Journal of Zhejiang University SCIENCE
ISSN 1009-3095
http://www.zju.edu.cn/jzus
E-mail: jzus@zju.edu.cn

JZUS

Isolation and characterization of a novel strain of *Natrinema* containing a *bop* gene*

XU Xue-wei (许学伟)[1], WU Min (吴 敏)[†1], HUANG Wei-da (黄伟达)[2]

([1]*School of Life Sciences, Zhejiang University, Hangzhou 310027, China*)

([2]*School of Life Sciences, Fudan University, Shanghai 200433, China*)

[†]E-mail: wumin@cls.zju.edu.cn

Received May 13, 2004; revision accepted Oct. 28, 2004

Abstract: A novel member of extremely halophilic archaea, strain AJ2, was isolated from Ayakekum Lake located in Altun Mountain National Nature Reserve of Xinjiang Uygur Autonomous Region in China. The strain AJ2 requires at least 10% (w/v) NaCl and grows 10% to 30% (optimum at 20%). Phylogenetic analysis based on 16S rDNA sequence comparison revealed that strain AJ2 clustered to three *Natrinema* species with less than 97.7% sequence similarities, suggesting AJ2 is a novel member of *Natrinema*. A bacteriorhodopsin-encoding (*bop*) gene was subsequently detected in the AJ2 genome using the polymerase chain reaction technique. The cloning and sequencing of a 401 base pairs fragment indicated the deduced amino acid sequence of *bop* from AJ2 is different from that reported for bacteriorhodopsins. This is the first reported detection of a *bop* gene in *Natrinema*.

Key words: Halophilic archaea, Phylogeny, Bacteriorhodopsin
doi:10.1631/jzus.2005.B0142 **Document code:** A **CLC number:** Q78

INTRODUCTION

Extremely halophilic archaea belonging to the order *Halobacteriales* had been isolated from various hypersaline environments such as the Dead Sea, the Great Salt Lake, sabkhas, and natural or artificial salterns (Radax *et al.*, 2001). Western China has many natural salt lakes, providing extremely abundant halophilic archaea resources. A novel haloarchaeal strain AJ2 containing a *bop* gene was documented in reports of molecular investigation on microbiological resources regarding their adverse effect on the environment.

Bacteriorhodopsin (BR), found in some extremely halophilic archaea, is a retinal-binding membrane protein with seven membrane-spanning segments and functions as a light-driven proton pump (Oesterhelt and Stoeckenius, 1971; Luecke *et al.*, 1999; Mukohata *et al.*, 1999). Their especial photoelectric properties and high thermal stability of BR proteins are useful in such as optical memory (Wise *et al.*, 2002), artificial retina (Frydrych *et al.*, 2000), photon switch, and so on (Lanyi and Pohorille, 2001; Margesin and Schinner, 2001). Because of the limitation of site-directed method, looking for native BR proteins, which could offer the best properties for materials improvement, become the available method today (Wise *et al.*, 2002).

In this study, we isolated and characterized a novel strain of extremely halophilic Altun Mountain archaeon containing a bacteriorhodopsin-encoding (*bop*) gene. This is the first reported detection of a *bop* gene in *Natrinema*.

MATERIALS AND METHODS

Archaeal strains and growth media

Strain AJ2 was isolated from water samples collected from Ayakekum Lake (37° N, 89° E, at

* Porject (No. 30370029) supported by the National Natural Science Foundation of China

altitude of 3880 m) located in Altun Mountain National Nature Reserve in Western China. Reference strain *Halobacterium salinarium* (*halobium*) R1M1 was obtained as a gift from Li Qing-guo (Shanghai, China). The strains were routinely cultured aerobically at 37 °C in rich medium (Oesterhelt and Stoeckenius, 1974) containing (L^{-1}) 250 g NaCl, 20 g $MgSO_4 \cdot 7H_2O$, 3 g trisodium citrate dihydrate, 2 g KCl, 0.26 g $CaCl_2$, 10 g peptone (Oxiod L37). The pH was adjusted to 7.0 with NaOH. And slants and plates were prepared by adding 20 g aga/L.

