Mutational Analyses of the Photosynthetic Reaction Center-Bound Triheme Cytochrome Subunit and Cytochrome c_2 in the Purple Bacterium *Rhodovulum sulfidophilum*†

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ABSTRACT: The purple photosynthetic bacterium *Rhodovulum sulfidophilum* has an unusual reaction center-(RC-) bound cytochrome subunit with only three hemes, although the subunits of other purple bacteria have four hemes. To understand the electron-transfer pathway through this subunit, three mutants of *R. sulfidophilum* were constructed and characterized: one lacking the RC-bound cytochrome subunit, another one lacking cytochrome c_2, and another one lacking both of these. The mutant lacking the RC-bound cytochrome subunit was grown photosynthetically with about half the growth rate of the wild type, indicating that the presence of the cytochrome subunit, while not indispensable, is still advantageous for the photosynthetic electron transfer to support its growth. The mutant lacking both the cytochrome subunit and cytochrome c_2 showed a slower rate of growth by photosynthesis (about a fourth of that of the wild type), indicating that cytochrome c_2 is the dominant electron donor to the RC mutationally devoid of the cytochrome subunit. On the other hand, the mutant lacking only the cytochrome c_2 gene grew photosynthetically as fast as the wild type, indicating that cytochrome c_2 is not the predominant donor to the RC-bound triheme cytochrome subunit. We further show that newly isolated soluble cytochrome *c*-549 with a redox midpoint potential of +238 mV reduced the photooxidized cytochrome subunit in vitro, suggesting that *c*-549 mediates the cytochrome c_2-independent electron transfer from the bc_1 complex to the RC-bound cytochrome subunit. These results indicate that the soluble components donating electrons to the RC-bound triheme cytochrome subunit are somewhat different from those of other purple bacteria.

The photosynthetic reaction center complex is the central component in the biological conversion of light energy, and it forms a cyclic electron-transfer system together with the cytochrome bc_1 complex,[1] soluble cytochromes, and membranous quinones (*1*−*3*). The RC complex of purple bacteria typically consists of L, M, H, and cytochrome subunits (*4*). The cytochrome subunit usually has four *c*-type hemes and is often referred to as a tetraheme cytochrome (*5*). The cytochrome subunit serves as the immediate electron donor to the photooxidized special pair of bacteriochlorophylls, and the oxidized hemes of this subunit are rereduced by soluble electron carriers such as cytochrome c_2 (*1*−*3*, *5*). Since some purple bacteria, such as *Rhodobacter* species, do not have this cytochrome subunit, the significance and the physiological importance of the cytochrome subunit are still not fully clarified. In such species, the soluble cytochromes carry electrons directly to the photooxidized bacteriochlorophyll dimer (*1*−*3*, *5*).

Our previous studies have shown that the heme-1 most distantly located from the special pair works as the direct electron acceptor from soluble electron carriers (*6*), indicating that all four hemes are involved in the electron transfer from soluble electron carrier proteins to the special pair in *Rubrivivax gelatinosus* and probably in *Blastochloris viridis.* However, in *Rhodovulum sulfidophilum*, we found that the RC-bound cytochrome subunit contains only three heme-binding motifs and the heme-1-binding motif is absent (*7*). Methionine residues that are the axial ligands to heme-1 and heme-2 irons in *B. viridis* were also not present in the cytochrome subunit of *R. sulfidophilum*. This unusual cytochrome subunit was shown to be able to donate electrons to the photooxidized special pair of bacteriochlorophyll (*7*).

Recently, a soluble cytochrome c_2 of *R. sulfidophilum* was shown to work as an electron donor to the photooxidized cytochrome subunit at least in vitro (*8*). The predicted amino acid sequence of *R. sulfidophilum* cytochrome c_2 was highly similar to that of a closely related *Rhodobacter* species (*8*), suggesting that the cytochrome c_2 of these two species is functionally similar. However, the in vivo significance of the cytochrome subunit and cytochrome c_2 in the photosynthetic electron transfer of this bacterium remains unclear.

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[1] Abbreviations: bc_1 complex, ubihydroquinone:cytochrome *c* oxidoreductase; DAD, 2,3,5,6-tetramethylphenylenediamine; PAGE, polyacrylamide gel electrophoresis; RC, reaction center; SDS, sodium lauryl sulfate; Triton X-100, polyoxyethylene(10) octylphenyl ether.

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In this study, we constructed and characterized the *R. sulfidophilum* mutants lacking the RC-bound cytochrome subunit, cytochrome c_2, and both of these to clarify the physiological role of the proteins for photosynthetic electron-transfer reactions. The results indicate that the unusual cytochrome subunit is involved in but not essential for photosynthetic growth in this bacterium. Cytochrome c_2 does not seem to be the dominant electron donor to RC in the wild-type cells. The electron transfer from a newly identified soluble cytochrome, *c*-549, to the RC-bound cytochrome was demonstrated.

