BioMetals **14:** 135–142, 2001.

Cloning and expression of the ferredoxin gene from extremely halophilic archaeon *Haloarcula japonica* strain TR-1

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Received 10 January 2001; accepted 6 March 2001

Key words: extremely halophilic archaeon, ferredoxin, gene cloning, gene expression, *Haloarcula japonica*, iron-sulfur cluster

Abstract

The gene encoding a ferredoxin (Fd) from *Haloarcula japonica* strain TR-1 was cloned and sequenced. Sequence analysis of the cloned *Ha. japonica* Fd gene revealed that the structural gene consisted of an open reading frame of 387 nucleotides encoding 129 amino acids. The deduced amino acid sequence of *Ha. japonica* Fd showed 84 to 98% identity with corresponding sequences in other extremely halophilic archaea. The *Ha. japonica* Fd gene was inserted into the shuttle vector pWL102 and used to transform *Ha. japonica*. *Ha. japonica* Fd could then be produced as a fusion with His·Tag (6xHis) in *Ha. japonica* host cells. The absorption and ESR spectra of the Fd/His·Tag fusion protein revealed the presence of a [2Fe-2S] cluster which is characteristic of native *Ha. japonica* Fd.

Abbreviations: haloarchaeon – extremely halophilic archaeon; Fd – ferredoxin; Fe-S – iron-sulfur; PCR – polymerase chain reaction; SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CBB – Coomassie brilliant blue R-250; IgG – immunoglobulin G; HRP – horse radish peroxidase.

Introduction

Ferredoxins (Fds) are ubiquitous small proteins involved in electron transfer reactions in a variety of metabolic pathways. They contain iron-sulfur (Fe-S) clusters of one of two different configurations: the [2Fe-2S] or the [4Fe-4S] ([3Fe-4S]) type. While some [2Fe-2S] Fds have been isolated from plants, algae and cyanobacteria, most of the described Fds are from (eu)bacteria (Hall *et al.* 1974; Thomson 1985).

Extremely halophilic archaea (haloarchaea) are extremophiles which can grow in hypersaline environments. They are currently divided into 15 genera including *Halobacterium, Haloarcula* and *Haloferax* (Dyall-Smith 2000). Haloarchaeal Fds from *Halobacterium salinarum* (formerly *Halobacterium halobium*) (Kerscher *et al.* 1976) and *Haloarcula marismortui* (formerly *Halobacterium* of the Dead Sea) (Werber & Mevarech 1978) have been purified and characterized. In all three cases, it was found that these Fds contain [2Fe-2S] clusters. While Fd proteins were characterized over 20 years ago, only recently was the Fd gene cloned from *Hb. salinarum* (Pfeifer *et al.* 1993) and *Haloferax volcanii* (Mevarech *et al.* 2000), and its crystal structure reported for *Ha. marismortui* (Frolow *et al.* 1996). The structural basis of protein halophilicity, however, remains unstudied.

Haloarcula japonica strain TR-1, isolated from a saltern soil at Noto peninsula in Japan, is a triangular disc-shaped haloarchaeon (for review see Horikoshi

et al. 1993). Its cell division (Hamamoto *et al.* 1988), taxonomy (Takashina *et al.* 1990), cell surface ultrastructure (Nishiyama *et al.* 1992), a cell surface glycoprotein (Nakamura *et al.* 1992; Nakamura *et al.* 1995; Wakai *et al.* 1997) and a proton pump (Yatsunami *et al.* 2000) have been studied extensively. Recently, a Fd was purified and characterized from *Ha. japonica* (Sugimori *et al.* 2000). The purified *Ha. japonica* Fd was found to contain a [2Fe-2S] cluster. The amino acid sequence of the *N*-terminal 30 residues was determined. These data have enabled us to clone and express the *Ha. japonica* Fd gene to facilitate future research on the structure-function relationship of *Ha. japonica* Fd. As a stepping-stone, we characterize a His-tagged recombinant Fd produced by *Ha. japonica*.