Growth characteristics

Optimal salt concentration conditions of strain AJ2 were determined by growing in DSMZ Meduim 97 regulated with 0, 5, 10, 12.5, 15, 17.5, 20, 22.5, 25 and 30% (w/v) NaCl. The pH tolerance was also tested in rich medium with pH values 3, 4, 5, 6, 7, 8, 9, 10, 11. All of the optimal conditions tests groups were incubated at 37 °C for every three days until day 14, and cell growth was monitored by OD_{600}. Cell shape was examined by optical microscopy (Olympus model BX40 microscope).

DNA extraction

Genomic DNAs from archaeal strains were extracted using the classic genomic DNA isolation kit (Sangon) according to the instructions of the manufacturer.

16S rDNA sequence PCR ampification

The 16S rDNA sequence primers, designed according to Gupta *et al.*(1983), were rDNA22F (5′-ATTCCGGTTGATCCTGC-3′, positions 6–22) and rDNA1521R (5′-AGGAGGTGATCCAGCCGCAG-3′, positions 1540–1521). The *E. coil* 16S rRNA sequence was used as the position reference. The PCR thermal cycling conditions were as follows: 25 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min.

Cloning and sequencing of PCR products

The desired PCR products were purified using the UNIQ-10 column DNA gel extraction kit (Sangon) according to the instructions of the manufacturer. The purified fragments were treated with T4 DNA ligase (MBI), then ligated to pUCm-T (Sangon), and transformed into *Escherichia coli* DH5α. Ampicillin (100 μg/ml) and a blue-white selection were used to identify the positive transformants. The plasmids were extracted using the alkaline lysis method. Subsequently, plasmids were digested by PstI and electrophoresed in agarose gel (1.5%) to select recombinants harboring plasmids with the correct insert of the 16S rDNA sequence. PCR amplifications, as done under above conditions, were performed using approximately 50 ng plasmid DNA as the template. Sequences were determined by automated dideoxynucleotide methods with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) on an ABI Prism 377XL DNA sequencer. The M13 universal sequencing primers (M13F, 5′-GTAAAACGACGGCCAGT-3′, and M13R 5′-GGAAACAGCTATGACCATG-3′) were used in the sequencing reaction.

Analysis on sequence data, and phylogenetic tree construction

The 16S rDNA sequence of strain AJ2 was fitted in the halophilic archaeal alignment with Clustal X 1.8, and a few minor corrections were made manually. Analyses were carried out with PHYLIP (version 3.6a3) (Felsenstein, 2003). The DNADIST programme was used to construct a Jukes and Cantor evolutionary distance matrix, then phylogentic tree was constructed using the neighbor-joining method with randomized input order. Bootstrap analysis (1000 replications) was performed using the additional programs SEQBOOT and CONSENSE.

Identification of *bop* gene

The *bop* PCR primers, designed on the basis of the conserved protein sequences PLLLLDL and KVGFGFI (Otomo *et al.*, 1992; Wang *et al.*, 2000), were bop331F (5′-CCGCTG(CT)TG(CT)TG(CT)T-(AC)GACCTCG-3′, positions 310–331) and bop686R (5′-AGGATGA(GA)(CG)CCGAA(CG)CCGACCTT-3′, positions 707–686). The *Hbt. salinarium bop* gene sequence was used as the position reference. The PCR amplification was performed in a 100 μl reaction mixture, for 25 cycles with denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min. PCR products were examined by gel electrophoresis in 4% agarose gel using the generulerTM 100 bp DNA ladder plus markers (Sangon) for size comparisons of PCR products. Cloning and se-

quencing of PCR products protocols, as described above, were used.

RESULTS

Growth characteristics and other features

At 37 °C, strain AJ2 grows at pH 6.0 to pH 8.0, and in media containing 10%–30% NaCl (pH 7.4) with an optimum at 20% NaCl. No growth was detected at NaCl concentrations below 10%. Microscopy indicated that cells lysed in less than 6% NaCl. In actively growing liquid cultures, the cells of strain AJ2 were rod shaped but were pleomorphic under unfavorable conditions. Colonies of stain AJ2 were red, circular and convex.

