Biochimica et Biophysica Acta 1807 (2011) 803–812

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

A heterogeneous tag-attachment to the homodimeric type 1 photosynthetic reaction center core protein in the green sulfur bacterium *Chlorobaculum tepidum*

Chihiro Azai [a], Kwang Kim [a], Toru Kondo [b], Jiro Harada [c], Shigeru Itoh [b], Hirozo Oh-oka [a,*]

[a] Department of Biological Sciences, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan
[b] Division of Material Science (Physics), Graduate School of Science, Nagoya University, Nagoya 464-8602, Japan
[c] Department of Medical Biochemistry, School of Medicine, Kurume University, Kurume, Fukuoka 830-0011, Japan

ARTICLE INFO

Article history:
Received 24 November 2010
Received in revised form 10 March 2011
Accepted 15 March 2011
Available online 21 March 2011

Keywords:
Green sulfur bacteria
Type 1 reaction center
Histidine tag
Electron paramagnetic resonance
LC/MS/MS
Iron–sulfur cluster

ABSTRACT

The *6xHis-tag-pscA* gene, which was genetically engineered to express N-terminally histidine (His)-tagged PscA, was inserted into a coding region of the *recA* gene in the green sulfur bacterium *Chlorobaculum tepidum* (*C. tepidum*). Although the inactivation of the *recA* gene strongly suppressed a homologous recombination in *C. tepidum* genomic DNA, the mutant grew well under normal photosynthetic conditions. The His-tagged reaction center (RC) complex could be obtained simply by Ni^{2+}-affinity chromatography after detergent solubilization of chlorosome-containing membranes. The complex consisted of three subunits, PscA, PscB, and PscC, in addition to the Fenna–Matthews–Olson protein, but there was no PscD. Low-temperature EPR spectroscopic studies in combination with transient absorption measurements indicated that the complex contained all intrinsic electron transfer cofactors as detected in the wild-type strain. Furthermore, the LC/MS/MS analysis revealed that the core protein consisted of a mixture of a His-/His-tagged PscA homodimer and a non-/His-tagged PscA heterodimer. The development of the *pscA* gene duplication method presented here, thus, enables not only a quick and large-scale preparation of the RC complex from *C. tepidum* but also site-directed mutagenesis experiments on the artificially incorporated *6xHis-tag-pscA* gene itself, since the expression of the authentic PscA/PscA homodimeric RC complex could complement any defect in mutated His-tagged PscA. This method would provide an invaluable tool for structural and functional analyses of the homodimeric type 1 RC complex.

1. Introduction

Green sulfur bacteria are obligatory anaerobic phototrophs that inhabit anoxic and sulfur-rich water, such as bottom sediments or deep layers of a water column, and deep-sea hydrothermal vents in the Pacific Ocean [1]. To survive under extremely low-light intensity, these bacteria have developed large light-harvesting apparatuses attached to the inner membrane surface, called "chlorosomes," which contain a very large amount of bacteriochlorophyll (BChl) *c*, *d*, and *e* molecules [2]. Green sulfur bacteria grow photoautotrophically utilizing reduced sulfur compounds, such as sulfide, elemental sulfur, and/or thiosulfate, as electron sources [3,4]. The solar energy captured by chlorosomes is finally converted to a highly reducing power, reduced ferredoxin, through a series of redox reactions in a homodimeric type 1 reaction center (RC), in which iron–sulfur (Fe–S) clusters serve as terminal electron acceptors, as in the case of photosystem (PS) I RC of oxygenic phototrophs, including cyanobacteria.

The green sulfur bacterial RC complex consists of four subunits, PscA, PscB, PscC, and PscD, and is additionally associated with the BChl *a*-containing Fenna-Matthews-Olson (FMO) protein [5]. It has a much simpler architecture than that of the PS I RC complex, which consists of more than 12 different protein subunits [6]. A pair of PscA makes up a homodimeric core protein that houses most electron transfer (ET) cofactors: P840, a special dimer of BChls *a* as the primary electron donor; A_0, a derivative of Chl *a*, Chl a_{PD}, as the primary electron acceptor; and F_X, a [4Fe–4S] center, as the secondary or tertiary electron acceptor. PscB is a functional homologue of PsaC in PS I, holding two [4Fe–4S] centers that serve as the terminal electron acceptors, F_A and F_B [7]. PscC, which is also called cytochrome (cyt) c_z, is a unique subunit of the green sulfur bacterial RC complex. Two molecules of cyt c_z are tightly bound to the RC complex and serve as the direct electron donor to the photooxidized $P840^+$ [8–10]. The ET rate from cyt c_z to $P840^+$ has unusual dependence on solvent viscosity due to the fluctuation nature of its C-terminal domain [11–13]. The FMO protein has a trimeric form *in vivo* and transfers excitation energy from chlorosomes to the RC core protein [14–16]. PscD is involved in its efficient energy transfer but is not essential for photosynthetic growth [17].

Abbreviations: RC, reaction center; PS, photosystem; cyt, cytochrome; ET, electron transfer; (B)Chl, (bacterio)chlorophyll; FMO, Fenna–Matthews–Olson; β-OG, n-octyl-β-D-glucopyranoside; ESP, Electron spin-polarization

* Corresponding author. Tel.: +81 6 6850 5423; Fax: +81 6 6850 5425.
E-mail address: ohoka@bio.sci.osaka-u.ac.jp (H. Oh-oka).

0005-2728/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2011.03.007

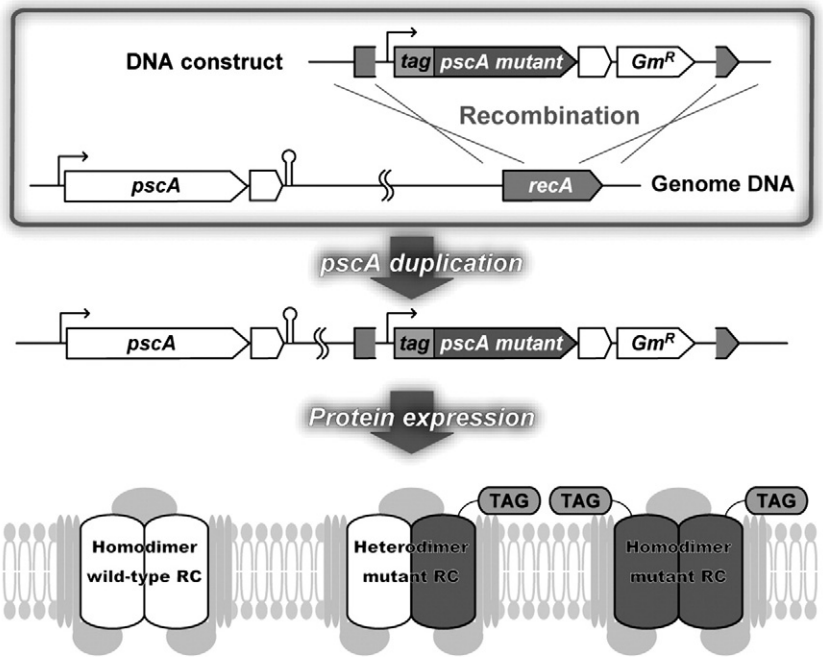

Fig. 1. Conceptual representation for the construction of the mutated PscA in *C. tepidum*. The *recA* gene is inactivated by an insertion of the mutated *tag-pscA* gene together with a selection marker (e.g., a gentamicin resistance cassette). The RC complex with the affinity tag attached to the mutated PscA could be isolated with conventional affinity chromatography. The authentic *pscA* gene is expected to express the wild-type RC complex and would complement any mutant defect in photosynthetic growth.

As to the ET pathway, whether the electron is transferred from A_0 directly to F_X without any involvement of the quinone molecule remains controversial [5,18]. Although menaquinone has been shown to be present in some RC preparations [19–21], no direct spectroscopic evidence for its function as a secondary electron acceptor (A_1) has been obtained [20,22,23]. At present, there is no three-dimensional structure of the green sulfur bacterial RC. The X-ray crystal structures of RCs so far determined, i.e., type 2 RCs and PS I RC, have demonstrated that they share common folding motifs of membrane-spanning α-helices of core proteins as well as the spatial configurations of ET cofactors [24]. Therefore, it would be reasonable to conclude that the structure modeling of the green sulfur bacterial RC, especially its central region, which coordinates ET cofactors, is quite similar to that of PS I RC [18]. However, the possible quinone-binding pocket is very hydrophilic compared to that in PS I RC and seems to loosely interact with a quinone molecule even if present [5,18]. The same situation is also applicable to the case for another homodimeric type 1 RC of heliobacteria [25], although the transient electron paramagnetic resonance (EPR) signal ascribable to the $P800^+A_1^-$ radical pair has recently been observed in it [26]. Moreover, the ET reaction is considered to proceed equivalently in two branches within the homodimeric RC, although the information on its symmetrical ET pathways is very scarce.

A molecular genetic study to modify the RC core protein would be the most promising approach for the elucidation of its detailed molecular constructions and physicochemical properties. Many mutagenesis experiments have already been executed in heterodimeric RCs of purple bacteria as well as oxygenic phototrophs, especially cyanobacteria (reviewed in Refs. [27–29]). Amino acid residues around cofactors were substituted intensively and systematically to explore their energy and ET processes. The recent work on the mutant PS I of a green alga, *Chlamydomonas reinhardtii*, has also revealed the bidirectional ET to quinone molecules in two branches and slightly different ET rates to the next cofactor F_X due to energetically different redox potentials [30,31].

