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The cytochrome c_8 involved in the nitrite reduction pathway acts also as electron donor to the photosynthetic reaction center in *Rubrivivax gelatinosus*☆

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ABSTRACT

The purple photosynthetic bacterium *Rubrivivax gelatinosus* has, at least, four periplasmic electron carriers, i.e., HiPIP, two cytochromes c_8 with low- and high-midpoint potentials, and cytochrome c_4 as electron donors to the photochemical reaction center. The quadruple mutant lacking all four electron carrier proteins showed extremely slow photosynthetic growth. During the long-term cultivation of this mutant under photosynthetic conditions, a suppressor strain recovering the wild-type growth level appeared. In the cells of the suppressor strain, we found significant accumulation of a soluble *c*-type cytochrome that has not been detected in wild-type cells. This cytochrome *c* has a redox midpoint potential of about +280 mV and could function as an electron donor to the photochemical reaction center *in vitro*. The amino acid sequence of this cytochrome *c* was 65% identical to that of the high-potential cytochrome c_8 of this bacterium. The gene for this cytochrome *c* was identified as *nirM* on the basis of its location in the newly identified *nir* operon, which includes a gene coding cytochrome cd_1-type nitrite reductase. Phylogenetic analysis and the well-conserved *nir* operon gene arrangement suggest that the origin of the three cytochromes c_8 in this bacterium is NirM. The two other cytochromes c_8, of high and low potentials, proposed to be generated by gene duplication from NirM, have evolved to function in distinct pathways.

1. Introduction

Photosynthetic proteobacteria (purple bacteria) possess not only photosynthetic ability but also various capabilities for dark metabolism, such as oxygen respiration, fermentation, and denitrification [1]. The initial step of the photosynthetic electron transfer pathway is a light-driven charge separation at the special pair of bacteriochlorophylls within the reaction center (RC) complex. In purple bacteria, this charge separation is followed by a cyclic electron transport between the RC complex and the cytochrome bc_1 (cyt bc_1) complex, which is connected by water-soluble electron carrier proteins in the periplasmic space and membranous quinones. This reaction is coupled with the formation of a transmembrane proton gradient used for NAD^+ reduction and ATP production.

Many species of photosynthetic purple bacteria have the RC-bound tetraheme cytochrome subunit containing two high-potential and two low-potential hemes, which re-reduce the photooxidized special pair. The re-reduction of the RC-bound cytochrome subunit (RC-bound cyt) occurs from a water-soluble *c*-type cytochrome, cytochrome c_2 (cyt c_2) or cytochrome c_8 (cyt c_8), or high-potential iron–sulfur protein (HiPIP) [2,3]. Cytochromes (cyts) c_2 have been found in the α-subclass of proteobacteria, and cyts c_8 and/or HiPIP have been found among β- and γ-proteobacteria [4–6]. The structural and functional properties of cyt c_8 are similar to those of cyt c_2. Some species posses additional electron carriers, such as cytochrome c_y in *Rhodobacter capsulatus* [7] and cytochrome c_{2m} in *Rhodovulum sulfidophilum* [8].

Apart from the function in photosynthesis, cyt c_8 is an ortholog of cytochrome c_{551} (NirM), which is a physiological electron carrier from the cyt bc_1 complex to the cytochrome cd_1-type nitrite reductase (cyt cd_1-Nir) in *Pseudomonas aeruginosa* [9–11]. The cyt cd_1-Nir catalyzes the conversion of nitrite to nitric oxide in the denitrifying pathway of bacteria. The gene for cyt c_{551}, *nirM*, is included in the operon *nirSMCFDLGHJEN* in *P. aeruginosa*, in which *nirS* gene codes for cyt cd_1-Nir and genes *nirCFDLGHJEN* codes for d_1 heme synthesis and assembly of cyt cd_1-Nir [12–14].

Hitherto, we have isolated and characterized a series of electron carrier proteins that can donate electrons to the RC complex in a β-subclass facultative photoheterotrophic bacterium, *Rubrivivax gelatinosus*, resulting in the identification of, at least, four possible donors to the RC: HiPIP, high- and low-midpoint potential cyts c_8 (HP cyt c_8 and LP cyt c_8), and cytochrome c_4 (cyt c_4) [15–19]. In these studies, we have demonstrated that HiPIP and LP cyt c_8 are predominantly expressed under

Abbreviations: HiPIP, high-potential iron–sulfur protein; cyt, cytochrome; RC, reaction center; cyt cd_1-Nir, cytochrome cd_1-type nitrite reductase; TMBZ, 3,3′5,5′-tetramethylbenzidine; DAD, diaminodurene (2,3,5,6-tetramethylphenylene diamine); MOPS, 3-morpholinopropanesulfonic acid; E_m, redox midpoint potential

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photosynthetic growth conditions and HiPIP is the major physiological electron donor to the RC, while LP cyt c_8 is expressed at low levels and may not function in the cyclic electron transport chain because its mid-point potential (+30 mV) is too low to accept an electron from the cyt bc_1 complex [17–19]. The HP cyt c_8, which is mainly expressed under aerobic growth conditions, has consequently been shown to be the main electron donor to the RC complex under these conditions [16,17]. The diheme cyt c_4 can maintain the photosynthetic cyclic electron transport pathway when the genes for HiPIP and cyts c_8 are knocked out [19]. Interestingly, a quadruple mutant lacking HiPIP, HP and LP cyts c_8, and cyt c_4 (ΔIHL4) was still viable under photosynthetic growth conditions, although its growth rate was very slow, indicating that other minor electron carriers should be working in *R. gelatinosus*.

