Extremophiles (2000) 4:109–114

ORIGINAL PAPER

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A novel bacteriorhodopsin-like protein from *Haloarcula japonica* strain TR-1: gene cloning, sequencing, and transcript analysis

Received: June 29, 1999 / Accepted: September 21, 1999

Abstract The gene encoding a novel bacteriorhodopsin (bR) -like protein from *Haloarcula japonica* strain TR-1 was cloned and sequenced. The nucleotide sequence of the gene contained an open reading frame that corresponded to a protein of 250 amino acids. The deduced amino acid sequence of *Ha. japonica* bR-like protein exhibited the highest homology to those of cruxrhodopsins (cRs) produced by members of the genus *Haloarcula*, suggesting that the bR-like protein of *Ha. japonica* belonged to the cR subfamily. The hydropathy analysis of *Ha. japonica* bR-like protein (cR) revealed that the *Ha. japonica* cR had a transmembrane heptahelical structure similar to that of bR. Furthermore, transcription of the cR gene in *Ha. japonica* was confirmed by the reverse transcription-polymerase chain reaction method.

Key words Bacteriorhodopsin · Halophilic archaeon · *Haloarcula japonica* · Gene cloning · Transcription

Introduction

Bacteriorhodopsin (bR) is the only protein found in the purple membrane of halophilic archaeon *Halobacterium (Hb.) salinarum*. It has been demonstrated to function as a light-driven proton pump creating an electrochemical proton gradient that can be used for ATP synthesis (Oesterhelt and Stoeckenius 1973). The protein is composed of a single polypeptide chain of 248 amino acids (bacterioopsin) (Khorana et al. 1979) and one molecule of the chromophore retinal (Bayley et al. 1981). Bacterioopsin consists of seven membrane-spanning helices, A to G, and the pigment retinal is bound covalently to Lys-216 via a protonated Schiff base (Kyte and Doolittle 1982). Upon photon absorption, retinal isomerizes around the C13–C14 bond and thermally reverts to the initial all-*trans* bR state, thereby passing a series of photocycle intermediates termed J, K, M, N, and O (Lanyi 1993). The transport of proton from the inside to the outside of the cell is coupled to this photocycle.

Several new members of the bR-like protein family (light-driven proton pump family) have been reported, for example, archaerhodopsin (aR)-1 (Sugiyama et al. 1989) and aR-2 (Uegaki et al. 1991) of *Halorubrum* spp., and cruxrhodopsin (cR)-1 (Tateno et al. 1994), cR-2 (Sugiyama et al. 1994), and cR-3 (Kitajima et al. 1996) of *Haloarcula* spp. The amino acid sequence of these proteins also indicated that all essential residues for proton pumping are conserved.

Haloarcula (*Ha.*) *japonica* strain TR-1 is a predominantly triangular disk-shaped halophilic archaeon (Horikoshi et al. 1993). Although taxonomic characteristics (Takashina et al. 1990), mode of cell division (Hamamoto et al. 1988), cell-surface glycoprotein (Nakamura et al. 1992; Wakai et al. 1997), and ferredoxin (Ikeda et al. 1997) have been extensively studied, the light energy-converting system in *Ha. japonica* has not yet been found.

Recently, we subjected the membrane fraction of *Ha. japonica* to flash-induced fluorescence spectroscopic analysis (Ohtani et al. 1992) and observed the O-like intermediate, found in the photocycle of bR (data not shown). This result strongly suggested the presence of a bR-like retinal protein on the cell surface of *Ha. japonica*, although bR activity was not detected.

In the present study, the gene encoding the bR-like protein was cloned and sequenced from *Ha. japonica*. The primary structure of the *Ha. japonica* bR-like protein, derived from the cloned gene, was then compared with those of other bR-like proteins from halophilic archaea. Transcript analysis of the *Ha. japonica* bR-like protein gene was also performed.