Materials and methods

Strains, plasmids, media and culture conditions

Haloarcula japonica strain TR-1 (JCM 7785) and *Haloferax volcanii* strain WDF11 (DSM 5716) were grown at 37 °C in liquid media as described previously (Nishiyama *et al.* 1995; DasSarma *et al.* 1995). *Escherichia coli* strain JM109 (Toyobo, Osaka, Japan) was cultivated at 37 °C in L broth (Sambrook *et al.* 1989). Plasmids pBluescript II SK(+) and pUC119, and pGEM-T Easy Vector System were obtained from Toyobo, Takara Shuzo (Kyoto, Japan) and Promega (Madison, Wisconsin, USA), respectively. Plasmid pWL102 is an *E. coli*-haloarchaea shuttle vector (Lam & Doolittle, 1989).

Isolation of chromosomal DNA

Ha. japonica chromosomal DNA was prepared according to the method described by Lechner and Sumper (1987).

Amplification of DNA by PCR

Polymerase chain reaction (PCR) primers were synthesized using an Applied Biosystems (Foster City, California, USA) model 391 (PCR-MATE) DNA synthesizer. The *TaKaRa Ex Taq* DNA polymerase used to amplify the DNA was purchased from Takara Shuzo. PCRs were carried out in Gene Amp PCR Systems 2400 and 9700 (Perkin-Elmer/Cetus, Forester City, California, USA).

Recombinant DNA techniques

Restriction endonucleases and T4 DNA ligase were used as specified by manufacturers (Takara Shuzo and Toyobo). Southern and colony hybridizations were performed according to the standard protocols (Sambrook *et al.* 1989) using a DIG DNA Labeling and Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany). DNA sequencing was carried out by the dideoxy chain termination method of Sanger *et al.* (1977) with a Shimadzu (Kyoto, Japan) model DSQ-2000L sequencer. Site-directed mutagenesis was done using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California, USA).

DNA sequence accession number and computer analysis

The DNA sequence data reported in this paper will appear in the DNA Data Bank of Japan (DDBJ), European Molecular Biology Laboratory (EMBL) and GenBank nucleotide sequence databases under the accession number AB029321. The DNA and predicted amino acid sequences were analyzed with the GENETYX-MAC set of programs (Software Development, Tokyo, Japan).

Transformation of haloarchaea

Transformations of *Hf. volcanii* and *Ha. japonica* were performed using the polyethylene glycol method (Charlebois *et al.* 1987; Cline *et al.* 1989; Dyall-Smith 2000) with some modifications (Yatsunami *et al.* unpublished). Transformants were selected on agar plates containing 8 μg/ml and 1 μg/ml plavastatin (a gift from Sankyo Co., Ltd., Tokyo, Japan) instead of mevinolin for *Ha. japonica* and *Hf. volcanii*, respectively.

SDS-PAGE and Western blotting analyses

Sodium dodecyl sulfate polyacrylamide (10%) gel electrophoresis (SDS-PAGE) was done following the method of Laemmli (1970). Prestained SDS-PAGE standards (low range, Bio-Rad, Hercules, California, USA) were used as molecular mass markers. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 (CBB).

Ha. japonica Fd was purified and then used for immunization of a rabbit by standard procedures (Ausubel *et al.* 1995). A mouse monoclonal antibody raised against His·Tag (6xHis) was purchased from

R & D Systems (Mineapolis, Minnesota, USA). Cell extracts of *Ha. japonica* transformants were prepared by sonication and then applied to SDS-PAGE. By employing the Western blotting method, proteins in the gel were transferred to a nitrocellulose filter (Burnette 1981). Recombinant Fd was stained using an antiserum against *Ha. japonica* Fd and an antibody against His·Tag as the primary antibodies, and anti-rabbit and anti-mouse immunoglobulin G (IgG) horse radish peroxidase (HRP) conjugates as the secondary antibodies. Protein A HRP conjugate (Bio-Rad) was also used with anti-His·Tag and anti-rabbit IgG HRP conjugates to enhance intensity of the assay.