A meso-thermophilic green sulfur bacterium, *Chlorobaculum* (*C.*) *tepidum*, is amenable to a genetic manipulation, and the information of its genome sequences is available [32–34]. However, there has been no practical method to examine amino acid substitution variants of the PscA core protein so far, since any substitution unfavorable for photosynthetic growth would be lethal or could easily be replaced or modified, resulting in the generation of a spontaneous revertant. We, here, propose a new strategy to obtain mutants in the core protein: "the *pscA* gene duplication method." The *6xHis-tag-pscA* gene encoding the PscA core protein with an affinity tag attached to its N-terminus is inserted into the *recA* locus, which causes the disruption of the *recA* gene concomitantly with a duplication of the *pscA* gene (see Fig. 1). This strategy incorporates three different ideas at once.

First, an affinity-tag attachment to the PscA subunit would make it possible to perform a brief and large-scale preparation of the RC complex [35–44]. Second, since the *recA* gene is responsible for homologous DNA recombination and repair [45], its disruption mutant could be a suitable host for the expression of the mutated *6xHis-tag-pscA* gene incorporated into the genome. Finally, the authentic *pscA* gene ensures the expression of the wild-type RC in any mutant. Any mutagenesis on the *6xHis-tag-pscA* gene has no critical influence on photoautotrophic growth. It would be even possible, in principle, to construct an artificial heterodimeric RC core protein consisting of the wild-type PscA and the His-tagged PscA with any given mutation on it (Fig. 1, lower).

In this study, we investigated the availability of this *pscA* gene duplication method without any mutagenesis on the *6xHis-tag-pscA* gene. The His-tagged RC complex was expressed in the *recA*$^-$ mutant that grew photoautotrophically under ordinary cultural conditions. Its spectroscopic and biochemical properties were studied in detail by EPR and LC/MS/MS analyses.

2. Materials and methods

2.1. Strains and growth conditions

The strain WT2321 [46] of *C. tepidum* was used as the wild-type and host strain for transformation. All *C. tepidum* strains were grown anaerobically in liquid CL media or solid CP media [32], basically as previously described [3]. Gentamicin (30 μg ml^{-1}) was added to

media as required to select mutant cells. The temperature for growth was set at 40 °C, and illumination was carried out at a light intensity of approximately 30 μmol photons m^{-2} s^{-1} with incandescent lamps.

Chemically competent *Escherichia coli* DH5α cells were used as a host for routine plasmid constructions. Ampicillin (100 μg ml^{-1}), chloramphenicol (20 μg ml^{-1}), and gentamicin (10 μg ml^{-1}) were used to select *E. coli* cells harboring desired plasmids.

2.2. Plasmid construction for recA gene disruption

Primers used for PCRs in the present study are shown in Table S1. Two DNA fragments of the upstream and downstream flanking regions of the *C. tepidum recA* gene (*CT1930*) were amplified by PCR with recA-4105F/5104R and recA-2203F/3208R primer sets, respectively. They were consecutively cloned into the *Stu*I/*Bam*HI and *Sma*I and *Sph*I sites of pKF3 (TaKaRa Bio Inc.), yielding plasmid pKF3-ΔrecA. A 0.8 kbp fragment containing a gentamicin resistance gene, the *aacC1* gene, which was excised from pUCGM [47] with *Sac*II followed by blunting and *Bam*HI digestion, was inserted between the *Bam*HI and *Sma*I sites of pKF3-ΔrecA, yielding plasmid pKF3-ΔrecA::aacC1. After excision of a 2.8 kbp fragment from pKF3-ΔrecA::aacC1 with *Stu*I, it was cloned into the *Sma*I site of pHP45 [48], yielding plasmid pHP45-ΔrecA::aacC1. A sufficient amount of pHP45-ΔrecA::aacC1 prepared from a large-scale culture was linearized with *Ahd*I, which was used for transformation to obtain the *recA*- mutant.

2.3. DNA construct for His-tagged PscA expression

To attach a His-tag to the N-terminus of PscA, a 3.1 kbp DNA fragment containing a *pscAB* gene cluster was amplified by PCR using a primer set of pscB-948F and pscA-4070BR and cloned into *Bam*HI and *Blp*I sites of pET15b (Novagen) in a proper reading frame, yielding plasmid pHisAB15b. This clone was obtained at an extraordinarily low frequency; only one colony harbored pHisAB15b when more than 1000 colonies resistant to ampicillin were checked. However, any further attempt to clone a full-length *C. tepidum pscA* gene into the multi-cloning site of conventional cloning vectors (e.g., pUC and pET) was unsuccessful, probably because PscA was potentially toxic to *E. coli*. Therefore, all plasmid constructions involving the *pscA* gene were made with caution so that their transcription level could be suppressed as much as possible hereafter.

A fragment amplified with pscB-1081F and pscA-4435R, which included a putative promoter region as well as the *pscAB* genes, was cloned into the blunt-ended *Bam*HI site of pHP45, yielding plasmid pHP45-AB. Using pHP45-AB as a template, a 5.7 kbp fragment was amplified by PCR with pscA-4072F and pscA-4070R. After digestion with *Nco*I and *Bgl*II, the fragment was ligated with a 0.3 kbp *Nco*I-*Bgl*II fragment of pHisAB15b, yielding plasmid pHP45-HisAB. A 3.5 kbp *Sma*I fragment of pHP45-HisAB was inserted into the *Sma*I site of pHP45-ΔrecA::aacC1 so that the *6xHis-tag-pscAB* gene cluster and the *aacC1* gene were transcribed in the same direction. However, all plasmids, as far as we checked, had a nonsense mutation somewhere within the coding region of *pscA*. The megaprimer PCR method [49] was thus introduced in order to design a correct DNA construct for the His-tagged PscA expression concomitantly with the *recA* inactivation. Using plasmid pHP45-ΔrecA::HisA*B/aacC1 (A* represents the *pscA* gene with any nonsense mutation), two megaprimers were amplified by PCR with the primer sets of pscA-4095F/HP45-blaF for the 5′-flanking region of *pscA* and pscB-1410R/HP45-ropR for the 3′-flanking region of *pscB*. A double megaprimer PCR was carried out in a reaction mixture that contained two gel-purified megaprimers, primers recA-2203F and recA-5104R, and pHP45-HisAB. After the gel purification of a 6.2 kbp PCR product, its entire DNA sequence was confirmed to be completely correct. The product was directly used for the transformation of *C. tepidum*.

2.4. Transformation and analytical PCR

Natural transformation was performed according to a previously described method [32]. Single colonies on gentamicin-containing CP plates were restreaked three times onto freshly prepared selective plates. To monitor the segregation of wild-type and mutant alleles in each transfer step, PCR was carried out directly using individual colonies as the templates (see below). Fully segregated transformants were inoculated into fresh CL media containing gentamicin and grown until the early stationary phase. After checking the segregation again, transformants were frozen-stored as original mutant strains for further research.

Genomic DNA preparation and analytical PCR were performed basically as previously described [17]. For a quick check, a colony or a cell suspension was used directly for PCR amplification. A colony was picked with a toothpick and directly soaked into a PCR reaction mixture, or cells were harvested by centrifugation and resuspended in water. An aliquot was directly added to a PCR reaction mixture at 50-fold dilution [49].

2.5. Measurement of homologous recombination frequency of C. tepidum

The homologous recombination frequency of *C. tepidum* was estimated as the insertional inactivation frequency of the *bchU* gene by the *aadA* streptomycin–spectinomycin resistance cassette [50]. After transformations of the wild-type and *recA*- mutant, each frequency was calculated by counting colonies on both selective and non-selective plates. The mean values and standard deviations were obtained from at least three independent experiments.

2.6. Preparation of the C. tepidum His-tagged RC complex

The *C. tepidum* cells expressing the His-tagged RC complex were grown in 1.2-l medium bottles. After 2 days of cultivation, cells were harvested by centrifugations at 12,000*g* for 5 min and stored at −80 °C until use. Unless otherwise indicated, the subsequent processes were carried out in an anaerobic chamber (Coy Laboratory Products) at room temperature. All buffers and resins used for preparation were fully degassed by leaving them in an anaerobic chamber overnight and supplemented with 10 mM L-cysteine and/or 2 mM dithiothreitol as appropriate reductants in order to remove residual oxygen. The preparation of crude membranes and subsequent solubilization treatment of them were carried out basically as previously reported [51] with a slight modification as follows: the membranes at 2.5 mg BChl $(a+c)$ ml^{-1} were solubilized with 30 mM n-octyl-β-D-glucopyranoside (β-OG, Sigma-Aldrich) in a buffer (50 mM Tris–HCl (pH 8.0), 300 mM NaCl, 2 mM dithiothreitol, and protease inhibitors). After centrifugation at 110,000*g* for 1 h, the resultant supernatant was mixed with a 3/10 volume of a 50% (V/V) suspension of the Ni^{2+}-immobilized sepharose resin, His-Accept (Nacalai Tesque, Inc.), which was pre-equilibrated with the same buffer as that used in solubilization. The mixture was gently stirred for 1 h and loaded into an open column. The column was washed with 8 to 10 column volumes of buffer A (50 mM Tris–HCl (pH 8.0), 2 mM sucrose monolaurate (SM-1200, Dojindo Laboratories), 300 mM NaCl, and 2 mM dithiothreitol) containing 10 mM imidazole to exchange the detergent. The His-tagged RC complex was eluted with buffer A containing 300 mM imidazole while forming a dark-green band.

The extraction procedure with the β-OG was much effective for the solubilization of the RC complex from chlorosome-containing membranes. Although other detergents, sucrose monolaurate and Triton X-100, were tried, they solubilized the RC complex with a relatively low yield compared to the β-OG and extracted a large amount of BChl *c*. In the present study, the β-OG was exchanged to the milder detergent sucrose monolaurate in the washing step of Ni^{2+} affinity chromatography.

