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Cytochrome c_4 Can Be Involved in the Photosynthetic Electron Transfer System in the Purple Bacterium *Rubrivivax gelatinosus*[†]

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ABSTRACT: Three periplasmic electron carriers, HiPIP and two cytochromes c_8 with low- and high-midpoint potentials, are present in the purple photosynthetic bacterium *Rubrivivax gelatinosus*. Comparison of the growth rates of mutants lacking one, two, or all three electron carrier proteins showed that HiPIP is the main electron donor to the photochemical reaction center and that high-potential cytochrome c_8 plays a subsidiary role in the electron donation in photosynthetically growing cells. However, the triple deletion mutant was still capable of photosynthetic growth, indicating that another electron donor could be present. A new soluble cytochrome *c*, which can reduce the photooxidized reaction center *in vitro*, was purified. Based on amino acid sequence comparisons to known cytochromes, this cytochrome was identified as a diheme cytochrome *c* of the family of cytochromes c_4. The quadruple mutant lacking this cytochrome and three other electron carriers showed about three times slower growth than the triple mutant under photosynthetic growth conditions. In conclusion, cytochrome c_4 can function as a physiological electron carrier in the photosynthetic electron transport chain in *R. gelatinosus*.

The class Proteobacteria (purple bacteria) is constituted of many species showing a variety of energy metabolisms, i.e., oxygen respiration, fermentation, denitrification, metal oxidation, and oxidation/reduction of sulfur compounds. Photosynthesis is one of the various energy metabolisms found in this class of bacteria. The basic process of photosynthesis is very common among purple bacteria and is known as the photosynthetic cyclic electron transfer pathway. This pathway is composed of four components, the photochemical reaction center (RC)[1] complex, the quinone pool, the cytochrome bc_1 complex, and mobile electron carriers. These components, with the exception of the RC complex, are shared by other energy metabolism pathways.

The RC complex of purple photosynthetic bacteria is composed of the L, M, and H subunits and, in many species, a cytochrome subunit. The photosynthetic electron transfer is initiated by light excitation of a bacteriochlorophyll dimer called a "special pair" in this complex, from which an electron is released. The electron moves among cofactors in the RC complex to reduce the quinone molecule in the membrane, and then it is transferred to the cytochrome bc_1 complex, which reduces in turn a soluble electron carrier protein located in the periplasmic space. To complete this cyclic pathway, the soluble electron carrier proteins reduce the hemes of the RC-bound cytochrome subunit. In some species lacking this subunit, the soluble electron carrier donates an electron directly to the photooxidized special pair.

So far, various electron carrier proteins, acting as the electron donor to the RC, have been identified in purple bacteria. For many species belonging to the α-subclass of purple bacteria, cytochrome c_2 is a popular electron donor to the RC. This cytochrome is a water-soluble protein with a molecular mass of approximately 10 kDa which contains one *c*-type heme with a redox midpoint potential around +300 mV. In *Rhodobacter sphaeroides*, the cytochrome c_2 is essential for photosynthesis, since its photosynthetic growth ability is lost by disruption of the gene coding this small cytochrome (*1*). Some species among the α-subclass have membrane-bound cytochromes *c*, which show high sequence similarities to cytochrome c_2 but possess a hydrophobic anchor stretch at the N-terminal region. These cytochromes, referred to as cytochrome c_y or c_{2m}, act as efficient electron donors to the RC in addition to the water-soluble cytochrome c_2 in *Rhodobacter capsulatus* (*2*) and *Rhodovulum sulfidophilum* (*3*).

Synthesis of cytochrome c_2 or its membrane-bound homologues has not been reported so far in any species belonging to the β- and γ-subclasses of purple bacteria. Instead, monoheme cytochromes c_8 have been found in some of these species. Cytochromes c_8 are similar to cytochromes c_2 in terms of size, structure, and electrochemical properties but distinct from these electron carriers in their amino acid sequences. In *Rubrivivax gelatinosus*, *Rhodocyclus tenuis*, and *Allochromatium vinosum*, cytochrome c_8 could act as the electron donor to the RC (*4*−*6*).

In addition to the *c*-type cytochromes, the high-potential iron−sulfur protein (HiPIP) is found as a soluble electron transfer protein accumulated in the periplasmic space. HiPIP is a soluble protein of 6−10 kDa in size which contains one 4Fe-4S cluster with an E_m ranging from 50 to 350 mV. Studies of several species belonging to the β- and γ-subclasses, such as *R. gelatinosus*, *Rhodoferax fermentans*, *R. tenuis*, and *A. vinosum*, have

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[1]Abbreviations: HiPIP, high-potential iron−sulfur protein; cyt, cytochrome; RC, reaction center; TMBZ, 3,3′,5,5′-tetramethylbenzidine; DAD, diaminodurene (2,3,5,6-tetramethylphenylenediamine); MOPS, 3-morpholinopropanesulfonic acid; E_m, redox midpoint potential.

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suggested that HiPIP works as a physiological electron donor to the RC (*6*−*9*). This has been clearly established in the β-purple bacterium, *R. gelatinosus*, by means of mutational analyses (*7*, *11*). In addition, *R. gelatinosus* produces, at least, two other soluble electron carrier proteins working as electron donors to the RC: a high-potential cytochrome c_8 (HP cyt c_8) and a low-potential cytochrome c_8 (LP cyt c_8), with redox midpoint potentials (E_m's) of +300 and +50 mV, respectively (*4*, *12*, *13*). The physiological importance of synthesizing different electron donors to the RC is, however, not clear.

