus pufL* in addition to the operative sequence for the PCR, which is identical to the sequence of the 5'-end region of the exogenous *pufL* gene. The reverse primers have a 15-base of the cohesive sequence to the 3' side of the M13 multiple cloning site in the *lacZ* gene, in addition to the operative sequence for PCR. Each of the amplified *pufLM(C)* fragments was mixed with the 1-kb *Rvi. gelatinosus* PCR product described above and with the *Eco*RI-digested pJP5603 suicide plasmid (Penfold and Pemberton 1992) DNA with a molar ratio of 1:1:1. The three DNA fragments were connected at their cohesive ends via homologous recombination using an In-Fusion HD Cloning Kit (TAKARA BIO INC., Otsu, Japan), generating a plasmid construct containing a chimeric *puf* operon consisting of *Rvi. gelatinosus pufBA* and the exogenous *pufLM(C)* (Fig. 1). This construct was cloned in *E. coli* S17-1 λ pir and introduced into the cells of *Rvi. gelatinosus* Δ pufLMC by conjugal transfer. *Rvi. gelatinosus* IL144RL2 mutants, in which the *pufLMC* was replaced by the exogenous *pufLM(C)* via homologous recombination, were screened by kanamycin resistance and named AR-1, AV-1, BV-1, PM-1, RD-1, RP-1, or RS-1, according to the source of the *pufLM(C)* gene, *Aph. rubrum*, *Ach. vinosum*, *Blc. viridis*, *Psp. molischianum*, *Rte.*

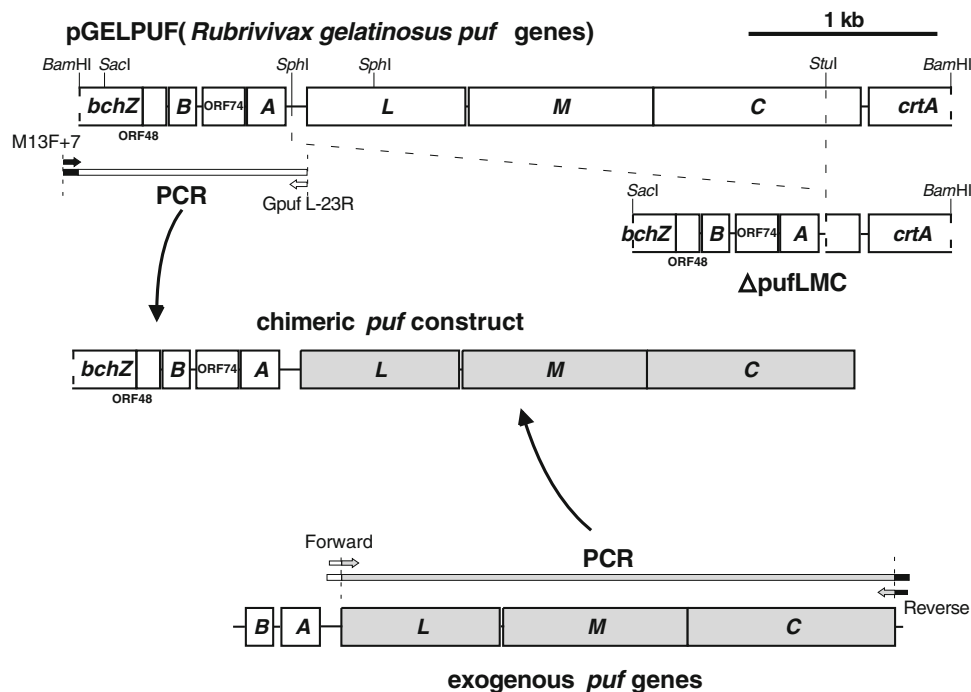


Fig. 1 Schematic representation of gene manipulations performed in this study. A 3-kb DNA region flanked by the *Sph*I and *Stu*I restriction sites, containing *pufL*, *pufM*, and *pufC*, was deleted from the *puf* operon DNA region of *Rvi. gelatinosus* IL144RL2. The mutant named Δ pufLMC, lacking this 3-kb DNA region containing *pufLMC*, was generated via a two-step homologous recombination method using a suicide vector, pJPCm, and had no insertions of antibiotics-resistant

cartridges. The PCR-amplified 1-kb *Rvi. gelatinosus* DNA region immediately upstream of the start codon of *pufL* was ligated with the PCR-amplified DNA region containing whole *pufLMC* or whole *pufLM* of each of seven other purple photosynthetic bacteria. Such a chimeric DNA construct was cloned in a pJP5603 suicide plasmid and inserted into the genomic DNA of the *Rvi. gelatinosus* Δ pufLMC mutant via single homologous recombination

depolymerans, *Rps. palustris*, or *Rba. sphaeroides*, respectively. A DNA fragment containing original *Rvi. gelatinosus* *pufBALMC* was also cloned in the pJP5603 suicide plasmid and introduced into the cells of Δ pufLMC to generate a control mutant, RG-LMC, in the same manner as used to generate the other mutants. Replacements of the genes in these mutants were confirmed by genomic Southern hybridization, PCR, and DNA sequencing experiments.