For the EPR measurements and further purification by gel filtration, the eluate was concentrated by an ultrafiltration unit (Minicon B15 concentrator, Millipore). SDS-PAGE was performed according to Laemmli's method [52]. The separated proteins on gel were stained with Coomassie Brilliant Blue.

2.7. Spectral measurements

The steady-state fluorescence emission and absorption spectra were measured with a spectrofluorophotometer RF-1500 (Shimadzu, Japan) and spectrophotometer UV-3101PC (Shimadzu, Japan), respectively. When measuring the whole cell fluorescence emission and absorbance spectra, the samples were prepared as previously described [17].

Flash-induced absorbance changes were measured with a home-built single-beam spectrophotometer. Samples in an air-tight cuvette (1 cm × 1 cm) were probed with a single-wavelength measuring beam isolated from a tungsten-halogen lamp with a 20 cm monochromator. Excitation flashes (decayed within 1 ms) were provided by a Xe flash lamp filtered by suitable band-pass filters. The sample was protected from the measuring beam by a shutter until 200 ms prior to the excitation flash. The transmitted measuring beam from the sample was detected by a photomultiplier through suitable long-pass filters and a 10 cm monochromator. The detected signal was amplified by an operational amplifier and stored in a digital oscilloscope. Collected signals were averaged 32–128 scans as required.

Low-temperature EPR measurements were performed using a Bruker ESP-300E spectrometer (Bruker Biospin) equipped with a liquid-helium flow cryostat and a temperature-control system (CF935, Oxford Instruments). Continuous white light for photoaccumulation was provided from a 500 W tungsten-halogen lamp through heat-cut glass filters. A Xe flash lamp with 1 Hz was used for measurements of flash-induced EPR signals. A field modulation frequency of 100 kHz was used for the transient and continuous wave EPR measurements. For EPR measurements, 10% (V/V) glycerol was added to the samples.

2.8. nLC/MS/MS

Approximately 5 μg of RC complexes, which were suspended in 50 mM Tris–HCl (pH 8.0) and 2 mM sucrose monolaurate, was digested with 0.1 μg of Trypsin Gold (Promega) at 37 °C overnight. The resultant peptides were separated by nanoscale liquid chromatography (nLC) using EASY-nLC (Proxeon, Denmark), and its mass spectra were analyzed with micrOTOF-QII (Bruker Daltonics, USA), as reported below in detail.

A tryptic sample of 0.5 μg was injected into the pre-column, NS-MP-10 BioSphere C18 (5 μm particle size, 120-Å pore size, 100 μm inner diameter, 20 mm length; NanoSeparations, Netherlands), at a 4 μl/min flow rate using autosampler. After washing the pre-column with 30 μl of Solution A (0.1% formic acid in double distilled water), the desalted peptides were subsequently separated by an analytical column, NS-AC-11-C18 BioSphere C18 (5 μm particle size, 120-Å pore size, 75 μm inner diameter, 150 mm length; NanoSeparations, Netherlands), which was developed at a 200 nl/min flow rate with a linear solvent gradient from 5% to 50% of Solution B (0.1% formic acid in acetonitrile) for 60 min. The separated peptides on a C18 column were introduced into the micrOTOF-QII mass spectrometer by electrospray ionization under a positive mode. The capillary voltage was −1.5 kV, and the drying gas temperature was set to 200 °C. The collision energy of the quadrupole for MS/MS fragmentation of each peptide using Ar gas was set from 20 to 40 eV depending on its size and charged state. The acquired mass spectra from micrOTOF-QII were analyzed using DataAnalysis 4.0 and Biotools 3.2 software (Bruker Daltonics).

3. Results

3.1. Inactivation of the recA gene and insertion of the 6xHis-tag-pscAB gene cluster

In various bacterial strains, RecA protein triggers an SOS response to DNA damage by its protease activity specific to the LexA protein, which is the direct repressor of SOS genes, including the *recA* gene [53]. However, there is neither a typical LexA homologue in the *C. tepidum* genome nor LexA-binding consensus sequences, such as SOS-box or LexA-box, in the upstream region of the *recA* gene [53]. Thus, the disruption of the *C. tepidum recA* gene was expected to affect only a homologous DNA recombination system and not the response system to DNA damage.

Two different *C. tepidum* mutants lacking a *recA* gene were constructed by the natural transformation method as previously described [32] (Fig. 2A). The *recA::aacC1* strain, in which the *aacC1* gentamicin resistance cassette was inserted into the *recA* region, was used as a control. In another mutant, the *recA::(HisAB-aacC1)* strain, the *6xHis-tag-pscAB* gene cluster was additionally ligated upstream of the *aacC1* cassette. The *6xHis-tag-pscAB* construct contains a putative 365-bp promoter region upstream of the intrinsic *pscAB* gene cluster in order to express the N-terminally His-tagged PscA along with the authentic PscA *in vivo*. Segregations of the wild-type and mutant alleles were confirmed by the PCR method using the *recA*-inside and -outside primer sets to amplify the *recA* locus (Fig. 2B). No fragment corresponding to the wild-type allele was detected in the amplified products from two mutant cells. The amplified products from the *recA::aacC1* cells were approximately 100-bp smaller than those from the wild-type cells. This size corresponds to the difference in length between the deleted *recA* gene and the inserted *aacC1* cassette. On the other hand, the amplified products from the *recA::(HisAB-aacC1)* cells were approximately 3.4 kbp larger than those from the *recA::aacC1* cells because of the additional insertion of the *6xHis-tag-pscAB* gene cluster. Direct sequencing of these products revealed no unintended alteration of nucleotides, indicating that the *recA* gene was insertionally inactivated with the desired constructs in these two mutants.

Duplication of the *pscAB* gene cluster in the *recA::(HisAB-aacC1)* strain was further confirmed by the PCR method using the *pscA* upstream primer set (Fig. 2A). The 450-bp fragments amplified from the wild-type, *recA::aacC1*, and *recA::(HisAB-aacC1)* cells were derived from the authentic *pscAB* gene cluster (Fig. 2B, panel b). However, an additional 520-bp fragment was also found only from the *recA::(HisAB-aacC1)* cells. This fragment corresponded in size to the extended region derived from the *6xHis-tag-pscAB* construct inserted into the *recA* locus. These results clearly indicated that the *pscAB* gene cluster was successfully duplicated in the *recA::(HisAB-aacC1)* strain. The *6xHis-tag-pscAB* construct was stably retained in the genome of the *recA::(HisAB-aacC1)* strain, since the same PCR results were reproducibly obtained from cultures after several generations (data not shown).

3.2. Phenotypes of C. tepidum mutant lacking the recA gene

Both *recA::aacC1* and *recA::(HisAB-aacC1)* strains could grow photoautotrophically and showed essentially the same whole cell absorption and fluorescence emission spectra as the wild-type strain (data not shown). Therefore, neither disruption of the *recA* gene nor insertion of the *6xHis-tag-pscAB* construct into the *recA* region seemed to have a serious effect on the photosynthetic system of the *C. tepidum*. The responsibility of the *recA* gene for the homologous recombination was further examined by estimating the transformation frequencies of the wild-type and *recA::aacC1* strains. When the wild-type strain was used as a host, about 1% of the total cells could be transformed