In the present study, we obtained a suppressor strain recovering the wild-type growth level (ΔIHL4-*spd*) by successive cultivation of the ΔIHL4 strain. In this strain, a large accumulation of an unknown *c*-type cytochrome at the level sufficient to account for photosynthetic growth was observed. From the sequence comparison, this cytochrome was assigned as a cyt c_8. Its gene is located in the well-conserved *nir* genes cluster and was therefore denoted NirM. Based on the phylogenetic analysis, its original function and the phylogenetic history in this bacterium are discussed.

2. Materials and methods

2.1. Bacterial strains and growth conditions

R. gelatinosus strain IL144 [20] and its variants were grown photosynthetically or aerobically in a PYS medium [21]. *Escherichia coli* JM109 was grown aerobically with a Luria-Bertani medium or an SOB medium at 37 °C to propagate plasmids or cosmids [22]. When needed, ampicillin was added to *Escherichia coli* at a final concentration of 50 μg/ml. The growth measurements were carried out as described previously [17,19]. Cultures were started with the addition of the dark aerobically grown preculture to a fresh PYS medium with 1/100 volume, and cells were grown at 30 °C. Illumination for photosynthetic growth was supplied by 60-W tungsten lamp.

2.2. Kinetic specrophotometry

Light-induced absorption changes were measured with laboratory-built spectrophotometers for intact cells [23] and membranes [24] as described previously. Membranes were suspended in a 1 mM MOPS buffer (pH 7.0) in the presence of 10 μM DAD, 1 mM sodium ascorbate, and 0.015% Triton X-100. The concentrations of whole cells and membrane samples were adjusted to give an absorbance of 1 at 875 nm (approximately 0.1 μM RC). The purified electron carriers were added at a final concentration of 1 μM. Myxothiazol was used at 1 μM.

2.3. Preparation of membranes and soluble electron donors

The membranes and the soluble fraction from cells of the *R. gelatinosus* wild-type and ΔIHL4-*spd* mutant strain grown under photosynthetic conditions were prepared as described previously [15]. The soluble redox proteins were partially purified from the soluble fraction by ammonium sulfate precipitation followed by cation-exchange chromatography (CM Sepharose Fast Flow, GE Healthcare) with a linear NaCl gradient in the 2 mM MOPS–NaOH buffer (pH 6.8). Chromatography was carried out with a chromatograph system BioChromatograph II (ATTO, Japan). Each fraction was dialyzed against 2 mM MOPS–NaOH pH 7.0 and purified by gel filtration on a Sephadex S-100 column (GE Healthcare) with the same buffer. The final purity of each protein was checked by SDS-PAGE.

2.4. Redox titration

The redox midpoint potential of the soluble cytochrome *c* was determined as described previously [19,25] using a double beam spectrophotometer UV-3000 (Shimadzu, Japan). The buffer for the measurements contained 20 mM MOPS–KOH (pH 7.0). The redox mediators used are as follows: 100 μM potassium ferrocyanide, 10 μM DAD, 1 mM Fe–EDTA, 10 μM phenazine methosulfate, 10 μM vitamin K_3. Oxygen was eliminated by a continuous stream of nitrogen gas. The redox poise in the titration cuvette (E_h) was changed oxidatively by addition of ferricyanide and reductively by dithionite.

2.5. SDS-polyacrylamide gel electrophoresis

SDS-PAGE was carried out according to Laemmli [26] with 18% polyacrylamide gel. Whole-cell lysate from *R. gelatinosus* cells were prepared as described previously [8]. Purified soluble proteins were denatured in a loading solution containing 2% SDS at boiling temperature for 2 min. Heme staining was performed by the method of Thomas et al. [27]. The protein content was determined with a Protein Assay Kit (Bio-Rad) using bovine serum albumin (fraction V) as a standard protein.

2.6. Cloning and mutagenesis of the gene encoding the soluble cytochrome c

A soluble cytochrome *c* polypeptide separated by SDS-PAGE was transferred to a polyvinylidene difluoride (PVDF) membrane (ATTO, Japan). The N-terminal amino acid sequence of the protein on the membrane was determined by the method of Edman degradation through a commercial service (Midwest Analytical Inc., St. Louis, MO). A DNA fragment containing a part of the gene coding for the novel cytochrome *c* was amplified by polymerase chain reaction (PCR) using the primers GISOHPC8F (5′-GCSATCACSAAGGCSGGCTGCAA-3′), designed on the basis of the determined N-terminal amino acid sequence, and GISOHPC8-250R (5′-ATCGGGATCGGGCCCCAGACGCC-3′), designed on the basis of the conserved sequences of cyts c_8 genes of various purple bacteria. The PCR product was labeled by digoxigenin using a DIG-High Prime kit (GE Healthcare) and was used as a probe for screening of the cosmid library of *R. gelatinosus* IL144 genomic DNA [28]. A positive clone was detected and denominated pGHiC8cos. The purified pGHiC8cos cosmid DNA was sequenced using specific primers designed with Primer3Plus software [29], and the gene for a novel cytochrome *c* was named *nirM* as described in Results section. Nucleotide sequence data were analyzed by a software package of DNASIS (HitachiSoft, Japan). The quintuple mutant ΔIHL4M, which was deleted the main part of the *nirM* gene of ΔIHL4 strain, was constructed as described previously [19]. An approximately 1.4 kb DNA PCR fragment containing *nirM* was amplified using primers nirS1277F-Xba (5'-TTTTTCTAGACACCGT-CATCGACGCCAAGG-3') and nirF251R-Kpn (5'-GCATGGTACCGGCC-GAAGACGTAGGCGTAGC-3'), and cloned in pUC118 plasmid. A region containing the sequence corresponding to the heme-binding motif was removed by digestion using *Eco*T14I endonuclease and self-ligation after end-filling to cause a frame shift in the backward sequence. The manipulated insert DNA was transferred to a suicide vector pJPCm [19]. A DNA cassette 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 plasmid obtained called pJP-ΔnirM-KmSac was introduced into the cells of *R. gelatinosus* ΔIHL4M strain by conjugal transfer from *E. coli* S17-1 λpir host cells. Cells growing on PYS-agar plates containing kanamycin and tetracycline, in which the plasmid was incorporated into *R. gelatinosus* genomic DNAs via a single cross-over homologous recombination, were selected. After sequential cultivation, sucrose-resistant but kanamycin-sensitive cells were selected on PYS-agar plates containing 15% sucrose to generate ΔIHL4M mutant. The lack of the gene was

confirmed by PCR and southern hybridization using a DNA fragment containing *nirM* gene.

