High-Potential Iron−Sulfur Protein (HiPIP) Is the Major Electron Donor to the Reaction Center Complex in Photosynthetically Growing Cells of the Purple Bacterium *Rubrivivax gelatinosus*‡

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ABSTRACT: A gene encoding the high-potential iron−sulfur protein (HiPIP) was cloned from the purple photosynthetic bacterium *Rubrivivax gelatinosus*. An insertional disruption of this gene by a kanamycin resistance cartridge resulted in a significant decrease in the growth rate under photosynthetic growth conditions. Flash-induced kinetic measurements showed that the rate of reduction of the photooxidized reaction center is greatly diminished in the mutant depleted in the HiPIP. On the other hand, mutants depleted in the low- and high-potential cytochromes c_8, the two other soluble electron carriers, which have been shown to donate an electron to the reaction center in *Rvi. gelatinosus*, showed growth rates similar to those of the wild type under both photosynthetic and respiratory growth conditions. It was concluded that HiPIP is the major physiological electron donor to the reaction center in *Rvi. gelatinosus* cells grown under photosynthetic conditions.

The reaction center complex of purple photosynthetic bacteria is a membrane protein converting light energy to electrochemical energy. The light energy captured by light-harvesting pigments is transferred to the special pair of bacteriochlorophylls in the reaction center complex. The excited special pair, localized near the periplasmic side of the membrane, releases an electron, which is transferred to the primary quinone acceptor, Q_A, via a bacteriopheophytin and finally to the secondary quinone, Q_B, near the cytoplasmic face of the membrane. This electron transfer process across the membrane generates an electrochemical potential utilized for ATP formation. In many species of photosynthetic purple bacteria, the photooxidized special pair is rapidly rereduced by the reaction center-bound cytochrome subunit. This cytochrome is bound to the L and M core subunits of the reaction center at the periplasmic side and contains four *c*-type hemes aligned perpendicularly to the membrane plane (*1*). In species belonging to the α-subclass of purple bacteria, the photooxidized hemes in the cytochrome subunit have been known to be reduced by cytochrome c_2, a soluble electron carrier localized in the periplasmic space (*2*). In species such as *Rubrivivax gelatinosus*, *Rhodoferax fermentans*, *Rhodocyclus tenuis*, and *Allochromatium vinosum*, high-potential iron sulfur protein, HiPIP, has been suggested to be a good electron donor to the reaction center (*3*−*7*).

In *Rvi. gelatinosus*, HiPIP forms a transient complex with the cytochrome subunit at the region surrounding the low-potential heme located at the most distal position from the special pair (*8*, *9*). This bacterium contains two other soluble electron carriers possibly reducing the reaction center, i.e., cytochromes c_8 with low- and high-midpoint potentials (*10*). The low-potential cytochrome c_8 is mainly synthesized under anaerobic conditions (*10*). Reconstitution experiments with purified membranes of *Rvi. gelatinosus* showed that the low-potential cytochrome c_8 could work as an electron donor to the reaction center (*4*). The biosynthesis of the high-potential cytochrome c_8 is highly enhanced under aerobic conditions (*10*). Flash-induced kinetic measurements suggested that this high-potential cytochrome c_8 is the main electron donor to the reaction center in the *Rvi. gelatinosus* cells grown under aerobic conditions (*10*). *Ach. vinosum*, a purple sulfur bacterium, also synthesizes a cytochrome c_8 with a relatively high midpoint potential. Recently, it was also shown in *Ach. vinosum* that growth conditions can affect whether HiPIP or cytochrome c_8 functions as the main electron donor to the reaction center (*7*). The physiological contributions of these electron carriers in growing cells, however, are still uncertain since the mutants suitable for such analyses have not been available. The difficulties entailed in the direct measurements of the redox changes of the HiPIP, due to its featureless spectral characteristic, further impede the clarification.

In this study, the relative contributions of the three electron carrier proteins, HiPIP, low-potential cytochrome c_8, and high-potential cytochrome c_8, to the photosynthetic electron transfer were examined by constructing gene-disrupted mutants of the respective soluble electron carriers.

‡ The sequence data appeared in this work have been submitted to the DDBJ/EMBL/GenBank databases under accession number AB088202.

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[1] Abbreviations: HiPIP, high-potential iron−sulfur protein; TNBT, tri-*n*-butyltin.