In this study, we constructed a series of *R. gelatinosus* mutants lacking three donor proteins, HiPIP, LP cyt c_8, and HP cyt c_8. The mutant lacking all three proteins still grows under photosynthetic conditions. Based on biochemical, spectrophotometric, and genetic analyses, a diheme cytochrome *c* was newly identified as a candidate of the electron donor to the RC in this triple mutant. The sequence comparison assigned this cytochrome as a member of the cytochrome c_4 family.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Media. *R. gelatinosus* IL144 and mutants derived from this strain were grown under aerobic-dark or anaerobic-light conditions at 30 °C with a PYS medium (*14*). Illumination was supplemented with 60 W tungsten lamps placed 20 cm apart from the culture bottles. *Escherichia coli* strains JM109, JM109 λpir, and S17-1 λpir were used as hosts for plasmids and grown aerobically with an LB medium or an SOB medium (*15*) at 37 °C. When needed, ampicillin, kanamycin, and tetracycline were added to the *E. coli* and *R. gelatinosus* cultures at the final concentration of 50 μg/mL.

Plasmid Construction. A 1.4 kbp DNA fragment containing the *R. gelatinosus* HiPIP gene (*hip*) had been cloned in a pUC119 plasmid (*11*). A region containing a part of the *hip* gene flanked by *Nco*I and *Stu*I restriction sites (225 bp) was removed from this plasmid. A DNA fragment containing a Km^r-marker gene and *sacB-sacR* genes of *Bacillus subtilis* was then inserted into the plasmid at the outside of the cloned fragment. The *sacB* gene encodes levansucrase, and its expression is lethal for the host in the presence of sucrose (*16*). The plasmid ΔHip-Skm was thus obtained and used to produce a mutant lacking HiPIP. Plasmids to construct mutants lacking HP cyt c_8 and LP cyt c_8 were created as well. A 1.2 kbp DNA fragment containing the HP cyt c_8 gene (*cyc8H*) and a 1.1 kbp DNA fragment containing the LP cyt c_8 gene (*cyc8L*) had been cloned in pHSG396 plasmids (*11*). A 244 bp *Apa*I−*Apa*I region including most of *cyc8H* and a 139 bp *Bsp*HI−*Apa*I region including the 5′-half of *cyc8L* were removed. The DNA fragment containing the Km^r-marker gene and the *sacB-sacR* genes was then inserted at the multicloning sites outside of the disrupted cytochrome genes. The plasmids obtained were named ΔHc8-Skm and ΔLc8BA-Skm, respectively, and used for the construction of the mutants.

Transformation and Screening. The plasmids, ΔHip-Skm, ΔHc8BA-Skm, and ΔLc8BA-Skm, were individually transferred into the cells of *R. gelatinosus* IL144 by electroporation as described previously (*17*). Cells growing on PYS-agar plates containing kanamycin were selected. The plasmids introduced are incapable of replication in *R. gelatinosus* cells; thus, kanamycin resistans means incorporation of the plasmid into their genomic DNAs via a single crossover homologue recombination. These cells were aerobically grown in a 3 mL PYS medium without kanamycin to allow second homologue recombination. One milliliter of the growing culture was added to a 3 mL PYS medium containing 15% sucrose and further grown for 2 days to eliminate cells with the incorporated plasmid. Sucrose-resistant but kanamycin-sensitive cells were selected on PYS-agar plates containing 15% sucrose. Two types of transformants could be predicted in these cells. One has the same gene arrangement as the host cell since the second homologue recombination occurred at the same site where the first recombination occurred. The other lacks the target gene since the site of the second recombination is different from that of the first. The latter type of transformant was selected by genomic Southern hybridization using a DNA fragment containing *hip*, *cyc8H*, or *cyc8L* as a probe. The lack of the gene was then further confirmed by PCR and DNA sequencing. Double- and triple-deletion mutants among the three genes were obtained by repetitions of the same procedure.

Membrane Preparation and Isolation of Cytochromes c. Cells of the *R. gelatinosus* wild type or mutants were photosynthetically grown and harvested by centrifugation at 7000 rpm with a RA-8R rotor in a 7820 centrifuge (Kubota, Japan) for 10 min at 4 °C. Harvested cells were washed with a buffer containing 5 mM MOPS−NaOH and 0.2 M NaCl (pH 7.0) and disrupted by sonication or passage through a French pressure cell at 1100−1200 kg/cm^2 in the presence of a few grains of DNase I. When needed, 0.1% octyl β-D-thioglucoside was added after the disruption. The disrupted cell suspension was centrifuged at 12000 rpm with a RA-3R rotor (Kubota, Japan) for 10 min at 4 °C to remove cell debris. Membrane fragments were collected by ultracentrifugation at 45000 rpm with a RP-50T rotor in a CP70G ultracentrifuge (Hitachi, Japan) for 90 min at 4 °C. The supernatant of the ultracentrifugation was dialyzed against 5 mM MOPS−NaOH (pH 7.0) and applied to a CM fast-flow ion-exchange column (Pharmacia LKB) equilibrated with the same buffer. Using a stepwise salt gradient, cytochromes *c* were separated and collected. Each fraction was further purified by gel filtration on a Sephadex S-100 (Pharmacia LKB, Sweden) column with a buffer containing 5 mM MOPS−NaOH (pH 7.0) and 25 mM NaCl. The final purity of each protein was checked by SDS−PAGE.

SDS−Polyacrylamide Gel Electrophoresis. SDS−PAGE was carried out according to the method of Laemmli (*18*). Purified proteins were denatured in 2% SDS and 5% β-mercaptoethanol at boiling temperature for 3 min. After an ultracentrifugation the supernatant was mixed with a 1/6 volume of a loading solution containing 40% glycerol and 0.02% bromophenol blue. After electrophoreses, the gel was treated with TMBZ/H_2O_2 for the detection of hemes according to the method of Thomas et al. (*19*).