2.7. In vivo measurement of nitrite reduction under photosynthetic conditions

Cells were grown to late-log phase (ca. 10^8 cells/1 ml) in PYS medium in 30-ml culture tubes under the photosynthetic conditions. The nitrite reduction reaction was initiated by the addition of $NaNO_2$ to the culture tubes at a final concentration of 50 μM. One-half milliliter of the growing culture was taken every 20 min and centrifuged to precipitate the cells. Nitrite in the supernatant was detected and quantified by the chromogenic reaction with sulfanilamide and *N*-[1-naphthyl]-ethylene-diamine dihydrochloride using a NITRITE/NITRATE colorimetric method kit (Roche Diagnostics GmbH, Manheim, Germany).

3. Results

3.1. Physiological properties of the suppressor strain

R. gelatinosus ΔIHL4 mutant lacking four major electron donors to the RC complex, i.e., HiPIP, HP and LP cyts c_8, and cyt c_4, showed about 10-fold lower photosynthetic growth rate than the wild type [19]. This observation suggests a minor synthesis of unknown electron donor(s) to the RC complex. In the present study, we noticed that prolonged cultivation of the mutant over 150 h under photosynthetic conditions often causes the generation of cells recovering a rapid photosynthetic growth. One such strain was selected on an agar plate and named ΔIHL4-*spd* (Fig. 1). Generation of revertants is often explained by the recovery of genes once knocked out. However, we can exclude this possibility since the functional parts of the four genes coding the donor proteins were not inactivated by simple insertions of antibiotics-resistant cassettes but completely removed from the genome. The other explanation is that the ΔIHL4-*spd* cell has developed a significant upregulation of synthesis of an electron carrier that is synthesized in low amount in the wild type but in a much larger amount in the revertant. This enhanced synthesis compensates for the lack of other known carriers. Such situation has already been observed in the case of *Rhodobacter sphaeroides* where the deletion of cyt c_2, the sole electron donor to the RC, is overcome by the synthesis of an isocyt c_2 in a revertant [30–32].

3.2. Flash-induced absorption changes in intact cells

To identify the physiological electron donor to the RC in the ΔIHL4-*spd* cells, flash-induced spectral changes were measured using intact cells grown under photosynthetic conditions (Fig. 2A). Photooxidation of the HP heme in the RC-bound cyt was observed immediately after the flash activation as a downward change. The minimum of this absorption change was then slightly shifted toward the shorter wavelength region, probably due to the re-reduction of the photooxidized RC-bound cyt by another *c*-type cytochrome. When the cells grown under the photosynthetic conditions are used for the measurements, α-absorption bands of cytochromes *c* were significantly distorted by spectral overlap with the electrochromic band shift of carotenoids in response to the membrane potential generated by the photosynthetic electron transfer as observed in Fig. 2A. Measurements using cells grown under dark semi-aerobic conditions, in which the carotenoid composition is drastically changed from that in the photosynthetically grown cells, showed a clear spectral shift (556 to 554 nm) possibly due to the electron transfer from unknown soluble cytochromes *c* to the RC-bound cyt. In this measurement, the absorption changes were less affected by spectral overlapping with a carotenoid band shift (Fig. 2B). Relative content of the cytochrome *c* possibly working as the electron donor to the RC was estimated at approximately 0.7 per RC based on these kinetic measurements and analyses on total cytochromes *c* photooxidized by a continuous light (data not shown).

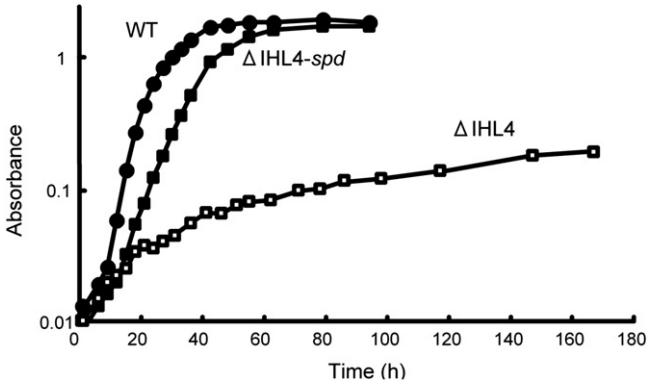

Fig. 1. Photosynthetic growth curves of *R. gelatinosus* wild-type and mutant strains. ΔIHL4 corresponds to the quadruple mutant lacking all four proteins, i.e., HiPIP, HP cyt c_8, LP cyt c_8, and cytochrome c_4. Light was supplied at an intensity of 5 μmol/m^2/s by placing the culture tubes 15 to 25 cm apart from 60-W tungsten lamp. ΔIHL4-*spd* refers to a spontaneous suppressor strain in ΔIHL4 that recovered the wild-type growth rate.