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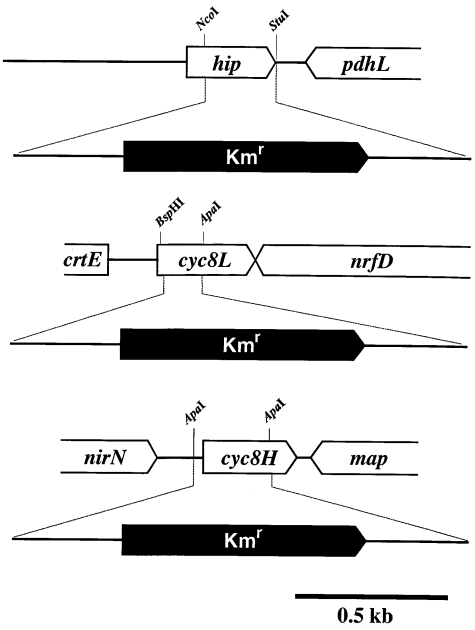

FIGURE 1: Construction of mutants of *Rvi. gelatinosus* deleted in genes coding for high-potential cytochrome c_8 (*cyc8H*), low-potential cytochrome c_8 (*cyc8L*), and HiPIP (*hip*). Genes are indicated by open arrows.

MATERIALS AND METHODS

Cloning of the HiPIP Gene. The gene coding for HiPIP (*hip*) was first screened from a cosmid (SuperCos1, Stratagene, La Jolla, CA) library for the *Rvi. gelatinosus* strain IL144 genomic DNA previously made (*11*). An oligonucleotide with a sequence of 5′-AAGGC(A or T)GA(A or G)-AAGGC(A or T)AAGTA(T or C)AAGCA(A or G)TTCGT-3′ designed from the known amino acid sequence of the purified HiPIP from *Rvi. gelatinosus* strain ATCC17011 (*12*) was labeled by digoxygenin-dUTP with a DIG oligonucleotide tailing kit (Boehringer-Mannheim GmbH, Germany) and used as a probe for colony hybridization. A positive colony was identified with a DIG nucleic acid detection kit (Boehringer-Mannheim GmbH, Germany). The purified cosmid was named pGHI1, digested by restriction enzymes *Pst*I and *Sma*I, and subcloned into pUC119 plasmids. Screening for plasmids containing the *hip* gene was carried out by the same method as mentioned above. The insert of the positive clone obtained was sequenced and confirmed to have an open reading frame coding for the amino acid sequence of the HiPIP. This clone was named pGHiPIP1.

Construction of Mutants. For construction of the mutant lacking HiPIP, most of the *hip* gene was replaced by a kanamycin-resistance gene derived from Tn5 (*13*) at the *Nco*I and *Stu*I restriction sites in the pGHiPIP1, as shown in Figure 1. For construction of the mutants lacking the low-potential and high-potential cytochromes c_8, the DNA fragments containing the genes coding for these cytochromes, *cyc8L* and *cyc8H*, which have already been cloned from *Rvi. gelatinosus* IL144 genome (*10*, *11*), were transferred into pUC plasmids and used as the parent plasmids pGLC1P and pGHC1P, respectively. In each of these plasmids, most of the part containing the region coding for the heme-binding site was replaced by a kanamycin-resistance gene at the *Bsp*HI and *Apa*I sites in the pGLC1P and at the *Apa*I sites in pGHC1P, respectively. These plasmids were individually introduced into the *Rvi. gelatinosus* IL144 cells by electroporation, according to the method described previously (*14*). The cells harboring the kanamycin-resistance gene on the genome by a homologous recombination were concentrated by growth in a PYS medium (*14*) containing 50 μg/mL kanamycin and plated on a solid medium with the same constitution. The colonies on the plate were transferred to a solid PYS medium containing 50 μg/mL ampicillin to test the presence or absence of the ampicillin-resistance gene derived from the pUC plasmid. When the strains showing kanamycin resistance and ampicillin sensitivity were selected, we expected that the *hip*, *cyc8L*, or *cyc8H* gene had been replaced by the kanamycin-resistance gene through a double-crossover recombination. These strains were further tested by genomic Southern hybridization against probes of the ampicillin-resistance gene, the kanamycin-resistance gene, and the DNA fragment containing the *hip*, *cyc8L*, or *cyc8H* gene. PCR with primers with sequences of the outside region from the insert DNA in pGHiPIP1, pGLC1P, or pGHC1P and nucleotide sequencing of the amplified DNA fragment were carried out for the confirmation of the genome construction in the strains obtained. Strains ΔHiPIP, ΔLPC, and ΔHPC were thus obtained as the mutants lacking the HiPIP, low-potential cytochrome c_8, and high-potential cytochrome c_8, respectively.

