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Genetic cloning and functional expression in *Escherichia coli* of an archaerhodopsin gene from *Halorubrum xinjiangense*

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Abstract Pairs of PCR primers that targeted the archae/bacteriorhodopsin gene were used to clone the archaerhodopsin (*aR*) gene of *Halorubrum xinjiangense* strain BD-1^T, and this gene was sequenced and functionally expressed in *Escherichia coli*. Recombinant *E. coli* cells harboring the plasmid carrying this gene became slightly purple or blue depending on whether they were supplemented with all- *trans* retinal or 3,4-dihydroretinal, respectively, during induction with IPTG. The purple and blue membranes from the recombinant *E. coli* showed maximal absorption at 555 and 588 nm, respectively, which are different from maximal absorption at 568 nm of the wild-type purple membrane. Purple membranes from the recombinant *E. coli* and from strain BD-1^T were investigated in parallel. The *E. coli* purple membrane was fabricated into films and photoelectric responses were observed that depended on the light-on and light-off stimuli.

Keywords Archaerhodopsin · Bacteriorhodopsin · Purple membrane · *Halorubrum*

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Introduction

Bacteriorhodopsins (bR) are of interest because they are potentially useful for the fabrication of biomaterial-based devices such as artificial retinas (Frydrych et al. 2000) and optical memories (Wise et al. 2002). Khodonov et al. (1997) listed many advantages of bR as material for fabrication of devices, and those advantages could be further improved by modifications either of the protein (Wise et al. 2002; Weetall et al. 2000), or of the chromophore (Druzhko and Chamorovsky 1995; Jussila et al. 2001; Drachev et al. 1989). Modification of the chromophore has been done by the replacement of the retinal with various retinal analogs, e.g., 3,4-dihydroretinal (Khodonov et al. 1997) or 4-keto-retinal (Druzhko and Chamorovsky 1995). The procedure for such modification includes usually, bleaching of the purple membrane, and reconstitution in the presence of the retinal analog.

A bR-like protein, archaerhodopsin (aR) from the *Halorubrum* sp. was identified (Mukohata et al. 1988; Sugiyama et al. 1989; Uegaki et al. 1991). This protein shows about 56–59% homology to the bR protein from *Halobacterium* spp., and exhibits similar photochemical properties. But aR and bR molecules showed differences in their absorbance maxima, the kinetics of the photocycles, and especially in alkaline-induced red-shifted absorption. In this study, we attempted to identify the *aR* in *Halorubrum xinjiangense* strain BD-1^T, and further functionally express the gene and modify the gene product in *Escherichia coli*.

Materials and methods

Bacterial strains, plasmids, medium, and cultivation

H. xinjiangense strain BD-1^T (CGMCC 1.3527^T = JCM 12388^T) was grown at 40°C in the medium as described previously (Feng et al. 2004). *E. coli* strains (Table 1)

Table 1 Strains, plasmids, and primers used in this study

	Characteristics or sequences	Sources (references) and notes
Bacterial/archael strains		
Halorubrum strain BD-1^T	From lab culture collection	Feng et al. (2004)
E. coli strain JM109	Cloning host	Novagen
E. coli strain BL21	Expressing host	Novagen
Plasmids		
pGEM-T easy vector	Cloning vector	Promega
pGEMaR-1	pGEM-T carrying a fragment of *aR* gene from strain BD-1^T	This study
pGEMaR-2	pGEM-T vector carrying the entire *aR* gene from strain BD-1^T	This study
pET28a	Expression vector	Novagen
pET28aR	pET28a carrying the *aR* gene of strain BD-1	This study
Primers		
1	5′GAC TGG (CT)TG TTC AC(GC) AC(AG) CC 3’	Primers 1 and 2 are general primers of screen bR-like genes. Primers 3 and 4 targeting the sequence of *Halorubrum* sp. aus-1 between −11 and 795. Primers 5 and 6 targeting the sequence of strain BD-1^T between 1 and 777. The underlined are *Nco*I and *Bam*HI sites, respectively
2	5′A(CG)G TC(GA) A(GT) (GC) ACC AT 3′	
3	5′(A,T)(C,A,G)G A(A,T)(G,A) TGG G(T,C)A TGG AC 3′	
4	5′(C,G)GC (T,C)(A,T)G CCG ATC AGT C 3′	
5	5′CAT G CC ATG GAC CCG ATAGCG CT 3′	
6	5′CGC GGA TCC GGC CAG CCG ATC AGT 3′	

were routinely grown at 37°C in Luria–Bertani (LB) medium. *E. coli* strains JM109 and BL21(DE3) (Novagen) and plasmids pGEM-T (Promega, WA, USA) and pET28a (Novagen) were used as hosts and vectors for gene sequencing and expression, respectively. When required, 100 mg of ampicillin per liter or 50 mg of kanamycin per liter was added to the culture medium.