MATERIALS AND METHODS

Bacterial Strains, Media, and Growth Conditions. The photosynthetic bacterium *R. sulfidophilum* strains W4 (wild type), PUFC1 (*pufC* mutant), C21 (*cycA* mutant), and DNC2 (*pufC-cycA* double mutant) were cultured at 30 °C in an RCV medium (*9*) supplemented with 0.35 M sodium chloride and 0.1% yeast extract (DIFCO). Illumination was provided by 60 W tungsten lamps (50 W/m^2) for photosynthetic growth. Aerobic growth was achieved by shaking a 5 mL culture in a 50 mL L-shaped tube at 100 strokes per minute in darkness. *Escherichia coli* strains DH5α, JM109 (*λpir*), S17-1, and S17-1 (*λpir*) (*10*, *11*) were grown at 37 °C in a Luria−Bertani medium. Antibiotics were added at the final concentrations given to *E. coli* cultures [ampicillin (100 μg/mL), kanamycin (25 μg/mL), streptomycin (50 μg/mL), or chloramphenicol (25 μg/mL)] and to *R. sulfidophilum* cultures [kanamycin (50 μg/mL) or streptomycin (20 μg/mL)] where necessary.

Construction of the R. sulfidophilum pufC Mutant Strain. The *Bam*HI−*Sac*I fragment of plasmid pUFS101 (*12*) containing the *pufC* gene encoding the RC-bound cytochrome subunit was blunt ended and then subcloned into a Km^r-suicide vector pJP5603 (*13*) at *Sma*I−*Hin*cII sites, yielding plasmid pUFC001. The 0.4 kb *Sal*I fragment of pUFC001 spanning the *pufC* gene was replaced by the 2.0 kb Sm^r/Sp^r Ω cassette (*14*), yielding plasmid pUFC001Ω. The plasmid was then transferred into *R. sulfidophilum* cells by conjugation with the mobilizing strain S17-1 lysogenized with *λpir* (*11*). Sm^r Km^s cells were selected as double-crossover candidates, and the chromosomal insertion was confirmed by Southern hybridization. The mutant strain was designated PUFC1.

Construction of the R. sulfidophilum cycA Mutant Strain. The 2.0 kb Sm^r/Sp^r Ω cassette (*14*) was inserted into the *Cla*I site of the *cycA* gene of *R. sulfidophilum* in plasmid pCYCA101, which contains a 2.3 kb *Pst*I insert (*8*), to create plasmid pCYCA101Ω (Figure 1). The pCYCA101Ω and the Km^r-suicide vector pJP5603 (*13*) were digested with *Kpn*I at polycloning sites and ligated together to construct plasmid pCYCA101ΩKm. The plasmid was then transferred into *R. sulfidophilum* cells by conjugation with the mobilizing strain S17-1 (*11*). Sm^r Km^s cells were selected as double-crossover candidates, and the chromosomal insertion was confirmed by Southern hybridization. The mutant strain was designated C21.

Construction of the R. sulfidophilum cycA-pufC Double Mutant Strain. The 1.3 kb Km^r cassette (*14*) was inserted into the *Cla*I site of the *cycA* gene of *R. sulfidophilum* in plasmid pCYCA101, which contains a 2.3 kb *Pst*I insert (*8*), to create plasmid pCYCA101ΔKm (Figure 1). The direction

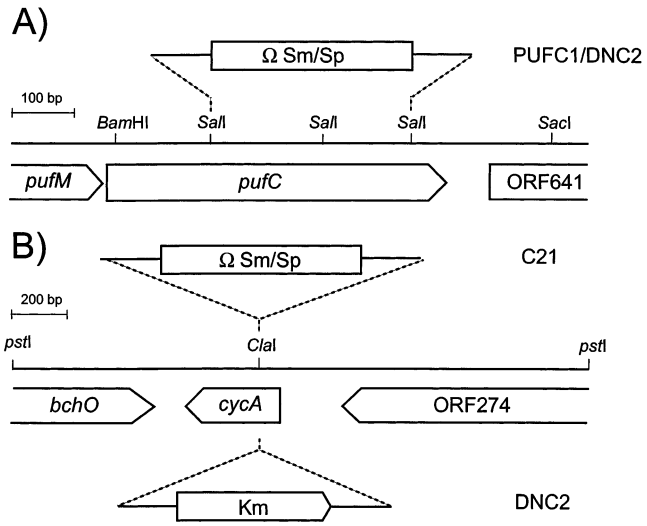

FIGURE 1: Physical and genetic maps of *R. sulfidophilum pufC* (A) and *cycA* (B) regions. ORFs and their directions of transcription are represented by open arrows. Insertions of the Sm^r/Sp^r Ω and Km^r cartridges are indicated. The constructions of PUFC1 (*pufC* mutant), C21 (*cycA* mutant), and DNC2 (*pufC-cycA* double mutant) are described in Materials and Methods.