Purification of recombinant Fd

All steps were performed at room temperature. Cell extracts of *Ha. japonica* that contained the expression vector were prepared according to Sugimori *et al.* (2000). The obtained cell extracts were dialyzed against 0.15 M Tris-HCl (pH 7.0) and loaded into a Q-Sepharose FF (Pharmacia, Uppsala, Sweden) column equilibrated with the same buffer. The adsorbed proteins were eluted using a linear gradient from 0 to 1 M NaCl in the same buffer used for dialysis. The Fd-containing (i.e. red colored) fractions were pooled and concentrated by ultrafiltration using an Amicon (Beverly, Massachusetts, USA) Diaflo with a PM10 membrane. The concentrated proteins were then applied to a Sephacryl S-200 HR (Pharmacia) column equilibrated with 0.15 M Tris-HCl (pH 7.0) and 0.1 M NaCl. The collected Fd-containing fractions were again concentrated by ultrafiltration, and then loaded into an Ni^{2+}-immobilized HiTrap Chelating (Pharmacia) column equilibrated with 0.02 M $Na_2HPO_4{\cdot}2H_2O$-$NaH_2PO_4{\cdot}H_2O$ (pH 7.4), 0.5 M NaCl, and 0.01 M imidazole. The column was washed continuously with ten volumes of the same buffer. The adsorbed proteins were then eluted with 0.02 M $Na_2HPO_4{\cdot}2H_2O$-$NaH_2PO_4{\cdot}H_2O$ (pH 7.4), 0.5 M NaCl, and 0.5 M imidazole. The Fd fractions were stored at 4 °C.

Protein concentrations were estimated by the BCA method (Pierce Chemicals, Rockford, Illinois, USA) with bovine serum albumin, fraction V (Pierce Chemicals) as a standard.

Spectroscopy

Absorption spectra were recorded on a Hitachi U-2000 spectrophotometer. An ESR spectrum was estimated by a JEOL (Tokyo, Japan) JES-RE3X spectrometer interfaced to an ESPRIT 375 ESR Data System, and equipped with a Liquid transfer HELI-TRAN model LTR-3 (APD Cryogenics, Allentown, Pennsylvania, USA). Absorption and ESR spectra were measured in 0.15 M Tris-HCl (pH 7.0) containing 0.33 NaCl and 3.1 M KCl. The samples were degassed under vacuum, replaced with argon gas, and then reduced by adding an oxygen-free solution of sodium dithionite.

Results and discussion

Generation of a DNA fragment by PCR

To generate a probe for cloning of the *Ha. japonica* Fd gene, PCR was performed using the chromosomal DNA of *Ha. japonica* as a template. The sense primer [5′-AA(T/C)TA(T/C)GA(A/G)GT(T/C/A/G)GT(T/C/A/G)GA(T/C)GA(T/C)AA(T/C)GG-3′] was designed from the *N*-terminal amino acid sequence of *Ha. japonica* Fd; the antisense primer [5′-GGCCA(G/A)TC(G/A)TA(G/A/C/T)CC(C/T)TG(G/A/C/T)GC-3′] was based on a polypeptide region conserved between *Hb. salinarum* and *Ha. marismortui* Fds (corresponding to the amino acid sequence Ala51-Pro60 in both Fds). The PCR product of an expected 160 bp, was first cloned into pBluescript II SK(+) and then sequenced. Since part of the PCR-generated DNA fragment had a deduced amino acid sequence that coincided with the *N*-terminal amino acid sequence of *Ha. japonica* Fd, the PCR product was concluded to have been derived from the *Ha. japonica* Fd gene.

Cloning of the Ha. japonica *Fd gene*

Chromosomal DNA of *Ha. japonica* was digested with several restriction enzymes and analyzed by Southern hybridization using the PCR fragment as a probe. Chromosomal DNA digested with *Sal* I showed a single hybridization band of about 3.1 kb. DNA fragments of about 3 kb were isolated from *Sal* I-digested chromosomal DNA by agarose gel purification, followed by ligation into the *Sal* I site of pUC119 and introduction into *E. coli*. Transformants were screened for the *Ha. japonica* Fd gene by colony hybridization. One positive clone was obtained from 230 colonies and were found to contain a recombinant plasmid with a 3.1 kb chromosomal insert. This plasmid was designated pJAF2.