Communicated by K. Horikoshi

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Materials and methods

Strains, plasmids, media, and DNA manipulation. *Haloarcula japonica* strain TR-1 (JCM 7785, ATCC 49778) was grown at 37°C in a liquid medium as described previously (Nishiyama et al. 1995). *Escherichia coli* strain JM109 was used as a host for all experiments and cultured at 37°C in L broth (Sambrook et al. 1989). Plasmids pUC119 and pCR2.1 were obtained from Takara Shuzo (Kyoto, Japan) and Invitrogen (San Diego, CA, USA), respectively. Restriction endonucleases and T4 DNA ligase were used as specified by manufacturers [Takara Shuzo and Toyobo (Osaka, Japan)].

Isolation of chromosomal DNA. Chromosomal DNA of *Ha. japonica* was prepared according to the method of Lechner and Sumper (1987).

Amplification of DNA by PCR. The polymerase chain reaction (PCR) primers were synthesized with an Applied Biosystems (Norwalk, CT, USA) model 391 (PCR-MATE) DNA synthesizer. The PCR mixture consisted of 100 ng *Ha. japonica* chromosomal DNA, 10 pmol each primer, 2.5 mM each deoxynucleotide triphosphate, and 2.5 U *Ex Taq* DNA polymerase (Toyobo) in 50 μl of the reaction buffer recommended by the manufacturer. PCR was carried out at 25 cycles under the following conditions: denaturation for 1 min at 94°C, annealing for 1.5 min at 55°C, and extension for 1.5 min at 72°C by using a Gene Amp PCR System 2400 (Perkin-Elmer/Cetus, Norwalk, CT, USA).

Hybridization analyses. Colony and Southern hybridizations were performed according to the standard protocols (Sambrook et al. 1989) using a DIG DNA Labeling and Detection Kit (Boehringer-Mannheim, Mannheim, Germany).

DNA sequencing and computer analysis. DNA sequencing was carried out by the dideoxy chain termination method of Sanger et al. (1977) with Shimadzu (Kyoto, Japan) models DSQ-500 and DSQ-1000 DNA sequencers. The DNA and predicted protein sequences were analyzed with the GENETYX-MAC set of programs (Software Development, Tokyo, Japan).

DNA sequence accession number. The DNA sequence data reported in this article will appear in the DNA Data Bank of Japan (DDBJ), European Molecular Biology Laboratory (EMBL), and GenBank nucleotide sequence databases under the accession number AB029320.

RNA extraction and RT-PCR for detection of mRNA. Total RNA of *Ha. japonica* was extracted by a modified procedure of RNeasy Mini Kit (Qiagen, Valencia, CA, USA) as follows. A culture sample of 2 ml was harvested in a microcentrifuge tube at 4200 × *g* for 10 min at room temperature. The supernatant was decanted, and any medium remaining above the pellet was removed by pipetting. Cells were resuspended in 100 μl of RNase-free water by vortexing. The remainder of the procedure is as described in the product brochure. The reverse transcription-PCR (RT-PCR) was performed by using an RT-PCR Kit (RT-PCR high -plus-; Toyobo). Total RNA (75 ng) was reverse transcribed at 60°C for 30 min in 50 μl of the reaction buffer containing 20 pmol each primer, 2.5 mM each deoxynucleotide triphosphate, 25 mM manganese (II) acetate, 20 U RNase inhibitor, and 5.0 U r*Tth* DNA polymerase. cDNA was then amplified by 40 cycles of PCR. The temperature profile for amplification was as follows: denaturation for 1 min at 94°C, annealing for 30 s at 45°C, and extension for 1.5 min at 60°C.

Results and discussion

Generation of a DNA fragment by PCR

A comparison of the amino acid sequence of bR-like proteins, such as bR, aRs, and cRs, reveals that amino acids in the helices C and G are highly conserved (Dunn et al. 1981) (see Fig. 2). To generate a probe for the cloning of the bR-like protein gene, PCR was performed on the *Ha. japonica* chromosomal DNA using oligonucleotide primers designed from the highly conserved regions. The sense and antisense primers used were 5′-GACTGG(T/C)TGTT CACGACGCC-3′ (corresponding to the amino acid sequence 98–103 in the helix C of the mature *Hb. salinarum* bR) and 5′-ATACCGAAGCCGA(T/C)CTTGGC-3′ (corresponding to the sequence 228–232 in the helix G of the mature bR), respectively. A PCR product of expected size of about 420 bp was obtained and then ligated into pCR2.1. Nucleotide sequencing analysis revealed that the deduced amino acid sequence encoded by the PCR fragment was 62%–94% identical to those of the bR-like proteins. Therefore, the PCR fragment was confirmed to be a part of the bR-like protein gene of *Ha. japonica.*