of transcription of the Km^r gene was the opposite of that of the *cycA* gene in this construction. Plasmid pCYCA101ΔKm and a Cm^r-suicide vector pJSC were digested with *Xba*I at polycloning sites and ligated together to construct plasmid pCYCA101ΔKmCm. Plasmid pJSC has a *sacB* gene encoding the lavansucrase of *Bacillus subtilis* (*15*). The expression of *sacB* in the presence of sucrose is lethal for many Gram-negative bacteria (*15*). The resulting plasmid, pCYCA101ΔKmCm, was transferred into the *R. sulfidophilum pufC* mutant strain, PUFC1, by conjugation with the mobilizing strain S17-1 (*11*). Km^r cells grown in the presence of 5% sucrose were selected as double-crossover candidates, and the chromosomal insertion was confirmed by Southern hybridization. The mutant strain was designated DNC2.

Purification of Soluble Cytochromes and Preparation of Membranes. Cells and membranes of *R. sulfidophilum* were prepared as described previously (*7*). The membrane preparations were treated with 0.01% Triton X-100 to exclude the effects of residual soluble cytochromes within closed chromatophores as described previously (*7*). The supernatant after ultracentrifugation of disrupted cells was subjected to ammonium sulfate precipitation. The fraction precipitated between 40% and 100% saturation of ammonium sulfate was collected, dialyzed against 5 mM Tris-HCl (pH 8.0), and applied to a DEAE-Sepharose Fast Flow (Pharmacia) column equilibrated with the same buffer. A stepwise gradient of sodium chloride was applied for elution. Cytochromes were eluted with 150−250 mM sodium chloride. The cytochrome samples were further purified by preparative electrophoresis. The buffer system was essentially according to Davis (*16*). Two collared bands were observed. These bands were excised separately and crushed in loosely fitted homogenizers. Phosphate buffer (10 mM) (pH 7.8) was added to the homogenizer to suspend the crushed gels. The cytochromes were eluted from the crushed gels placed on glass filters fitted with paper filters by washing. The two purified cytochromes were run on SDS−PAGE gels (18%). The *c*-type cytochromes were stained for their heme peroxidase activity using

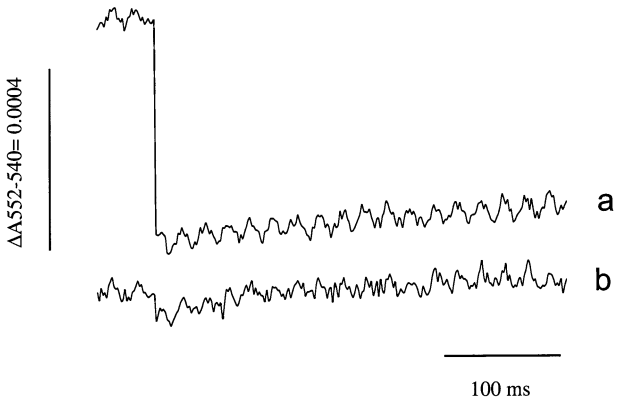

FIGURE 2: Flash-induced absorbance changes in purified membrane fragments isolated from the *R. sulfidophilum* wild type (a) and PUFC1 mutant (b). Membrane preparations free of soluble components were suspended with 5 mM Tris-HCl (pH 7.8) supplemented with 0.1% Triton X-100, 0.5 mM ascorbate, and 20 μM DAD.

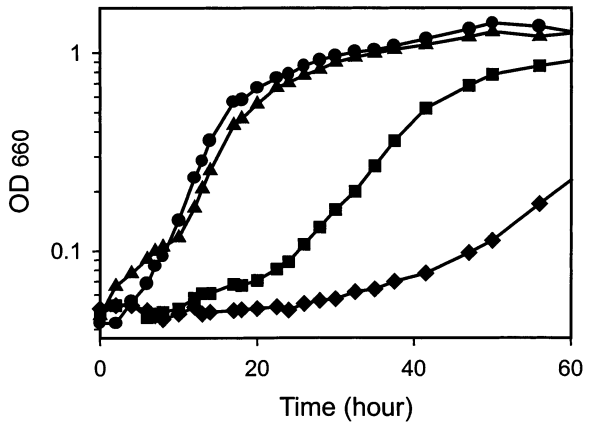

FIGURE 3: Growth profiles of wild type (circles), PUFC1 (squares), C21 (triangles), and DNC2 (diamonds) of *R. sulfidophilum* under photosynthetic conditions (50 W/m^2). The measurements were started by inoculation of $^1/_{30}$th volume of late log phase cells grown aerobically.