Growth Tests for *Rvi. gelatinosus* Mutants

Rvi. gelatinosus IL144RL2 and mutants were aerobically grown to mid-exponential growth phases in the dark at 30 °C in a PYS medium and used as precultures. Measurements were started with an addition of 0.3 ml of the preculture to a fresh PYS medium in a screw cap test tube with a diameter of 18 mm and a volume of 30 ml. The tube was filled with the growth medium and placed in a transparent water bath under light supplemented with a 60 W halogen lamp 15–25 cm away from the tubes. The temperature was kept at 30 °C by water circulation. The bacterial growth was monitored as the optical density at the wavelength of 660 nm. The minimum and maximum values among the five

independent measurements were omitted, and the average of the other three values was plotted against the time.

Biochemical and Spectrophotometric Assays

Rvi. gelatinosus mutants grown by aerobic respiration in the dark have been used for assays on the protein content and pigment accumulation. Three to five colonies on a plate culture were picked up (or 40 μ l of the liquid culture was taken) and added to a 4 ml PYS growth medium in an open-top glass tube with a diameter of 18 mm and a height of 130 mm. The tube was covered by a loosely fitted aluminum cap and shaken in a reciprocal shaker with 140 cycles/min at 30 °C in the dark. A ten microliter of the liquid culture grown to a rate exponential to stationary growth phase was mixed with the same volume of a buffer containing 40 mM Tris (pH 7.0), 1 % Tween20, 0.2 % Nonidet P-40, and 0.2 mM EDTA to lyse the bacterial cells. A protein content in the lysate was assayed by a method of pyrogallol red-molybdate complex reaction using a Protein Assay Rapid Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Bovine serum albumin (BSA) was used as the protein standard. Pigments were extracted from the cells in the liquid culture by mixed with

Table 1 Primers used to amplify genes coding reaction center subunits, *pufLM* or *pufLMC*

Source of DNA template	Forward primer	Reverse primer
<i>Allochromatium vinosum</i>	5'-CGGAGGAATCAATATGGCCATGCTCAGTTTGTGAGAG	5'-TACCGAGCTCGAATTCCTACAGCTGTGCGGAGCCG
<i>Acidiphilium rubrum</i>	5'-CGGAGGAATCAATATGGCAATGCTCAGTTTGTGAAAG	5'-TACCGAGCTCGAATTCCTATTGCCCCGTTGCCCCG
<i>Blastochloris viridis</i>	5'-CGGAGGAATCAATATGGCACTGCTCAGCTTTGAGAG	5'-TACCGAGCTCGAATTCCTACTTCGCAGCAGCCTTGATCG
<i>Pheospirillum molischianum</i>	5'-CGGAGGAATCAATATGGCGATGCTCAGTTTTCGAGAAG	5'-TACCGAGCTCGAATTCGGAAAGGATTAATTCCTTGCTCGTC
<i>Roseateles depolymerans</i>	5'-CGGAGGAATCAATATGGCCATGCTGAACCTTCGAGAGG	5'-TACCGAGCTCGAATTCACGCCAATTTGCTCAGCTGGG
<i>Rhodospseudomonas palustris</i>	5'-CGGAGGAATCAATATGGCAATGCTCAGCTTTGAGAAG	5'-TACCGAGCTCGAATTCGATAGACGCTGCGGTTGTATTACCG
<i>Rhodobacter sphaeroides</i>	5'-CGGAGGAATCAATATGGCACTGCTCAGCTTCGAGCG	5'-TACCGAGCTCGAATTCGCCGAGACTTGTCTCAGACGAGATG

Cohesive sequences to the 5'-end of *pufL* and to the 3'-end of *pufM* or *pufC* are underlined

a 25 volume of acetone/methanol (7:2) solution. The absorption spectrum of the supernatant after a centrifugation at $10,000\times g$ for 1 min was recorded with a UV-2600 spectrophotometer (Shimadzu Co., Kyoto, Japan). The concentration of BChl *a* was determined based on the absorbance at 770 nm, applying the extinction coefficient of 75 mM^{-1} in a cuvette with a 1-cm optical path-length. Membranes of the *Rvi. gelatinosus* strains were prepared by a passage through a French pressure cell and ultracentrifugation, according to the procedure described previously (Maki et al. 2003) and used for measurements of absorption spectra.