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   1  CGCCTGAGTGGTAATCGGCCCCGAACAACTGCCGTCAGTTTCCGACGAATCCCCGCGTTG   60

  61  TCCCTCATTATTTTGCACGGCCTAAAACCGAAGGGCAAAGTCTAACAGGGCGGGGTTCGA  120

 121  ACCAATCAGGTGATGCCCACGGTAGAGTACCTTAACTACGAAGTAGTGGACGATAACGGC  180
                 M  P  T  V  E  Y  L  N  Y  E  V  V  D  D  N  G
 181  TGGGACATGTACGACGACGACGTCTTCGCAGAGGCGTCAGATATGGACCTCGACGGTGAG  240
      W  D  M  Y  D  D  D  V  F  A  E  A  S  D  M  D  L  D  G  E
 241  GACTACGGGTCCCTCGAGGTGAACGAAGGCGAGTACATCCTGGAAGCCGCCGAGGCGCAG  300
      D  Y  G  S  L  E  V  N  E  G  E  Y  I  L  E  A  A  E  A  Q
 301  GGCTACGACTGGCCCTTCTCGTGTCGCGCCGGTGCCTGTGCGAACTGTGCCGCCATCGTT  360
      G  Y  D  W  P  F  S  C  R  A  G  A  C  A  N  C  A  A  I  V
 361  CTCGAAGGCGACATCGACATGGACATGCAGCAGATCCTCAGCGACGAGGAAGTCGAAGAC  420
      L  E  G  D  I  D  M  D  M  Q  Q  I  L  S  D  E  E  V  E  D
 421  AAGAACGTTCGCCTGACCTGTATCGGCAGCCCGGACGCCGACGAGGTCAAGATCGTCTAC  480
      K  N  V  R  L  T  C  I  G  S  P  D  A  D  E  V  K  I  V  Y
 481  AACGCCAAGCACCTCGATTACCTGCAGAACCGCGTCATCTAACGCGGCTCCTGCAGCGCA  540
      N  A  K  H  L  D  Y  L  Q  N  R  V  I  *
 541  CTACACTTTCTTCTTCCAGCAGCAACTGCATCCGACAGCCGTCGTACCGGAACCATTAGG  600

 601  GGCACCCTGTATTGCGTATAGCCGTGCTCGGGTCGATACCGGACCTGCTTCGGCTGGTCG  660
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Fig. 1. Nucleotide sequence of the *Ha. japonica* Fd gene with its deduced amino acid sequence. The nucleotide sequence was determined in forward and reverse directions. Numbers denote nucleotide positions. The *N*-terminal amino acid sequence that was determined from purified *Ha. japonica* Fd is underlined.

DNA sequence and protein structure

The DNA sequence of the cloned fragment revealed that the *Ha. japonica* Fd gene contains an open reading frame of 387 nucleotides that are predicted to encode a polypeptide of 129 amino acids (Figure 1). The deduced amino acid sequence at the *N*-terminus is identical to that determined by protein sequencing, assuming a processing the initial methionine. The amino acid composition, as predicted from the nucleotide sequence, corresponds to that determined by direct amino acid analysis of purified *Ha. japonica* Fd (Sugimori *et al.* 2000). However, the calculated molecular mass of 14,362 Da is much lower than that observed after SDS-PAGE (30 kDa; see Figure 4, lane 1). It is plausible that the discrepancy is due to the reduced ability of *Ha. japonica* Fd to bind SDS because of its unusual acidic amino acid content. In support of this notion, reduction in electrophoretic mobility due to high acidic amino acid content has been observed in other haloarchaeal proteins (Wakai *et al.* 1997).

The primary structures of *Ha. japonica* Fd, four other haloarchaeal Fds, and a non-halophilic [2Fe-2S] Fd from spinach, are aligned in Figure 2. *Ha. japonica* Fd showed about 98%, 89%, 84% and 29% identity in amino acid sequence to Fds from *Ha. marismortui* (Kerscher *et al.* 1976), *Hb. salinarum* (Werber & Mevarech 1978), *Hf. volcanii* (Mevarech *et al.* 2000) and spinach (Werber & Mevarech 1978), respectively. Four cysteine residues in *Ha. japonica* Fd most likely serve as ligands to the iron atoms of the [2Fe-2S] cluster. The relative positions of these four cysteine residues at positions 63, 68, 71 and 102 are the same as those in other haloarchaeal and spinach Fds. A recent X-ray crystallographic analysis of *Ha. marismortui* Fd (Frolow *et al.* 1996) has shown that its [2Fe-2S] cluster is covalently bound to the sulfur atoms at Cys63, Cys68, Cys71 and Cys102.