3,3′,5,5′-tetramethylbenzidine (TMBZ) and H_2O_2 by the method of Thomas et al. (*17*).

Spectral and Protein Analysis. Optical densities and absorption spectra were measured with a UV-160 or UV-3000 spectrometer (Shimazu). Protein content was determined with a Bradford assay kit (Bio-Rad Laboratories).

Flash-Induced Absorbance Change Measurements. The flash-induced absorbance changes were recorded with a single-beam spectophotometer (*18*). In each measurement the concentration of membranes was adjusted to $A_{855} = 2.0$.

RESULTS

Construction and Phenotypes of Mutants Lacking the RC-Bound Cytochrome and Cytochrome c_2. The *R. sulfidophilum pufC* gene encoding the RC-bound cytochrome subunit was interrupted by exchanging a 0.4 kb fragment containing *pufC* sequences with a Sm^r cartridge (Figure 1), and the mutant strain was named PUFC1. Figure 2 shows the flash-induced oxidation kinetics of *c*-type cytochromes in the Triton X-100-treated membrane preparations of wild type and PUFC1. By using the detergent during preparations and measurements, the effects of soluble cytochromes enclosed and attached to the chromatophore membranes became negligible (*7*). In the wild type, fast photooxidation of cytochromes was observed as an absorbance decrease at 552−540 nm, suggesting the oxidation of the RC-bound cytochrome subunit. On the other hand, no photooxidation of cytochromes was observed in the kinetics of PUFC1 membranes, indicating that the RC-bound cytochrome subunit was absent in the PUFC1 strain.

To identify the photosynthetic electron-transfer components in *R. sulfidophilum*, the cytochrome c_2 gene (*cycA*) was deleted in the wild-type and PUFC1 strains and named C21 and DNC2, respectively (see Figure 1 and Materials and Methods). Figure 3 shows the photosynthetic growth curves of PUFC1, C21, DNC2, and wild-type strains. PUFC1, lacking the RC-bound cytochrome subunit, was able to grow photosynthetically, although its doubling time (6.8 h) was longer than that of the wild type (3.3 h). This indicates that the cytochrome subunit is not essential for photosynthetic growth but is definitely significant for the photosynthetic cyclic electron transfer in growing cells. On the other hand, there was no significant difference in photosynthetic growth between the wild type and C21 lacking cytochrome c_2. This suggests that cytochrome c_2, encoded by the *cycA* gene, does not have a major role in the photosynthetic electron transfer in the wild type of *R. sulfidophilum*. However, the DNC2 strain showed very slow growth (doubling time of about 11.5 h), indicating that cytochrome c_2 is the major electron donor to the photosynthetic reaction center complex mutationally devoid of the cytochrome subunit. The growth rate of the DNC2 was not changed after re-inoculation in fresh medium after 4 days of growth, indicating no suppressor effect in the mutant during the photosynthetic growth. When grown under aerobic dark conditions, the doubling times of all the mutants and that of the wild-type strain were identical (about 2.5 h) (data not shown).

Purification of Soluble Cytochromes from the Cytochrome c_2 Mutant. To elucidate soluble components in the photosynthetic electron-transfer system of *R. sulfidophilum*, the soluble fractions after the disruption of cells of the wild type and C21 grown photosynthetically were examined by redox-difference absorbance spectra. The changes in the α-peaks of *c*-type cytochromes were monitored after reduction by ascorbate as shown in Figure 4A. The spectra obtained were very different between these two strains. In the wild type, a sharp α-peak was observed at 551 nm. This peak seems to be due to cytochrome c_2 because the α-peak was absent in the spectrum of C21. Although the content of soluble cytochromes in C21 was largely decreased, compared to that of the wild type, significant amounts of *c*-type cytochromes seemed to be still present, and the spectrum showed that the α-peak wavelength is different from the major peak in the spectrum of the wild type. These results indicated that *c*-type cytochromes other than cytochrome c_2 are present in *R. sulfidophilum*. These cytochromes may be functionally active to reduce the RC-bound cytochrome subunit because C21 had the ability to grow photosynthetically at the same rate as the wild type (Figure 3).

The soluble fractions from the wild type and C21 were separately adsorbed to DEAE-cellulose columns and eluted with a gradient of NaCl. Pink bands were eluted between fractions from number 4 to number 6, which corresponded to the elution with 150−250 mM NaCl. Panels B and C of Figure 4 show the absorption spectra of these fractions reduced by ascorbate from wild type and C21, respectively.