Kinetic Measurements for Intact Cells

Kinetic measurements of flash-induced absorbance changes of the cytochromes were carried out using a double beam spectrophotometer as described previously (Schoepp et al. 1995).

Phylogenetic Tree Construction

Phylogenetic trees were constructed using the programs ClustalX (Thompson et al. 1997) and MEGA 3.1 (Kumar et al. 2004). Amino acid sequences of the L and M subunits were concatenated for each species and used for generating a sequence alignment. Unnatural gaps in the alignment were located by eyes and corrected. Construction of the tree was performed by the neighbor-joining method, applying the *p*-distance as a distance estimator, in which all gaps in the sequence alignment were completely omitted in calculations. The sequences were obtained from the DDBJ/EMBL/GenBank databases.

Results

Rvi. gelatinosus IL144RL2, which is a spontaneous mutant generated from the wild-type strain IL144, shows a greatly depressed production of the light-harvesting 2 (LH2 or B800–860) complex but synthesizes a significant amount of reaction center (RC) and light-harvesting 1 (LH1 or B875) complexes, even under dark-aerobic respiratory conditions (Maki et al. 2003). Therefore, using this strain as a parent for gene manipulation experiments, it can be expected that the mutated RC-LH1 complex is expressed enough to be analyzed even if the mutation caused a functional deficiency of photosynthesis. In the present study, the genes coding the L, M, and cytochrome subunits of the photochemical reaction center complex (*pufLMC*) were deleted from the genomic DNA of this strain, IL144RL2, to generate the mutant strain $\Delta pufLMC$, which is, of course, not able to grow under light-anaerobic

(photosynthetic) conditions. The PCR-amplified *pufLMC* genes from the five species of purple photosynthetic bacteria, *Aph. rubrum*, *Ach. vinosum*, *Blc. viridis*, *Psp. molischianum*, and *Rte. depolymerans*, and *pufLM* genes of the two species without the cytochrome subunit, *Rps. palustris* and *Rba. sphaeroides*, have been independently incorporated *in cis* into the genomic DNA of the Δ *pufLMC* mutant cells. The growth rates of these “*puf*-exchanged” mutants grown under the dark respiratory conditions were almost identical (about 2 h of a doubling time) to that of the parent strain, IL144RL2, and the all mutants showed much amount of syntheses of BChl *a* and LH1 complex (Table 2), comparable to those in the parent strain. Possibly, the chimeric *puf* operons are similarly transcribed and translated as that in the parent strain. Three of such *puf*-exchanged mutants, PM-1 (*Psp. molischianum*), RD-1 (*Rte. depolymerans*), and AV-1 (*Ach. vinosum*), were capable of photosynthetic growth under light-anaerobic conditions. Figure 2 shows their photosynthetic growth curves as measured by the optical densities (at 660 nm) of their liquid cultures placed under light provided by halogen lamps (photon flux density $\approx 30 \mu\text{mol m}^{-2} \text{s}^{-1}$). The RD-

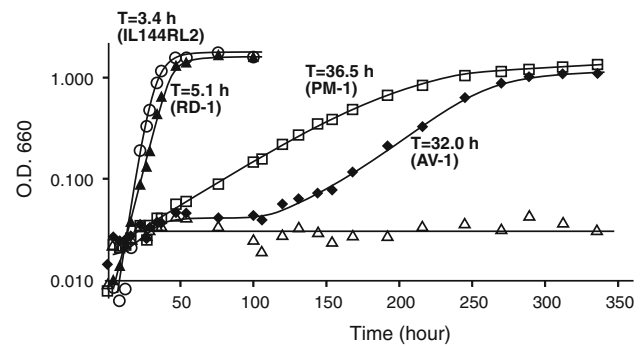


Fig. 2 Growth curves of *Rvi. gelatinosus* mutant cells grown under photosynthetic (light-anaerobic) conditions. The measurements were started by inoculation of 1/100 volume of exponential-phase cells grown aerobically. The absorbance at 660 nm was measured in a screw cap tube with a diameter of 18 mm. Symbols used are open circles for the original *Rvi. gelatinosus* strain IL144RL2, open triangles for Δ *pufLMC*, closed triangles for RD-1(*Rte. depolymerans*), open squares for PM-1(*Psp. molischianum*), and closed diamonds for AV-1 (*Ach. vinosum*). The doubling time, T, of each strain or mutant was calculated by exponential fitting to the data points ranging from 0.08 to 0.8 of the optical densities