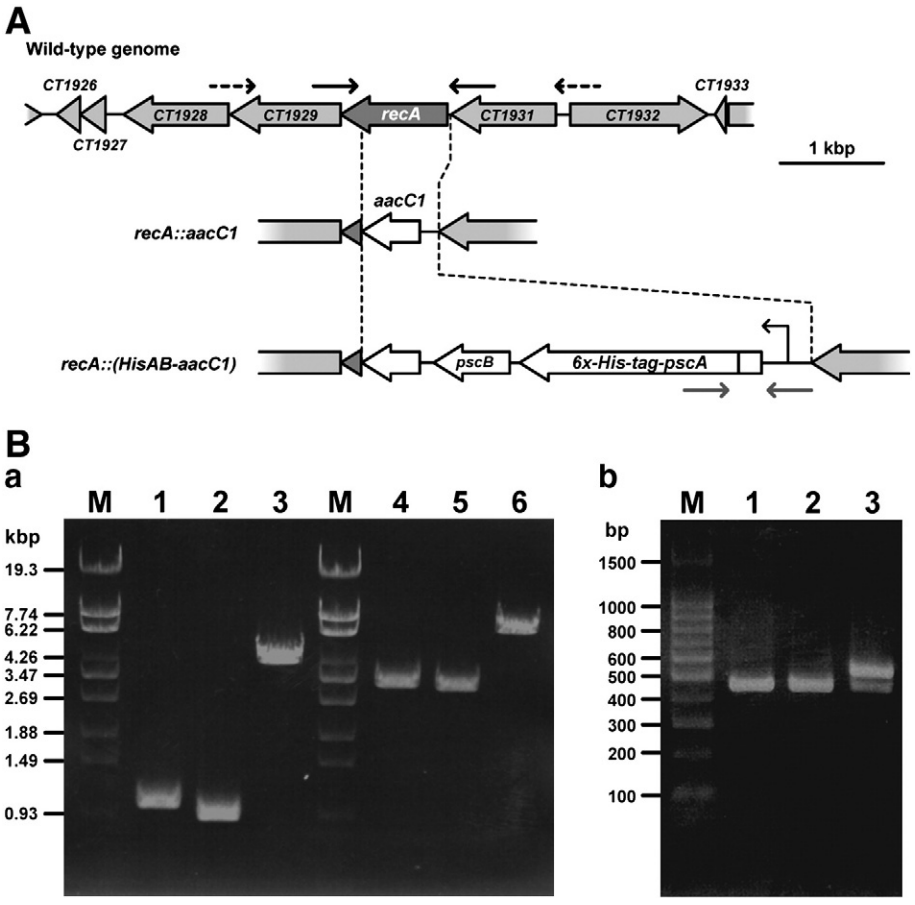

Fig. 2. Insertional inactivation of the *recA* gene and duplication of the *pscA* gene in *C. tepidum.* (A) Schematic representation of the construction of *recA*-disrupted mutants. A genomic region around the *recA* gene is shown along with DNA constructs used for homologous recombination. Genes are indicated by rectangles. The arrows represent the oligonucleotide primers used for the PCR analyses. (B) (a) PCR analyses of the genomes from the wild-type (lanes 1 and 4), the *recA::aacC1* (lanes 2 and 5), and the *recA::(HisAB-aacC1)* (lanes 3 and 6) strains. Lanes 1–3 and 4–6 represent the PCR products amplified by the *recA*-inside (black arrows in A) and the *recA*-outside (black broken arrows in A) primers, respectively. The λ/*Sty*I digests are used as molecular-weight markers in lanes M. The numbers indicate the lengths of DNA fragments in kilobase pairs. (b) PCR analyses for verifying the *pscA* gene duplication. Lanes 1, 2, and 3 represent the PCR products amplified by *pscA* upstream primers (gray arrows in A) using the genomes of the wild-type, the *recA::aacC1*, and the *recA::(HisAB-aacC1)* strains, respectively. The 100 bp DNA ladder is used as a molecular-weight marker in lane M. The numbers indicate the lengths of the DNA fragments in base pairs.

into the *bchU*$^-$ strain (Table 1). In contrast, when the *recA::aacC1* strain was used as a host, its transformation frequency was found to be 10^{-5} to 10^{-6} smaller than the wild type. Since the BchU-less mutant appeared to exhibit a normal phenotype in its growth compared to the wild type [50], the significant decrease of the transformation frequency obtained in the *recA::aacC1* strain was attributable to the complete deficiency of the double-crossover DNA recombination. This result clearly indicates that *CT1930* annotated as the *recA* gene is actually involved in the homologous recombination and that no *orf* other than *CT1930* would serve as a *recA*-like gene in *C. tepidum* [34].

Table 1
Comparison of transformation efficiencies for the *bchU* gene inactivation between the wild-type and the *recA::aacC1* mutant strains of *C. tepidum.*

Strain	Amount of DNA for the transformation	Number of vial cells (cfu/ml) [a]	Transformation frequency [b]
Wild-type	0.1 μg	$5.6 \pm 0.4 \times 10^9$	$2.5 \pm 1.2 \times 10^{-4}$
	1 μg	$8.0 \pm 2.0 \times 10^9$	$4.9 \pm 1.6 \times 10^{-3}$
	10 μg	$6.2 \pm 2.4 \times 10^9$	$1.1 \pm 0.6 \times 10^{-2}$
recA::aacC1	0.1 μg	$4.4 \pm 1.6 \times 10^9$	$1.1 \pm 1.9 \times 10^{-9}$ [c]
	1 μg	$9.6 \pm 2.0 \times 10^9$	$7.7 \pm 5.7 \times 10^{-9}$ [c]
	10 μg	$6.0 \pm 1.6 \times 10^9$	$3.1 \pm 0.6 \times 10^{-8}$ [c]

[a] The number of cells in the liquid culture at late exponential phase.
[b] The ratio of the number of drug-resistant colonies on the selective plate to the total number of colonies (see Materials and methods).
[c] $P<0.05$, for comparison to the wild-type within the same conditions.

3.3. Purification of the His-tagged RC complex

A crude fraction containing the RC complex was obtained after extraction with β-OG from chlorosome-containing membranes of the *recA::(HisAB-aacC1)* strain followed by its isolation using Ni^{2+} affinity chromatography, as described in Materials and methods section. As expected, dark-green components were adsorbed onto the Ni^{2+}-immobilized resin. Most of them were retained after a wash procedure with 10 mM imidazole and then eluted with 300 mM imidazole. The eluate contained four major polypeptides of the green sulfur bacterial RC [5], that is, PscA, FMO, PscB, and PscC, while no band corresponding to the PscD was observed, judging from its apparent molecular mass (Fig. 3A, lane 1). The further purification step by gel filtration chromatography with Sephacryl S-300 revealed that these four polypeptides were coeluted from the column and formed a stable complex as the His-tagged RC complex (Fig. 3A, lane 2). The recovery of the RC complex in a single fraction strongly indicated that the complex formed a monodisperse protein/detergent complex (data not shown), although a precise estimation of its molecular mass needs a further examination using reference samples such as RC core complexes from purple bacteria and/or PS I with known sizes. About a 40–60% fraction of the total extracted RC was

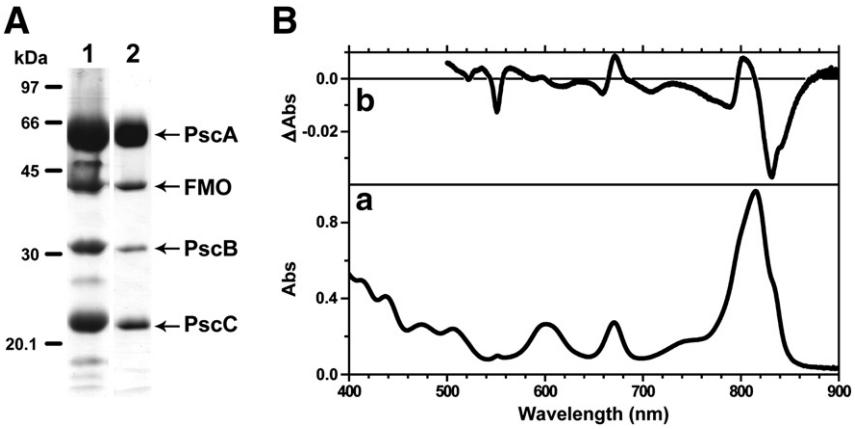

Fig. 3. SDS-PAGE analysis of the His-tagged RC complex and its absorption and chemically oxidized-minus-reduced different spectra. (A) Lane 1, His-tagged RC complex eluted from the Ni^{2+}-immobilized resin; lane 2, purified His-tagged RC complex after gel filtration with Sephacryl S-300. The numbers on the left indicate molecular masses estimated by using an LMW protein marker (GE Healthcare): phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and trypsin inhibitor (20.1 kDa). (B) The absorption spectrum (trace a) was measured using the RC complex from the Ni^{2+}-resin. The difference spectrum (trace b) was obtained by oxidizing the sample with a small amount of ferricyanide, followed by reducing it again with an excess amount of ascorbate. The sample used for the difference spectrum was the same as that for trace a.

adsorbed onto the resin, given the absorbance difference between the extract and non-adsorbed fraction. Contrary to this, no RC complex extracted from the wild-type membrane was bound to the immobilized Ni^{2+} resin (data not shown). Thus, the His-tagged RC complex was successfully expressed in the *recA::(HisAB-aacC1)* strain and could be obtained in a single step as well as considerably high purity by Ni^{2+} affinity chromatography.

The absorption spectrum and the chemically induced oxidized-minus-reduced difference spectrum of the His-tagged RC complex are shown in Fig. 3B. Assuming the extinction coefficient for BChl *a* at the Q_y peak and the difference extinction coefficient for P840 at 830 nm to be 100 mM^{-1} cm^{-1} [54] and the difference extinction coefficient of the *c*-type heme of cytochrome c_z at 551 nm to be 20 mM^{-1} cm^{-1} [55], the present RC preparation contained about 26 BChls *a* and 1.7 hemes per P840. The antenna size was almost the same as that of 1FMO-RCC complex comprising a monomer of the FMO protein and an RC core protein, as inferred from their spectral similarity in the Q_y region (data not shown) [56]. The flash-induced absorption changes of P840 in the His-tagged preparation showed that the rapid electron donation from cytochrome c_z to the photooxidized $P840^+$ and the charge recombination between the $P840^+$ and the photoreduced Fe–S centers, $(F_A/F_B)^-$, in the absence and presence of 60% glycerol, respectively, as previously demonstrated in the isolated RC complex (Figs. S1 and S2) [8,11]. These data suggest that the His-tagged RC complex contains all ET components essential for the light-energy conversion reaction to produce reducing power.

3.4. Light-induced EPR signals of Fe–S centers in the His-tagged RC complex

The EPR measurements were carried out to clarify the intactness of iron–sulfur centers in the His-tagged RC fraction. The illumination for 20 min at 5 K induced an irreversible signal of the reduced Fe–S center with apparent *g*-values of $g_z = 2.077$, $g_y = 1.907$, and $g_x = 1.859$ when measured at 10 K (Fig. 4A). Their *g*-values and temperature dependence (data not shown) closely resembled those of the F_B^- reported in isolated RC complexes [51,57] as well as membranes [58].

After illumination for 20 min at 210 K followed by subsequent cooling to 5 K under illumination, two other signals with apparent *g*-values of $g_x = 1.886$ and $g_y = 1.948$ at 10 K and $g_x = 1.754$ and $g_y = 1.928$ at 5 K were observed (Fig. 4B). These signals exhibited different temperature dependences (Fig. 4C). The former signal could be assigned to the spin-interacting state of F_A-/F_B-, and the latter one, to the F_X-, as previously characterized in both membranes [17,58,59] and isolated RC complexes [51,57,59]. The EPR signal of semiquinone radicals ($g = 2.0066$; bandwidth, 1.2 mT) was also detected using the same photoaccumulated sample (Fig. S3), as previously reported in the membrane of *C. parvum* [19] and the RC core complex of *Heliobacterium modesticaldum* [26].

