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Isolation and functional expression of the *bop* gene from *Halobiforma lacisalsi*

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Data storage material

Summary

A novel *bop* gene was described from *Halobiforma lacisalsi* strain AJ5^T, an extremely halophilic archaeon isolated from Ayakekum Lake, China. Following six rounds of PCR amplification based on the conserved fragment of the *bop* gene, the complete sequence of the *bop* gene, including the 5′ and 3′ flanking regions of the conserved fragment, was obtained by the ligation-mediated PCR amplification (LPA) approach. The data presented provide us with further insight into the distribution of *bop*-like genes in the family Halobacteriaceae. This is the first example of a *bop*-like gene in halophiles found in the high-pH environment. Alignment and hydropathy analysis of the deduced amino acid sequence identified the conserved functional sites as well as some variations compared with other bacterio-opsins. Molecular phylogenetic analysis revealed the position of the bacterio-opsin of strain AJ5, which is closest to that of *Haloterrigena* sp. arg-4 with 85% identity. In the presence of all-*trans* retinal, recombinant *Escherichia coli* cells expressing the gene turned dark purple. The purple membrane from the recombinant *E. coli* showed maximal absorption at 540 nm.

Introduction

Bacteriorhodopsin (bR), encoded by the *bop* gene, is a membrane protein with seven membrane-spanning segments that functions as a light-driven proton pump (Oesterhelt and Stoeckenius, 1971; Luecke et al., 1999; Mukohata et al., 1999) and consists of bacterio-opsin and retinal with a ratio of 1:1. The presence of bR enables cells to transform light energy into a transmembrane electric potential, which can then be used to

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generate ATP by ATP synthase. In-depth studies of *Halobacterium* sp. NRC-1 as a model organism have shown that bR plays an important role in the organism's adaptation to fluctuating environmental conditions (Ng et al., 2000; Baliga et al., 2002).

Based on its photoelectric properties, bR has been found to have many interesting applications in the engineering of artificial retinas (Frydrych et al., 2000), optical memories (Wise et al., 2002), and photon switches (Lanyi and Pohorille, 2001; Margesin and Schinner, 2001). Till now, some *bop* genes have been successfully expressed in *Escherichia coli* (Feng et al., 2006; Kamo et al., 2006). Identification of additional natural bRs or their mutant forms may offer us useful biomolecules for material improvement (Wise et al., 2002).

bR is present in some, but not all, representatives of the family Halobacteriaceae. Thus far, only 19 complete and confirmed *bop* genes (GenBank, April 23, 2007) have been isolated from cultured halophilic archaea, among which five were achieved from complete genome sequencing (Ng et al., 2000; Baliga et al., 2004; Bolhuis et al., 2006). Previously, a conserved fragment of the *bop* gene was obtained from *Natrinema altunense* strain AJ2, isolated from Ayakekum Lake, China, which grows in pH range 6.0–8.0 (Xu et al., 2005a, b).

Halobiforma lacisalsi strain AJ5^T, isolated from the same lake, can grow in the pH range 6.5–9.0, with 7.5 being the optimum (Xu et al., 2005c). Therefore, we tested whether the moderately alkaliphilic strain AJ5 may contain the *bop* gene. After the presence of the gene was confirmed, we managed to obtain the complete ORF using the ligation-mediated PCR amplification (LPA) method (Isegawa et al., 1992; Wang et al., 2003), and expressed it in *E. coli*. In this paper, we present the properties of the *H. lacisalsi* bR.

Materials and methods

Strains, plasmids, and cultivation conditions

H. lacisalsi strain AJ5^T (= CGMCC 1.3738^T = JCM 12983^T) was cultured aerobically at 42 °C in rich medium as described previously (Oesterhelt and Stoeckenius, 1974). *E. coli* strain JM109 and pMD18T (TAKARA, Dalian, China) were used as host and vector for cloning and sequencing. *E. coli* strain Rosetta and pET28a (Merck, Darmstadt, Germany) were used as host and vector for gene expression, respectively. *E. coli* strains were routinely grown at 37 °C in Luria–Bertani (LB) medium. When required, the concentration for ampicillin and kanamycin used were 60 and 10 mg/L, respectively.