Table 2 Accumulation of photosynthetic components in cultures of *R. gelatinosus pufLMC*-exchanged mutant strains

Strains	Protein content (mg/ml of growth medium)	Amount of BChl <i>a</i> ($\mu\text{g}/\text{mg}$ of protein)	Extent of LH1 spectral band ($A_{878-920}/A_{660}$) ^a	Photosynthetic growth ability ^b
IL144RL2	1.4 (0.9)	3.3 (6.0)	0.39 (0.81)	+
RD-1	2.0 (0.7)	2.0 (5.7)	0.43 (0.48)	+
PM-1	1.9 (0.9)	2.3 (4.1)	0.48 (0.68)	+
AV-1	1.7 (0.6)	2.7 (10.5)	0.49 (0.55)	+
RG-LMC	1.5 (1.0)	1.7 (3.0)	0.31 (0.36)	+
Δ <i>pufLMC</i>	2.2	2.2	0.49	–
AR-1	1.3	2.3	0.31	–
BV-1	1.4	3.5	0.37	–
RP-1	1.4	1.4	0.31	–
RS-1	1.9	2.2	0.46	–

Liquid cultures grown to the stationary growth phase (optical density at 660 nm = 2.0–3.5) in a PYS medium under the dark respiratory conditions were used for the measurements. Values measured for the photosynthetically grown cultures are shown in parentheses

^a Absorbance at the peak (878 nm) of the spectral band derived from the LH1, subtracted by absorbance around 920 nm as the baseline, was measured for the culture medium and normalized by the cell densities (optical density at 660 nm = 1.0)

^b When placed under the light (20 cm apart from a 60 W tungsten lamp) and anaerobic growth conditions after an inoculation with a 1/100 volume of the respiratory-grown culture, the culture of the strain showing an optical density at 660 nm (in a screw-capped glass tube with a diameter of 18 mm) of less than 0.1 was judged as negative (–) in photosynthetic growth

1 mutant grew faster than the other two mutants and showed a doubling time comparable to that of the original strain, IL144RL2. This shows that the reaction center subunits of *Rte. depolymerans* can fully compensate for the deletion of those in *Rvi. gelatinosus*. This may not be surprising, since the amino acid sequences of the L and M core subunits of *Rvi. gelatinosus* show the highest identities to those of *Rte. depolymerans*, 84.0 and 78.2 %, respectively, among those of the seven species used as donors in this study (Table 3). An interesting point is that *Rte. depolymerans* is a so-called “aerobic photosynthetic bacterium” and cannot grow under light-anaerobic conditions, contrary to what is observed for the RD-1 conjugant. The reaction center subunits of *Psp. molischianum* and *Ach. vinosum* also compensated for the deletion of these subunits of *Rvi. gelatinosus*, although the photosynthetic growth rates were significantly slower than that of the original strain, IL144RL2. These two species are distantly related to *Rvi. gelatinosus*, based on the phylogenetic analysis of 16S rRNA sequences, although their amino acid sequences of the L and M subunits show relatively high identities to those of *Rvi. gelatinosus* (Table 3).

Figure 3 shows the absorption spectra of membranes purified from the *Rvi. gelatinosus pufLMC*-exchanged mutants, RD-1, PM-1, and AV-1, grown under photosynthetic conditions. The spectrum of the membrane of Δ *pufLMC*, the direct parent strain of these mutants, is also shown for comparison. A large absorption band that peaked at 877 nm (at 874 nm only in Δ *pufLMC*) was seen in all of the spectra, attributable to the Q_y band of BChl *a* bound to the LH1 complex. Around the shorter wavelength region of

Table 3 Identities of amino acid sequences between *Rvi. gelatinosus* and other species used in this study

Species	PufL	PufM	PufC	PuhA	Accession numbers of sequence information used
<i>Allochrochromatium vinosum</i>	81.8	72.3	51.1	47.2	AB011811, CP001896
<i>Acidiphilium rubrum</i>	75.6	72.0	44.4	nd	AB005218
<i>Blastochloris viridis</i>	66.2	61.7	45.9	44.6	X03915, X05768, X02659
<i>Pheospirillum molischianum</i>	83.3	67.6	48.2	49.6	D50654, CAHP01000001.1
<i>Roseateles depolymerans</i>	84.0	78.2	58.2	nd	AB028938
<i>Rhodopseudomonas palustris</i>	75.0	73.4	none	50.6	BX571963
<i>Rhodobacter sphaeroides</i>	69.5	65.9	none	41.4	AF195122 (for strain 2.4.1)