Redox Titration. Redox titration for the isolated cytochrome *c* was carried out using a method described previously (*20*) with a double-beam spectrophotometer UV-3000 (Shimadzu, Japan). The buffer for the measurements contained 100 mM KCl and 20 mM MOPS−KOH (pH 7.0). The redox mediators used were 100 μM potassium ferrocyanide, 10 μM diaminodurene (DAD), 1 mM ferric-EDTA, 10 μM phenazine methosulfate (PMS), and 10 μM vitamin K_3. At the beginning of the measurements, the suspension was reduced by the addition of sodium ascorbate and sodium dithionite. Subsequently, the redox potential was raised stepwise by the addition of potassium ferricyanide.

Growth Analysis. Before the measurements, *R. gelatinosus* wild type and mutants were aerobically grown to midexponential growth phases in the dark at 30 °C in a PYS medium. Measurements were started with the addition of the preculture to a fresh

PYS medium with 1/100 volume, and cells were grown at 30 °C. For the measurement of the respiratory growth, a 5 mL culture in a 20 mL test tube with a diameter of 18 mm was shaken in a reciprocal shaker at 120 strokes min^{-1} in the dark. Photosynthetic growth was measured with a screw-capped test tube with a diameter of 18 mm filled with the PYS growth medium and placed under the light supplemented with a 60 W tungsten lamp 15−25 cm apart from the tubes. The cell density was monitored by the absorbance at 660 nm. The minimum and the maximum values among the five independent measurements were omitted, and the average of the other three values was plotted against the time.

Flash-Induced Kinetic Measurements. Kinetic measurements of flash-induced absorbance changes of the cytochromes were carried out using a single beam spectrophotometer (*21*) or a double beam spectrophotometer (*7*) as described previously. Membranes or whole cells were suspended in 5 mM MOPS−NaOH, pH 7.0, supplemented with 20 μM DAD and 100 μM sodium ascorbate. The concentrations of the whole cells and membranes were adjusted to give an absorbance of 1 at 875 nm (approximately 0.1 μM RC). The concentrations of the purified cytochromes *c* were adjusted to give an absorbance of 0.02 at the α-absorption band (approximately 1 μM cytochrome *c*).

Cloning and Mutagenesis of the Gene Coding the Newly Isolated Cytochrome c. The N-terminal amino acid sequence of the isolated cytochrome *c* was determined by the method of Edman degradation. Based on the sequence, an oligonucleotide hybridization probe with a sequence of 5′-GARGC-SAAGCCSGCSAAGCCSGACYTSGCS was synthesized. The genomic DNA library of *R. gelatinosus* produced in our previous study (*22*) was screened by this probe. A cosmid DNA showing a positive signal was cloned and digested by *Pst*I endonuclease and subcloned in pHSG298 plasmids. This mini-DNA library was again screened by the same probe. The plasmid obtained had an insert DNA with approximately 2.4 kb in length. The nucleotide sequence of this insert DNA was determined by a dye terminator cycle sequencing kit and a 310A genetic analyzer (Applied Biosystems, CA) using oligonucleotide primers designed to generate overlapping DNA sequences. A gene coding a possible cytochrome carrying two binding motifs to *c*-type hemes was found in the sequence and named *cyc4*. A region containing the sequence corresponding to the first heme-binding motif was removed by digestion using *Eco*T14I endonuclease and self-ligation after end-filling. This manipulation caused a frame shift in the backward sequence of the gene and rendered the sequence for the second heme-binding motif nonsense. The manipulated insert DNA was transferred to a suicide vector pJPCm, a derivative of the plasmid pJP5603 (*23*), in which the kanamycin-resistance gene is replaced by a chloramphenicol-resistance gene derived from the plasmid pHSG396. A DNA cassette containing a Km^r-marker gene and *sacB-sacR* genes was then inserted into this plasmid at the outside of the insert DNA. The plasmid obtained, called pJP-ΔGC553-KmSac, was introduced into the cells of *R. gelatinosus* by conjugal transfer from *E. coli* S17-1 λpir host cells. First, cells growing on PYS-agar plates containing kanamycin and tetracycline were selected. *E. coli* host cells are tetracycline-sensitive, but *R. gelatinosus* cells are naturally tetracycline-resistant. Screening for the mutant lacking the main part of the diheme cytochrome *c* gene was then performed with the same procedure described in the Transformation and Screening section. For gene complementation tests, a pHSG396 plasmid containing the entire *cyc4* gene was connected with a pJRD215 plasmid (*24*), which can be maintained in *R. gelatinosus* cells. This plasmid was named pJRD-C4Cm and introduced into the mutant lacking the *cyc4* gene by conjugal transfer from *E. coli* S17-1 host cells. The DNA sequence reported in this paper is deposited in the DDBJ (DNA Data Bank of Japan) and is available in GenBank/EMBL/DDBJ (accession no. AB462891).