3.3. Purification and characterization of the cytochromes c

C-type cytochromes synthesis in cells of the wild-type and ΔIHL4-*spd* strains grown under photosynthetic conditions were analyzed by SDS-PAGE followed by heme-specific staining. As shown in Fig. 3, bands with apparent molecular masses of around 46, 30, 17, and 12 kDa were detected in the wild-type cell sample and assigned to the RC-bound cyt, cytochrome c_1 of the cyt bc_1 complex, cyt c_4, and cytochrome *c′* (cyt *c′*), respectively [19,33]. The HP and LP cyts c_8 with molecular masses of around 10 kDa [16] were not clearly resolved under the experimental conditions used in this study. The content of cyts c_8 was known to be significantly low (0.2–0.5 per RC) in the wild-type cells grown under the photosynthetic conditions [16]. In ΔIHL4-*spd* cells, the band of cyt c_4 disappeared but three novel bands are detected at around 60, 36, and 10 kDa. When the absorption spectrum of the soluble fraction prepared from the disrupted cells of ΔIHL4-*spd* was measured, accumulation of cytochromes *c* with an α-band peak at 552 nm was observed (data not shown). It was suspected that this cytochrome corresponds to, at least, one of the three cytochromes *c* newly detected in the SDS-PAGE analysis and could be the electron carrier participating in the photosynthetic electron transfer in ΔIHL4-*spd* cells.

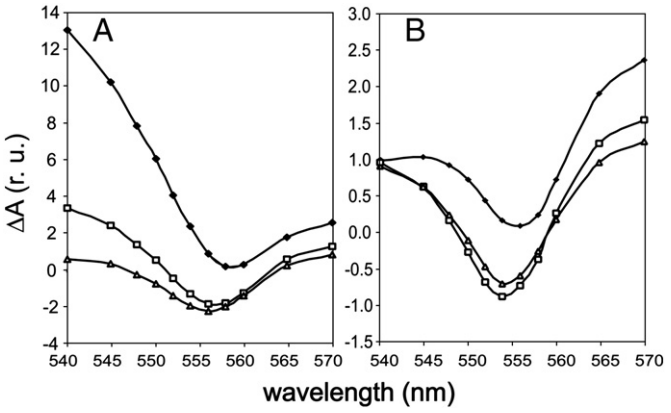

Fig. 2. Flash-induced spectral changes in the α-band region of cytochromes in intact cells of the ΔIHL4-*spd* mutant grown under photosynthetic conditions (A) and aerobic dark conditions (B). The absorption changes were sampled at 100 μs (*filled diamond*), 11 ms (*open square*), and 40 ms (*open triangle*) after the actinic flash. Note that the absorption change of photosynthetically grown cells in the 540- to 570-nm regions is overlapped by the carotenoid bandshift. *r.u.*, Relative units.

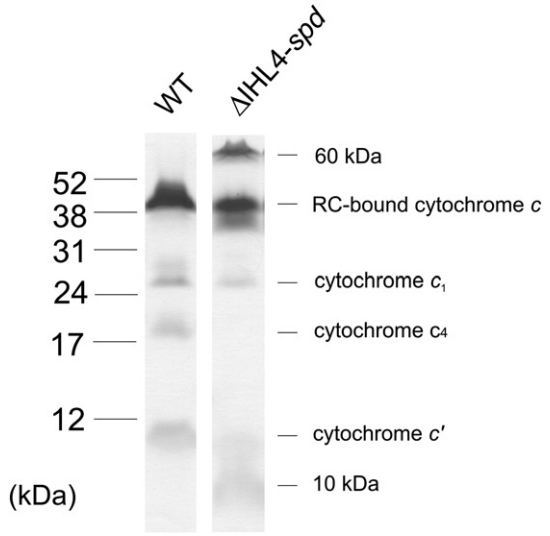

Fig. 3. SDS-PAGE analysis of cytochromes *c* in photosynthetically grown cells of *R. gelatinosus* wild-type (*left*) and ΔIHL4-*spd* mutant (*right*). The gel was stained for hemes by TMBZ/H_2O_2.

The *c*-type cytochromes were then partially purified from the soluble fraction of the ΔIHL4-*spd* cells by ammonium sulfate precipitation and cation-exchange column chromatography. Two fractions with reddish colors were eluted by 15 and 100 mM NaCl and further purified by gel filtration. As shown in Fig. 4A, these fractions contained a 10-kDa cytochrome *c* (10-kDa cyt *c*) and cyt *c*′, respectively. The absorption spectrum of the 10-kDa cyt *c* showed an α-band that peaked at 552 nm in the reduced state (Fig. 4B). The redox midpoint potential (E_m value) of this cytochrome was estimated to be +280 mV (Fig. 4C), i.e., only 20–50 mV lower than those of the HP cyt c_8 and HiPIP [16]. The 60-kDa cytochrome (Fig. 3) fractionated by a CM-fast flow ion-exchange column as a brown-colored band had a peak around 630 nm in addition to approximately 553 nm peak, which is characteristic of cyt cd_1-Nir (data not shown), but was very unstable and therefore difficult to purify. The 36-kDa band of cytochrome was also detected in the wild type depending on the sample conditions and was not further analyzed in this study.

3.4. Electron transfer from 10-kDa cyt c to the RC complex

Flash-induced absorbance changes of the RC-bound cyt in the purified membrane of *R. gelatinosus* wild type were recorded before and after the addition of the 10-kDa cyt *c*. The measurement was performed under the condition of reducing the high-potential hemes in the RC-bound cyt prior to flash activation by the addition of 1 mM ascorbate. As shown in Fig. 5A, the photooxidized RC-bound cyt was re-reduced upon the addition of the 10-kDa cyt *c*. The effects of the additions of HiPIP and HP cyt c_8 purified from the wild-type cells are also shown for comparison. These results indicate that the 10-kDa cyt *c*, which is less effective than HiPIP and HP cyt c_8, can work as an electron donor to the photooxidized RC-bound cyt.