Accession number of sequence information used for *Rvi. gelatinosus* IL144 is AP012320
 nd sequence is not determined

this large band, a small band peaked at 803 nm, and a faint absorption around 760 nm could be found in the spectra of the *pufLMC*-exchanged mutants. These two bands could be assigned to be contributions from the accessory BChl *a* and bacteriopheophytin *a* bound to the RC complex, respectively. Note, however, that the absorption band that peaked at 803 nm may also include a contribution from the BChl *a* in the very small amount of LH2 complex, as seen in the spectrum of the Δ pufLMC membrane. Even taking into account the presence of this LH2, the ratio between the LH1 and RC complexes is very similar to those among PM-1, AV-1, RD-1, and IL144RL2. Therefore, the significant difference in the photosynthetic growth rates among these strains could not be due to a difference in the LH1/RC ratio. In Fig. 3, the absorption spectra of membranes purified from the dark-aerobically grown cells of the mutants showing no growths under the photosynthetic conditions, RS-1 (*Rba. sphaeroides*), BV-1 (*Blc. viridis*), AR-1 (*Aph. rubrum*), and RP-1 (*Rps. palustris*), are also shown. It is noteworthy that the absorption bands possibly derived from the BChl *a* and Bphe *a* in the RC complex are detected although their signals are weaker than those detected in the spectra of the membranes from the photosynthetically grown mutants. This suggests that the process of expression of exogenous *puf* genes is basically reconstructible in *Rvi. gelatinosus*.

To assess the reason for the difference in the photosynthetic growth rates among PM-1, AV-1, RD-1, and IL144RL2, the photosynthetic electron transfer in *Rvi. gelatinosus* has been analyzed by flash-induced kinetic measurements for redox changes of cytochromes *c* in intact cells (Fig. 4). The measurements were performed by applying a low-intensity continuous light to eliminate participation of low-potential hemes in the RC-bound cytochrome subunit. This keeps the low-potential hemes oxidized and allows recording of the light-induced changes of the high-potential hemes solely related to the cyclic electron transfer. Under these conditions, the kinetic trace obtained for the IL144RL2 original strain showed a flash-

induced rapid photo-oxidation of cytochrome *c* and biphasic reduction with a fast ($t_{1/2} \approx 300 \mu\text{s}$) and a slow ($t_{1/2} \approx 8 \text{ ms}$) phase of approximately equal amplitudes. These changes were nearly identical to those reported for the cells of the *Rvi. gelatinosus* wild-type strain IL144 (Menin et al. 1999; Nagashima et al. 2002). The fast phase was assigned to the fast reduction of the photo-oxidized heme in the RC-bound cytochrome subunit by the high-potential iron sulfur protein (HiPIP) bound to this subunit. Due to the low amount of cytochrome *bc*₁ complex in comparison to the amount of RCs, the complete reduction of the tetraheme cytochrome requires several turnovers of the cytochrome *bc*₁ complex; this is conducted to the slow reduction phase of the flash-oxidized high-potential heme in the RC-bound cytochrome subunit. The kinetic trace measured for the RD-1 intact cells also showed a similar biphasic reduction of photo-oxidized cytochrome *c*, although the contribution of the fast phase (about 75 %) was higher than that estimated (about 50 %) in IL144RL2 cells. This suggests that the ratio of HiPIP to RC is higher in the RD-1 conjugant than that in the IL144RL2 strain. Alternatively, the difference in midpoint potential between the RC-bound high-potentialheme and the HiPIP could be higher for the RD-1 conjugant than that for *Rvi. gelatinosus*. For the other two mutants, AV-1 and PM-1, the fast rereduction of the photo-oxidized cytochromes observed in IL144RL2 and RD-1 was not detected. Most of the photo-oxidized cytochromes bound to the RC in these two mutant cells were rereduced with $t_{1/2} \approx 4 \text{ ms}$ in PM-1 and $t_{1/2} \approx 10 \text{ ms}$ in AV-1, which is comparable to the rate of the slow phase in IL144RL2. The main electron donor to the RC-bound cytochromes in these two mutants is suspected to be the HiPIP but not a soluble cytochrome *c*, since no clear spectral shifts were detected during the process of the reaction (data not shown). In any case, the slower rereduction of the RC-bound cytochromes in AV-1 and PM-1 is certainly related to the slower growth of these mutants under photosynthetic conditions. This interpretation is in agreement with results obtained in our previous studies, in