Cloning of the *Ha. japonica* bR-like protein 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 at about 5.5 kb. DNA fragments of 5–6 kb were isolated from *Sal*I-digested chromosomal DNA by preparative agarose gel electrophoresis, ligated into the *Sal*I site of pUC119, and then introduced into *E. coli*. Transformants were screened for the bR-like protein gene by colony hybridization. Seven positive clones were obtained from 270 colonies and were found to contain recombinant plasmids with an identical 5.5-kb genomic insert. One such plasmid was designated pJCR3.

DNA sequence and protein structure

The DNA sequence of the cloned fragment revealed that the putative bR-like protein gene of *Ha. japonica* contained

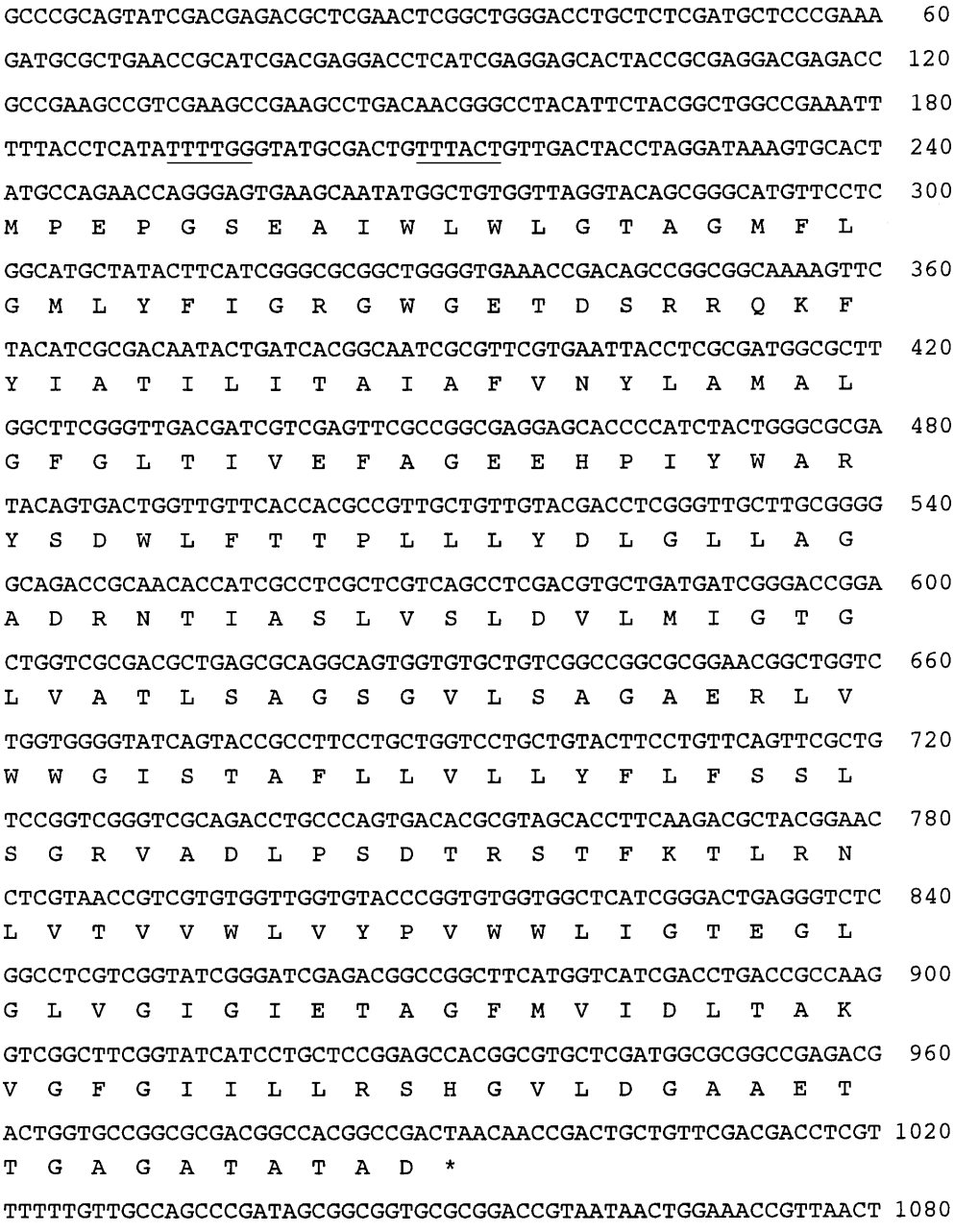

Fig. 1. Nucleotide and deduced amino acid sequences of the gene encoding the bacteriorhodopsin-like (bR-like) protein of *Haloarcula* (*Ha.*) *japonica*. The numbers on the *right* refer to the nucleotides. *Underlined nucleotides* correspond to the segment with high homology to the eukaryotic box-A motif

an open reading frame of 750 nucleotides that encoded a polypeptide of 250 amino acids (Fig. 1). The sequences 5′-TTTTGG-3′ and 5′-TTTACT-3′, which resemble the eukaryotic TATA-like "box A" motif [consensus: 5′-TTTA(A or T)A-3′] (Reiter et al. 1990), were observed at 49 and 32 bp upstream of the possible initiation codon, respectively.

The primary structures of the *Ha. japonica* and other bR-like proteins are aligned in Fig. 2. The deduced amino acid sequence of the *Ha. japonica* bR-like protein is more closely related to cRs than bR and aRs. This result suggested that the bR-like protein of *Ha. japonica* belonged to the cR subfamily. The port-bR, which has the highest identity with the *Ha. japonica* cR, might also be a member of the cR subfamily. The hydropathy profile of the *Ha. japonica* cR showed a significant similarity with that of bR (Fig. 3), suggesting the transmembrane heptahelical structure of the *Ha. japonica* cR like that of bR.