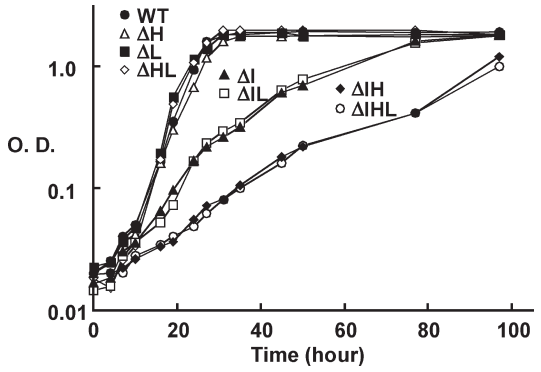

FIGURE 1: Photosynthetic growth curves of *R. gelatinosus* wild type and mutants lacking soluble electron carrier proteins. ΔI, ΔH, and ΔL refer to mutants devoid of genes coding HiPIP, HP cyt c_8, and LP cyt c_8, respectively. ΔIL, ΔIH, and ΔHL indicate double mutants lacking HiPIP/LP cyt c_8, HiPIP/HP cyt c_8, and HP cyt c_8/LP cyt c_8, respectively. ΔIHL means a triple mutant lacking all three proteins. The measurements were started by inoculation of 1/100 volume of late exponential phase cells grown aerobically in PYS medium. Then the cultures were photosynthetically grown at 30 °C in screw-capped tubes filled with the medium. The optical density measured at 660 nm for the culture tube (diameter of 18 mm) was plotted against the time.

RESULTS

Photosynthetic Growth Rates of Mutants Lacking HiPIP, HP cyt c_8, and LP cyt c_8. In this study, the genes coding HiPIP, HP cyt c_8, and LP cyt c_8 were deleted without any insertions of external DNAs like an antibiotic-resistance cartridge. First, three mutants lacking each of these electron carrier proteins were obtained and named ΔI, ΔH, and ΔL, respectively. Next, a series of double mutants lacking two of the three proteins was constructed using the single mutants as the hosts. They were named ΔIH (lacking HiPIP and HP cyt c_8), ΔIL (lacking HiPIP and LP cyt c_8), and ΔHL (lacking HP cyt c_8 and LP cyt c_8). Finally, a mutant lacking all three proteins (ΔIHL) was generated.

Under aerobic-dark conditions, the growth rates of the wild type and the seven mutants were almost identical (data not shown). In contrast, under anaerobic-light (photosynthetic) conditions, the mutants showed different growth rates that could be divided into three groups (Figure 1). In the first group, the mutants lacking cytochromes c_8 but retaining HiPIP (ΔH, ΔL, and ΔHL) showed almost the same growth rates as the wild type. The second group, the mutants lacking HiPIP but retaining HP cyt c_8 (ΔI and ΔIL), showed about one-half of the growth rate of the wild type. These observations clearly indicate, as has already been concluded in a previous study (*11*), that HiPIP is the most efficient electron donor to the RC under photosynthetic growth conditions in *R. gelatinosus*.

The third group included ΔIH and ΔIHL mutants lacking HiPIP and HP cyt c_8. The growth rates of these mutants were about one-fourth that of the wild type. This implies that HP cyt c_8 can contribute to the photosynthetic electron transfer in *R. gelatinosus* but not LP cyt c_8. This inability may be related to its E_m, which is too low to accept an electron from the cyt bc_1 complex. Another important finding is that the mutant lacking all

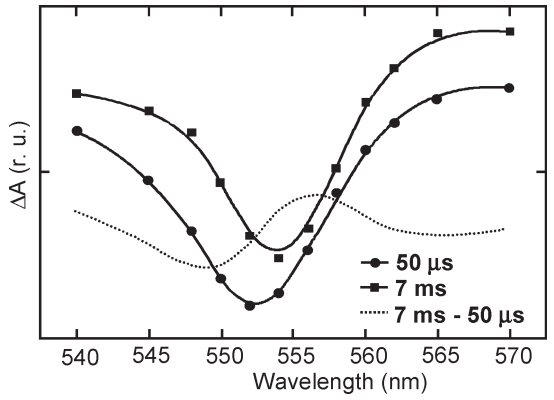

FIGURE 2: Flash-induced spectral changes of cytochromes *c* in intact cells of the *R. gelatinosus* ΔIHL triple mutant. Cells were grown under aerobic-dark conditions. Flash-induced absorbance changes at 50 μs (closed circles) and 7 ms (closed squares) were plotted against the wavelength. The spectral difference between the two data is shown by a broken line.

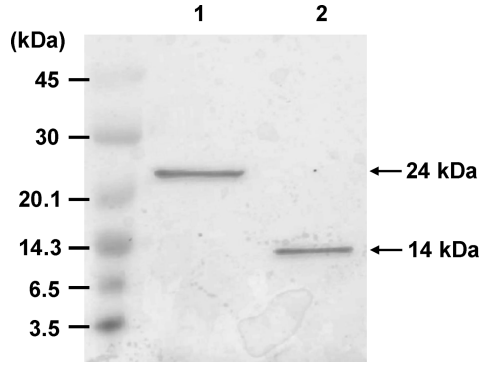

FIGURE 3: SDS−PAGE analysis of cytochromes *c* purified from the *R. gelatinosus* ΔIHL triple mutant. Cytochromes *c* were isolated from a soluble fraction after cell disruption in the presence of 0.1% octyl thioglucoside. Two fractions containing cytochromes *c* were obtained by a CM fast-flow ion-exchange column. Lane 1: Elution with 40 mM NaCl. Lane 2: Elution with 100 mM NaCl. The gel was stained for hemes by TMBZ/H_2O_2. Molecular mass marker proteins were also applied to the most left lane. The marker proteins were preliminarily colored and their molecular masses are indicated on their left. The estimated molecular masses of the two cytochromes *c* are also indicated with arrows.

of the three soluble proteins, ΔIHL, still grows under photosynthetic conditions. It is evident that other electron carrier(s) working in photosynthetic electron transfer is (are) present in this triple mutant of *R. gelatinosus*. This prompted us to find out such electron transfer proteins.