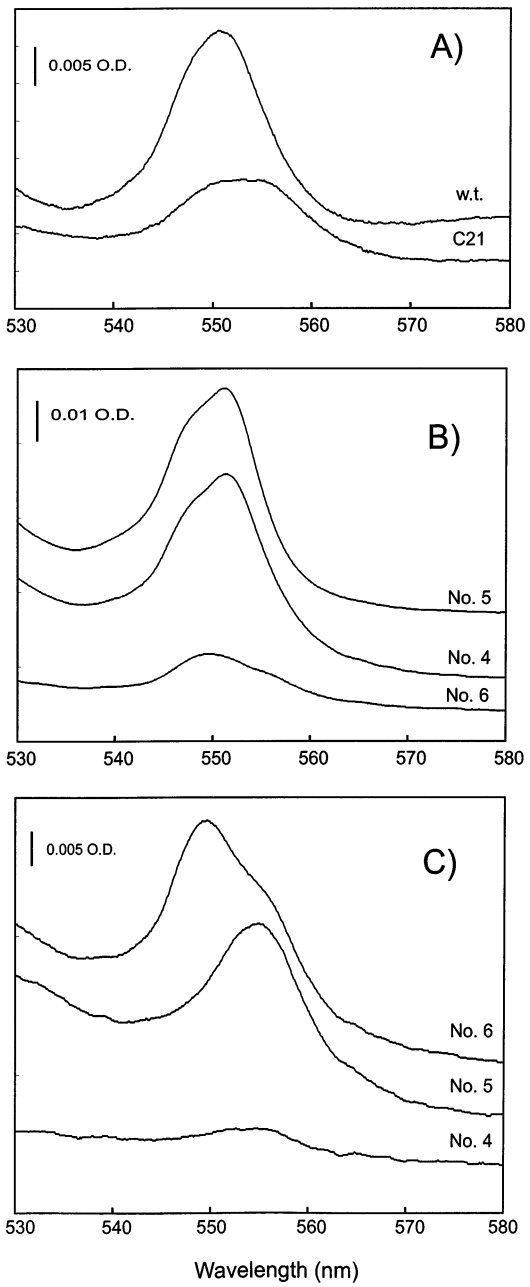

FIGURE 4: Detection and purification of soluble cytochromes from the *R. sulfidophilum* wild type and C21 mutant. (A) Ascorbate-reduced minus ferricyanide-oxidized difference spectra of chromatophore supernatants obtained from the wild type (wt) and from C21. The protein concentrations for the soluble extracts analyzed were adjusted to 2 mg/mL. (B and C) Ascorbate-reduced spectra of elution samples from the DEAE column containing cytochromes from the wild type and C21 mutant, respectively. Numbers 4, 5, and 6 indicate the order of the collected elution samples (see text). Conditions for absorption of the supernatants to the DEAE column and elution of cytochromes by sodium chloride were exactly the same in experiments B and C.

In the wild type, α-peaks of *c*-type cytochromes were observed at around 549 and 551 nm in fractions 4 and 5 and at around 549 nm in fraction 6 (Figure 4B). A small shoulder could be observed at around 556 nm in fraction 6 (Figure 4B), indicating that three cytochromes with peaks at 549, 551, and 556 nm seemed to be present in the wild type. In the C21 strain, an α-peak was observed at around 556 nm in fractions 4 and 5 and at around 549 and 556 nm in fraction 6 (Figure 4C). Two soluble cytochromes, *c*-549 and *c*-556,

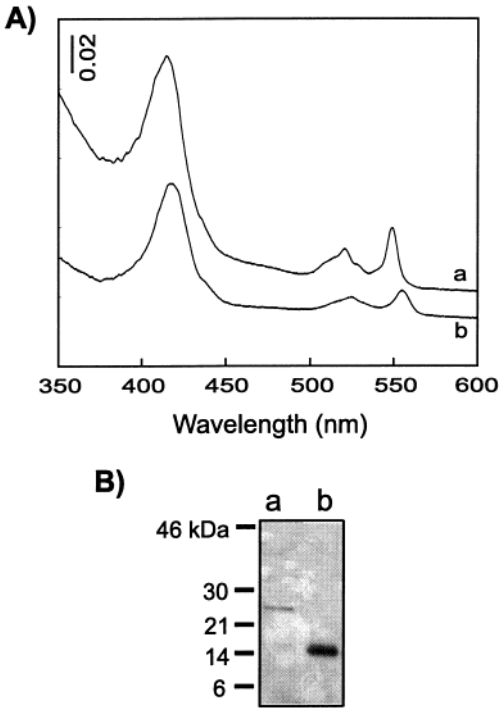

FIGURE 5: Properties of soluble *c*-type cytochromes isolated from the C21 mutant. (A) Ascorbate-reduced absorption spectra of two cytochromes purified from C21. The α-peaks of the cytochromes were 549 (a) and 556 nm (b). (B) SDS−PAGE and heme staining of cytochromes *c*-549 (a) and *c*-556 (b) purified from strain C21. Molecular mass markers are in kilodaltons.

were still present in C21. On the other hand, the α-peak at 551 nm was absent in C21, indicating that this peak was derived from cytochrome c_2.