Identification of the conserved sequence of the *bop* gene

Genomic DNA of strain AJ5 was extracted and used as template for PCR according to the standard methods (DasSarma et al., 1995). Primers targeting PLLLLDL and KVGFGFI (Otomo et al., 1992; Wang et al., 2000), the conserved regions in bR, were named bopF and bopR. Sequences of primers and other oligonucleotides used in this study are shown in Table 1. Thermal cycling of PCR was set for denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min for a total of 25 cycles using *LA Taq* DNA polymerase with GC buffer (TAKARA, Dalian, China). The recombinant plasmid generated by ligation of the amplified DNA fragments and vector pMD18T was transformed into JM109. Ampicillin resistance and blue–white selection were used to identify positive transformants. The sequencing of the recombinant plasmid was carried out in both directions using an ABI

Table 1. Oligonucleotides used in this study

Name	Sequence
bopF	5′CCGCTG(CT)TG(CT)TG(CT)T(AC)GACCTCG3′
bopR	5′AGGATGA(GA)(CG)CCGAA(CG)CCGACCTT3′
Oligonucleotide LP	5′GGGATCCTTCACTCTCAAGTGC3′
CG linker	5′CACGGC3′
GATC linker	5′CACGCTAG3′
Primer C1	5′CGCGTTCATGATCCTCGACC3′
Primer C2	5′CATGATCCTCGACCTCTCGG3′
Primer N1	5′GAGCAGCGTCGCGATCGTGT3′
Primer N2	5′CGATCGTGTTGCGGTTCGCT3′
Primer Complete1	5′CCGGAGGAGAAACAACGACCCG3′
Primer Complete2	5′GGATCCAGCGGGAGGCCGACGC3′
Expression 1	5′GACCATGGCAACTGTAGGTCCAGAG3′
Expression 2	5′CGGAATTCGTACGAGCGGCCTTACG3′

PRISM® 3730 automated DNA sequencer and the BigDye™ Terminator cycle-sequencing ready-reaction kits, Version 3.0 (PE Corporation, Foster City, CA). The conserved sequence was then used as the known fragment to obtain the 5′ and 3′ flanking regions using the LPA approach.

PCR amplification of the 5′ unknown region

Primers N1 and N2 (Table 1) were designed based on the 5′ end of the partial CDS sequence that had been identified above. Primer N2 is upstream of Primer N1 by 11 bp (see Figure 1b). Oligonucleotide LP and a CG linker (Table 1) were mixed at a molecular ratio of 1:1, incubated at 70 °C for 10 min, and then the mixture was gradually cooled to room temperature to enable the formation of CG adaptors with sticky ends of CG, the structure of which is shown in Figure 1a. At the same time, genomic DNA of AJ5 was partially digested by the restriction enzyme *Msp* I (TAKARA, Dalian, China), yielding mainly fragments of 1–2 kbp in size with sticky ends complementary to those of the CG adaptors. The CG adaptors and partially digested genomic DNA were then ligated and the ligation solution was used as template for a semi-nested 1st round PCR using Primer N1 and Oligonucleotide LP under conditions described above. The amplified fragments were recovered and purified individually following agarose gel electrophoresis, which were subsequently confirmed by semi-nested 2nd round PCR with Primer N2 and Oligonucleotide LP.