RESULTS

The 1.4 kb DNA fragment containing a gene coding for HiPIP (*hip*) was cloned from the *Rvi. gelatinosus* genome. The *Rvi. gelatinosus hip* gene coded for a polypeptide of 101 amino acid residues. Twenty-six of those residues from the N-terminus were assumed to be a signal peptide since they had not been found in a previous report on the amino acid sequence obtained from the purified protein (*10*). Southern hybridization experiments with the *hip* gene as a probe showed that no homologues to the *hip* gene are present in the *Rvi. gelatinosus* genome. The direct downstream region of the *Rvi. gelatinosus hip* gene was shared by an ORF that showed a 77% sequence identity to the 3′ region of the *Alcaligenes eutrophus pdh* gene coding for the dihydrolipoamide dehydrogenase. We did not find any other ORFs showing significant identities to the known genes in the 1.4 kb DNA fragment.

The genes coding for the two cytochromes c_8, *cyc8L*, and *cyc8H*, have already been cloned from the *Rvi. gelatinosus* genome (*10*, *11*). As shown in Figure 1, these genes and the *hip* gene were disrupted by a replacement by a kanamycin resistance gene. All three of the mutants obtained, ΔLPC, ΔHPC, and ΔHiPIP, showed nearly the same growth rates as the wild type under aerobic dark conditions (data not shown). Under anaerobic photosynthetic conditions, however, the growth rate of the mutant defective in the synthesis of the HiPIP (ΔHiPIP) was reduced to less than a half that of the wild type (Figure 2). Growth rates under photosynthetic conditions are not affected in mutants defective in the synthesis of the high- and low-potential cytochromes c_8 (Figure 2).

Figure 3 shows flash-induced kinetics of the reaction center-bound cytochrome in whole cells of the wild type and the ΔHiPIP mutant grown under photosynthetic conditions. The cells were placed under anaerobic conditions and

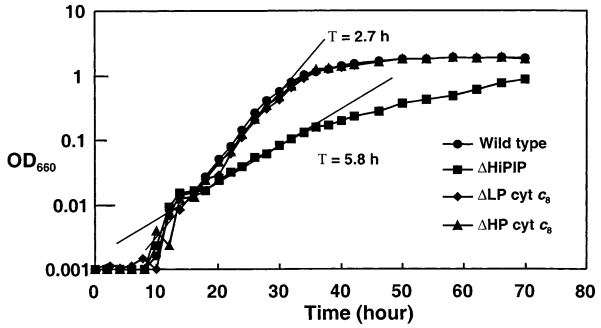

FIGURE 2: Growth curves of *Rvi. gelatinosus* wild type and mutants lacking soluble electron carriers. The measurements were started by inoculation of 1/1000 volume of late log-phase cells grown aerobically in a PYS medium. Cultures were grown at 30 °C. Cultivation was performed in screw-capped tubes filled with the medium under a light supplied by a 60 W tungsten lamp placed 20 cm from the culture. The absorbance at 660 nm was measured in the tube with a diameter of 18 mm. The average of three out of five independent measurements, omitting the minimum and the maximum, was plotted against the time.

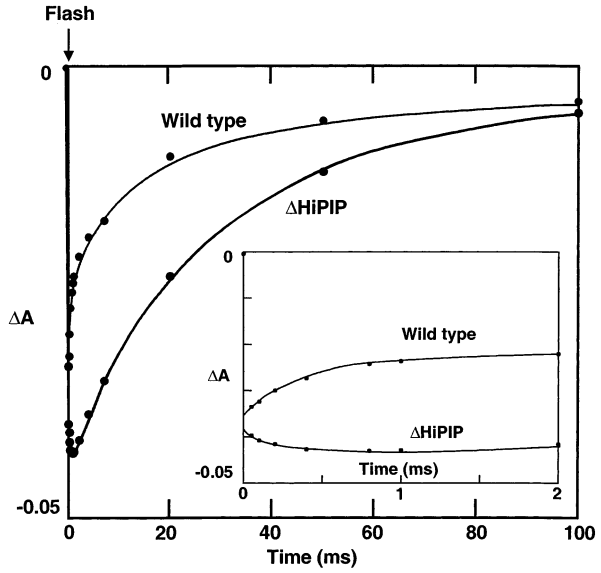

FIGURE 3: Flash-induced kinetics of RC-bound cytochrome rereduction in intact cells of the wild type and the ΔHiPIP mutant. Absorption changes were measured at 427 nm. The measurements were performed under anaerobiosis, applying a weak continuous light in order to photooxidize the low-potential hemes in the RC-bound cytochrome subunit.