The two newly identified cytochromes were further purified from the eluted samples of C21 by preparative electrophoresis. Figure 5A shows the absorption spectra of the purified cytochromes from C21. The α-band peak wavelengths of the reduced forms were at 549 nm (a) and 556 nm (b). The redox midpoint potentials of the newly isolated cytochromes *c*-549 and *c*-556 were +238 and +73 mV, respectively, based on redox titrations in the α-band region (data not shown). On SDS gel electrophoresis, cytochromes *c*-549 and *c*-556 appeared to be about 25 and 14 kDa, respectively (Figure 5B).

Photosynthetic Electron Transport via a Newly Identified Cytochrome, c-549. The purified cytochromes, *c*-549 and *c*-556, were added separately to membrane fractions of *R. sulfidophilum* to measure the electron transfer from soluble cytochromes to the reaction center in the membranes. Figure 6 shows the light-induced changes of the spectra recorded before and after the addition of *c*-549. The spectra at 1 ms after the flash (Figure 6A) showed a peak at around 554 nm due to the photooxidation of the RC-bound cytochrome both in the absence and in the presence of *c*-549. On the other hand, the spectrum at 100 ms after the flash in the presence of *c*-549 showed a shift of the peak wavelength to around 548 nm (Figure 6B, squares), although that in the absence of the cytochrome showed no such shift. This peak shift corresponded to the oxidation of *c*-549, indicating that the cytochrome is able to reduce the photooxidized RC-bound cytochrome subunit. The second-order rate constant of the reaction was calculated to be 2.3×10^7 M^{-1} s^{-1}. At that

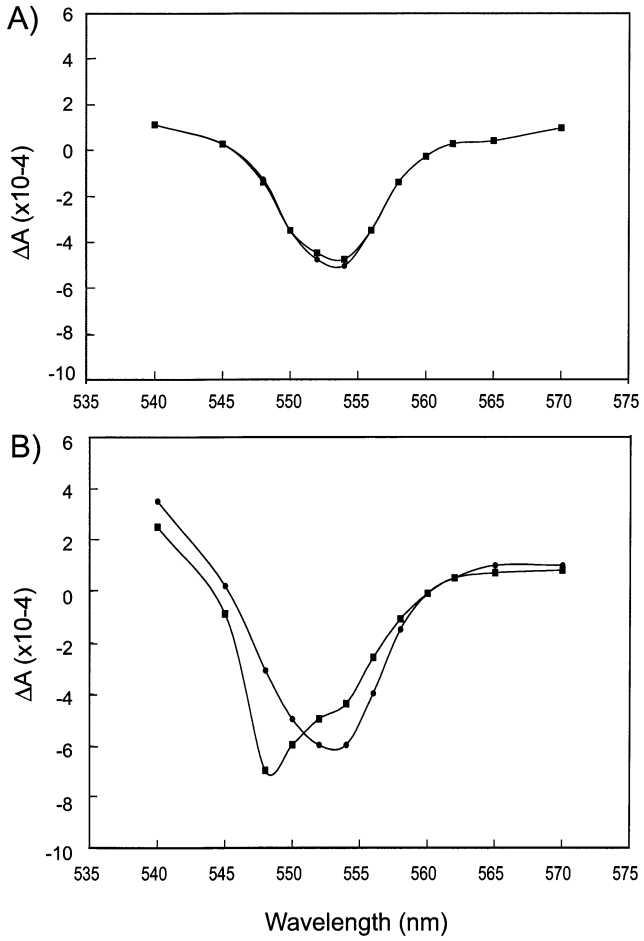

FIGURE 6: Electron transfer mediated by cytochrome *c*-549 in membrane preparations of the *R. sulfidophilum* wild-type strain. Flash-induced absorbance change spectra of membrane preparations of *R. sulfidophilum* in the absence (A) and in the presence (B) of 1 μM cytochrome *c*-549 are shown. Spectra at 1 ms (circles) and 100 ms (squares) after the actinic flash are shown. The mixtures were supplemented with 5 mM Tris-HCl (pH 7.8) supplemented with 0.1% Triton X-100, 0.5 mM ascorbate, and 20 μM DAD.

condition, cytochrome c_2 was shown to reduce the RC-bound cytochrome subunit with a rate constant of 3.5×10^6 M^{-1} s^{-1} (data not shown), indicating that *c*-549 is actually a better electron donor to the RC-bound cytochrome subunit than cytochrome c_2.