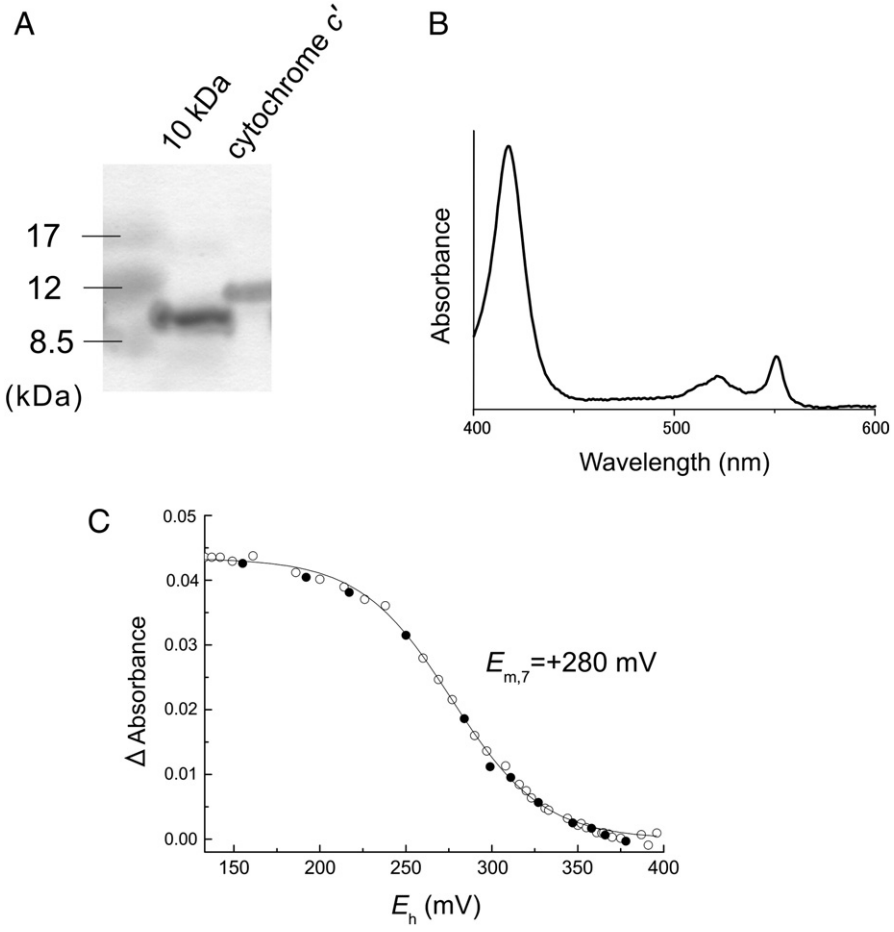

Fig. 4. Properties of the 10-kDa cyt *c* purified from *R. gelatinosus* ΔIHL4-*spd* mutant. (A) SDS-PAGE analysis, (B) measurement of absorption spectrum, and (C) redox titration. (A) Cytochromes *c* were separated by a CM-fast flow ion-exchange column with 15 mM NaCl (10-kDa cytochrome *c*) and with 100 mM NaCl (cytochrome *c*′). The gel was stained for hemes by TMBZ/H_2O_2. Molecular-weight marker proteins were also applied to the leftmost *lane*. (B) The absorption spectrum of 10-kDa cyt *c* was recorded after addition of ascorbate to reduce the cytochrome *c*. (C) The redox poise in the titration cuvette (E_h) was changed oxidatively by addition of ferricyanide (closed circle) and reductively by dithionite (open circle).

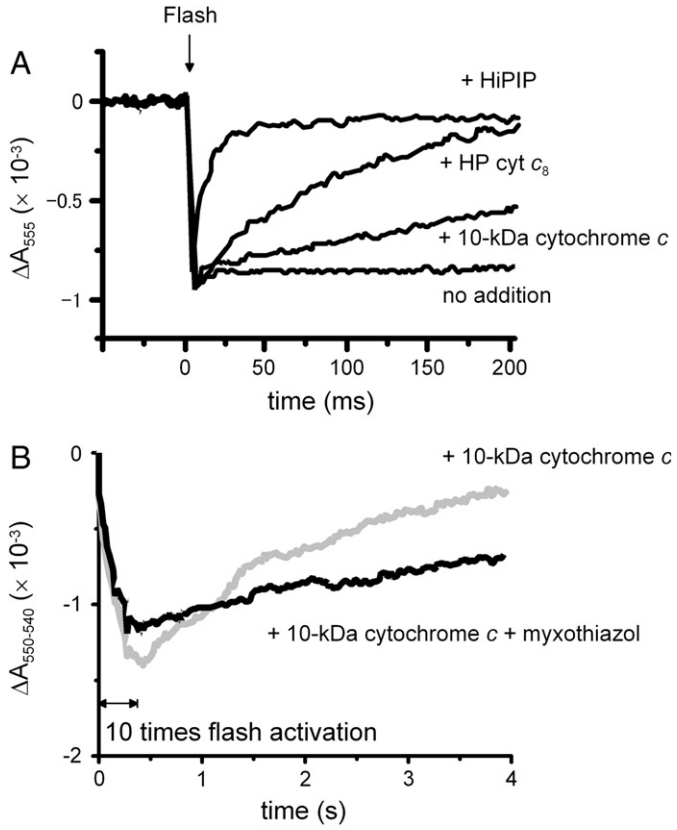

Fig. 5. Single flash-induced absorbance changes at 555 nm of the RC-bound cytochrome in membranes in the presence or absence of HiPIP, HP cyt c_8, or 10-kDa cyt *c* (A) and multiple flash-induced oxidation of the 10-kDa cyt *c* and re-reduction by the cyt bc_1 complex in the presence or absence of 1 μM myxothiazol (B). The membrane from the *R. gelatinosus* wild type was prepared to give an absorbance of 1 at 875 nm in 2 mM MOPS–NaOH (pH 7.0) containing 1 mM sodium ascorbate, 10 μM DAD, and 0.015% Triton X-100. The electron carrier proteins were added at 1 μM.