Furthermore, the electron spin-polarized (ESP) signal with E/A (E, emission; A, absorption) pattern around 339.4 mT was observed in the His-tagged RC complex by flash-induced transient EPR measurements at 10 K as in the membrane of the wild-type strain (Fig. S4A). It was almost identical to that in the membrane of the wild-type strain (Fig. S4B). The same ESP signal has already been assigned to the charge-separated state of $P840^+F_X$-, although measurements were carried out at 100 K [22]. This clearly indicates that electron transfer reaction from P840 to F_X proceeded so rapidly event at cryogenic temperature as to detect the ESP signal formed between them. The His-tagged RC complex, therefore, seems to have the intrinsic physicochemical nature so far described in the wild-type one.

3.5. nLC/MS/MS analyses of the His-tagged RC preparation

In principle, the *recA::(HisAB-aacC1)* mutant cells can express three kinds of RC complexes: the non-/non-tagged PscA homodimer, the His-/His-tagged PscA homodimer, and the non-/His-tagged PscA heterodimer (Fig. 1, lower). The His-tagged RC preparation obtained in the present study would thus be a mixture of the His-/His-tagged PscA homodimer and the non-/His-tagged PscA heterodimer. This heterogeneity was explored by the nLC/MS/MS analysis of peptides produced by a tryptic digestion of the His-tagged RC preparation under non-denaturing conditions (Fig. S5). The N-terminal tryptic peptide of the His-tagged PscA was detected as a 1767.86 Da fragment ($m/z = 884.93$, accuracy < 10 ppm) at the retention time of 8.2 min in the nLC/MS analysis (Fig. S6). The same ion peak was not observed in the RC preparation isolated from the wild-type strain according to our previous methods [8]. The product ion spectrum of the 1767.86 Da fragment by tandem mass spectrometry (MS/MS) further confirmed that it corresponded to peptide 2–13 (GSSHHHHHHSSGLVPR) in the His-tagged PscA (Fig. S7). On the other hand, no ion peak corresponding to every possible N-terminal peptide of the non-tagged PscA could be identified at all in the MS/MS data. There were two conceivable explanations for this: 1) the non-tagged PscA

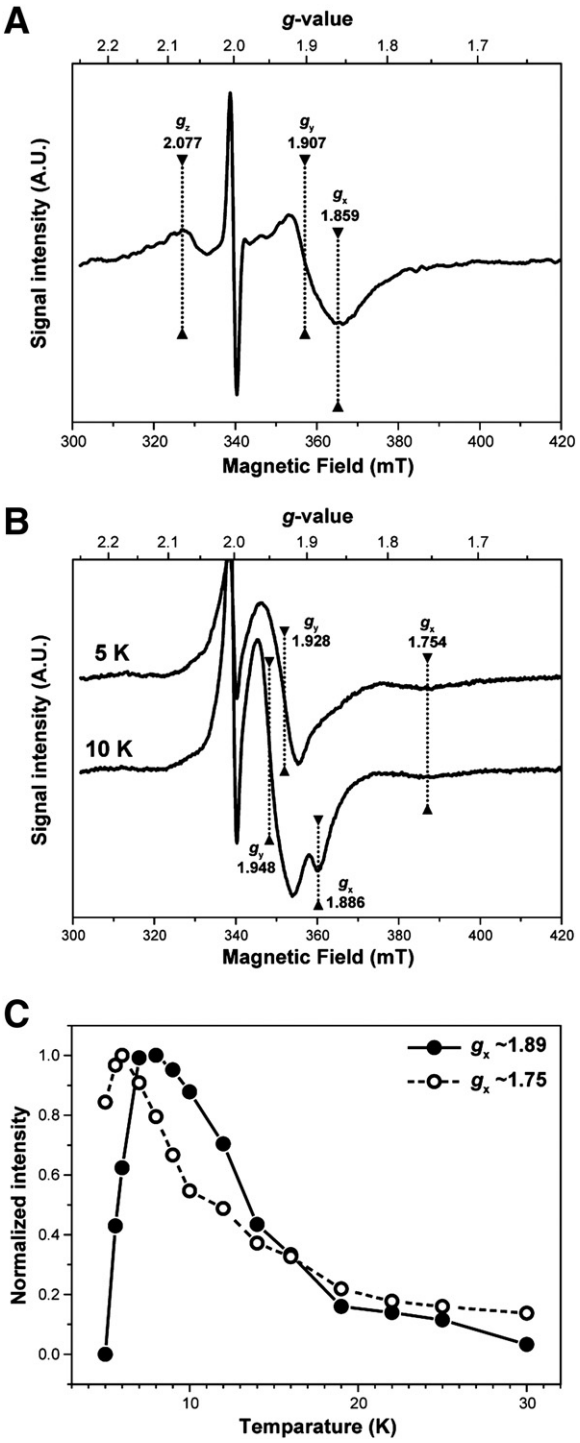

Fig. 4. EPR spectra of iron–sulfur centers and their temperature dependency in the His-tagged RC. (A) The sample was frozen to 5 K in the dark in the presence of an excess dithionite. The light-minus-dark difference spectrum was obtained by subtracting the spectrum measured without illumination from the spectrum measured after illumination for 20 minutes at 5 K. Conditions of EPR measurements: temperature, 10 K; microwave power, 10 mW; microwave frequency, 9.518 GHz; modulation amplitude, 2.0 mT; time constant, 20 ms; and modulation frequency, 100 KHz. (B) The photoaccumulated sample was prepared by preillumination for 20 minutes at 210 K in the presence of an excess dithionite and subsequent cooling to 5 K under illumination. The light-minus-dark difference spectra at 5 (upper) and 10 K (lower) were obtained by subtracting the spectra measured in the sample frozen in the dark from the spectra measured after illumination for 20 minutes at 5 K in the photoaccumulated sample. The conditions of EPR measurements are the same as in (A) except for the temperature. (C) Temperature dependency of EPR signals obtained in (B). The intensities of g_x signals corresponding to g_x values of approx. 1.89 (closed circle) and 1.75 (open circles) were normalized and plotted against temperature.

polypeptide was never present in the His-tagged RC preparation, or 2) the amount of the N-terminal peptide recovered from a tryptic digestion of the non-tagged PscA polypeptide was too small to be analyzed by MS/MS.

To resolve this issue, we tried to determine the retention time and the accurate mass of the N-terminal tryptic peptide of the non-tagged PscA using the RC complex isolated from the wild-type strain [8]. The nLC/MS/MS analysis revealed that peptide 2–16 (AEQVKPAGVKPK) in the authentic PscA, which was eluted at 17.8 min from the C18 column, was acetylated at the N-terminal alanine residue, giving a molecular mass of 1292.74 Da ($m/z = 647.37$, accuracy < 10 ppm) (Fig. 5A, inset, and Fig. S8). This fragment was regarded as the sole N-terminal tryptic peptide of the non-tagged PscA because no peptide fragment subjected to any other potential modification, e.g., oxidation, formylation, and/or acetylation of the first methionine residue, was ever detected. A small but obvious peak with the same mass and retention time could be detected in the tryptic digests of the His-tagged RC preparation in the nLC/MS analysis (Fig. 5B, inset). This result clearly indicated that the *recA::(HisAB-aacC1)* mutant cells expressed the non-/His-tagged PscA heterodimeric RC complex in addition to the His-/His-tagged PscA homodimeric one.

The amounts of these two kinds of RC complexes in the His-tagged RC preparation were roughly estimated by comparing the intensity of the ion peak corresponding to the non-tagged N-terminal peptide from the His-tagged preparation with that from the authentic RC one. Two major peaks in MS spectra at 17.8 min (Fig. 5), which were attributable to peptides 185–188 (FLGK, $m/z = 464.29$) and 656–659 (FLNR, $m/z = 549.32$), were used as the internal standard to calculate the relative amount of the non-tagged N-terminal peptide recovered from each preparation. The intensities of the 647.37 m/z peak normalized by either standard peak (464.29 or 549.32 m/z) were compared between the His-tagged and the authentic RC preparations. The non-tagged PscA polypeptide content in the His-tagged RC preparation was thus estimated to be $11.1 \pm 4.4\%$ compared to that in the authentic RC one in three independent measurements. This value did not vary significantly depending on what peak was taken as the internal standard. A similar estimation (approx. 10%) was also obtained by the SDS-PAGE analysis; non- and His-tagged PscA polypeptides could be almost, but not completely, separated on the gel containing a high concentration of urea to improve its resolution (data not shown). Since the non-tagged PscA polypeptide in the His-tagged RC preparation should be derived only from the non-/His-tagged PscA heterodimer, the estimated value implies that the artificial heterodimeric RC complex constitutes about one fifth of the total amount in this preparation. This estimation could be verified more directly by electron microscopic analysis. An attempt to visualize the heterogeneously attached His-tag to the RC complex using an antibody against the $(His)_6$-tag is currently in progress.