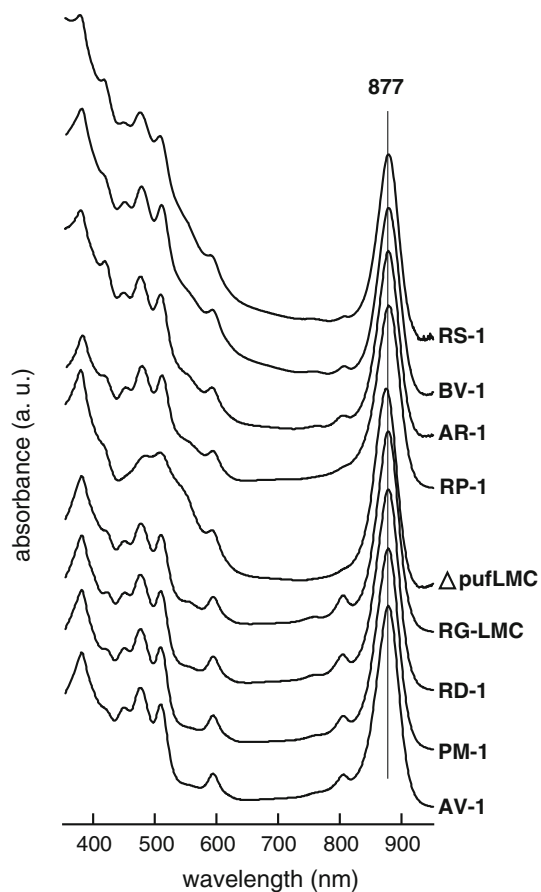


Fig. 3 Absorption spectra of membranes prepared from *Rvi. gelatinosus* mutants in which the reaction center genes (*pufLMC*) have been replaced by those from other species. The membranes of AV-1, PM-1, RD-1, and RG-LMC were prepared from the photosynthetically grown cells. The membranes of RS-1, BV-1, AR-1, RP-1, and Δ pufLMC were prepared from cells grown under dark-aerobic conditions. The membranes were suspended in a buffer containing 50 mM potassium phosphate (pH 7.0) and 50 mM potassium chloride to be a concentration of 100 μ g of protein/ml. The absorption spectra were normalized at 877 nm which is the peak position of LH1 Q_y-band

which the lack of fast (<1 ms) rereduction of the RC due to genetic deletions of HiPIP and/or the RC-bound cytochrome made the cell growth more than twice as slow of that of the wild-type cells under photosynthetic conditions (Nagashima et al. 1996, 2002; Verméglio et al. 2012).

Discussion

In the present study, the exchange of genes coding for the L, M, and cytochrome subunits (*pufL*, *pufM*, and *pufC*) of the photosynthetic reaction center has been tried using the β -purple bacterium *Rvi. gelatinosus* as a host and various species of purple bacteria as donors. Introduction of the *puf* genes from three of the seven species tested, *Rte*.

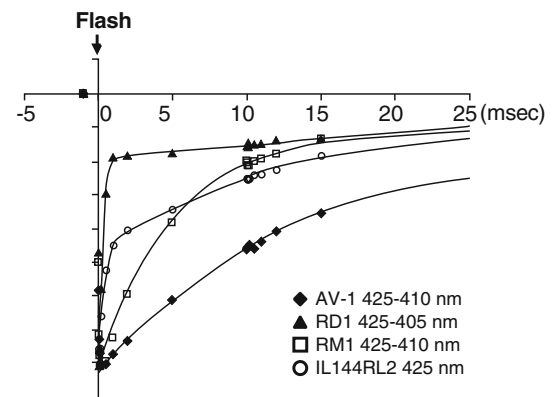
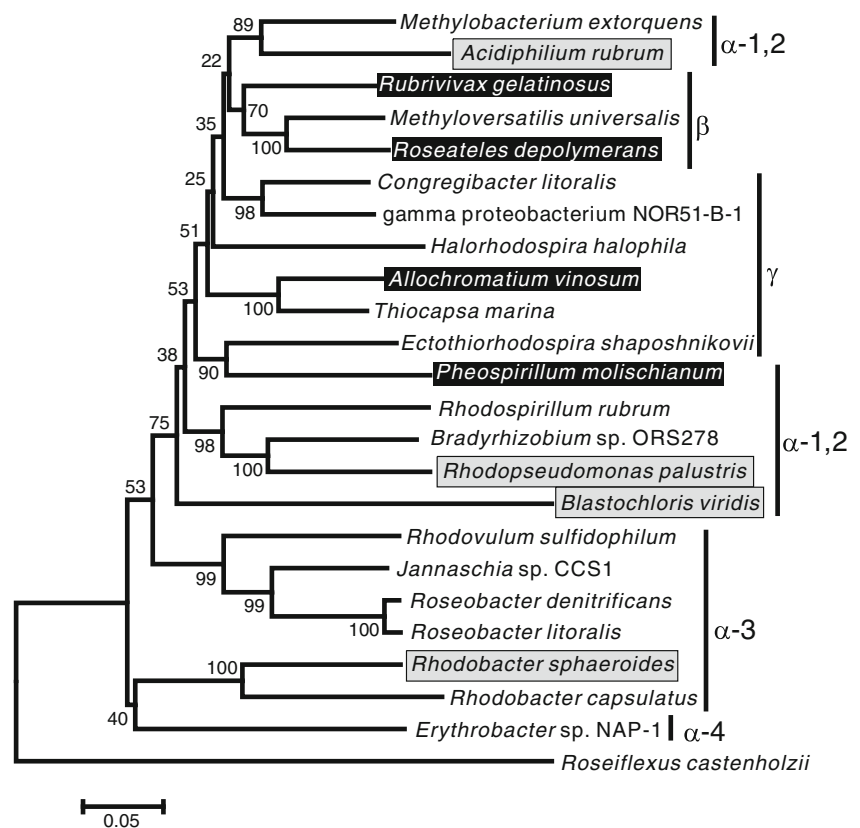