subjected to a weak background illumination so that the low-potential hemes in the reaction center-bound cytochrome would be photooxidized prior to the flash activation (*10*). The kinetic trace obtained from the wild-type cells was multiphasic, consistent with the results in a previous report according to which it can be decomposed into three exponentials with halftimes of 240 μs, 7 ms, and 50 ms (*10*). The fastest phase was completely absent in the kinetics for the ΔHiPIP mutant cells. The absence of the fastest phase in the ΔHiPIP mutant was apparent also in the flash-induced spectra around the Soret-band region of the cytochromes (Figure 4), in which no changes took place between 50 μs and 7 ms after the flash activation, although a decrease of the absorption band was apparent in the same measurements in the wild-type cells (Figure 4). The fastest phase was thus assigned as the rereduction of the reaction center-bound cytochrome by HiPIP. The contribution of the fastest phase to the reaction center-bound cytochrome rereduction in the wild-type cells was estimated to be approximately 50%.

Although the rate of rereduction of the photooxidized reaction center in the ΔHiPIP mutant was highly diminished, the mutant cells still retained the ability for photosynthetic growth. This could only be explained if an efficient photo-induced cyclic electron transfer were occurring in this mutant, which implies that soluble electron donors to the reaction center other than the HIPIP are present in the ΔHiPIP mutant. To characterize the nature of electron transfer in the ΔHiPIP cells, the flash-induced carotenoid band shift, which is an electrochromic indicator of the membrane potential, was analyzed. Figure 5 shows the kinetics of the flash-induced carotenoid band shift measured for the cells of the ΔHiPIP mutant. After a rapid absorbance increase caused by the electron transfer in the reaction center, a slow increase of the shift within 100 ms, which is concomitant with the rereduction of the reaction center-bound cytochrome (Figure 5), was observed. When an inhibitor to the electron transfer in the cytochrome bc_1 complex, stigmatellin, was added, this slow electrogenic phase was completely lost (Figure 5, ●). This suggests that the cyclic electron transfer through the cytochrome bc_1 complex is still operative in the ΔHiPIP cell. However, the electron carrier between the reaction center and the cytochrome bc_1 complex in this mutant was not identified by spectroscopic measurements.

The steady-state level of the membrane potential caused by the photosynthetic electron transfer in the ΔHiPIP cell was also measured to estimate the physiological significance of the HiPIP deletion in *Rvi. gelatinosus*. Figure 6 compares the kinetics of the carotenoid bandshift induced by continuous illumination to the cells of the wild type and the ΔHiPIP mutant. The extents of the shift were normalized for the contents of the reaction centers. When the extents of the cytochrome photooxidation after excitation by a saturating flash were equal between the two samples, the extent of the carotenoid band shift was slightly higher for the ΔHiPIP mutant (a factor of 1.08). This slight difference, probably due to a difference in their antenna size, was also taken into account in the normalization. The extent of the shift in the ΔHiPIP cell was about half that of the wild-type cell. In addition, the time required for an establishment of the steady-state potential in the ΔHiPIP cell was rather long. The slower growth of the ΔHiPIP cell under the photosynthetic condition may be ascribed to the slower turnover of the cyclic electron transfer and the insufficient generation of the steady-state membrane potential.