Phylogenetic analysis of 16S rDNA sequence

The 16S rDNA sequence of strain AJ2, 1474 bp was determined. The GenBank accession number was AY208972. Phylogenetic tree based on 16S rDNA sequence was constructed using the neighbor-joining method with bootstrap values calculated from 1000 trees subsequently (Fig.1). On the phylogenetic tree strain AJ2 clustered to the three described *Natrinema* species. Similarity percentages between the AJ2 sequence and the type strains of *Nnm. pallidum* (NCIMB 777 and NCIMB 784), *Nnm. pellirubrum* and *Nnm. versiforme* were 97.1%, 97.1%, 96.6% and 97.7%, respectively.

Detection and analysis of *bop* gene by PCR

The nucleotide sequence of the partial *bop* gene from strain AJ2 (GenBank accession No. AY279548), 401 bp in total, was sequenced and the deduced amino acid sequence was compared to the previously determined sequence of *Hbt. salinarium*, which was the first discovered as BR protein, and strain arg-4, which processes the highest similarity sequence with AJ2 among all extremely halobacterial archaea (Fig.2). The result of sequence alignment confirmed that the nucleotide fragment from PCR reaction is just the part of the *bop* gene that encodes the retinal protein from helix C to helix G. The hydropathic indices from helix C to G region of BR proteins (Fig.3) were calculated according to the method of Kyte and Doolittle (1982). The similar hydropathic profiles between *Hbt. salinarium* BR and retinal protein of strain AJ2,

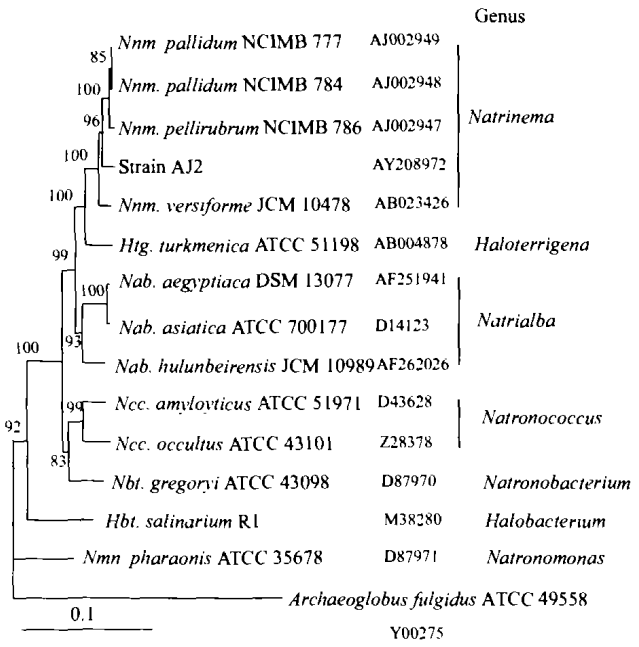

Fig.1 Phylogenetic tree based on the nucleotide sequences of 16S rDNA from fourteen halobacterial strains, where the 16S rDNA sequence from *Archaeoglobus flugidus* was used as outgroup. The tree was constructed using the neighbor-joining method with bootstrap values calculated from 1000 trees. The number at each branch point represents the percentage bootstap support. Bar, 0.1 sequence divergence. GenBank accession numbers of nucleotide sequences are listed on the side of strains name

as illustrated in Fig.3, could mean that both proteins fold their polypeptide chains in a similar way.