Isolation of Cytochromes c from the Triple Mutant ΔIHL. We measured flash-induced absorbance changes of cytochromes *c* in the whole cells of the triple mutant ΔIHL. When cells grown under photosynthetic conditions were used, the changes were not clear due to spectral overlapping with a carotenoid band shift. However, rapid photooxidation of the RC-bound cytochrome followed by rereduction of cytochromes *c* with a half-time of several milliseconds was observed when the cells grown under aerobic-dark conditions were used. During this rereduction period, a slight shift of the α-absorption band toward a shorter wavelength region was detected (Figure 2). When spheroplasts were used for the measurements, such a rereduction phase disappeared (data not shown). Therefore, this series of experiments suggests that the unidentified electron carrier acting as an electron donor to the RC in the triple ΔIHL mutant is a *c*-type cytochrome located in the periplasmic space.

When the periplasmic fraction obtained from the ΔIHL mutant was applied to cation-exchange column chromatography, two fractions with reddish colors were eluted by 40 and 100 mM NaCl. These putative cytochromes *c* were further purified by gel filtration and analyzed by SDS−PAGE (Figure 3). When stained for hemes, these proteins showed bands with apparent molecular masses of around 24 and 14 kDa, respectively. The latter was identified as cytochrome *c′*, as already shown in a previous study using the *R. gelatinosus* IL144 wild type (*13*). In *R. gelatinosus*, cytochrome *c′* has been tested as a possible electron carrier participating in photosynthesis. However, no indications of the oxidation of this protein by the RC-bound cytochrome were found (*4*). The presence of a cytochrome *c* of 24 kDa was not noticed in our previous studies using *R. gelatinosus*, mainly because of its low yield of extraction. In the present study, however, we improved the extraction of this cytochrome by a moderate treatment of the membrane with detergent (0.1% octyl β-D-thioglucoside).

A reduced-minus-oxidized difference spectrum of the 24 kDa cytochrome *c* is shown in Figure 4A. The α-absorption band peaks at 553 nm, but it is asymmetric with a clear shoulder at the shorter

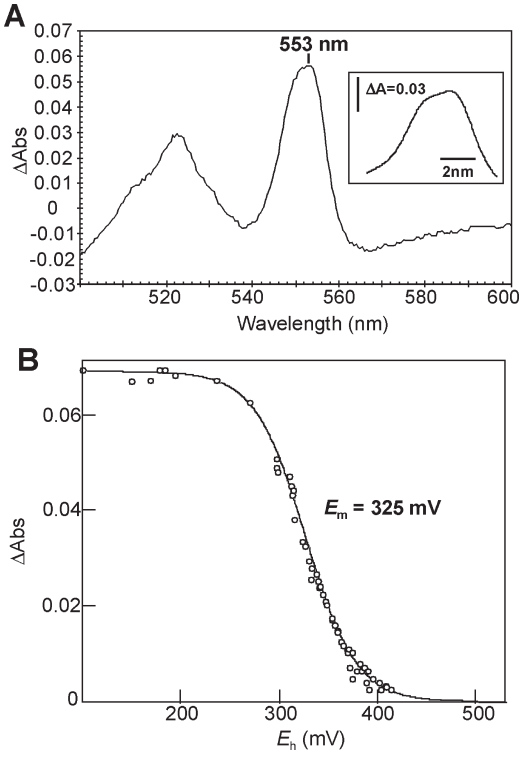

FIGURE 4: Redox difference spectra (A) and redox titration curve (B) of the newly isolated cytochrome *c* from *R. gelatinosus* ΔIHL. The cytochrome was reduced by addition of a small grain of sodium ascorbate. The inset shows an enlarged image of the α-absorption band that appeared around 553 nm. The titration curve was obtained by plotting the absorbance at 553 nm against the ambient redox potentials.

wavelength region (Figure 4A inset). This cytochrome will be referred to as cytochrome c_{553} (cyt c_{553}). Figure 4B shows a result of redox titration for this cytochrome obtained by plotting the peak heights of its α-absorption band against the ambient potentials (E_h). The data points could be fitted with a Nernst curve when the presence of one redox component was postulated. The redox midpoint potential (E_m) of the heme was calculated to be +325 mV.

In Vitro Electron Transfer from Cytochrome c_{553} to the RC-Bound Cytochrome. To examine whether cyt c_{553} could work as an electron donor to the RC-bound cyt, reconstitution experiments using this cytochrome and the purified membranes were performed. Figure 5 shows kinetic traces of flash-induced absorbance changes at 555 nm measured for the RC-bound cyt in the membrane (about 0.1 μM) in the presence and absence of various electron donor proteins (1 μM each). A rapid photooxidation of the RC-bound cytochrome was commonly observed immediately after the flash activation. In the absence of any electron donors, the RC-bound cytochrome remained oxidized over several hundred milliseconds. In contrast, a second-order rate constant for the rereduction of the photooxidized RC-bound cytochrome equal to 1.8×10^8 M^{-1} s^{-1} or 3.0×10^7 M^{-1} s^{-1} is measured in the presence of HiPIP or HP cyt c_8, respectively, in full agreement with the values previously reported (*4*). In the presence of cyt c_{553}, the RC-bound cytochrome was also rereduced although less efficiently, with a second-order rate constant of approximately 1.2×10^7 M^{-1} s^{-1}. Although this rate constant is lower than those for HiPIP and HP cyt c_8, our result shows that cyt c_{553} can serve as a physiological electron donor to the *R. gelatinosus* RC. We have previously shown that the photosynthetic growth of *R. gelatinosus* is not inhibited even when the rate constant of the electron transfer from HiPIP to the RC was dropped more than 20 times (*25*). There are, therefore, no reasons to exclude the possibility that cyt c_{553} supports the photosynthetic growth of the *R. gelatinosus* ΔIHL mutant.