4. Discussion

4.1. The C. tepidum recA mutant as a host for homologous gene expression

The *recA* gene is involved in homologous recombination to repair the damaged DNA in bacteria [60–62]. Indeed, the disruption of the *C. tepidum recA* gene caused a drastic decrease of the homologous recombination efficiency (Table 1). A defect in the *recA*-related repair system is, however, considered to be lethal for oxygenic photosynthetic organisms, since exposure to light produces reactive oxygen radicals, including singlet oxygen, which would damage chromosomal DNA as well as the D1 protein in PS II. The *recA*$^-$ mutant of a cyanobacterium *Synechocystis* sp. PCC6803 accumulated nonviable cells even under low-light intensity (about 10 μmol photons $m^{-2}\,s^{-1}$) [62]. In contrast, the *recA*$^-$ mutant of *C. tepidum* was found to be viable judging from the colony counts on plates. This may simply imply that

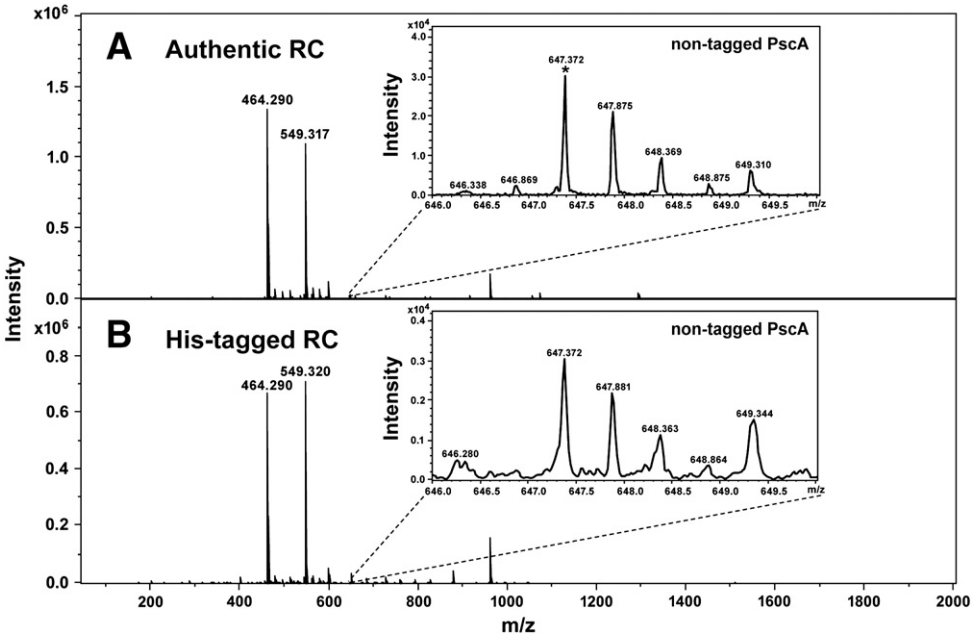

Fig. 5. nLC/MS/MS analyses of N-terminal tryptic peptides of non-tagged PscA from authentic and His-tagged RC preparations. Mass spectra of tryptic peptides from authentic (A) and His-tagged (B) RC preparations. Enlarged spectra in the 646–650 *m/z* region are shown in insets. These peptides were eluted at 17.8 min with a C18 column. The asterisk indicates the double-charged ion peak of the N-terminal tryptic peptide of non-tagged PscA, whose amino acid sequence was determined by subsequent MS/MS analysis (see Fig. S8).

no oxidative stress exceeds a harmful threshold level in the mutant because *C. tepidum* requires a strictly anaerobic condition for its growth.

A recent study has demonstrated that antioxidant enzymes were constitutively expressed to defend against oxidative stress in *C. tepidum* [63]. Other DNA repair systems by non-homologous recombination could thus be operative in response to such stress because the phenotype of the *recA*$^{-}$ mutant was stably maintained even after multiple subcultures. Although the RecA protein also serves as a trigger for the SOS response to the damaged DNA in many bacterial lineages, the typical RecA–LexA-mediated SOS response system is missing in *C. tepidum* [53]. In conclusion, the *recA*$^{-}$ strain constructed in the present study would be a desirable host for mutagenesis experiments of essential genes for the photosynthetic growth and the stable expression of externally incorporated homologous genes as well.

4.2. The C. tepidum His-tagged RC complex

This is the first report on the affinity purification of the green sulfur bacterial RC complex. The His-tagged RC complex contained a series of functional ET cofactors and exhibited a stable charge separation state between P840 and terminal acceptors F_A/F_B. The addition of a $(His)_6$-tag to the N-terminus of PscA was thus effective for the convenient and short-time preparation of the photoactive *C. tepidum* RC complex. Gulis et al. reported a successful purification of PS I by introducing the N-terminal His-tag to the PsaA core protein in a green alga, *C. reinhardtii* [40]. They noted that the N-terminus of PsaA was a desirable portion for the attachment of tags from the point of view of the 3D structure; it protrudes into an aqueous phase on the cytoplasmic/stromal side, and the N-terminal 12 residues was invisible due to a disorder, as seen in the crystal structure of the PS I complex of a cyanobacterium *Thermosynechococcus elongatus*. On the other hand, when the $(His)_6$-tag was attached to the C-terminus of PscA, no RC complex could be adsorbed onto the Ni^{2+}-immobilized resin, presumably because the tag was embedded in the complex (data not shown). The C-terminal water-soluble domain of cytochrome c_z might have covered the tag, which was anticipated to reside on the periplasmic side. Similarly, Tang and Chitnis also failed to purify PS I with affinity chromatography by attaching the $(His)_6$-tag to the C-termini of PsaK and PsaL on the luminal side in cyanobacterium *Synechocystis* sp. PCC6803 [44]. However, in the case of type 2 RC, some favorable results have been obtained by His-tag attachment on both sides [35,36,39,42,43].

The PscD subunit was completely absent in the *C. tepidum* His-tagged RC complex. The whole cell fluorescence emission spectrum of the *recA::(HisAB-aacC1)* mutant did not show any increase in the fluorescence intensity derived from chlorosomes, as observed in the PscD-less mutant (data not shown) [17], suggesting that PscD was present *in vivo* but would have dissociated during the preparation of the His-tagged RC complex. Since PscD is supposed to be a highly hydrophilic and basic protein on the surface of the cytoplasmic side [5,17], the electrostatic repulsion between PscD and the six histidine residues in the N-terminus of PscA would have destabilized PscD and loosely bound it to the RC complex. In fact, the PscD seemed to have been dissociated from the complex after the β-OG treatment because lower-MW (< 20 kDa) proteins in the eluate from the Ni^{2+} resin (see Fig. 3A, lane 1), even if the PscD was contained in a small amount, were completely removed by ultrafiltration with a 15-kDa cutoff membrane filter before gel filtration chromatography (data not shown). The FMO protein, which was expected to be somehow associated with PscD on the cytoplasmic side, would thus have been easily released from the complex because the stoichiometry of FMO protein per RC was roughly estimated to be one from the SDS-PAGE and spectral analyses (see Fig. 3) [5,14]. However, the loss of the PscD subunit never induced any serious defect in the kinetics of ET reactions, as revealed by the present spectroscopic analyses.

The His-tagged RC preparation was a mixture of the His-/His-tagged PscA homodimeric and the non-/His-tagged PscA heterodimeric RC complexes as indicated from LC/MS/MS analysis. This heterogeneity would hinder the growth of appropriate crystals for the 3D structural study of the RC complex. The His-tag can easily be

removed by thrombin digestion, as its recognition sequence (LVPRGS) is present four residues behind the 6xHis sequence. However, the N-terminally extended sequence (GSHMLEDPM) still remains after thrombin cleavage (see Fig. S5). Another method would thus be the replacement of the authentic *pscA* gene with the *6xHis-tag-pscA* gene. In fact, the *6xHis-tag-pscA* mutant strain could be obtained and the crystallization of the His-/His-tagged PscA homodimeric RC complex is now being tried.

4.3. A methodological proposal for site-directed mutagenesis in *C. tepidum*

In this paper, we examined the availability of *pscA* gene duplication as a mutagenesis method for the *C. tepidum* RC (Fig. 1). All results clearly demonstrated that the *recA* locus was suitable for the introduction of the second *pscA* gene. As approximately one half of the total RCs solubilized with the detergent did not adsorb onto the Ni^{2+}-immobilized resin, the *recA::(HisAB-aacC1)* mutant strain appeared to also express the non-tagged homodimeric core protein. This non-tagged complex, which is supposed to have the same nature *in vivo* as the wild-type one, could complement any growth defect due to a nonfunctional mutant core protein. This method, gene replacement concomitant with disruption of the *recA* gene, would be applicable to other essential genes for growth in order to characterize the biochemical properties of their products. Affinity-tag attachment to them is helpful for their convenient purification as well.

The success of *pscA* gene duplication could propose a novel methodology to alter a homodimeric RC core protein into a heterodimeric one by a site-directed mutation of the *6xHis-tag-pscA* gene. In fact, the nLC/MS/MS analyses revealed that the His-tagged RC preparation contained the His-/His-tagged PscA homodimer as well as the non-/His-tagged PscA heterodimer, although the latter content was estimated to be 20% at most. Therefore, two issues must be overcome for the practical application of this method to investigate electron transfer mechanisms in the artificial heterodimeric RC.

First, it is necessary to isolate only the non-/His-tagged PscA heterodimer from the His-tagged RC preparation. The most promising method would be the attachment of a different tag to the authentic *pscA* gene and purification with tandem affinity chromatography. Relatively small tags, such as a His-tag and a strep-tag, are considered not to cause any critical damage to structures and/or functions [64]. The mutant in which the authentic *pscA* gene is replaced with the *strep-pscA* gene has already been obtained and appears to show no deleterious effect on the photosynthetic apparatuses (data not shown). Detailed results about the strep-/His-tagged PscA heterodimer will be discussed elsewhere. In the present study, a fraction of about 15–25% of the total amount of the His-tagged RC adsorbed onto the Ni^{2+}-immobilized resin was eluted in a washing step with 10 mM imidazole. Since the non-/His-tagged PscA heterodimer was expected to be more loosely bound to the Ni^{2+}-immobilized resin than the His-/His-tagged PscA homodimer, the fraction might contain a higher content of the heterodimer. The strategy of tandem affinity chromatography could also be available for the recovery of the strep-/His-tagged PscA heterodimer, which was discarded in a washing step with 10 mM imidazole, resulting in the improvement of the total yield.