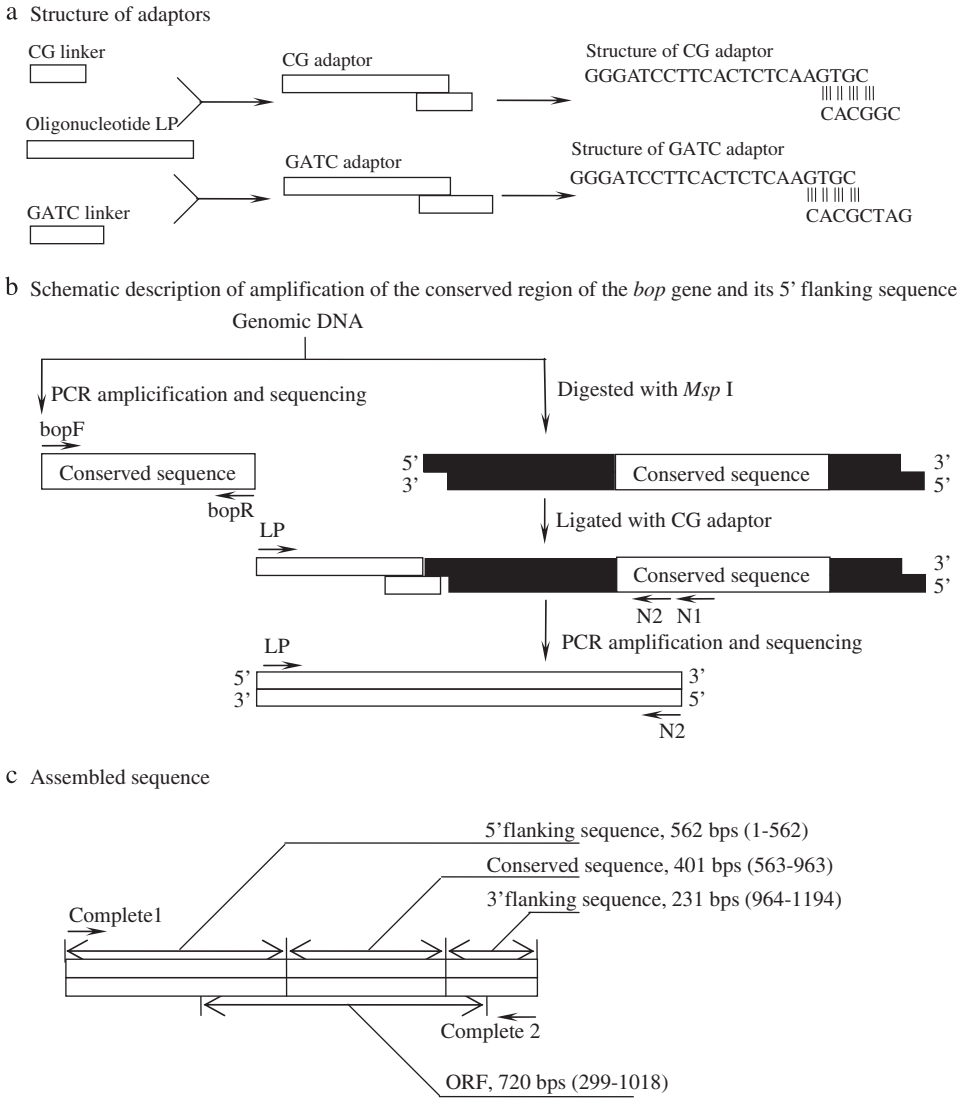

Figure 1. (a) Structures of CG and GATC adaptors. (b) Schematic description of amplification of the conserved region of the *bop* gene and its 5′ flanking sequence, with positions of the primers used. (c) Assembled sequence. Sequences from different amplifications are shown with their length and positions in the entire fragment in parentheses.

The longest fragment identified was cloned and sequenced as described above. Figure 1b gives a schematic description of the amplification of the 5′ unknown region.

PCR amplification of the 3′ flanking region

Based on the conserved sequence identified above, Primers C1 and C2 (Table 1) were designed based on the 3′ end of the identified sequence. Primer C2 is downstream of Primer C1 by 6 bps (see Figure 1b). Genomic DNA of AJ5 partially digested by the restriction enzymes *Sau3A* I (TAKARA, Dalian, China) was ligated with GATC adaptors, which were composed of Oligonucleotide LP and GATC linker (Table 1) and complementary to those genomic fragments digested by *Sau3A* I. The structure of the GATC adaptors is shown in Figure 1a. Similar to the cloning of the 5′ flanking region, the 3′ flanking region was amplified by semi-nested PCR, the 1st round PCR using Primer C1 and Oligonucleotide LP, the 2nd round PCR using Primer C2 and Oligonucleotide LP. The longest fragment amplified by 2nd round PCR was cloned and sequenced as above.

