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Interspecific replacement of the gene coding for the reaction center-bound cytochrome subunit in purple bacteria

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Introduction

The photosynthetic reaction center (RC) of purple bacteria contains three subunits, L, M and H. The membrane-spanning L and M subunits are encoded by *pufL* and *pufM* genes, respectively, which form an operon called *puf*. In many species such as *Blc. viridis* and *Rvi. gelatinosus* the cytochrome subunit is also bound to the RC, which contains four *c*-type hemes. It is coded by *pufC* gene located immediately downstream of the *pufM* gene. The cytochrome subunit donates an electron to the photo-oxidized special pair in the RC. The water-soluble electron carrier proteins such as cytochrome c_2 in the periplasmic space work as electron donors to the oxidized cytochrome subunit. Some species such as *Rhodobacter sphaeroides* do not have the cytochrome subunit, in which the cytochrome c_2 donates an electron directly to the oxidized special pair. Physiological significance of the cytochrome subunit has not been fully understood.

The fine structure of the *Blc. viridis* RC with the cytochrome subunit has been clarified with a resolution of 2.3 Å (Deisenhofer et al. 1995). The four *c*-type hemes are arranged in a roughly linear manner almost perpendicular to the membrane. The thermodynamic properties of the four hemes and their arrangement in the cytochrome subunit have been extensively studied in *Blc. viridis*. *Rvi. gelatinosus* is a purple bacterium belonging to the β-subclass and can grow under both aerobic-dark and anaerobic-light conditions. The characteristics of four hemes in the cytochrome subunit of *Rvi. gelatinosus* have been shown to be similar to those of *Blc. viridis*. However, the major physiological electron donor to the cytochrome subunit in *Rvi. gelatinosus* is a high-potential iron-sulfur protein (HiPIP) but not cytochromes *c*. We have demonstrated by means of site-directed mutagenesis that the HiPIP donates an electron to the low-potential heme located at the most distant position from the special pair (Osyczka et al. 1999). It is likely that all four hemes are involved in the electron transfer from the soluble electron carrier to the special pair.

Mutants of *Blc. viridis* with the modified cytochrome subunit by site-specific mutagenesis would be very useful for the studies on the structure-function relationships in the electron transfer reaction. Gene manipulation systems for *Blc. viridis* have been established previously (Laußermair and Oesterhelt 1992), and some studies had been done using site-directed mutagenesis manipulated into the cytochrome subunit (Chen et al. 2000) of *Blc. viridis*. However, *Blc. viridis* grows very slowly under micro-aerobic respiratory conditions. Establishment of a better

system for the site-directed mutagenesis in the *Blc. viridis* reaction center will contribute to the studies on the structure-function relationships in RCs.

Materials and methods

Rvi. gelatinosus and *Blc. viridis* were grown under anaerobic-light conditions at 30 °C. *Escherichia coli* strain JM109, grown aerobically with a SOB medium at 37 °C, was used as a host for plasmids. When needed, ampicillin or kanamycin was added to the cultures at the final concentration of 50 μg/ml.