Second, detailed biochemical and spectroscopic analyses demand a large amount of the heterodimer. The simple way to overcome this difficulty is to use a preparation from large-scale culturing. A manipulation under anaerobic conditions, however, makes it a very daunting task. Another possible solution might be to regulate the expressions of both authentic *pscA* and *6xHis-tag-pscA* genes at the same level. In the *recA::(HisAB-aacC1)* mutant, the *6xHis-tag-pscA* gene could be transcribed by not only the promoter of the *pscA* gene but also the intrinsic promoter of the *recA* gene. The same expression level would thus be achieved by a tandem arrangement of the authentic *pscA* and *6xHis-tag-pscA* genes, the latter of which is inserted into the *pscAB* gene cluster in the genome.

In conclusion, the *pscA* gene duplication method described here would enable investigating the direct effect of a single mutation of an amino acid residue on the electron transfer reaction. It has been impossible, so far, to precisely estimate the degree of such an effect in the case of the PS I, since the latter has to exhibit a side effect derived from inherent heterogeneous properties in two branches. Moreover, this novel method could provide a useful tool to experimentally verify the electron transfer theory as well. We are now trying to execute a site-directed mutagenesis in the *6xHis-tag-pscA* gene to study the availability of this method.

Acknowledgments

This work was financially supported by Grants-in-Aid for Scientific Research (C) (no. 21570168) (to H.O.) and (B) (no. 19370064) (to S.I.), by the COE program "The Origin of the Universe and Matter" (to S.I.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by postdoctoral fellowships from the Japan Society for the Promotion of Science (no. 211578 to C.A., no. 21008983 to T.K.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbabio.2011.03.007.

References

[1] J.T. Beatty, J. Overmann, M.T. Lince, A.K. Manske, A.S. Lang, R.E. Blankenship, C.L. Van Dover, T.A. Martinson, F.G. Plumley, An obligately photosynthetic bacterial anaerobe from a deep-sea hydrothermal vent, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 9306–9310.
[2] J.M. Olson, Chlorophyll organization and function in green photosynthetic bacteria, Photochem. Photobiol. 67 (1998) 61–75.
[3] C. Azai, Y. Tsukatani, J. Harada, H. Oh-oka, Sulfur oxidation in mutants of the photosynthetic green sulfur bacterium *Chlorobium tepidum* devoid of cytochrome c-554 and SoxB, Photosynth. Res. 100 (2009) 57–65.
[4] N.U. Frigaard, D.A. Bryant, Genomic insight into the sulfur metabolism of phototropic green sulfur bacteria, in: H. Rüdiger (Ed.), Sulfur Metabolism in Phototrophic Organisms, Springer, New York, 2008, pp. 337–355.
[5] G. Hauska, T. Schoedl, H. Remigy, G. Tsiotis, The reaction center of green sulfur bacteria(1), Biochim. Biophys. Acta 1507 (2001) 260–277.
[6] P. Jordan, P. Fromme, H.T. Witt, O. Klukas, W. Saenger, N. Krauss, Three-dimensional structure of cyanobacterial photosystem I at 2.5 A resolution, Nature 411 (2001) 909–917.
[7] B. Jagannathan, J.H. Golbeck, Unifying principles in homodimeric type I photosynthetic reaction centers: properties of PscB and the FA FB and FX iron–sulfur clusters in green sulfur bacteria, Biochim. Biophys. Acta 1777 (2008) 1535–1544.
[8] H. Oh-oka, S. Kamei, H. Matsubara, M. Iwaki, S. Itoh, Two molecules of cytochrome c function as the electron donors to P840 in the reaction center complex isolated from a green sulfur bacterium. *Chlorobium tepidum*, FEBS Lett. 365 (1995) 30–34.
[9] H. Oh-oka, M. Iwaki, S. Itoh, Membrane-bound cytochrome cz couples quinol oxidoreductase to the P840 reaction center complex in isolated membranes of the green sulfur bacterium *Chlorobium tepidum*, Biochemistry 37 (1998) 12293–12300.
[10] Y. Tsukatani, C. Azai, T. Kondo, S. Itoh, H. Oh-Oka, Parallel electron donation pathways to cytochrome c(z) in the type I homodimeric photosynthetic reaction center complex of *Chlorobium tepidum*, Biochim. Biophys. Acta 1777 (2008) 1211–1217.
[11] H. Oh-oka, M. Iwaki, S. Itoh, Viscosity dependence of the electron transfer rate from bound cytochrome c to P840 in the photosynthetic reaction center of the green sulfur bacterium *Chlorobium tepidum*, Biochemistry 36 (1997) 9267–9272.
[12] M. Higuchi, Y. Hirano, Y. Kimura, H. Oh-oka, K. Miki, Z.Y. Wang, Overexpression, characterization, and crystallization of the functional domain of cytochrome c(z) from *Chlorobium tepidum*, Photosynth. Res. 102 (2009) 77–84.
[13] Y. Hirano, M. Higuchi, C. Azai, H. Oh-Oka, K. Miki, Z.Y. Wang, Crystal structure of the electron carrier domain of the reaction center cytochrome c(z) subunit from green photosynthetic bacterium *Chlorobium tepidum*, J. Mol. Biol. 397 (2010) 1175–1187.
[14] J.M. Olson, The FMO Protein, Photosynth. Res. 80 (2004) 181–187.
[15] R.E. Fenna, B.W. Matthews, Chlorophyll arrangement in a bacteriochlorophyll protein from *Chlorobium limicola*, Nature 258 (1975) 573–577.
[16] D.E. Tronrud, J. Wen, L. Gay, R.E. Blankenship, The structural basis for the difference in absorbance spectra for the FMO antenna protein from various green sulfur bacteria, Photosynth. Res. 100 (2009) 79–87.