Amplification of the entire DNA fragment after assembling

The sequences obtained as described above were assembled in Vector NTI (InforMax Inc., USA), as shown in Figure 1c. Primers Complete1 and Complete2 (Table 1) were used to amplify the entire sequence. PCR, cloning, and sequencing were performed as described above.

Sequence analysis

BLASTP was carried out using http://www.ncbi.nlm.nih.gov/BLAST/ (Altschul et al., 1997; Schäffer et al., 2001). Phylogenetic analysis of all 19 complete *bop* genes available in GenBank was constructed using the MEGA program, version 3.1 (Kumar et al., 2004). Transmembrane segments and hydrophobicity of the *H. lacisalsi* bR were analyzed using DNAMAN Version 5.2.2 (Lynnon Biosoft, USA), setting the window at 15 and scale from −3 to 3.

Functional expression of the *bop* gene from AJ5 in *E. coli*

The *bop* gene from AJ5 was amplified by PCR using primers Expression 1 and Expression 2 (Table 1). The products were digested with *Nco* I and *Eco*R I (TAKARA, Dalian, China) and then ligated into pET28a vector at the sites of *Nco* I and *Eco*R I. The generated recombinant plasmid, named pET28aAJ5bop, was transformed into Rosetta. Positive transformants were identified as kanamycin resistant and verified by sequencing. To induce synthesis of bR protein, IPTG (Sangon, Shanghai, China) was added to recombinant *E. coli* cultures at a final concentration of 1 mM after the OD_{600} of the culture had reached 0.61. Cultures were also supplemented with 10 μM all-*trans* retinal (SIGMA-ALDRICH, St. Louis, USA). After additional incubation for 6 h at 25 °C, cells (designated RbopIA) were harvested by centrifugation at 6000*g* for 10 min at 4 °C. The same protocol was used for Rosetta harboring pET28a with a supplement of IPTG and all-*trans* retinal (designated RblankIA) and Rosetta harboring pET28aAJ5bop with IPTG but without retinal (designated RbopI) as controls.

Measurement of absorption spectra of purple membranes

After cells of RbopIA and RblankIA were sonicated in an ice bath (120 W, 3 s pulses separated by 5 s pauses, 99 cycles) in Tris–HCl buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, pH 8.0), membrane suspensions were subjected to centrifugation at 18,000*g* for 10 min at 4 °C. The supernatant was retrieved for further analysis. Before recording the absorption spectra, membrane suspensions were light-adapted by illumination for 1 h under a white fluorescent tube (Philips TLD 18W/54) at a distance of 5 cm. Absorption spectra were measured in a Vis–UV spectrophotometer (Beckman Coulter DU800) in the visible range (400–700 nm) (Feng et al., 2006).

Results

Amplification and analysis of the entire *bop* gene

Using primers designed based on the conserved sequence within the *bop* gene, a partial CDS of 401 bp was obtained (GenBank accession number AY279551). A fragment of about 630 bp was specially amplified from the CG adaptor-derived ligation reaction. By aligning these 630 bp to the 5′ end of the conserved region, we were able to extend 562 bp from the conserved region, containing the start codon ATG. Similarly, a fragment of 231 bp was extended toward 3′ direction of the conserved region, including an in-frame stop codon, TAA, at position 1019. After assembling the sequences derived, primers Complete1 and Complete2 were synthesized to verify the contig of

the three sequences. We used these primers to amplify a fragment of 1194 bp, containing the complete ORF (720 bp) of the *bop* gene of AJ5, 298 bp upstream from the start codon and 173 bp downstream of the stop codon (GenBank accession number AY607024.2). The details of the sequence are shown in Figure 1c.