DISCUSSION

The fastest phase in the rereduction kinetics of the photooxidized reaction center in *Rhodocyclus tenuis* ($t_{1/2}$ = 300 μs) and in *Rvi. gelatinosus* ($t_{1/2}$ = 240 μs) cells grown under photosynthetic conditions has been assigned to the electron transfer from the HiPIP to the reaction center (*10*, *15*). This rate of electron transfer is comparable to that observed between the cytochrome c_2 and the reaction center, $t_{1/2}$ = 110 μs, in the cells of *Blastochloris viridis* (*16*). This fast electron-transfer reaction was lost in the mutant cells of *Rvi. gelatinosus* lacking the HiPIP. In addition, the photosynthetic growth rate of this mutant was significantly lower than that of the wild type. On the other hand, the deletion of

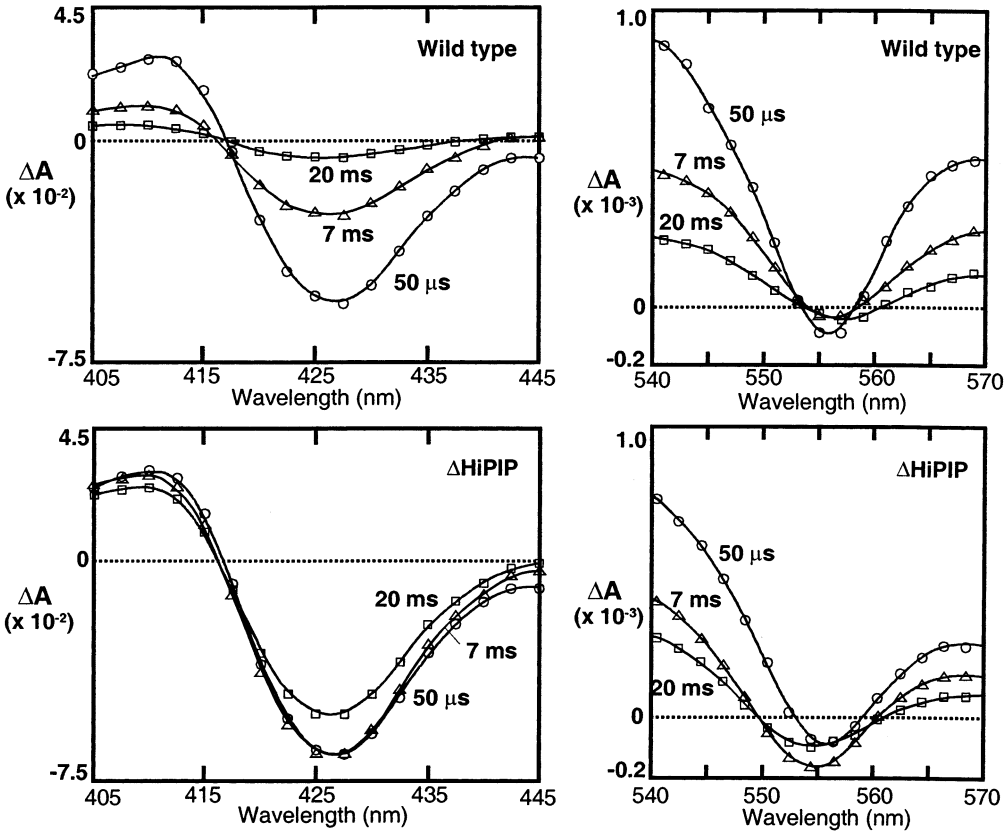

FIGURE 4: Flash-induced spectra detected at 50 μs, 7 ms, and 20 ms after the actinic flash in the α- and γ-band regions for intact cells of the wild type and the ΔHiPIP mutant.

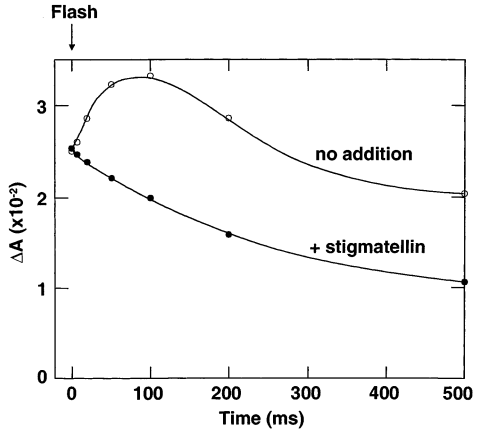

FIGURE 5: Flash-induced carotenoid band shift in intact cells of the ΔHiPIP mutant. Absorption changes measured at 505-minus-480 nm were recorded. The measurements were performed under the presence (●) or absence (O) of 5 μM stigmatellin. TNBT (10 mM) was added to avoid the effect of ATPase.