DISCUSSION

The results showing that cells of strain AJ2 lyse in low NaCl concentration (<6%) medium, morphology and growth characteristics agreed with descriptions of *Natrinma* (McGenity *et al.*, 1998; Xin *et al.*, 2000), AJ2 clustered to the three described *Natrinema* species in phylogenetic tree based on 16S rDNA sequence, and the cluster was supported by a high bootstrap value (100%), indicating strain AJ2 is a member of *Natrinma* belonging to extremely halophilic archaea. Moreover, compared to 16S rDNA sequences among *Natrinma* strains, AJ2 had more than 95% and less than 97.7% sequence similarity with others. According to the current classification suggestion, strains whose 16S rDNA sequence homology values is below about 97.5% are related at the species level (Stackebrandt and Goebel, 1994). Therefore, AJ2

```
01: PLLLLDLALLVDADQGTILALVGADGIMIGTGLVGALTKVYSYRFVWWAI   50
02: **********AG*NRN**AT*I*L*VF*****AIA**SSTPGA*IA****
03: *******S**AG*NRN**AT*I*L*VF*****AIA**SSTPGT*IA****

01: STAAMLYILYVLFFGFTSKAESMRPEVASTFKVLRNVTVVLWSAYPVVWL   100
02: **G*L*TLP*I*VGTLSEN*R*RA*****L*GR***LVIA**FL*****1
03: **G*L*AL****VGTLSEN*RNRA*****L*GR***LVIA**FL*****1

01: IGSEG-AGIVPLNIETLLFMVLDVSAKVGFGLI    Hbt. salinarium    132
02: L*T**TF**L**YW**AA*****L*******F*    Strain AJ2
03: L*T**TF**L**YW**AA*****L*******V*    Strain arg-4
```

Fig.2 Comparison of deduced amino acid sequence among bacteriorhodopsin of strain AJ2, arg-4 [6] and *Hbt. salinarium* (Dunn *et al.*, 1981). The same sequences are indicated by asterisk (*)

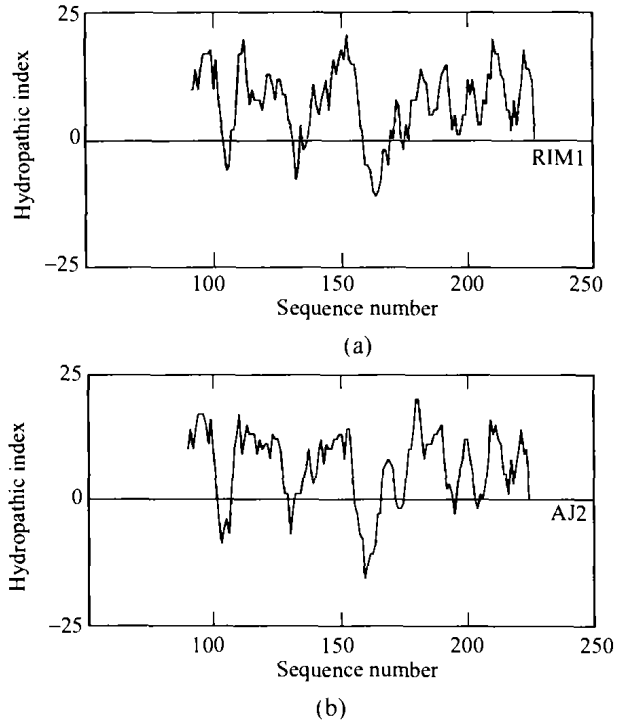

Fig.3 Hydropathic profile from amino acid residue 91 to 223 of *Hbt. salinarium* R1M1 BR (a) and AJ2 BR (b). The profile was calculated according to Kyte and Doolittle (1982). All plots utilize a span setting of 9

is a novel member of *Natrinma*.

The fragment of *bop* gene encoding BR protein from helix C to helix G contains the main gene primary structure, and this part of protein domain include the main part of amino acid residues which line the retinal binding pocket or proton channel. Accordingly, the partial protein sequence from helix C to helix G region is more conservative than the whole protein sequence. However, compared to BR protein sequences from different strains, about 94.7% of amino acid residues are identical between strain AJ2 and arg-4, but less than 60% homology exists between AJ2 and other reported strains (Ihara *et al.*, 1999; Mukohata *et al.*, 1999). The results indicated strain AJ2 should be closer to the strain arg-4, which belong to *Haloterrigena*, than to other strains, such as *Hbt. salinarium*. The phylogenetic tree (Fig.1) also makes clear this viewpoint.

Among different genera of *Halobacteriaceae*, five have been found to possess *bop* gene: *Halobacterium*, *Haloarcula*, *Halomicrobium*, *Halorubrum* and *Haloterrigena* (Ihara *et al.*, 1999; Mukohata *et al.*, 1999). This is the first reported detection of a *bop* gene in *Natrinema*. The study extends the range of strains in other genera that harbor *bop* gene, and provides the base for using of species and BR proteins resources.