Sequence analysis

The *H. lacisalsi* bR is composed of 240 amino acids, with a molecular mass of 26 kDa. The alignment of the *H. lacisalsi* bR with 18 other *bop* genes showed conservation in the main functional sites involving proton transport, including Arg-80, Asp-83, Asp-94, Asp-211, and the retinal binding site, Lys-215 (Subramaniam and Henderson, 2000; Werner, 2000). In the phylogenetic tree, bRs isolated from AJ5 and *Haloterrigena* sp. arg-4 formed a branch (Figure 2). BLASTP analysis showed that the *H. lacisalsi* bR is closely related to that of *Haloterrigena* sp. arg-4 with 85% identity. Hydrophobicity profile of the *H. lacisalsi* bR is shown in Figure 3, together with seven predicted transmembrane segments and their positions and length.

Expression of the *bop* gene and the absorption spectra of bR-membranes in recombinant *E. coli* cells

Cells of RbopIA turned dark purple during cultivation in LB broth, while RbopI and RblankIA remained light yellow (see Supplementary Material I). The absorption spectrum of the purple membrane fraction isolated from RbopIA, having its absorption peak at 540 nm, is shown in Figure 4.

Discussion

Using approaches developed earlier (Otomo et al., 1992; Wang et al., 2003), we have successfully cloned the complete *bop* gene of *H. lacisalsi* AJ5. The gene was cloned by using six rounds of PCR in total: one round for the conserved sequence, two rounds for the 5′ unknown region, another two rounds for the 3′ unknown region, and finally the last one for verification of the entire fragment. The identified regions flanking the ORF are particularly useful for identifying the transcriptional regulation of the *bop* gene in further studies. This method can also be used for cloning complete ORFs of other genes starting from conserved sequences, such as many partial *bop*-like genes deposited in GenBank. We are currently utilizing this method in an attempt to characterize more opsins from other halophilic strains (from the Altun Mountain National Nature Reserve, China), to analyze the evolutionary relationships among opsins (Ihara et al., 1999) and their photoelectric properties for engineering applications.

This is the first report on the presence of the *bop* gene in the genus *Halobiforma*, extending the number of genera known to harbor *bop* genes and the diversity of bacterio-opsins. More interestingly, strain AJ5, which is reported to survive at pH 9.0, is the first known alkali-tolerant representative of the Halobacteriaceae having a *bop*-like gene.

Differences in amino acid composition could provide different thermal stability. It is of interest

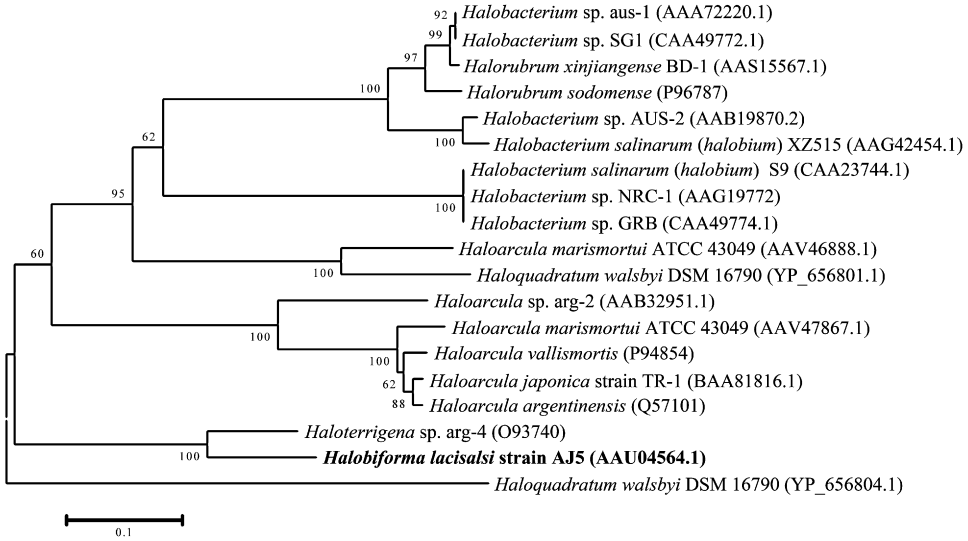

Figure 2. Phylogenetic tree of 19 bacterio-opsins (GenBank accession number shown in brackets). The tree was constructed using the neighbor-joining method. Bootstrap values, based on 1000 replications are shown at the nodes.