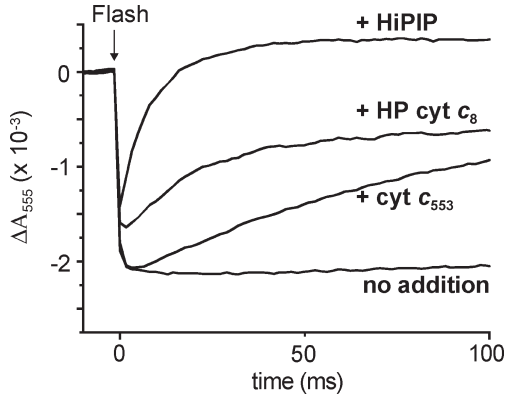

FIGURE 5: Flash-induced absorbance changes of the RC-bound cytochrome in membranes in the presence or absence of HiPIP, HP cyt c_8, or newly purified cyt c_{553}. The samples were suspended in 5 mM MOPS−NaOH (pH 7.0) containing 0.1 mM sodium ascorbate and 20 μM DAD. The concentration of the membrane was adjusted to A_{860} = 1.0. The electron carrier proteins were added at 1 μM.

Cloning of the Gene Coding Cytochrome c_{553}. The amino acid sequence of the N-terminal 10 residues of cyt c_{553} was directly determined for the purified protein. Using an oligonucleotide probe having a sequence to code this amino acid sequence, a gene coding this cytochrome was screened from the cosmid-based DNA library of the *R. gelatinosus* genome (*22*). The deduced amino acid sequence of cyt c_{553} consisted of 224 residues and contained two heme-binding motifs (Figure 6), showing that this cytochrome is a diheme cytochrome *c*. Its asymmetric α-absorption band shown in Figure 4 possibly reflects such a diheme nature.

The sequence of the 10 residues determined for the purified protein was found to start at the 42nd amino acid from the N-terminus in the deduced sequence. The N-terminal sequence up to 41st amino acid was predicted to have a hydrophobic stretch that can span the membrane once and some basic residues just ahead of the stretch, suggesting that this region is processed as a signal peptide during maturation. A molecular weight of 20360 was calculated for the mature form of this cytochrome when the presence of two *c*-type hemes was taken into account, in agreement with the molecular mass estimated in the SDS−PAGE analysis.

The amino acid sequence of cyt c_{553} showed strong identities to those of cytochromes c_4. When compared with the sequence of *Pseudomonas stutzeri* cyt c_4 whose structure has been revealed at 2.2 Å resolution (*26*), 39% identity was obtained. Two methionine residues serving as the sixth axial ligands to the hemes, as well as two -C-X-X-C-H- heme-binding motifs, were also conserved. Therefore, we concluded that cyt c_{553} found in *R. gelatinosus* should be included in the cyt c_4 family. The rather high E_m value (325 mV) of this cytochrome also shows the

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   1                                                                  M  G  H  C  K  I  L  R  F  D  T
1201 CGCGGTGTGCGCCCAGGCCAGAGCACTCTTTTCAGCGTCGCTTGGCACATCTCGAAAGGTAGACGTCATGGGCCATTGTAAAATCCTGCGGTTTGACACC
                                                                        S.D.
  12  R  T  P  A  D  R  G  P  R  R  M  K  F  A  V  A  L  T  L  A  A  L  A  A  G  P  A  F  A  Q  E  A  K
1301 CGAACACCTGCCGACCGAGGCCCCCGCCGCATGAAGTTCGCCGTCGCCCTGACCCTCGCCGCGCTCGCCGCCGGCCCCGCCTTCGCCCAGGAAGCGAAGC

  45  P  A  K  P  D  L  A  K  G  Q  I  T  A  A  T  C  Q  A  C  H  T  A  D  G  S  R  G  V  P  T  Y  P  I  L
1401 CCGCCAAGCCGGACCTGGCCAAGGGCCAGATCACGGCCGCCACCTGCCAGGCCTGCCACACGGCCGACGGGTCGCGTGGTGTGCCGACCTACCCGATCCT

  78   Q  G  Q  H  A  A  Y  I  V  K  Q  L  T  E  F  K  A  G  K  R  M  N  P  I  M  M  G  M  A  A  P  L  S
1501 GCAAGGCCAGCACGCGGCCTACATCGTCAAGCAGCTCACCGAGTTCAAGGCCGGCAAGCGCATGAACCCCATCATGATGGGCATGGCCGCGCCGCTGAGC

 112  P  E  D  M  V  N  V  A  A  F  F  A  S  K  A  P  V  K  G  E  S  T  S  K  E  T  L  A  L  G  Q  S  I
1601 CCGGAAGACATGGTCAACGTCGCCGCGTTCTTCGCGTCGAAGGCGCCGGTCAAGGGCGAGTCGACCTCCAAGGAGACGCTGGCCCTGGGCCAATCGATCT

 145  F  R  G  G  I  A  S  K  K  V  P  A  C  A  G  C  H  S  P  N  G  A  G  I  P  A  Q  Y  P  R  L  G  G  Q
1701 TCCGCGGCGGCATCGCCTCGAAGAAGGTCCCGGCCTGCGCCGGCTGCCACAGCCCGAACGGCGCCGGCATCCCCGCGCAGTACCCGCGCCTGGGTGGCCA

 178   H  V  E  Y  T  T  T  Q  L  N  T  F  R  S  G  E  R  A  N  S  I  Q  M  T  A  I  A  A  K  L  S  D  A
1801 GCACGTCGAATACACGACGACGCAGCTGAACACCTTCCGCTCGGGCGAACGCGCCAACAGCATCCAGATGACGGCCATCGCCGCCAAGCTGAGCGACGCC

 212  E  I  K  A  V  A  D  Y  I  A  G  L  H  *
1901 GAGATCAAGGCCGTGGCCGACTACATCGCCGGCCTGCACTGATCGCACACAGCGCATGCTCGACGGGCCGGCTCATGCCGGCCCGTTTTGTTTGGGGCGC
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FIGURE 6: Nucleotide sequence of the gene coding cytochrome c_4 found in *R. gelatinosus* and its translation. The amino acid sequence directly determined from the purified protein is surrounded by a rectangle. Binding motifs to the *c*-type heme are hatched. Restriction sites recognized by *Eco*T14I endonuclease are indicated by upward arrows. Head-to-head arrows show a presumed hairpin structure possibly working as a transcription terminator.