To examine the participation of the 10-kDa cyt *c* in the cyclic electron transfer *via* the cyt bc_1 complex, the multiple-flash-induced absorbance changes were measured in the presence or absence of myxothiazol, an inhibitor of the cyt bc_1 complex (Fig. 5B). The RC-bound cyt and the 10-kDa cyt *c* were fully oxidized by 10 flashes with 40-ms intervals and then slowly re-reduced. When myxothiazol was added, the re-reduction of cytochromes *c* was significantly slowed down, suggesting that the 10-kDa cyt *c* was re-reduced by the cyt bc_1 complex in the photosynthetic cyclic electron transfer.

3.5. Sequencing of the gene coding the 10-kDa cytochrome c

The amino acid sequence of the N-terminal 15 residues of the purified 10-kDa cyt *c* was determined to be APEDAITKAGCNACH. Based on this sequence, a part of the gene coding the 10-kDa cyt *c* was amplified by PCR and used as a DNA probe to screen the cosmid-based DNA library of the *R. gelatinosus* genome. The nucleotide sequences of the genes included in the selected cosmid were then determined. The amino acid sequence deduced from the gene for 10-kDa cyt *c* showed identities of 65% and 56% to those of the HP and LP cyts c_8, respectively. Residues characteristic among cyts c_8, such as a typical heme attachment sequence motif (–Cys–X–Y–Cys–His–), were conserved. The N-terminal 20 residues in the deduced amino acid sequence, which is lost in the purified protein, correspond to a signal peptide as already observed for the HP cyt c_8 [16]. The molecular mass of 9471.8 Da, calculated taking the signal peptide and binding of a *c*-type heme into account, was almost equal to that of HP cyt c_8 (9472 ± 2 Da). The p*I* value predicted from the amino acid sequence of the mature 10-kDa cyt *c* was 9.62, which was similar to those for the HP and LP cyts c_8, 9.79 and 10.07, respectively [16].

To our surprise, the gene for the 10-kDa cyt *c* was found located in the putative cyt cd_1-Nir gene cluster. The gene for the 10-kDa cyt *c* corresponded to *nirM* when the ten open reading frames (ORFs) were annotated as *nirESMCFDLGHJN* on the basis of their similarities with *nir* genes in other bacteria. The amino acid sequence of the 10-kDa cyt *c* was 37% identical to that of NirM of *P. aeruginosa*, which is the electron donor to cyt cd_1-Nir encoded by the *nirS* gene. Thus, we denote the novel 10-kDa cyt *c* as NirM. The gene arrangement of *nir* genes found in *R. gelatinosus* was almost the same as that in *P. aeruginosa*, except for *nirE* and *nirQ*, both of which are needed for the expression and maturation of cyt cd_1-Nir, respectively. A putative *nirE* gene in *R. gelatinosus* was located in the region upstream of putative *nirS*, with a transcriptional orientation opposite to *nirS*, and no genes corresponding to *nirQ* were found in this cluster. It appears that the 5′ flanking region of *R. gelatinosus nirS* has a potential upstream promoter (TTGAACCAGTTTAA) similar to the FNR box (TTGATNNNNATCAA) conserved in Fnr-dependent promoters [34] and to the DNR box (TTGATNNNNGTCAA) conserved in *nirS* promoters of *P. stutzeri* and *P. aeruginosa* [35]. The putative *nirSMCFDLGHJN* gene cluster found in *R. gelatinosus* possibly forms an operon, as reported in *P. aeruginosa* [12]. We then examined the ability of the ΔIHL4-*spd* cell to reduce nitrite. Fig. 6 shows the time course of nitrite consumption in the growing cultures of the wild-type and ΔIHL4-*spd* cells after the addition of 50 μM $NaNO_2$ under the photosynthetic conditions. Nitrite was significantly consumed in the ΔIHL4-*spd* culture as compared with that in the wild-type culture (Fig. 6). This strongly suggests that *nirM* is overexpressed with other genes included in the *nir* operon in the ΔIHL4-*spd* cells. The gene for HP cyt c_8, *cyc8H*, was positioned immediately after the putative *nirN gene*, as has been already reported in our previous study [16]. A consensus FNR box (TTGACCGGACTCAA) was also found between the *nirN* and *cyc8H* genes, suggesting that *cyc8H* is not included in the putative *nir* operon.

3.6. Mutant lacking NirM

To confirm the physiological function of NirM, quintuple mutant ΔIHL4M lacking this cytochrome was created using the *R. gelatinosus* quadruple mutant ΔIHL4 as the parent. When grown in a PYS medium under aerobic-dark conditions, the ΔIHL4M mutant showed similar growth rates to the wild type (data not shown). However, under the photosynthetic conditions with high-light illumination as shown in Fig. 7, the quintuple mutant ΔIHL4M showed approximately 2 times slower growth than the parental strain, ΔIHL4 mutant. This result

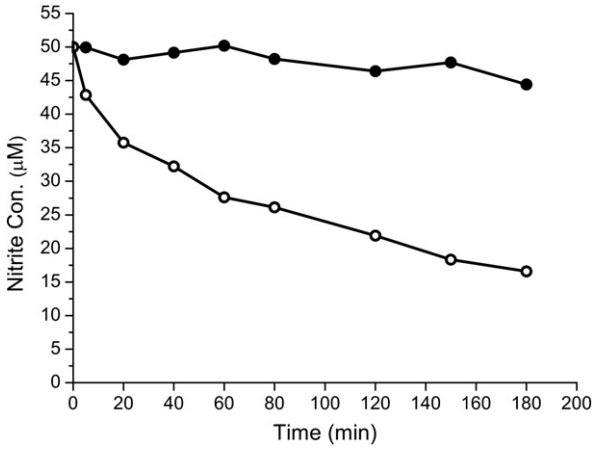

Fig. 6. Nitrite reduction by whole cells of the wild-type and ΔIHL4-*spd* strains under the photosynthetic conditions. Concentrations of nitrite in the cultures of wild type (filled circle) and ΔIHL4-*spd* (opened circle) were plotted against the time. Sodium nitrite was added at a final concentration of 50 μM at time zero.