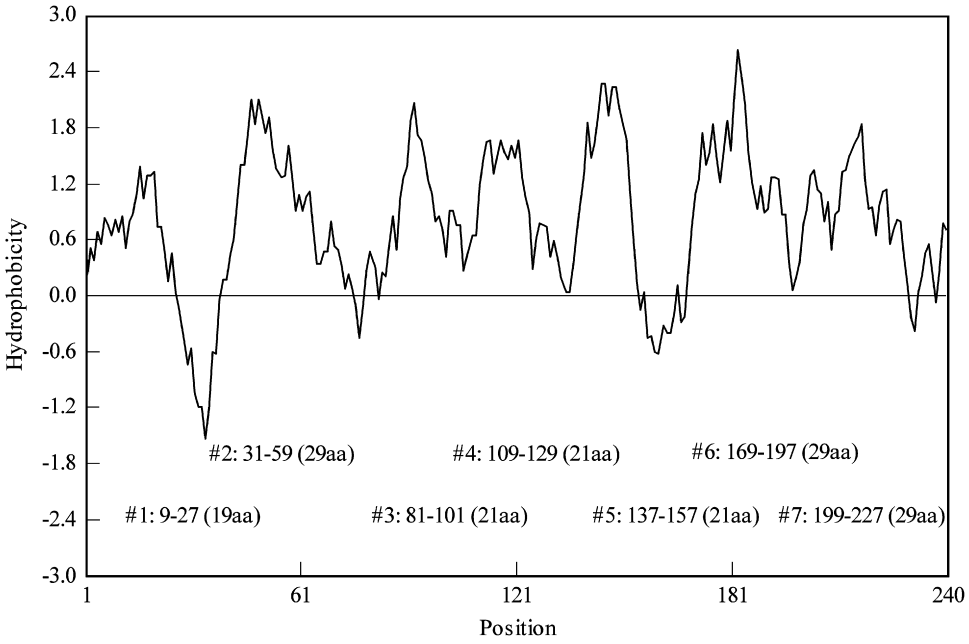

Figure 3. Hydrophobicity profile of the bacterio-opsin from strain AJ5. Seven predicted transmembrane segments are shown with their position and length in parentheses.

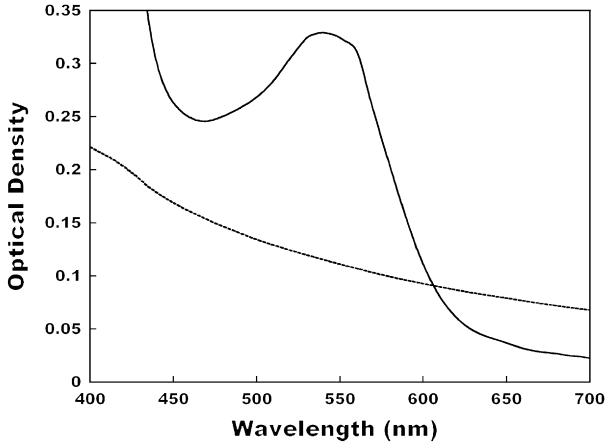

Figure 4. Absorption spectra of membranes from RbopIA (solid line) and membranes from RblankIA (dashed line).

to elucidate the potential of this bacterio-opsin protein as a photoelectric material in further biophysical studies. The maximal absorption of the purple membrane from RbopIA is at 540 nm, which is different from the maximal absorption at 568 nm for purple membrane from other halophilic archaea (Lukashev et al., 1994) and 555 nm for purple membrane from recombinant *E. coli* (Feng et al., 2006). Even though the question whether the *H. lacisalsi* bR functions as proton pump remains open, the different absorption maximum of the purple membrane from RbopIA suggests that this bR protein may have a potential as photoelectric material, and may be applied as photosensitive storage material in combination with other bR proteins with different absorption to improve the ability of data storage.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2007.06.004

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