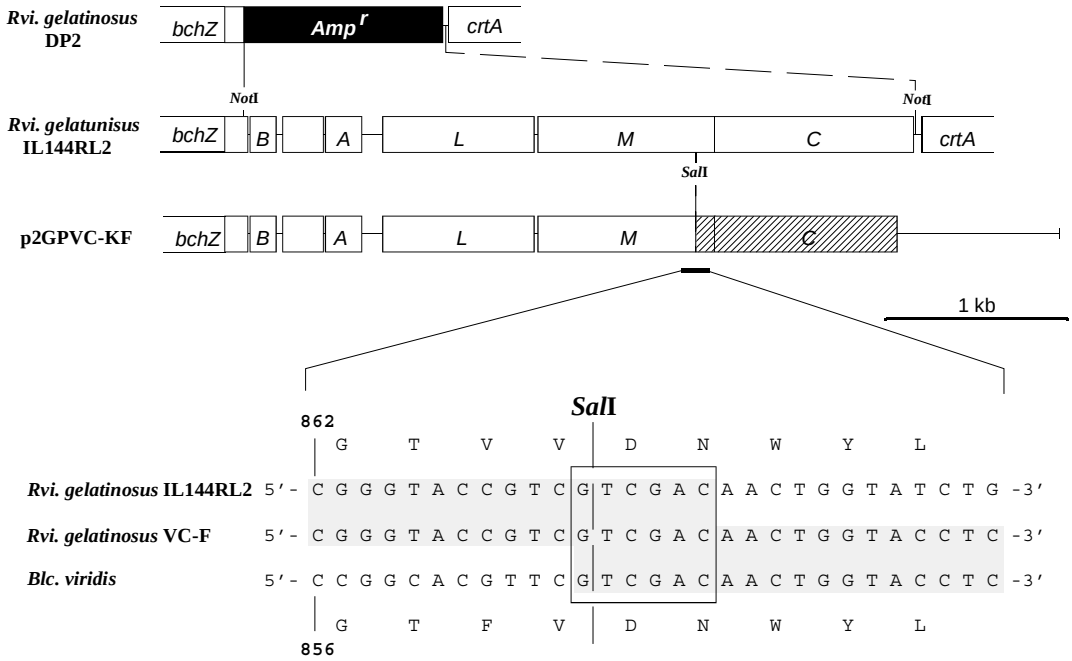

Fig. 1. Gene manipulation to *Rvi. gelatinosus puf* operon performed in this study. The DNA region derived from *Blc. viridis* are hatched.

The 4.6 kb DNA fragment containing the whole *puf* operon of *Rvi. gelatinosus* has already been cloned (Nagashima et al. 1994). A region flanked by *Not*I restriction sites on this fragment was replaced by an ampicillin resistance gene (Fig. 1). This DNA construct was introduced into cells of *Rvi. gelatinosus* strain IL144RL2 which is a spontaneous mutant derived from the wild-type strain IL144 and shows highly reduced production of the light-harvesting 2 (LH2) complex. The cells showing ampicillin-resistance and no production of the RC-LH1 complex were picked up and named strain DP2. Southern hybridization analyses and DNA sequencing verified the disruption of the *puf* operon in the genome of the strain DP2. The whole *puf* operon of *Rvi. gelatinosus* cloned into a plasmid pHSG298 (kanamycin-resistant) was digested at the *Sal*I site to remove the *pufC* gene. The region removed was replaced by a *Sal*I-*Bam*HI-digested 2.1-kb DNA fragment containing a part of *pufM* and the entire *pufC* of *Blc. viridis*. This chimeric structure caused no insertions and deletions within the homologous *pufM* genes of both species as shown in Fig. 1. A plasmid p2GPVC-KF was thus constructed and introduced by electroporation into *Rvi. gelatinosus* strain DP2 cells. The strain showing production of the RC-LH1 under the pressure of kanamycin was named VC-F. *Rvi. gelatinosus* strain ΔC lacking the *pufC* gene (Osyczka et al., 1999) was also used in this study.

SDS-polyacrylamide gel electrophoresis of membranes was carried out according to a modified method of Laemmli. The solublized membrane containing 10 μg proteins was applied on each lane of the gel. The gel was stained for hemes according to the method of Thomas et al. Xenon flash-induced absorption changes in whole cells were measured with a single-beam spectrophotometer. The *Rvi. gelatinosus* cells were suspended in a 20 mM MOPS buffer (pH7.0) containing 100 mM KCl, 1 mM sodium ascorbate and 10 μM DAD to give an absorbance of 1 at 875 nm.

Results

The *Rvi. gelatinosus* VC-F mutant was able to grow under photosynthetic conditions, suggesting that the RC coded by the *puf* operon containing the *Blc. viridis pufC* gene functions in the photosynthetic electron transfer. The expression of the *Blc. viridis* cytochrome was confirmed by SDS-PAGE of the membrane preparations obtained from the strains of *Rvi. gelatinosus* and *Blc. viridis* as shown in Fig. 2. In the membrane of the strain IL144RL2, a peptide with a molecular mass of 43 kDa was detected and assigned to the cytochrome subunit. In the membrane of the strain ΔC, no heme-stained peptides were observed due to the lack of the cytochrome subunit. In the membrane of the strain VC-F, a heme-stained peptide was detected at the 39-kDa molecular mass, which was identical to that of the cytochrome subunit of *Blc. viridis*. This indicates that the cytochrome subunit derived from *Blc. viridis* was synthesized in the *Rvi. gelatinosus* strain VC-F. The reduced-minus-oxidized difference spectrum of the cytochromes *c* in the strain VC-F membrane was nearly identical to that obtained from the *Blc. viridis* membrane, suggesting that molecular environments of the hemes in the cytochrome subunit in the strain VC-F is almost the same as those in the *Blc. viridis* subunit (data not shown).