On the other hand, cytochrome *c*-556 is not a good electron donor to the RC-bound cytochrome subunit. Since both *c*-556 and the RC-bound cytochrome subunit absorb at nearly the same wavelength, it is almost impossible to detect an electron transfer between these two cytochromes by spectral analysis of light-induced absorption changes as shown for cytochrome *c*-549 (Figure 6). We, instead, measured the multiple flash-induced absorption changes of the membrane preparations in the absence and presence of soluble cytochromes. Typically, we excited the membrane preparations with eight successive flashes of 20 ms intervals. The absorption changes of cytochromes are almost identical between the presence and absence of *c*-556, whereas the absorbance decrease was observed after each flash in the presence of *c*-549 (data not shown). These observations suggest that there is no apparent electron transfer from *c*-556 to the RC-bound cytochrome subunit within times of milliseconds.

Reduction of the Photooxidized RC Lacking the Cytochrome Subunit. The photosynthetic electron transfer from the soluble cytochromes to the RC lacking the cytochrome subunit was also measured in the prepared membrane. The purified cytochromes were added to membrane fractions of the *R. sulfidophilum* mutant lacking the RC-bound cytochrome subunit (PUFC1). Figure 7 shows the light-induced changes of the spectra recorded at 20 ms after the flash in the absence and presence of 5 μM cytochrome c_2 of *R. sulfidophilum.* The spectrum in the presence of cytochrome c_2 (squares) showed a peak at around 551 nm due to the photooxidation of cytochrome c_2, while that in the absence of cytochrome c_2 (circles) did not show any peaks. The difference of the spectra confirmed the electron-transfer reactions from cytochrome c_2 to the RC complex mutationally devoid of the bound cytochrome subunit. On the other hand, the spectra in the presence of the same concentration of *c*-549 and/or *c*-556 did not show any peaks, indicating no photooxidation of these soluble cytochromes by the membrane fractions in the time range (data not shown). These results suggest that cytochrome c_2 is the major electron donor to the RC mutationally devoid of the cytochrome subunit. This agrees with the growth properties (Figure 3) showing that the strain lacking both the cytochrome subunit and cytochrome c_2 resulted in the very slow rate of the photosynthetic growth.

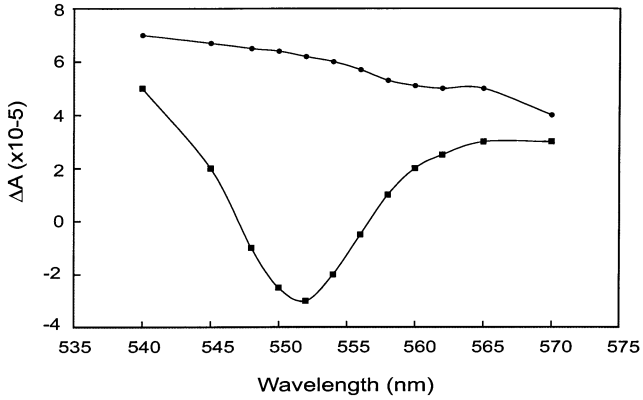

FIGURE 7: Spectra of flash-induced changes in the membrane fragment isolated from the PUFC1 mutant of *R. sulfidophilum*. Absorbance changes recorded 20 ms after the flash in the absence of soluble electron donors (circles) and after addition of 5 μM cytochrome c_2 (squares) were plotted versus wavelength. The mixtures were supplemented with 5 mM Tris-HCl (pH 7.8) supplemented with 0.1% Triton X-100, 0.5 mM ascorbate, and 20 μM DAD.

DISCUSSION

We previously reported a unique cytochrome subunit bound to the RC in *R. sulfidophilum* in terms that it contains only three heme-binding motifs instead of the usual four (*7*). To elucidate the photosynthetic electron transfer through this subunit, three mutants of *R. sulfidophilum* were constructed and characterized: one lacking the RC-bound cytochrome subunit, another one lacking cytochrome c_2, and another one lacking both of these. The PUFC1 strain lacking the RC-bound cytochrome subunit grew twice as slowly as the wild-type strain under anaerobic light conditions (Figure 3), indicating that the loss of the cytochrome subunit is disadvantageous but not absolutely required for the photosynthetic growth in *R. sulfidophilum.* This growth inhibition may be caused by a lack of rapid rereduction of the photooxidized special pair by the bound cytochrome subunit

(Figure 2). The similar phenotypic effect caused by the mutation on the RC-bound cytochrome subunit was reported in another purple bacterium, *Rubrivivax gelatinosus*, which has a usual RC-bound tetraheme cytochrome subunit (*19*). These results directly show that the RC-bound cytochrome subunit containing only three hemes still mediates the photosynthetic electron transfer in this bacterium.