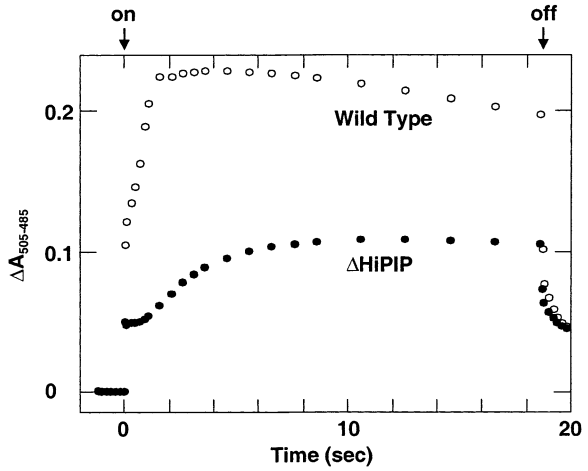

FIGURE 6: Light-induced carotenoid band shift in intact cells of wild type and ΔHiPIP mutant of *Rvi. gelatinosus*. Continuous illumination was applied from 0.005 to 18.6 s. Absorption changes were measured at 505-minus-485 nm. The measurements were performed under anaerobiosis. The two traces were normalized for the reaction center content and the field effects.

the two other possible electron donors to the reaction center, the low-potential and high-potential cytochromes c_8, did not affect the growth rates. We concluded that the HiPIP is the most efficient electron donor to the reaction center in *Rvi. gelatinosus* cells grown photosynthetically.

Rhodobacter sphaeroides cells mutationally devoid of cytochrome c_2 cannot grow photosynthetically, showing that cytochrome c_2 is an essential electron donor to the reaction center in the cells of this species (*17*). However, *Rvi. gelatinosus* cells lacking the HiPIP showed photosynthetic growth, although the rate was greatly reduced to about 50%, indicating that electron carriers other than HiPIP can mediate the electron transfer to the reaction center. The kinetics of generation of the flash-induced membrane potential monitored by the carotenoid band shift in the ΔHiPIP cells of *Rvi. gelatinosus* showed that a slow electrogenic phase concomitant with the rereduction of the reaction center-bound cytochrome is abolished when the inhibitor to the electron transfer in the cytochrome bc_1 complex was added (Figure 5). It is, therefore, likely that the cyclic pathway of photosynthetic electron transfer is maintained in the ΔHiPIP cells of *Rvi. gelatinosus*. The steady-state level of the membrane potential caused by the cyclic electron transfer in the ΔHiPIP cells, however, was significantly lower than

that in the wild-type cells, possibly due to the slower turnover of the electron transfer from the bc_1 complex to the reaction center. This indicates that the rereduction of the reaction center-bound cytochrome is the rate-limiting step of the cyclic photoinduced electron transfer in the ΔHiPIP mutant.

The high-potential cytochrome c_8 may be a possible electron carrier between the reaction center and the cytochrome bc_1 in the ΔHiPIP cells since its midpoint potential of 300 mV (*10*), close to that of the HiPIP, 330 mV (*4*), is in the redox range suitable to oxidize the cytochrome c_1. Indeed, under aerobic conditions, the high-potential cytochrome c_8 has been shown to serve as a main electron donor to the photooxidized reaction center in *Rvi. gelatinosus* cells (*10*). However, we could not detect the spectral changes due to the high-potential cytochrome c_8 after the flash activation in ΔHiPIP cells grown under photosynthetic conditions (Figure 4). This may be due to the low level of synthesis of the high-potential cytochrome c_8 under photosynthetic conditions. It is possible that no significant amount of photooxidized high-potential cytochrome c_8 was accumulated during the electron transfer from the bc_1 complex to the reaction center. A preliminary observation of a mutant devoid of both HiPIP and high-potential cytochrome c_8 was consistent with these interpretations. The growth rate of this double mutant was about half that of the ΔHiPIP mutant under photosynthetic growth conditions although their respiratory growth rates were nearly identical (data not shown). The significant decrease in the growth rate for the double mutant shows that the high-potential cytochrome c_8 acts as an efficient electron donor to the reaction center in the ΔHiPIP mutant. On the other hand, the ability of the double mutant to grow under photosynthetic conditions indicates that another unidentified electron carrier is able to mediate the electron transfer between the reaction center and the bc_1 complex in *Rvi. gelatinosus*.