The multiple alignment also shows that *Ha. japonica* Fd and other haloarchaeal Fds have an *N*-terminal domain of 24 amino acids, as well as a *C*-terminal region of 6 amino acids, that are not present in spinach Fd. Spinach Fd is an acidic protein carrying a negative net charge of −20. Haloarchaeal Fds are even more acidic, having negative net charges of −30 to −34. These relatively higher negative charges are due to the additional *N*-terminal domains. To maintain biological function under hypersaline conditions, the proteins of halophilic organisms must compete with the excess inorganic ions for water binding. The *N*-terminal domain conserved in haloarchaeal Fds may provide the means for larger solvent-accessible surface areas, thus enabling efficient water binding in their hypersaline biological niches. This idea is supported by the

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                    1         10          20          30          40
Ha. japonica        PTVEY LNYEV VDDNG WDMYD DDVFA EASDM DLDGE DYGSL
Ha. marismortui     PTVEY LNYEV VDDNG WDMYD DDVFG EASDM DLDDE DYGSL
Hb. salinarum       PTVEY LNYET LDDQG WDMDD DDLFE KAADA GLDGE DYGTM
Hf. volcanii        PTVEY LNYEV LDDNG WDLDD DGLFE QAADA GLDAE DYGEM
Spinach             ----- ----- ----- ----- ----A AYKVT LVTPT GNVEF

                         50          60   *     *70 *         80
Ha. japonica        EVNEG EYILE AAEAQ GYDWP FSCRA GACAN CAAIV LEGDI
Ha. marismortui     EVNEG EYILE AAEAQ GYDWP FSCRA GACAN CAAIV LEGDI
Hb. salinarum       EVAEG EYILE AAEAQ GYDWP FSCRA GACAN CASIV KEGEI
Hf. volcanii        EVNQG EYILE AAEAQ GYDWP FSCRA GACAN CAAIV KEGEI
Spinach             QCPDD VYILD AAEEE GIDLP YSCRA GSCSS CAGKL KTGSL

                         90          100  *      110         120
Ha. japonica        DMDMQ QILSD EEVED KNVRL TCIGS PDADE VKIVY NAKHL
Ha. marismortui     DMDMQ QILSD EEVED KNVRL TCIGS PDADE VKIVY NAKHL
Hb. salinarum       DMDMQ QILSD EEVEE KDVRL TCIGS PAADE VKIVY NAKHL
Hf. volcanii        DMDMQ QILSD EEVNE KNVRL TCIGS PVEDE VKIIY NAKHL
Spinach             NQDDQ SFLDD DQIDE GWV-L TCAAY PVSDV TIETH KEEEL