[17] Y. Tsukatani, R. Miyamoto, S. Itoh, H. Oh-Oka, Function of a PscD subunit in a homodimeric reaction center complex of the photosynthetic green sulfur bacterium Chlorobium tepidum studied by insertional gene inactivation. Regulation of energy transfer and ferredoxin-mediated NADP + reduction on the cytoplasmic side, J. Biol. Chem. 279 (2004) 51122–51130.
[18] P. Heathcote, M.R. Jones, P.K. Fyfe, Type I photosynthetic reaction centres: structure and function, Philos. Trans. R. Soc. Lond. B Biol. Sci. 358 (2003) 231–243.
[19] B. Kjaer, N.U. Frigaard, F. Yang, B. Zybailov, M. Miller, J.H. Golbeck, H.V. Scheller, Menaquinone-7 in the reaction center complex of the green sulfur bacterium *Chlorobium vibrioforme* functions as the electron acceptor A1, Biochemistry 37 (1998) 3237–3242.
[20] N. Kusumoto, P. Setif, K. Brettel, D. Seo, H. Sakurai, Electron transfer kinetics in purified reaction centers from the green sulfur bacterium *Chlorobium tepidum* studied by multiple-flash excitation, Biochemistry 38 (1999) 12124–12137.
[21] S. Takaichi, H. Oh-oka, Pigment composition in the reaction center complex from the thermophilic green sulfur bacterium. *Chlorobium tepidum*: carotenoid glucoside esters, menaquinone and chlorophylls, Plant Cell Physiol. 40 (1999) 691–694.
[22] A. van der Est, C. Hager-Braun, W. Leibl, G. Hauska, D. Stehlik, Transient electron paramagnetic resonance spectroscopy on green-sulfur bacteria and heliobacteria at two microwave frequencies, Biochim. Biophys. Acta 1409 (1998) 87–98.
[23] M. Iwaki, S. Itoh, S. Kamei, H. Matsubara, H. Oh-oka, Time-resolved spectroscopy of chlorophyll-a like electron acceptor in the reaction center complex of the green sulfur bacterium *Chlorobium tepidum*, Plant Cell Physiol. 40 (1999) 1021–1028.
[24] P. Heathcote, P.K. Fyfe, M.R. Jones, Reaction centres: the structure and evolution of biological solar power, Trends Biochem. Sci. 27 (2002) 79–87.
[25] H. Oh-oka, Type 1 reaction center of photosynthetic heliobacteria, Photochem. Photobiol. 83 (2007) 177–186.
[26] R. Miyamoto, H. Mino, T. Kondo, S. Itoh, H. Oh-Oka, An electron spin-polarized signal of the P800 + A1(Q)- state in the homodimeric reaction center core complex of *Heliobacterium modesticaldum*, Biochemistry 47 (2008) 4386–4393.
[27] B.A. Diner, F. Rappaport, Structure, dynamics, and energetics of the primary photochemistry of photosystem II of oxygenic photosynthesis, Annu. Rev. Plant Biol. 53 (2002) 551–580.
[28] M.C. Wakeham, M.R. Jones, Rewiring photosynthesis: engineering wrong-way electron transfer in the purple bacterial reaction centre, Biochem. Soc. Trans. 33 (2005) 851–857.
[29] A.N. Webber, V.M. Ramesh, Mutagenesis of Ligands to the Cofactors in Photosystem I, in: J.H. Golbeck (Ed.), Photosystem I: The Light-Driven Plastcyanin:Ferredoxin Oxidoreductase, Springer, 2006, pp. 193–204.
[30] Y. Li, A. van der Est, M.G. Lucas, V.M. Ramesh, F. Gu, A. Petrenko, S. Lin, A.N. Webber, F. Rappaport, K. Redding, Directing electron transfer within Photosystem I by breaking H-bonds in the cofactor branches, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 2144–2149.
[31] S. Santabarbara, K. Reifschneider, A. Jasaitis, F. Gu, G. Agostini, D. Carbonera, F. Rappaport, K.E. Redding, Interquinone electron transfer in photosystem I as evidenced by altering the hydrogen bond strength to the phylloquinone(s), J. Phys. Chem. B 114 (2010) 9300–9312.
[32] N.U. Frigaard, D.A. Bryant, Chromosomal gene inactivation in the green sulfur bacterium Chlorobium tepidum by natural transformation, Appl. Environ. Microbiol. 67 (2001) 2538–2544.
[33] N.U. Frigaard, D.A. Bryant, Seeing green bacteria in a new light: genomics-enabled studies of the photosynthetic apparatus in green sulfur bacteria and filamentous anoxygenic phototrophic bacteria, Arch. Microbiol. 182 (2004) 265–276.
[34] J.A. Eisen, K.E. Nelson, I.T. Paulsen, J.F. Heidelberg, M. Wu, R.J. Dodson, R. Deboy, M.L. Gwinn, W.C. Nelson, D.H. Haft, E.K. Hickey, J.D. Peterson, A.S. Durkin, J.L. Kolonay, F. Yang, I. Holt, L.A. Umayam, T. Mason, M. Brenner, T.P. Shea, D. Parksey, W.C. Nierman, T.V. Feldblyum, C.L. Hansen, M.B. Craven, D. Radune, J. Vamathevan, H. Khouri, O. White, T.M. Gruber, K.A. Ketchum, J.C. Venter, H. Tettelin, D.A. Bryant, C.M. Fraser, The complete genome sequence of Chlorobium tepidum TLS, a photosynthetic, anaerobic, green-sulfur bacterium, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 9509–9514.
[35] E.C. Abresch, H.L. Axelrod, J.T. Beatty, J.A. Johnson, R. Nechushtai, M.L. Paddock, Characterization of a highly purified, fully active, crystallizable RC-LH1-PufX core complex from *Rhodobacter sphaeroides*, Photosynth. Res. 86 (2005) 61–70.
[36] T.M. Bricker, J. Morvant, N. Masri, H.M. Sutton, L.K. Frankel, Isolation of a highly active photosystem II preparation from Synechocystis 6803 using a histidine-tagged mutant of CP 47, Biochim. Biophys. Acta 1409 (1998) 50–57.
[37] M. Cullen, N. Ray, S. Husain, J. Nugent, J. Nield, S. Purton, A highly active histidine-tagged *Chlamydomonas reinhardtii* Photosystem II preparation for structural and biophysical analysis, Photochem. Photobiol. Sci. 6 (2007) 1177–1183.
[38] H. Fey, D. Piano, R. Horn, D. Fischer, M. Schmidt, S. Ruf, W.P. Schroder, R. Bock, C. Buchel, Isolation of highly active photosystem II core complexes with a His-tagged Cyt b559 subunit from transplastomic tobacco plants, Biochim. Biophys. Acta 1777 (2008) 1501–1509.
[39] J.O. Goldsmith, S.G. Boxer, Rapid isolation of bacterial photosynthetic reaction centers with an engineered poly-histidine tag, Biochim. Biophys. Acta 1276 (1996) 171–175.
[40] G. Gulis, K.V. Narasimhulu, L.N. Fox, K.E. Redding, Purification of His6-tagged Photosystem I from *Chlamydomonas reinhardtii*, Photosynth. Res. 96 (2008) 51–60.
[41] H. Kubota, I. Sakurai, K. Katayama, N. Mizusawa, S. Ohashi, M. Kobayashi, P. Zhang, E.M. Aro, H. Wada, Purification and characterization of photosystem I complex from *Synechocystis* sp. PCC 6803 by expressing histidine-tagged subunits, Biochim. Biophys. Acta 1797 (2010) 98–105.
[42] M. Sugiura, Y. Inoue, J. Minagawa, Rapid and discrete isolation of oxygen-evolving His-tagged photosystem II core complex from *Chlamydomonas reinhardtii* by Ni2+ affinity column chromatography, FEBS Lett. 426 (1998) 140–144.
[43] M. Sugiura, F. Rappaport, K. Brettel, T. Noguchi, A.W. Rutherford, A. Boussac, Site-directed mutagenesis of *Thermosynechococcus elongatus* photosystem II: the O2-evolving enzyme lacking the redox-active tyrosine D, Biochemistry 43 (2004) 13549–13563.
[44] H. Tang, P.R. Chitnis, Addition of C-terminal histidyl tags to PsaL and PsaK1 proteins of cyanobacterial photosystem I, Indian J. Biochem. Biophys. 37 (2000) 433–440.
[45] S.C. Kowalczykowski, D.A. Dixon, A.K. Eggleston, S.D. Lauder, W.M. Rehrauer, Biochemistry of homologous recombination in *Escherichia coli*, Microbiol. Rev. 58 (1994) 401–465.
[46] T.M. Wahlund, M.T. Madigan, Genetic transfer by conjugation in the thermophilic green sulfur bacterium Chlorobium tepidum, J. Bacteriol. 177 (1995) 2583–2588.
[47] H.D. Schweizer, Small broad-host-range gentamycin resistance gene cassettes for site-specific insertion and deletion mutagenesis, Biotechniques 15 (1993) 831–834.
[48] P. Prentki, H.M. Krisch, In vitro insertional mutagenesis with a selectable DNA fragment, Gene 29 (1984) 303–313.
[49] N.U. Frigaard, Y. Sakuragi, D.A. Bryant, Gene inactivation in the cyanobacterium Synechococcus sp. PCC 7002 and the green sulfur bacterium *Chlorobium tepidum* using in vitro-made DNA constructs and natural transformation, Methods Mol. Biol. 274 (2004) 325–340.
[50] J.A. Maresca, A. Gomez Maqueo Chew, M.R. Ponsati, N.U. Frigaard, J.G. Ormerod, D.A. Bryant, The bchU gene of *Chlorobium tepidum* encodes the c-20 methyltransferase in bacteriochlorophyll c biosynthesis, J. Bacteriol. 186 (2004) 2558–2566.
[51] H. Oh-oka, S. Kakutani, H. Matsubara, R. Malkin, S. Itoh, Isolation of the photoactive reaction center complex that contains three types of Fe–S centers and a cytochrome *c* subunit from the green sulfur bacterium *Chlorobium limicola* f. *thiosulfatophilum*, strain Larsen, Plant Cell Physiol. 34 (1993) 93–101.
[52] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[53] I. Erill, S. Campoy, J. Barbe, Aeons of distress: an evolutionary perspective on the bacterial SOS response, FEMS Microbiol. Rev. 31 (2007) 637–656.
[54] J.M. Olson, K.D. Philipson, K. Sauer, Circular dichroism and absorption spectra of bacteriochlorophyll-protein and reaction center complexes from *Chlorobium thiosulfatophilum*, Biochim. Biophys. Acta 292 (1973) 206–217.
[55] T.E. Meyer, R.G. Bartsch, M.A. Cusanovich, J.H. Mathewson, The cytochromes of *Chlorobium thiosulfatophilum*, Biochim. Biophys. Acta 153 (1968) 854–861.
[56] C. Francke, H.P. Permentier, E.M. Franken, S. Neerken, J. Amesz, Isolation and properties of photochemically active reaction center complexes from the green sulfur bacterium *Prosthecochloris aestuarii*, Biochemistry 36 (1997) 14167–14172.
[57] U. Feiler, W. Nitschke, H. Michel, Characterization of an improved reaction center preparation from the photosynthetic green sulfur bacterium Chlorobium containing the FeS centers FA and FB and a bound cytochrome subunit, Biochemistry 31 (1992) 2608–2614.
[58] W. Nitschke, U. Feiler, A.W. Rutherford, Photosynthetic reaction center of green sulfur bacteria studied by EPR, Biochemistry 29 (1990) 3834–3842.
[59] I.R. Vassiliev, M.T. Ronan, G. Hauska, J.H. Golbeck, The bound electron acceptors in green sulfur bacteria: resolution of the g-tensor for the F(X) iron–sulfur cluster in *Chlorobium tepidum*, Biophys. J. 78 (2000) 3160–3169.
[60] A.J. Clark, A.D. Margulis, Isolation and characterization of recombination-deficient strains of *Escherichia coli* K-12, Proc. Natl. Acad. Sci. U.S.A. 53 (1965) 451–459.
[61] I.P. Chen, H. Michel, Cloning, sequencing, and characterization of the recA gene from *Rhodopseudomonas viridis* and construction of a recA strain, J. Bacteriol. 180 (1998) 3227–3232.
[62] R. Minda, J. Ramchandani, V.P. Joshi, S.K. Bhattacharjee, A homozygous recA mutant of Synechocystis PCC6803: construction strategy and characteristics eliciting a novel RecA independent UVC resistance in dark, Mol. Genet. Genomics 274 (2005) 616–624.
[63] H. Li, S. Jubelirer, A.M. Garcia Costas, N.U. Frigaard, D.A. Bryant, Multiple antioxidant proteins protect *Chlorobaculum tepidum* against oxygen and reactive oxygen species, Arch. Microbiol. 191 (2009) 853–867.
[64] A. Skerra, T.G. Schmidt, Applications of a peptide ligand for streptavidin: the Strep-tag, Biomol. Eng. 16 (1999) 79–86.