Our previous study using the purified low-potential cytochrome c_8 and the membrane of *Rvi. gelatinosus* showed that the rate constant of the electron transfer from this soluble electron carrier to the reaction center-bound cytochrome is more than 3.1×10^7 M^{-1} s^{-1}, which is comparable to the value for the electron donation from the HiPIP, 1.1×10^8 M^{-1} s^{-1}. This suggests that the low-potential cytochrome c_8 is potentially a good electron donor to the reaction center (*4*). However, the mutant lacking the low-potential cytochrome c_8 showed almost the same growth rate as the wild type under both respiratory and photosynthetic conditions (Figure 2). The gene coding for this cytochrome c_8 is located between the photosynthesis gene cluster and the unknown genes encoding a putative membrane-bound electron-transfer complex (*11*). The redox midpoint potential of the low-potential cytochrome c_8, 50 mV (*4*), seems to be too low to accept an electron from the cytochrome bc_1 complex (*2*). This low-potential cytochrome might work in an electron-transfer pathway through the putative electron-transfer complex but not through the cytochrome bc_1 complex.

In this study, we provided concrete evidence that the cyclic electron transfer in *Rvi. gelatinosus* grown under photosynthetic conditions is essentially mediated by the HiPIP. However, this electron carrier can be replaced by, at least, the high-potential cytochrome c_8. Detailed analyses of mutants devoid of both HiPIP and other putative electron carriers to define photosynthetic electron-transfer pathways in *Rvi. gelatinosus* are in progress.

REFERENCES

1. Deisenhofer, J., Epp, O., Miki, K., Huber, R., and Michel, H. (1985) *Nature 318*, 618−624.
2. Meyer, T. E., and Donohue, T. J. (1995) in *Anoxygenic Photosynthetic Bacteria* (Blankenship, R. E., Madigan, M. T., and Bauer, C. E., Eds.) pp 725−745, Kluwer Academic Publishers, Dordrecht, The Netherlands.
3. Schoepp, B., Parot, P., Menin, L., Gaillard, J., Richaud, P., and Vermeglio, A. (1995) *Biochemistry 34*, 11736−11742.
4. Osyczka, A., Yoshida, M., Nagashima, K. V. P., Shimada, K., and Matsuura, K. (1997) *Biochim. Biophys. Acta 1321*, 93−97.
5. Hochkoeppler, A., Ciurli, S., Venturoli, G., and Zannoni, D. (1995) *FEBS Lett. 357*, 70−74.
6. Hochkoeppler, A., Zannoni, D., Ciurli, S., Meyer, T. E., Cusanovich, M. A., and Tollin, G. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 6998−7002.
7. Verméglio, A., Li, J., Schoepp-Cothenet, B., Pratt, N., and Knaff, D. B. (2002) *Biochemistry 41*, 8868−8875.
8. Osyczka, A., Nagashima, K. V. P., Shimada, K., and Matsuura, K. (1999) *Biochemistry 38*, 2861−2865.
9. Osyczka, A., Nagashima, K. V. P., Sogabe, S., Miki, K., Shimada, K., and Matsuura, K. (1999) *Biochemistry 38*, 15779−15790.
10. Menin, L., Yoshida, M., Jaquinod, M., Nagashima, K. V. P., Matsuura, K., Parot, P., and Verméglio, A. (1999) *Biochemistry 38*, 15238−15244.
11. Igarashi, N., Harada, J., Nagashima, S., Shimada, K., Matsuura, K., and Nagashima, K. V. P. (2001) *J. Mol. Evol. 52*, 333−341.
12. Tedro, S. M., Meyer, T. E., and Kamen, M. D. (1976) *J. Biol. Chem. 251*, 129−136.
13. Saeki, K. Suetsugu, Y., Tokuda, K., Miyatake, Y., Young, D. A., Marrs, B. L., and Matsubara, H. (1991) *J. Biol. Chem. 266*, 12889−12895.
14. Nagashima, K. V. P., Shimada, K., and Matsuura, K. (1996) *FEBS Lett. 385*, 209−213.
15. Menin, L., Schoepp, B., Parot, P., and Vermeglio, A. (1997) *Biochemistry 36*, 12183−12188.
16. Garcia, D., Richaud, P., and Vermeglio, A. (1993) *Biochim. Biophys. Acta 1144*, 295−301.
17. Donohue, T. J., McEwan, A. G., Van Doren, S., Crofts, A. R., and Kaplan, S. (1988) *Biochemistry 27*, 1918−1925.

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