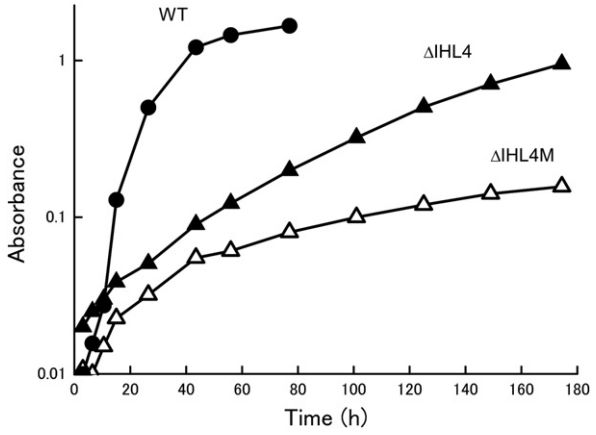

Fig. 7. Photosynthetic growth curves of *R. gelatinosus* wild-type and mutant strains. ΔIHL4M corresponds to the quintuple mutant deleted for NirM protein from quadruple mutant ΔIHL4. An illumination of 10 to 15 μmol/m^2/s was obtained from 60-W tungsten lamp placed at a distance of 10 to 15 cm from the tubes.

suggests that NirM can compensate for the lack of other soluble electron carriers in ΔIHL4 mutant.

4. Discussion

In this study, a suppressor mutant of *R. gelatinosus* recovering from the lack of photosynthetic electron transfer components was obtained and named ΔIHL4-*spd*. This mutant showed wild-type level cell growth under photosynthetic conditions, although the genes for the four known electron carrier proteins – HiPIP, HP and LP cyts c_8, and cyt c_4 – had been deleted (ΔIHL4) [19]. The cells of ΔIHL4-*spd* accumulated a large amount of the monoheme cytochrome *c*, which showed the highest sequence identity to the HP cyt c_8 and to the *P. aeruginosa* NirM. From biochemical and spectral properties and the location of the gene in the well-conserved *nir* gene cluster, we identified this cytochrome as NirM, which functions as an electron donor to cyt cd_1-Nir.

In vitro flash-induced measurements showed that the newly identified *R. gelatinosus* NirM could accept electrons from the cyt bc_1 complex and re-reduce the photooxidized RC-bound cytochrome with a second-order rate constant of 1.6×10^7 M^{-1} s^{-1}. Under the same experimental conditions, the second-order rate constants for the re-reduction of the photooxidized RC-bound cytochrome by HiPIP and HP cyt c_8 were estimated at 3.2×10^8 and 4.8×10^7 M^{-1} s^{-1}, respectively, in agreement with values previously reported [15,19]. We propose that the expression level of *nirM*, which is usually uninduced in the parent strains, has been significantly upregulated in the ΔIHL4-*spd* cells without any effectors like nitrogen oxide. The relatively large amount of NirM enables sufficient electron transfer from the cyt bc_1 to the RC in spite of the low rate constant. The flash-induced spectral shift at the α-absorption band region of cytochromes *c* in intact cells of ΔIHL4-*spd* (Fig. 2) may reflect such an electron transfer. The gene location showed that *nirM* is included in *nir* operon and should be co-expressed with other *nir* genes. In this operon, three genes coding cytochromes *c*, *nirM*, *nirC*, and *nirN*, and a gene coding cytochrome cd_1, *nirS*, are included. We suspect that the two cytochrome bands with apparent molecular masses of around 10 and 60 kDa resolved in the SDS-PAGE analysis for the ΔIHL4-*spd* cells (Fig. 3) resulted from overexpression of these genes. The 10-kDa band could be assigned to the NirM and NirC proteins based on their estimated molecular masses of about 10 and 12 kDa, respectively. Such speculations were strongly supported by the *in vivo* nitrite reduction experiments (Fig. 6), in which the ΔIHL4-*spd* cells showed a significantly high level of nitrite reductase activity. The 60-kDa cytochrome band is ascribable to an overlap of NirN and NirS with the estimated molecular masses of about 58 and 63 kDa, respectively.

Photosynthesis and denitrification are both energy metabolisms that take place under anaerobic conditions. Bacteria possessing both abilities are reported in some strains of *R. sphaeroides* and *Roseobacter* species [36]. The finding of a *nir* gene cluster in *R. gelatinosus* was surprising, since denitrification ability had not been reported in this bacterium. However, the predicted products of these genes, in addition to the fairly conserved gene arrangement in the cluster, showed very high sequence identity (37 to 66%) to those in the non-phototrophic denitrifier, *P. aeruginosa*, suggesting that reduction of nitrite to nitric oxide is possible in *R. gelatinosus* if these genes are expressed. In addition, we found that the *R. gelatinosus* genome contains complete sets of *nor* (NO to N_2O) and *nos* (N_2O to N_2) operons (to be published). However, *nar* and *nap* genes (NO_3^- to NO_2^-) are lacking. This implies that *R. gelatinosus* can reduce NO_2^- to N_2 gas but cannot use NO_3^- as electron donor (to be published). An important observation is that *R. gelatinosus* possesses three different cyts c_8: the newly found cyt c_8; NirM, which has been called *Pseudomonas* cytochrome or cytochrome *c*-551; and the LP and HP cyts c_8. While cyt cd_1-Nir is involved in the denitrification pathway, LP and HP cyts c_8 have been thought to act in the photosynthetic electron transport pathway in *R. gelatinosus*. Phylogenetic analyses on their sequences will provide some insight into the origin and evolution of these small monoheme cytochromes *c*.