                    128
Ha. japonica        DYLQN RVI
Ha. marismortui     DYLQN RVI
Hb. salinarum       DYLQN RVI
Hf. volcanii        DYLQN RVI
Spinach             TA--- ---
```

Fig. 2. Multiple amino acid sequence alignment of *Ha. japonica* Fd, other haloarchaeal Fds (e.g. *Ha. marismortui*, *Hb. salinarum* and *Hf. volcanii*), and a non-halophilic spinach Fd. Cysteine residues thought to be involved in cluster binding are marked by asterices.

finding that aspartic acid and glutamic acid are good water binders (Kuntz 1971); both of which are found abundant in the *N*-terminal region of *Ha. japonica* Fd.

Expression of the Ha. japonica *Fd gene in haloarchaea*

First, expression of the *Ha. japonica* Fd gene in *E. coli* was performed. *Ha. japonica* Fd was produced as a fusion with glutathione *S*-transferase in *E. coli*. The resulting fusion protein, however, did not contain a [2Fe-2S] cluster (data not shown). Since a [2Fe-2S] cluster-containing non-halophilic Fd from *Chlamydomonas reinhardtii* has been produced in an *E. coli* system (Rogers *et al.* 1992), it is plausible that high salt concentrations are required for Fe-S cluster assembly with *Ha. japonica* Fd.

Guided by this notion, the expression of the *Ha. japonica* Fd gene was tried in haloarchaea. A mutant *Ha. japonica* Fd gene encoding the *C*-terminally His-tagged Fd was constructed by site-directed mutagenesis using the following mutation primer sets: 5′-CAGAACCGCGTCATCCACCACCACCACCAC CACTAAAGCTGCTCCTGCATCGCA-3′ and 5′-TG CGCTGCAGGAGCAGCTTTAGTGGTGGTGGTGG TGGTGGATGACGCGGTTCTG-3′. In this construct, six histidine codons were inserted between the *Ha. japonica* Fd structural gene and its stop codon. Next, a *Bam* HI restriction site was introduced downstream of the His-tagged Fd gene by PCR using an M13 sense primer (5′-AGTCACGACGTTGTA-3′) and a mutation-generating antisense primer (5′-CGGGATCCCGTCGACACGAATATCAGCGA-3′). The PCR product was subcloned into pGEM-T Easy vector, digested with *Bam* HI, and then ligated into the *Bam* HI site of the shuttle vector pWL102 to obtain pJAF18.

The novel haloarchaeal expression plasmid, pJAF18, was introduced into both *Ha. japonica* and *Hf. volcanii*. Cell extracts of the successful transformants were separated by SDS-PAGE and then West-

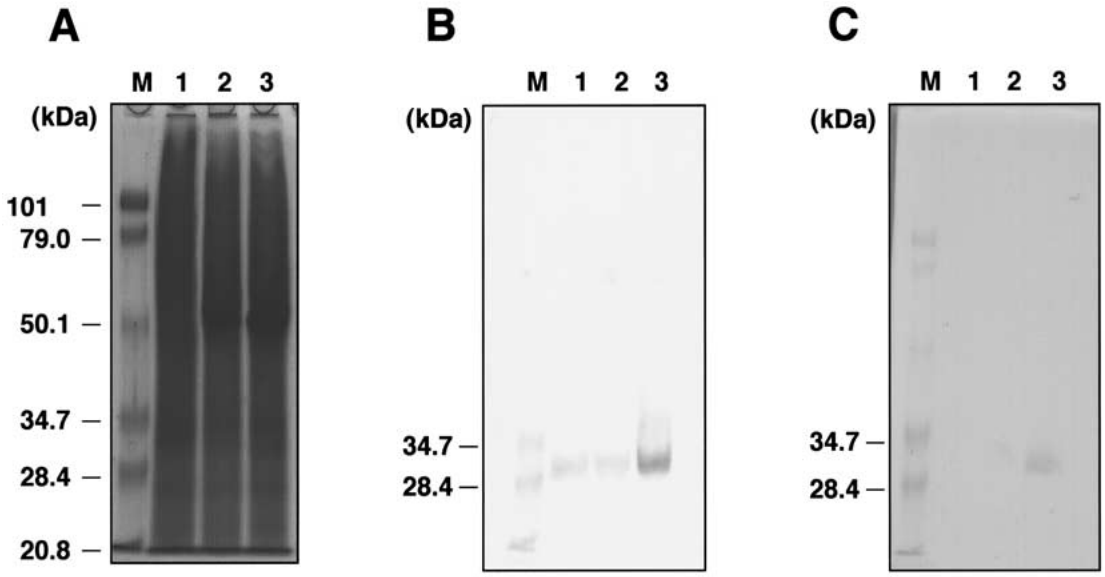

Fig. 3. SDS-PAGE of the cell extracts of *Ha. japonica* transformants. Ten percent (w/v) polyacrylamide gels were used. After electrophoresis, the gels were stained with CBB (panel A), or subjected to Western blotting using anti-*Ha. japonica* Fd (panel B) or anti-His·Tag antibodies (panel C). Lanes 1, cell extracts of *Ha. japonica*; lanes 2, cell extracts of *Ha. japonica* carrying pWL102; lanes 3, cell extracts of *Ha. japonica* carrying pJAF18. M indicates the molecular mass markers.