In the *Ha. japonica* cR, Lys-216 (the number refers to that of bR, hereafter), which binds retinal in bR, is conserved as well as in other bR-like proteins. By site-directed mutagenesis study of bR, Arg-82, Asp-85, and Asp-96 in the helix C and Asp-212 in the helix G are thought to be responsible for proton translocation during the photocycle. All these residues are also common to the *Ha. japonica* cR along with other bR-like protein homologs. Sugiyama et al. (1994) have pointed out that the loop region connection helices D and E in cR-1 and -2 has six extra amino acid

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                    -13            1                  20                  40                  60                  80
bR                  MLELLPTAVEGVS--QAQITGRPEWIWLALGTALMGLGTLYFLVKGMGVSDPDAKKFYAITTLVPAIAFTMYLSMLLGYGLTMVPFG--GEQNPIYW
aR-1                   MDPIALTAAVGADLLGDGRPETLWLGIGTLLMLIGTFYFIVKGWGVTDKEAREYYSITILVPGIASAAYLSMFFGIGLTEVQVG--SEMLDIYY
aR-2                   MDPIALQAGF--DLLNDGRPETLWLGIGTLLMLIGTFYFIARGWGVTDKEAREYYAITILVPGIASAAYLAMFFGIGVTEVELAS-GTVLDIYY
aR-3                   MDPIALQAGY--DLLGDGRPETLWLGIGTLLMLIGTFYFLVRGWGVTDKDVREYYAVTILVPGIASAAYLSMFFGIGLTEVTVG--GEMLDIYY
mex-bR                 MDPIALQAGY--DLLGDGRPETLWLGIGTLLMLIGTFYFIARGWGVTDKKAREYYAITILVPGIASAAYLSMFFGIGLTTVEVAGMAEPLEIYY
cR-1                                 MPEPGSEAIWLWLGTAGMFLGMLYFIARGWGETDSRRQNFYIATILITAIAFVNYLAMALGFGLTIVEFA--GEEHPIYW
cR-2                         MLQSGM---STYVPGGESIFLWVGTAGMFLGMLYFIARGWSVSDQRRQKFYIATIMIAAIAFVNYLSMALGFGVTTIELG--GEERAIYW
cR-3                                 MPAPEGEAIWLWLGTAGMFLGMLYFIARGWGETDSRRQKFYIATILITAIAFVNYLAMALGFGLTIVEIA--GEQRPIYW
port-bR                               MEPGSEAIWLWLGTAGMFLGMLYFIARGWGETDSRRQKFYIATILITAIAFVNYLAMALGFGLTIVEFA--GEEHPIYW
Ha. japonica cR                      MPEPGSEAIWLWLGTAGMFLGMLYFIGRGWGETDSRRQKFYIATILITAIAFVNYLAMALGFGLTIVEFA--GEEHPIYW
dR-1                         MCCAALAPPMAATVGPESIWLWIGTIGMTLGTLFYVGRGRGVRDRKMQEFYIITIFITTIAAAMYFAMATGFGVTEVMVG--DEALTIYW
                                                 Helix A                        Helix B