In our recent study, cytochrome c_2 was shown to be able to donate electrons to the RC complex in vitro in *R. sulfidophilum* (*8*). However, the mutational loss of cytochrome c_2 does not have any effect on the photosynthetic growth (Figure 3), indicating that cytochrome c_2 is not a dominant electron donor to the RC-bound cytochrome subunit in this bacterium. Two soluble cytochromes, *c*-549 and *c*-556, were identified in the cytochrome c_2 mutant (Figure 5A), and one of the cytochromes, *c*-549, reduced the photooxidized RC-bound cytochrome subunit with a higher second-order rate constant (2.3×10^7 M^{-1} s^{-1}) (Figure 6) than cytochrome c_2 did (3.5×10^6 M^{-1} s^{-1}) (data not shown). These results suggest that *c*-549 is an actual electron-transfer component in the photosynthetic electron-transfer system of *R. sulfidophilum*.

In the phylogenetically related purple bacterium *Rhodobacter capsulatus*, a mutant lacking cytochrome c_2 could also grow photosynthetically (*20*). Genetic and biochemical studies have revealed that the membrane-bound cytochrome c_y mediates electrons from the bc_1 complex to the RC (*21*). We thought that the cytochrome c_y homologue might also be present in *R. sulfidophilum* and mediate photosynthetic electron transfer in the cytochrome c_2 mutant. However, rereduction of the photooxidized RC-bound cytochrome subunit was not observed in the membrane preparations of the wild-type cells (Figure 2a), indicating the absence of an active cytochrome c_y homologue in *R. sulfidophilum*.

The newly identified cytochrome *c*-549 was unique in the sense that the size of this protein is much larger than that of other soluble electron donors to the RC in purple bacteria studied so far (Figure 5B) (*8*). Our recent study showed that the low-potential heme-1 most distantly located from the special pair in the RC-bound tetraheme subunit works as a direct electron acceptor from the soluble electron carriers (*6*). However, this is not the case in *R. sulfidophilum* since heme-1 is missing in this bacterium (*7*). Therefore, an alternative docking site for soluble electron carrier proteins should be present in the RC-bound triheme cytochrome subunit, and the alternative docking site may be suitable for an alternative electron donor, possibly an unusual cytochrome *c*-549. Further characterization of cytochrome *c*-549 is in progress to clarify its functional role as the electron donor to the RC-bound cytochrome in *R. sulfidophilum*.

Mutational loss of cytochrome c_2 from the PUFC1 strain lacking the RC-bound cytochrome subunit resulted in a significant reduction in its ability to grow photosynthetically, although the C21 mutant lacking only cytochrome c_2 did not show any effects concerning the photosynthetic growth (Figure 3). In vitro experiments of photosynthetic electron transfer revealed that the excited membranes of PUFC1 lacking the RC-bound cytochrome were able to oxidize cytochrome c_2 (Figure 7). These results indicate that cytochrome c_2 is the dominant electron donor to the RC complex mutationally devoid of the cytochrome subunit in *R. sulfidophilum*. This situation is similar to that of the closely related *Rhodobacter* species which lacks the cytochrome subunit even in the wild type. In these organisms, cytochrome c_2 (and an isocytochrome c_2 in *R. sphaeroides*) is the sole soluble electron donor to the RC complexes (*1*−*3*).

Three classes of cytochrome c_2 (*c*-551) are known in purple bacteria. Each class is named S (short), M (medium), or L (long) depending on the number of chain insertions (*22*, *23*). Interestingly, RC complexes in species that have the L-type cytochrome c_2 tend to lack a cytochrome subunit (e.g., *Rhodobacter* species), suggesting that the L-type cytochrome c_2 of purple bacteria has evolved from the S-type to have insertions (*1*, *22*−*24*), which may have advantages for an electron donor to the RC complexes lacking the cytochrome subunit. The alignment of amino acid sequences of cytochrome c_2 from several purple bacteria revealed that cytochrome c_2 of *R. sulfidophilum* is an L-type (*8*), suggesting that it could not be a good electron donor to the RC-bound cytochrome subunit. Previous studies with the *Rhodobacter* species have shown that cytochrome c_2 is also involved in other electron-transfer pathways than the cyclic electron transfer during photosynthesis (*1*−*3*). It may be that the major role of *R. sulfidophilum* cytochrome c_2 is not to donate electrons to the RC-bound cytochrome subunit containing only three hemes but to donate them to other electron-transfer components involved in aerobic and anaerobic respiratory pathways. In this sense, cytochrome c_2 is also suggested to be involved in sulfur metabolism during photosynthesis, since this bacterium can grow photolithotrophically with electron donors such as sulfide and dimethyl sulfide as electron donors (*25*−*27*). Further studies concerning the photosynthetic electron-transfer components in *R. sulfidophilum* may allow us to understand the physiological meaning of the presence of the RC-bound cytochrome subunit as well as the evolution of photosynthetic and respiratory electron-transfer systems in purple bacteria.

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