ern blotted. By this type of immunological detection of His-tagged *Ha. japonica* Fd in cell extracts of *Ha. japonica* transformants which employed anti-*Ha. japonica* Fd or anti-His·Tag antibody, a positive band at about 30 kDa was revealed (Figure 3B, lane 3 and Figure 3C, lane 3). On the other hand, no positive bands were detected from cell extracts of *Hf. volcanii* transformants as evidenced by Western blotting analyses (data not shown). Therefore, it is suggested that the promoter sequence of the *Ha. japonica* Fd gene works more efficiently in *Ha. japonica* than in *Hf. volcanii*.

Purification and characterization of the His-tagged recombinant Fd

The His-tagged recombinant Fd was purified from cell extracts of *Ha. japonica* that carried pJAF18. When cell extracts were applied directly to Ni^{2+}-chelating columns, not only the His-tagged Fd, but also some other proteins were adsorbed to the column. This required the His-tagged recombinant Fd and the native Fd to be once co-purified using the anion-exchange chromatography and gel filtration methods, before the His-tagged Fd could be separated from the native Fd by affinity chromatography. The purified His-tagged Fd showed a single protein band at about 30 kDa on SDS-PAGE (Figure 4, lane 2). About 2.2 mg of purified His-tagged recombinant Fd was obtained from 80 g (wet-weight) of transformant cells. The purity index (A_{420}/A_{280}) of the final preparation was 0.28. This value supports a previously reported A_{420}/A_{280} value (0.29) for purified native *Ha. japonica* Fd (Sugimori *et al.* 2000).

Haloarchaea are known to contain very high concentrations of K^+ (about 3 M), instead of Na^+, in their cytoplasm (Lanyi 1974; Meseguer *et al.* 1995). Therefore, absorption and ESR spectra of the His-tagged recombinant Fd were measured in a buffer containing 3.1 M KCl. The absorption spectral patterns for both oxidized and reduced recombinant Fds (Figure 5) are almost identical to those of native *Ha. japonica* Fd (Sugimori *et al.* 2000), Fds from other haloarchaea (Kerscher *et al.* 1976; Werber & Mevarech 1978), and even those of spinach (Tagawa & Arnon 1962; Palmer *et al.* 1967; Hall *et al.* 1974). The ESR spectrum of the dithionite-reduced recombinant Fd recorded at approx. 25 K (Figure 6) was rhombic with three *g*-values of 1.89, 1.96 and 2.06, and an average *g*-value of 1.97. The signals were also similar to those of reduced native Fds from *Ha. japonica* (Sugimori *et al.* 2000), *Hb. salinarum* (Kerscher *et al.* 1976) and spinach (Hall *et al.* 1974). The absorption and ESR spectra clearly indicate that the recombinant Fd had a similar chromophore to native *Ha. japonica* Fd, as well as to other haloarchaeal and spinach Fds. Therefore,