                     *  *        * 100                120                   140                 160
bR                  ARYADWLFTTPLLLLDLALLVDADQGTILALVGADGIMIGTGLVGALT------KVYSYRFVWWAISTAAMLYILYVLFFGFTSKAESMRPEVASTF
aR-1                ARYADWLFTTPLLLLDLALLAKVDRVSIGTLVGVDALMIVTGLVGALS------HTPLARYTWWLFSTICMIVVLYFLATSLRAAAKERGPEVASTF
aR-2                ARYADWLFTTPLLLLDLALLAKVDRVTIGTLIGVDALMIVTGLIGALS------KTPLARYTWWLFSTIAFLFVLYYLLTSLRSAAAKRSEEVRSTF
aR-3                ARYADWLFTTPLLLLDLALLAKVDRVTIGTLVGVDALMIVTGLIGALS------HTAIARYSWWLFSTICMIVVLYFLATSLRSAAKERGPEVASTF
mex-bR              ARYADWLFTTPLLLLDLALLANADRTTIGTLIGVDALMIVTGLIGALS------HTPLARYTWWLFSTIAFLFVLYYLLTVLRSAAAELSEDVQTTF
cR-1                ARYSDWLFTTPLLLYDLGLLAGADRNTITSLVSLDVLMIGTGLVATLSPGSGVLSAGAERLVWWGISTAFLLVLLYFLFSSLSGRVADLPSDTRSTF
cR-2                ARYTDWLFTTPLLLYDLALLAGADRNTIYSLVGLDVLMIGTGALATLSAGSGVLPAGAERLVWWGISTGFLLVLLYFLFSNLTDRASELSGDLQSKF
cR-3                ARYSDWLFTTPLLLYDLGLLAGADRNTISSLVSLDVLMIGTGLVATLSAGSGVLSAGAERLVWWGISTAFLLVLLYFLFSSLSGRVADLPSDTRSTF
port-bR             ARYSDWLFTTPLLLYDLGLLAGADRNTITSLVSLDVLMIGTGLVATLSAGSGVLSAGAERLVWWGISTAFLLVLLYFLFSSLSGRVADLPSDTRSTF
Ha. japonica cR     ARYSDWLFTTPLLLYDLGLLAGADRNTIASLVSLDVLMIGTGLVATLSAGSGVLSAGAERLVWWGISTAFLLVLLYFLFSSLSGRVADLPSDTRSTF
dR-1                ARYADWLFTTPLLLLDLSLLAGANRNTIATLIGLDVFMIGTGAIAALS------STPGTRIAWWAISTGALLALLYVLVGTLSENARNRAPEVASLF
                           Helix C               Helix D                          Helix E