References

Dunn, R., McCoy, J., Simsek, M., Majumdar, A., Chang, S.H., RajBhandary, U.L., Khorana, H.G., 1981. The bacteriorhodopsin gene. *Proc Natl Acad Sci USA*, **78**:6744-6748.

Felsenstein, J., 2003. PHYLIP. http://evolution.genetics.washington.edu/phylip.html.

Frydrych, M., Silfsten, P., Parkkinen, S., Parkkinen, J., Jaaskelainen, T., 2000. Color sensitive retina based on bacteriorhodopsin. *Biosystems*, **54**:131-140.

Gupta, R., Lanter, J.M., Woese, C.R., 1983. Sequence of the 16S ribosomal RNA from *Halobacterium volcanii*, an archaebacterium. *Science*, **221**:656-659.

Ihara, K., Umemura, T., Katagiri, I., Kitajima-Ihara, T., Sugiyama, Y., Kimura, Y., Mukohata, Y., 1999. Evolution of the archaeal rhodopsins: evolution rate changes by gene duplication and functional differentiation. *J Mol Biol*, **285**:163-174.

Kyte, J., Doolittle, R.F., 1982. A simple method for displaying the hydropathic character of a protein. *J Mol Biol*, **157**:105-132.

Lanyi, J.K., Pohorille, A., 2001. Proton pumps: mechanism of action and applications. *TRENDS Biotechnol*, **19**: 140-144.

Luecke, H., Schobert, B., Richter, H.T., Cartailler, J.P., Lanyi, J.K., 1999. Structural changes in bacteriorhodopsin dur-

ing ion transport at 2 angstrom resolution. *Science*, **286**:255-261.

Margesin, R., Schinner, F., 2001. Potential of halotolerant and halophilic microorganisms for biotechnology. *Extremophiles*, **5**:73-83.

McGenity, T.J., Gemmell, R.T., Grant, W.D., 1998. Proposal of a new halobacterial genus *Natrinema* gen. nov., with two species *Natrinema pellirubrum* nom.nov. and *Natrinema pallidum* nom. nov. *Int J Syst Bacteriol*, **48**:1187-1196.

Mukohata, Y., Ihara, K., Tamura, T., Sugiyama, Y., 1999. Halobacterial rhodopsins. *J Biochem*, **125**:649-657.

Oesterhelt, D., Stoeckenius, W., 1971. Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. *Nature*, **233**:149-152.

Oesterhelt, D., Stoeckenius, W., 1974. Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane. *Methods Enzymol*, **31**:667-678.

Otomo, J., Urabe, Y., Tomioka, H., Sasabe, H., 1992. The primary structures of helices A to G of three new bacteriorhodopsin-like retinal proteins. *J Gen Microbiol*, **138**:2389-2396.

Radax, C., Cruber, C., Stan-Lotter, H., 2001. Novel haloarchaeal 16S rRNA gene sequences from Alpine Permo-Triassic rock salt. *Extremophiles*, **5**:221-228.

Stackebrandt, E., Goebel, B.M., 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol*, **44**:846-849.

Wang, H., Zhan, S., Sun, Q., Xu, D., Zhao, W., Huang, W., Li, Q., 2000. Primary structure of helix C to helix G of a new retinal protein in H.sp.xz515. *Chinese Sci Bull*, **45**:1108-1112 (in Chinese).

Wise, K.J., Gillespie, N.B., Stuart, J.A., Krebs, M.P., Birge, R., 2002. Optimization of bacteriorhodopsin for bioelectronic devices. *TRENDS Biotechnol*, **20**:387-394.

Xin, H., Itoh, T., Zhou, P., Suzuki, K.I., Kamekura, M., Nakase, T., 2000. *Natrinema versiforme* sp. nov., an extremely halophilic archaeon from Aibi salt lake, Xinjiang, China. *Int J Syst Evol Microbiol*, **50**:1297-1303.