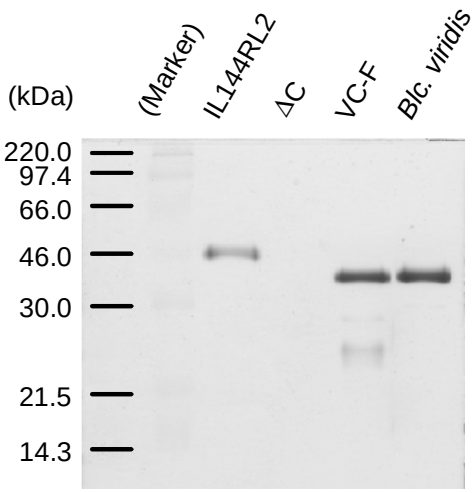

Fig. 2. SDS-PAGE of membrane preparation from *Rvi. gelatinosus* strains and *Blc. viridis*. The gel was stained for hemes.

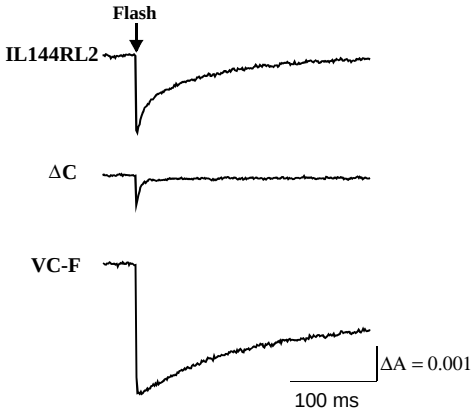

Fig. 3. Flash-induced redox change of cytochromes in whole cells of *Rvi. gelatinosus* strains. Absorption changes were measured at 551-minus-

Figure 3 shows the kinetics of flash-induced electron transfer through the cytochrome subunit in whole cells of *Rvi. gelatinosus* strains under the conditions that the high-potential hemes in the subunit were reduced prior to the flash-activation. In the strain IL144RL2, a flash excitation elicited a rapid oxidation and a subsequent re-reduction with a $t_{1/2}$ of 15 ms. The strain VC-F also showed a

rapid photo-oxidation of the hemes although the re-reduction was significantly slow. In the case of the strain ΔC no distinct oxidation of the *c*-type cytochromes was observed.

Discussion

In this study, a recombinant strain of *Rvi. gelatinosus* which synthesizes a chimeric RC complex consisting of the LM core of *Rvi. gelatinosus* and the cytochrome subunit of *Blc. viridis* was obtained and characterized. The flash-induced kinetic measurements indicated that the cytochrome subunit derived from *Blc. viridis* functions as a direct electron donor to the oxidized special pair in the chimeric RC. However, the rate of re-reduction of the hemes was much slower than that in the *Rvi. gelatinosus* cells containing the native RC. The major electron donor to the RC in *Rvi. gelatinosus*, HiPIP, may not be an efficient donor to the *Blc. viridis* cytochrome subunit. This was possibly due to the difference in sets of amino acids involved in the recognition of the soluble electron carriers (Osyczka et al. 1999).

The amino acid sequence identities of the L, M and cytochrome subunits between *Rvi. gelatinosu*s and *Blc. viridis* are 66, 61 and 45%, respectively. The amino acid residues connecting the LM core and the cytochrome subunit in *Blc. viridis* by hydrogen-bonds or salt bridges were not well conserved in *Rvi. gelatinosus*. A factor, which may contribute to the formation of the chimeric RC, is that the 107-bp sequence of the 3' end of the *pufM* gene was derived from *Blc. viridis*. The C-terminus region of the M subunit coded by the 3' end had been suggested to associate strongly with the cytochrome subunit (Nitschke and Dracheva, 1995).

Further physiological and kinetic studies and additional site-directed mutagenesis on the chimeric RC may be useful for the clarifications of the precise mechanisms of the electron transfer and of the physiological and evolutionary roles of the cytochrome subunit. The system established in this study, together with the available structural and kinetic information in *Blc. viridis*, will contribute to obtain new information about the relationships between the structure and function in the electron transfer proteins.

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