Fig. 4 Flash-induced redox changes of cytochrome *c* in intact cells of *Rvi. gelatinosus* IL144RL2 and mutants grown under light-anaerobic conditions. Cell suspensions were placed under anaerobic conditions. A low-intensity continuous light was used to photo-oxidize the low-potential hemes in the RC-bound cytochrome subunit. Flash excitation reveals solely the photo-oxidation of the high-potential hemes in this subunit

depolymerans, *Psp. molischianum*, and *Ach. vinosum*, into the cells of the deletion mutant of these genes recovered the photosynthetic growth phenotype. The latter two species are α - and γ -purple bacteria, respectively, distantly related to *Rvi. gelatinosus* in the phylogenetic relationship based on 16S rRNA sequences (Woese 1987). However, a phylogenetic tree based on the amino acid sequences of the L and M subunits of the reaction center placed these species closer to *Rvi. gelatinosus*, as shown in Fig. 5. Such an inconsistency, observed among phylogenetic trees, has been explained by the horizontal transfer of photosynthesis genes between the ancestral species of α -1 or α -2 subclasses and the ancestral species of β - and γ -classes (Nagashima et al. 1997a; Igarashi et al. 2001; Nagashima and Nagashima 2013). The results obtained in the present study may reflect such an evolutionary process. Indeed, the amino acid sequences of the L and M subunits used in the mutants recovering photosynthetic growth showed higher identities to those of *Rvi. gelatinosus*, which are over 81 % for the L subunits and over 67 % for the M subunits (Table 2). Possibly, these “carried-in” L and M subunits form a functional reaction center complex with the native H subunit of *Rvi. gelatinosus*. The carried-in cytochrome subunit was also incorporated into these chimeric reaction center complexes and showed a fast oxidation to reduce the special pair of the BChls oxidized by a flash illumination. However, the rate of rereduction of the hemes in the cytochrome subunit was significantly slowed in the two mutants expressing the *pufLMC* of *Psp. molischianum* (PM-1) and *Ach. vinosum* (AV-1). This slow rereduction can cause the significantly slower growth of these two mutants under photosynthetic conditions and may be

Fig. 5 Phylogenetic tree based on amino acid sequences of L and M subunits of RC. Species for which the genes coding for the L, M, and cyt subunits were introduced into the *Rvi. gelatinosus* mutant lacking these subunit genes are in boxes. When such gene exchanges resulted in the PS⁺ phenotype, the names of the donor species are highlighted. Strain names and accession numbers of the sequence data used in the tree construction can be found in (Nagashima and Nagashima 2013)



due to differences in the distribution of charged amino acids on the surface of the cytochrome subunits that provide binding sites to the soluble electron donor proteins (Nagashima et al. 1998). The amino acid sequence identities of the cytochrome subunits between *Rvi. gelatinosus* and these two species are 48 and 51 %, respectively. On the other hand, the mutant expressing the *Rte. depolymerans* *pufLMC* (RD-1) showed a comparable growth rate with the parent *Rvi. gelatinosus* strain and fast rereduction of the hemes in the cytochrome subunit after the photo-oxidation by flash-activation. Likewise, the amino acid sequence identity between the cytochrome subunits of *Rvi. gelatinosus* and *Rte. depolymerans* is not very high, 58 %. However, the local structure for the binding sites to the electron donor proteins may be well conserved between these two species, since they are closely related in β -purple bacteria.