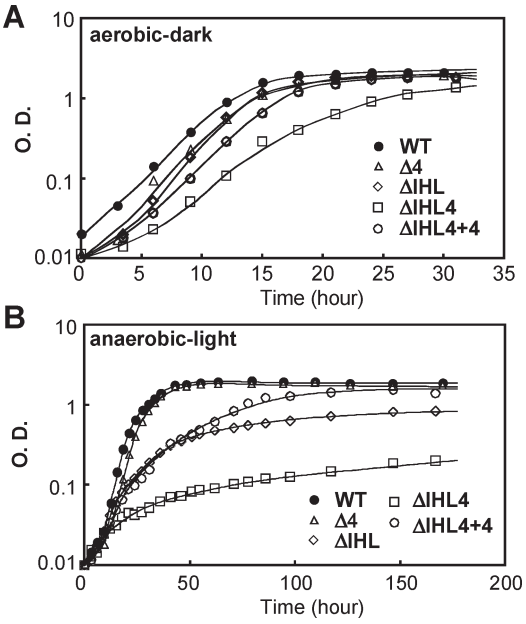

FIGURE 7: Growth curves of *R. gelatinosus* mutants lacking cytochrome c_4 under aerobic-dark (A) and anaerobic-light (B) conditions. Δ4, ΔIHL, and ΔIHL4 indicate mutants lacking cyt c_4 (single), HiPIP/HP cyt c_8/LP cyt c_8 (triple), and HiPIP/HP cyt c_8/LP cyt c_8/cyt c_4 (quadruple), respectively. ΔIHL4 + 4 means a complementary mutant strain in which the cyt c_4 gene was introduced *in trans* into the ΔIHL4 mutant cell. Measurements of cell growth were carried out in the same way as those described in Figure 1. Aerobic-dark cultivation was carried out by shaking the 4 mL culture in a 20 mL volume test tube (diameter of 18 mm) with a reciprocal shaker at 140 strokes per minute.

common feature of cyts c_4 known so far, i.e., E_m's ranging from 190 to 510 mV. The presence of a clear shoulder at the shorter wavelength side of the α-band of cyt c_{553} (Figure 4A) is further evidence for the dihemic character of this cytochrome. However, the two hemes could not be distinguished in the redox titration curve (Figure 4B). The absorption spectra measured at the fully reduced, half-reduced, and slightly reduced conditions showed an identical shape of the α-band. Probably, two hemes have nearly the same E_m's. Hereafter, we refer to the name of this cytochrome found in *R. gelatinosus* as cytochrome c_4.

Mutant Lacking Cytochrome c₄. To clarify the physiological function of cyt c_4, mutants lacking this cytochrome were created using the *R. gelatinosus* wild type and the triple mutant ΔIHL as the parents and named Δ4 and ΔIHL4, respectively. These mutations were confirmed by a SDS−PAGE/TMBZ analysis (data not shown). When grown in a PYS medium under aerobic-dark conditions, these mutants showed no significant decrease in growth rates from the wild type, as shown in Figure 7A. However, under photosynthetic conditions, the quadruple mutant ΔIHL4 showed approximately three times slower growth than the triple mutant, as shown in Figure 7B, while the Δ4 single mutant showed nearly the same growth rate as the wild type. This suggests that cyt c_4 is not essential for photosynthetic growth but can compensate for the lack of other soluble electron carriers in *R. gelatinosus*. When the gene coding cyt c_4 was introduced into the ΔIHL4, the photosynthetic growth rate was recovered to the level of that of the triple mutant. This clearly shows that cyt c_4 can function as the electron donor to the photooxidized RC in growing cells of *R. gelatinosus*. Another finding in the growth tests is that the quadruple mutant ΔIHL4 still retained the ability of photosynthetic growth, although the cell density in the stationary growth phase was quite low. This means that other electron carriers are able to sustain, although at a very low rate, a light-induced cyclic electron transfer in *R. gelatinosus*.

DISCUSSION

In this study, a series of mutants lacking three electron carrier proteins was constructed without the insertion of marker genes. On the basis of the growth tests, we confirmed that HiPIP is the main physiological electron donor to the RC in *R. gelatinosus* as already concluded in a previous study (*11*). It was also shown that HP cyt c_8 functions as the alternate electron carrier between cyt bc_1 and RC as has been predicted in previous studies (*11*, *13*). In contrast, LP cyt c_8 probably makes almost no contributions to the photosynthetic cyclic electron transfer since its deletion has no effects on the cell growth. Its low midpoint potential (+50 mV) may be too low to accept electrons from the cytochrome bc_1 complex. The most striking result obtained from these mutants is that the mutant lacking all of the three proteins (ΔIHL strain) can still grow under photosynthetic conditions, implying that another unidentified electron carrier is working. We purified a candidate for such an electron carrier protein and identified it as a diheme cytochrome c_4.