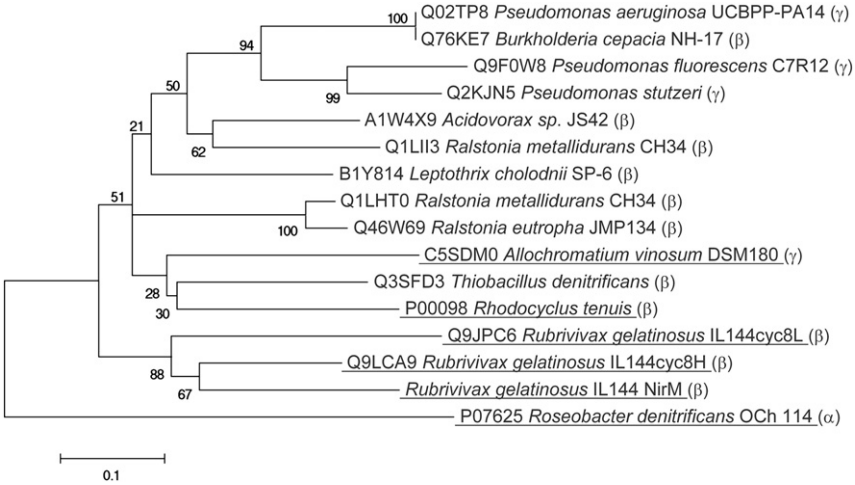

Fig. 8. Phylogenetic tree based on the amino acid sequences of NirM/cyt c_8. The 16 sequences of NirM/cyt c_8 were collected using BLAST analysis. Cytochrome c_2 of *Roseobacter denitrificans* OCh114, which has a cd_1-Nir, was used as an outgroup. The accession numbers of UniProtKB are attached with strain names. The α-, β-, and γ-subgroup of proteobacteria are shown in parentheses. Photosynthetic bacteria are underlined. The obtained bootstrap values are presented at the corresponding nodes. The alignment was carried out using ClustalW [38]. The trees were constructed on the basis of the neighbor-joining method applying the *p*-distance determined in MEGA 4.0 [39].

Fig. 8 shows a phylogenetic tree based on the amino acid sequences of the cytochromes annotated as NirM in DNA/protein databases and cyts c_8 from photosynthetic bacteria. The three cytochromes of *R. gelatinosus* form an exclusive cluster, implying that these duplications are the result of successive gene duplications that occurred rather recently. Since the gene locus in the *nir* operon is almost fixed in β- and γ-proteobacteria and conserved in *R. gelatinosus* (Fig. S1), the most likely interpretation is that *nirM* is the original copy. The phylogenetic tree based on the amino acid sequences of NirS, an essential component in nitrite reductase (cyt cd_1-Nir), and a possible electron acceptor from NirM, is very similar to the phylogenetic tree based on 16 S rRNA (Fig. S1). This suggests that the *nir* operon has evolved in parallel with the differentiation of proteobacteria species. Some species have a potentially duplicated gene of *nirS*, but *R. gelatinosus* does not.

The most recent gene duplication event in the course of the evolution of the three cytochrome genes is assumed to have occurred between *nirM* and *cyc8H*, since these two genes show the highest sequence identity. This speculation is also consistent with the gene location of *cyc8H* directly adjacent to the putative *nir* operon in *R. gelatinosus*. Probably, HP cyt c_8 was generated by a recent duplication of *nirM* and has evolved to increase the rate constant of electron transfer to the RC. The control mechanism for the expression of *cyc8H* might have been changed from that for the *nir* operon, although both include similar promoter sequences.

In the wild-type cells of *R. gelatinosus*, HP cyt c_8 accumulates under aerobic conditions and works as an electron donor to the RC [16]. On the other hand, NirM is not detected in the wild type under photosynthetic conditions and is only observed in the cells of the ΔIHL4-*spd* mutant. This suggests that the *nir* operon including *nirM* is usually repressed in the wild-type cells. Probably, in the ΔIHL4-*spd* mutant, the control mechanism for the expression of *nirM* was changed by an unknown mutation and resulted in overexpression of NirM.

5. Conclusions

In this study, we propose that the origin of the various cyts c_8 in *R. gelatinosus* is NirM. Probably, cyts c_8 of *R. gelatinosus* are paralogs and have been specialized toward photosynthetic electron transfer by tuning their redox potentials and gene expression mechanisms after gene duplication events. The driving force for or selection pressure against these alterations can be correlated with the course of evolution of photosynthesis in this bacterium. Phylogenetic analyses on the RC proteins and pigment biosynthesis enzymes have demonstrated that the photosynthesis gene clusters of β- and γ-proteobacteria, including *R. gelatinosus*, were derived from an ancestral α-proteobacterium through horizontal gene transfer [21,37]. Increased synthesis of a low-affinity but putative electron carrier such as NirM by gene multiplication and upregulation of its gene expression might allow the incorporation of such exogenous photosynthetic function. Then optimization of the structures and functions of the electron carrier proteins has facilitated the photosynthetic electron transfer into the network of the preexisted metabolic pathways. Other electron donors to the RC in *R. gelatinosus*, i.e., HiPIP and cyt c_4, may also have been integrated into photosynthetic electron transfer after the horizontal gene transfer event. Further analyses of the evolution of multiple electron carriers will be useful for a comprehensive mapping of the electron transfer network of photosynthesis in purple photosynthetic bacteria including *R. gelatinosus*.

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