Recovery of the photosynthetic growth ability in the *Rvi. gelatinosus* mutant expressing the reaction center genes from *Rte. depolymerans* is particularly interesting. *Rte. depolymerans* is an aerobic bacterium and does not grow phototrophically under light-anaerobic conditions in spite of the potential production of photosynthetic apparatuses (Suyama et al. 1999). Synthesis of BChl *a* has been reported to be induced by a sudden decrease in the concentration of carbon sources in the growth medium and to

be advantageous in maintaining the viability of the starved cells in the presence of light (Suyama et al. 2002). Through studies on many other species of aerobic photosynthetic bacteria, it has been suggested that their photosynthetic apparatuses help generate membrane potential and give them advantages for surviving in natural environments (Shimada 1995; Yurkov and Csotonyi 2009). However, biochemical or thermodynamical reasons for their inability to grow photosynthetically have not been straightforward. One possible explanation is that the primary quinone, Q_A, with relatively high redox midpoint potential might be over-reduced under anaerobic conditions (Okamura et al. 1985). This explanation is consistent with a study of the expression of *puf* genes of the aerobic photosynthetic species, *Rsb. denitrificans*, of the *puf*-deleted mutant of the genuine phototrophic species, *Rba. capsulatus*, in which the transconjugants did not grow under light-anaerobic conditions, although the reaction center was synthesized and showed a charge separation by flash activation (Kortlücke et al. 1997). However, the present study clearly shows that the reaction center L, M, and cytochrome subunits of the aerobic photosynthetic species *Rte. depolymerans* are perfectly functional in *Rvi. gelatinosus*, showing that the midpoint potentials of quinones, special pair of BChls *a*, and hemes *c* are adjusted to function in the cyclic electron flow of the genuine phototrophic species placed under

light-anaerobic growth conditions. Possibly, the aerobic photosynthetic bacteria's inability to grow photosynthetically is related to an electron transfer step other than at the reaction center level. Measurements of the electron transfer to the cytochrome bc_1 and the concomitant electrogenic phase in the RC-exchanged mutant, RD-1, as well as those in the original *Rte. depolymerans* under various redox conditions will be required to clarify the importance of appropriate equilibrium among the Q_A , Q_B , and quinone pool.

Four of the seven *puf*-exchanged mutants, AR-1, RP-1, BV-1, and RS-1, in which the *pufLMC* or *pufLM* genes of *Aph. rubrum*, *Rps. palustris*, *Blc. viridis*, and *Rba. sphaeroides*, respectively, are introduced into the *Rvi. gelatinosus* $\Delta pufLMC$ mutant, showed no apparent growth under light-anaerobic photosynthetic conditions for at least 10 days. These mutants showed synthesis of a large amount of LH1 complexes, even under aerobic respiratory growth conditions, as in the parent strains of *Rvi. gelatinosus*. A small absorption band that peaked at 803 nm and approximately 760 nm could also be seen in the spectra of the membranes of these mutants (Fig. 3). However, flash-induced kinetic measurements using intact cells of these four mutants grown under aerobic conditions showed no apparent signals linked to a charge separation between P^+ and Q_A^- (data not shown). This suggests that the reaction center complex, composed of the introduced LM core subunits and the H subunit of *Rvi. gelatinosus*, is not correctly assembled or is unstable in these mutants. Related to this possibility, the Q_A site in the chimeric reaction center may not be occupied by an appropriate quinone molecule. It has been shown that the deletion of the gene coding for the H subunit, *pufA*, results in deficiency in the synthesis of the reaction center complex and abolishment of the photosynthetic growth ability (Wong et al. 1996; Lupu and Ghosh 2004). Sequence identities between the RC subunits of *Rvi. gelatinosus* and the four species might be too low to support the tight interaction between the H subunit of *Rvi. gelatinosus* and the introduced LM core. Concerning the AR-1 and BV-1 mutants, in which the *pufLMC* genes are derived from *Aph. rubrum* and *Blc. viridis*, respectively, differences in pigment preferences might be another reason why the reaction center becomes deficient. They use Zn-BChl *a* and BChl *b*, respectively, different from BChl *a* used in the other species, including *Rvi. gelatinosus*.

The present study shows that reaction center genes are exchangeable without a loss of the photosynthetic phenotype if the sequence identities between the donor and acceptor species are within some threshold, i.e., more than 70 or 80 % identical. The introduced reaction center can be incorporated into the electron transfer pathway in the acceptor species. This means that the process of the evolution of photosynthesis is possible to trace by experiments,

and that we may create new metabolic pathways driven by light energy.

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