The biochemical and spectroscopic characteristics of cyt c_4 have been thoroughly studied in nonphotosynthetic species, i.e., *Pseudomonas aeruginosa*, *Ps. stutzeri*, and *Azotobacter vinelandii* (*27*, *28*). Recently, several homologues of cyt c_4 were found in *Acidithiobacillus ferroxidans* and have been precisely characterized in terms of their biophysical properties (*29*). Cytochrome c_4 was also recently isolated and characterized in the purple photosynthetic bacterium *Thiocanpsa roseopersicina* (*30*). Common features among these cyts c_4 are the high midpoint potential of the two hemes and relatively low ratio between the extinction coefficients of the α and β absorption bands. Such features are conserved in *R. gelatinosus* cyt c_4, as shown in Figure 4.

However, a biological role common in the cyt c_4 family has not clearly been defined. In *A. vinelandii*, cyt c_4 shows a significant tendency to form aggregation with cytochrome *o* (*31*). Studies on mutants devoid of cyt c_4 have suggested that cyt c_4, as well as the monoheme cyt c_5, transfers electrons from a quinone pool via the cyt bc_1 complex to the terminal oxidase, cyt *o* or cbb_3, in this species (*32*−*34*). Such a pathway seems to be reasonable when considering the high-potential nature of cyt c_4. A similar role is also possible in *R. gelatinosus*, since the synthesis of cyt c_4 is actively maintained even under respiratory conditions. Northern hybridization experiments showed that the accumulation levels of the mRNA derived from the cyt c_4 gene are nearly the same in cells grown under aerobic-dark and anaerobic-light conditions (data not shown). However, the mutant devoid of cyt c_4 (Δ4) showed no difference in growth rate from the wild type under respiratory conditions as well as photosynthetic conditions. Even the quadruple mutant (ΔIHL4) showed only a minor decrease in the growth rate under respiratory conditions relative to the wild type. It is likely that cyt c_4 is not the main contributor in the electron transfer to the terminal oxidase in growing cells of *R. gelatinosus*.

Nevertheless, it was clearly demonstrated in the present study that cyt c_4 has the potential to donate electrons to the RC in photosynthetically grown cells. This cytochrome works as the main electron carrier in the photosynthetic electron transport pathway if HiPIP and HP cyt c_8 are missing, by connecting the

cyt bc_1 to the RC. *In vitro* flash-induced experiments showed that the second-order rate constant for the electron transfer from cyt c_4 to the RC ($1.2 \times 10^7\ M^{-1}\ s^{-1}$) is about 10 times lower than that from HiPIP but not different from the rate constant of the electron transfer from HiPIP to the mutated RC-bound cytochrome ($1.1 \times 10^7\ M^{-1}\ s^{-1}$), which is high enough to still support the wild-type level of photosynthetic growth in *R. gelatinosus* (*25*). However, the photosynthetic growth rates of the triple mutant ΔIHL and the complementary strain ΔIHL4 + 4 were about one-fourth that of the wild type. This can be explained by the significant decrease in the reduction rate of *in vivo* flash-induced photooxidation of the RC, for which the half-time was 240 μs when HiPIP was working (*13*) but over a millisecond in the triple mutant ΔIHL. Such a decrease may be ascribed to the relatively small accumulation of cyt c_4 and the association constant between cyt c_4 and the RC-bound cytohcrome. On the other hand, we do not exclude a possibility that membrane-anchored cyt c_4 works in growing cells. Our previous study on the marine purple non-sulfur bacterium *R. sulfidophilum* showed that the membrane-bound cyt c_{2m} is converted into the soluble form, cyt c_{549}, by an unknown proteolytic reaction when the cell was aged (*3*). If similar conversion occurs on *R. gelatinosus* cyt c_4, the rate of electron transfer to the RC may be changed according to the cell growth conditions. A recent study on the other membrane-bound cytochrome, cyt c_y, in *Rhodobacter* species showed that the soluble form exhibits slower electron transfer to the RC than the membrane-bound form (*35*). Although we have not detected a membrane-bound form of cyt c_4 in *R. gelatinosus* cells in this study, such possibility should be taken into account in future studies. From this series of results, it is unlikely that cyt c_4 is a major contributor to the photosynthetic cyclic electron transport chain in the wild-type cells of *R. gelatinosus*. The original function of cyt c_4 is still unclear. Nevertheless, we provided in this study the first evidence that diheme cytochrome c_4 can mediate photosynthetic cyclic electron transfer.

Cytochrome c_4 has a wide distribution among purple photosynthetic bacteria (*10*). Possibly, cyt c_4 can completely compensate the role of HiPIP and monoheme cytochromes *c*, such as cyt c_2 and cyt c_8, in photosynthetic electron transfer if the expression level is sufficiently enhanced to overcome its relatively low rate constant. It would not be surprising if some species use cyt c_4 as the physiologically main electron carrier in the photosynthetic cyclic electron transport.

In summary, *R. gelatinosus* synthesizes, at least, three identified electron carriers working in photosynthesis, HiPIP, HP-cyt c_8, and cyt c_4. In addition, other minor carrier(s) should be found, since the mutant lacking these three proteins was still capable of photosynthetic growth. HiPIP is the major carrier but can be replaced by others in *R. gelatinosus*. Such multiplicity will give this species a guarantee of photosynthetic energy conversion. The presence of the RC-bound cytochrome subunit may allow to transfer electrons from various carrier proteins by providing different binding sites. Indeed, a previous study showed that HP-cyt c_8 and HiPIP bind the RC-bound cytochrome at two distinct but partially overlapping areas in *R. gelatinosus* (*36*). Anyway, it can also be suspected that these carriers will mediate connections between the photosynthetic electron transfer and electron flows of other metabolic pathways rather than only give redundancy. Precise analyses on such an electron transfer network powered by photosynthesis will be useful for studies on the ecological significance of photosynthetic bacteria and bioremediation using light energy.

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