                           180                 200          *   * 220                   240      249      Identity(%)
bR                  KVLRNVTVVLWSAYPVVWLIGSEGAGIVP-LNIETLLFMVLDVSAKVGFGLILLRSRAIFGEAEAPEPSAGDGAAATSD       52.3
aR-1                NTLTALVLVLWTAYPILWIIGTEGAGVVG-LGIETLLFMVLDVTAKVGFGFILLRSRAILGDTEAPEPSAGAEASAAD        47.4
aR-2                NTLTALVAVLWTAYPILWIVGTEGAGVVG-LGIETLAFMVLDVTAKVGFGFVLLRSRAILGETEAPEPSAGADASAAD        49.6
aR-3                NTLTALVLVLWTAYPILWIIGTEGAGVVG-LGIETLLFMVLDVTAKVGFGFILLRSRAILGDTEAPEPSAGADVSAAD        48.9
mex-bR              NTLTALVAVLWTAYPILWIIGTEGAGVVG-LGVETLAFMVLDVTAKVGFGFVLLRSRAILGETEAPEPSAGAEAQAAD        49.1
cR-1                KTLRNLVTVVWLVYPVWWLIGTEGIGLVG-IGIETAGFMVIDLTAKVGFGIILLRSHGVLDG---AAETTGTGATPADD       96.4
cR-2                STLRNLVLVLWLVYPVLWLVGTEGLGLVG-LPIETAAFMVLDLTAKIGFGIILLQSHAVLDE----GQTASEGAAVAD        75.4
cR-3                KTLRNLVTVVWLVYPVWWLVGTEGIGLVG-IGIETAGFMVIDLVAKVGFGIILLRSHGVLDG---AAETTGAGATATAD       95.6
port-bR             KTLRNLVTVVWLVYPVWWLIGTEGIGLVG-IGIETAGFMVIDLTAKVGFGIILLRSHGVLDG---AAESTGAGATATAD       98.0
Ha. japonica cR     KTLRNLVTVVWLVYPVWWLIGTEGLGLVG-IGIETAGFMVIDLTAKVGFGIILLRSHGVLDG---AAETTGAGATATAD      100.0
dR-1                GRLRNLVIALWFLYPVVWILGTEGTFGILPLYWETAAFMVLDLSAKVGFGVILLQSRSVLERVATPTAAPT               51.3
                           Helix F               Helix G
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Fig. 2. Alignment of the amino acid sequences of the *Ha. japonica* cruxrhodopsins (cR) (bR-like protein) and other bR-like proteins. The names of bR-like proteins are as follows: *bR*, bR from *Halobacterium* (*Hb.*) *salinarum*; *aR-1*, aR-1 from *Halorubrum* sp. aus-1; *aR-2*, aR-2 from *Halorubrum* sp. aus-2; *aR-3*, aR-3 from *Halorubrum sodomense* (Mukohata et al. 1999); *mex-bR*, bR-like protein from strain mex (Mukohata et al. 1999); *cR-1*, cR-1 from *Ha. argentinensis*; *cR-2*, cR-2 form *Ha. mukohataei*; *cR-3*, cR-3 from *Ha. vallismortis*; *port-bR*, bR-like protein from strain port (Mukohata et al. 1999); *dR-1*, dR-1 from strain sp. arg-4 (Mukohata et al. 1999). The residues are numbered referring to bR, and the segments of the seven transmembrane helices (*A–G*) suggested for bR are also indicated. Lys-216, Arg-82, Asp-85, Asp-96, and Asp-212 are shown by *asterisks*

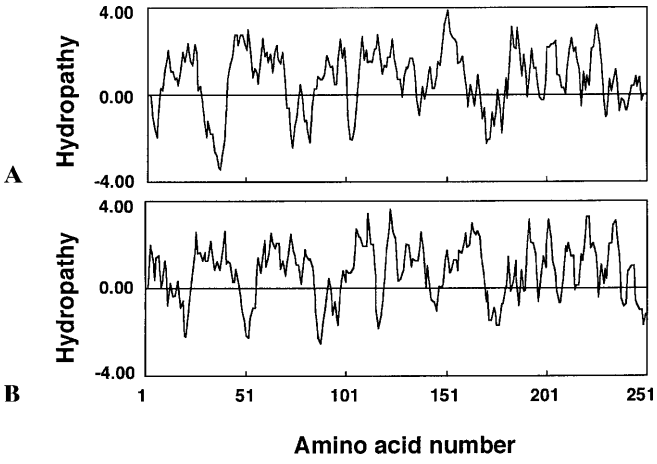

Fig. 3A,B. Hydropathy plots of *Ha. japonica* cR (**A**) and *Hb. salinarum* bR (**B**). The curve plots the hydrophobicity index for each residue as the average hydrophobicity index for the window of five amino acids centered around that residue

residues compared to bR and aRs. This insertion of six amino acids was observed also in the *Ha. japonica* cR, suggesting the common feature of the cR subfamily.