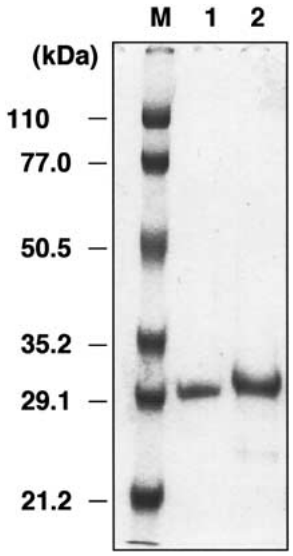

Fig. 4. SDS-PAGE of purified native and recombinant Fds of *Ha. japonica*. A 10% (w/v) polyacrylamide gel was used. After electrophoresis, proteins in the gel were stained with CBB. Lane 1, purified native *Ha. japonica* Fd; lane 2, purified recombinant *Ha. japonica* Fd with His·Tag. M indicates the molecular mass markers.

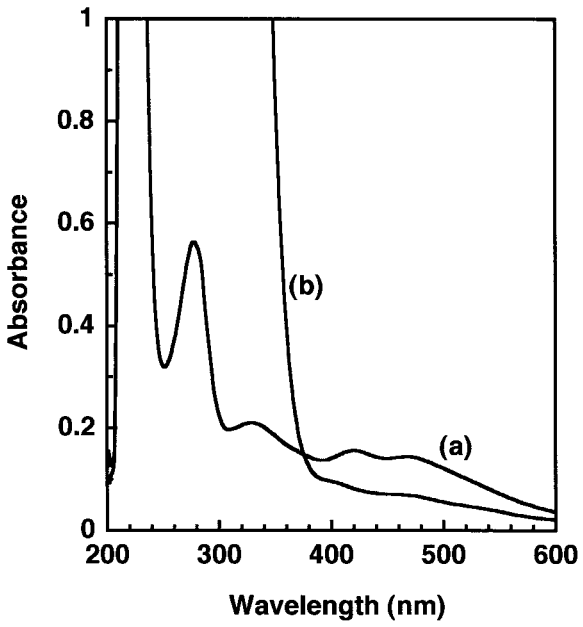

Fig. 5. Absorption spectra of recombinant *Ha. japonica* Fd. (a) Oxidized recombinant Fd (0.586 mg/ml) in a 0.15 M Tris-HCl (pH 7.0) buffer containing 0.33 M NaCl and 3.1 M KCl. (b) The same sample reduced with sodium dithionite.

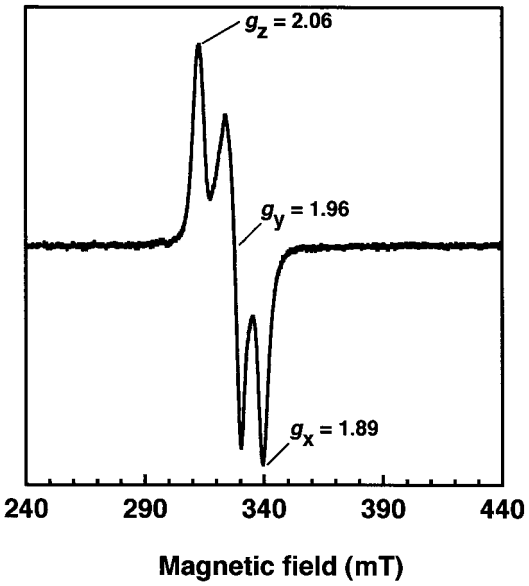

Fig. 6. ESR spectrum of dithionite-reduced recombinant Fd of *Ha. japonica*. The purified recombinant Fd [0.586 mg/ml in 0.15 M Tris-HCl (pH 7.0), 0.33 M NaCl and 3.1 M KCl] was reduced with sodium dithionite. The spectrum was recorded at 23.8 ± 1 K by using 1 mW of microwave power. The spectrometer settings were as follows: gain 63; modulation frequency, 100 kHz; modulation amplitude, 0.5 mT; time constant, 30 ms; sweep time, 240 s; microwave frequency, 9.02 GHz.

it was concluded that the His-tagged recombinant Fd of *Ha. japonica* indeed contained the [2Fe-2S] cluster and that its protein structure, especially near the Fe-S cluster, was not influenced by the *C*-terminal His·Tag. The successful expression of the *Ha. japonica* Fd gene in *Ha. japonica* now enable mutational studies to characterize the halophilicity of this Fd.

Acknowledgements

We thank Prof. W.F. Doolittle of Dalhousie University for providing *Hf. volcanii* strain WDF11 and plasmid pWL102. We are also indebted to Dr M. Kamekura and Mr Y. Seno of Noda Institute for Scientific Research for their kind instruction on haloarchaeal transformation, and to Sankyo Co., Ltd. for providing plavastatin. Thanks are further due to Prof. I. Okura, Dr T. Kamachi and Mr A. Miyaji of Tokyo Institute of Technology for facilitating ESR and absorption spectra measurements and for providing helpful discussions. This study was partially supported by a Grant-in-Aid for Scientific Research on Priority Areas (A) 'Dynamic Control of Strongly Correlated

Soft Materials' to S. Nakamura from the Ministry of Education, Culture, Sports, Science and Technology.

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