Transcription of *Ha. japonica* cR gene

To determine whether the *Ha. japonica* cR gene is transcribed, RT-PCR was performed for the detection of the *Ha. japonica* cR mRNA. Total RNA was extracted from a stationary-phase culture of *Ha. japonica*, because Yang and DasSarma (1990) observed an increase in transcription of about 20 fold of the *Hb. salinarum* bacterioopsin gene (*bop*) between midexponential and stationary phases. The primers for RT-PCR were designed on the basis of the determined DNA sequence of the *Ha. japonica* cR gene. The sense and antisense primers were 5′-CAGAACCAGGGAGTGAAG-3′ (corresponding to the amino acid sequence 2–6 of the *Ha. japonica* cR) and 5′-TCTCGATCCCGATACCGAC-3′ (corresponding to the amino acid sequence 219–224 of the *Ha. japonica* cR), respectively. As shown in Fig. 4, a single band of the expected size of about 620 bp was obtained on agarose gel electrophoresis when total RNA was amplified by RT-PCR with a set of primers for the *Ha. japonica* cR gene. The cDNA band was confirmed to hybridize with the *Ha. japonica* cR gene fragment by Southern analysis (data not shown). This result suggested that the *Ha. japonica* cR gene was transcribed.

Kamekura et al. (1998) have reported that species of the genera *Haloferax* and *Halococcus* do not posses bR activity and detectable bR-like protein genes. They also showed that extreme halophiles strain HT and strain GSL11 (now designated as *Haloterrisena turkmenicus* JCM 9743 and strain GSL11, respectively) (Ventosa et al. 1999) have genes encoding bR-like proteins, although both strains show no bR activity. In this report, we have provided the evidence for existence and transcription of the bR-like protein (cR)

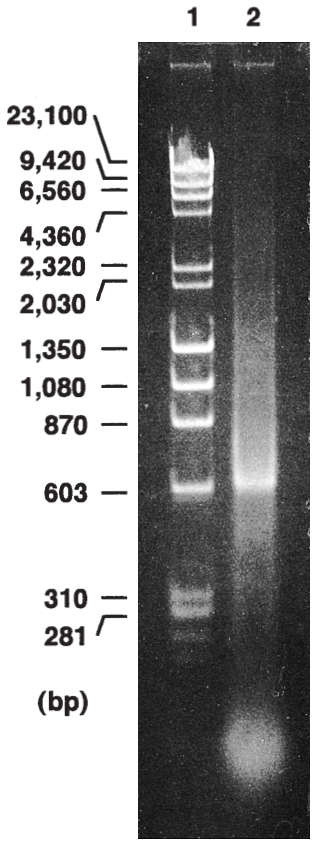

Fig. 4. Agarose gel electrophoresis of RT-PCR products separated on a 2.0% (w/v) agarose gel. *Lane 1*, *Hin*dIII digest of λ DNA-*Hae*III digest of ΦX174 DNA markers; *lane 2*, RT-PCR products

gene in *Ha. japonica*. These results suggested that the *Ha. japonica* cR gene was not dormant, although bR activity had not yet been detected in *Ha. japonica*. To date, bR-like proteins have been found in representatives of three genera: *Halobacterium*, *Halorubrum*, and *Haloarcula*.

As was mentioned, we detected the photocycle intermediate in the *Ha. japonica* membrane fraction although bR activity was not detected. The amount of cR in *Ha. japonica* cells seemed to be relatively small, or perhaps the *Ha. japonica* cR might show lower proton pumping activity than that of bR. To clarify the function of the *Ha. japonica* cR, we are trying to overexpress the *Ha. japonica* cR gene in a bR-deficient *Hb. salinarum* host using the *bop* promoter.

Acknowledgment This work was partially supported by CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation (JST).

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