DNA extraction and restriction enzyme treatment

Genomic DNA and plasmid DNA extraction, and restriction enzyme treatment on plasmid DNA and amplified DNA fragments were carried out according to Sambrook and Russell (2001).

Cloning and sequencing the *aR* gene from strain BD-1^T

A fragment of and the entire *aR* gene were PCR-amplified with two pairs of primers, (Primers 1, 2 and 3, and 4, Table 1). The PCR was carried out at 30 cycles under the following conditions: denaturation for 1 min at 94°C, annealing for 1 min at 54°C, and extension for 1 min at 72°C. The amplified DNA fragments were inserted into pGEM-T easy vectors and the generated pGEMaR-1 and pGEMaR-2 were transformed into *E. coli* JM109. DNA sequences were determined by Beijing Genome Institute (Huada Corp., Beijing, China).

Functional expression of *aR* gene from strain BD-1^T in *E. coli* in the presence of all- *trans* retinal and 3,4-dihydroretinal

The PCR-amplified *aR* gene from strain BD-1^T was ligated into pET28a at the sites of *Nco*I and *Bam*HI. The resulting plasmid, pET28aR, was transformed into *E. coli* BL21(DE3) by electroporation at the following conditions: 25 μF, 12.5 kV/cm, and 200 Ω (ECM630, BTX, USA). Synthesis of aR proteins in recombinant *E. coli* cells harboring pET28aR were induced by the addition of 1 mM IPTG and 10 μM all- *trans* retinal or 3,4-dihydroretinal when the culture reached OD_{600} of 0.4–0.6. After further continuous cultivation for 2 h, cells were harvested by centrifugation at 10,000 *g* for 5 min at 4°C.

Preparation of aR membranes from strain BD-1^T and recombinant *E. coli* cells

The aR membranes of strain BD-1^T were fractionated by sucrose density gradient centrifugation, according to the method described by Oesterhelt and Stoecknius (1974). Purification of aR-membrane fractions from recombinant *E. coli* were performed in the same way except that the recombinant *E. coli* cells were broken up by sonification at 4°C (160 W, 3 s sonifying vs. 5 s break, 99 cycles) in Tris–HCl buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, pH 8.0).

Measurement of absorption spectra

The membranes were suspended in a buffer of 50 mM Tris–HCl (pH 8.0). Before recording the absorption of spectra, membrane suspensions were light-adapted for 20 min. Absorption spectra were recorded with a Vis–UV spectrophotometer (Beckman Coulter DU800) with wavelength interval of 0.5 nm in the visible range (400–700 nm). Data were treated using origin 6.0 Software (Microcal Software Inc., Northampton, MA, USA).

Determination of photoelectric properties of fabricated films made from aR-membrane fractions of strain BD-1^T and recombinant *E. coli*

Indium-tin-oxide (ITO) glass slide after negative-charged treatment was immersed into Poly(allylamine hydrochloride) (PAH) aqueous solution (2 mg/mL, pH 6.4) for 5 min, rinsed with doubly distilled water, and then dried by nitrogen flow. This modified glass slide was immersed in aR-membrane suspension (pH 9.4) for 5 min, rinsed with doubly distilled water, and then dried with nitrogen again. In this way, we obtained one bilayer of aR-membrane/PAH (M/PAH) films that were marked as $(M/PAH)_1$. This process was repeated six times. The ITO glass with M/PAH films was used as a working electrode and platinum wire as a counter electrode. The electrolyte solution was 0.5 M KCl, with pH 7.3. To test the photoelectric property of the fabricated M/PAH films, the films were irradiated with light from a 150 W xenon lamp and through the filter (560 nm). Photocurrent generated by the film was measured by using a picoamper with the sensitivity of 0.1 nA and digitalized by using a digital storage oscilloscope (20 MHz, Gold Star) (Chu et al. 2003).

Chemicals and reagents

DNA restriction enzymes, DNA ligase, and DNA polymerase were purchased from Takara or Promega. All- *trans* retinal was purchased from Sigma. The 3,4-dihydroretinal was synthesized from all- *trans* retinal by following the method of Drachev et al. (1989). PAH was purchased from Aldrich Chemicals.

The Genbank accession numbers

The Genbank accession number for the *aR* gene of strain BD-1^T is AY510709. Other Genbank numbers of aR or bR proteins are given in Fig. 1.

Results

Genetic cloning and characterization of *aR* gene from strain BD-1^T

With Primers 1 and 2 (Table 1), a DNA fragment of 388 bp was PCR-amplified from genomic DNA of strain

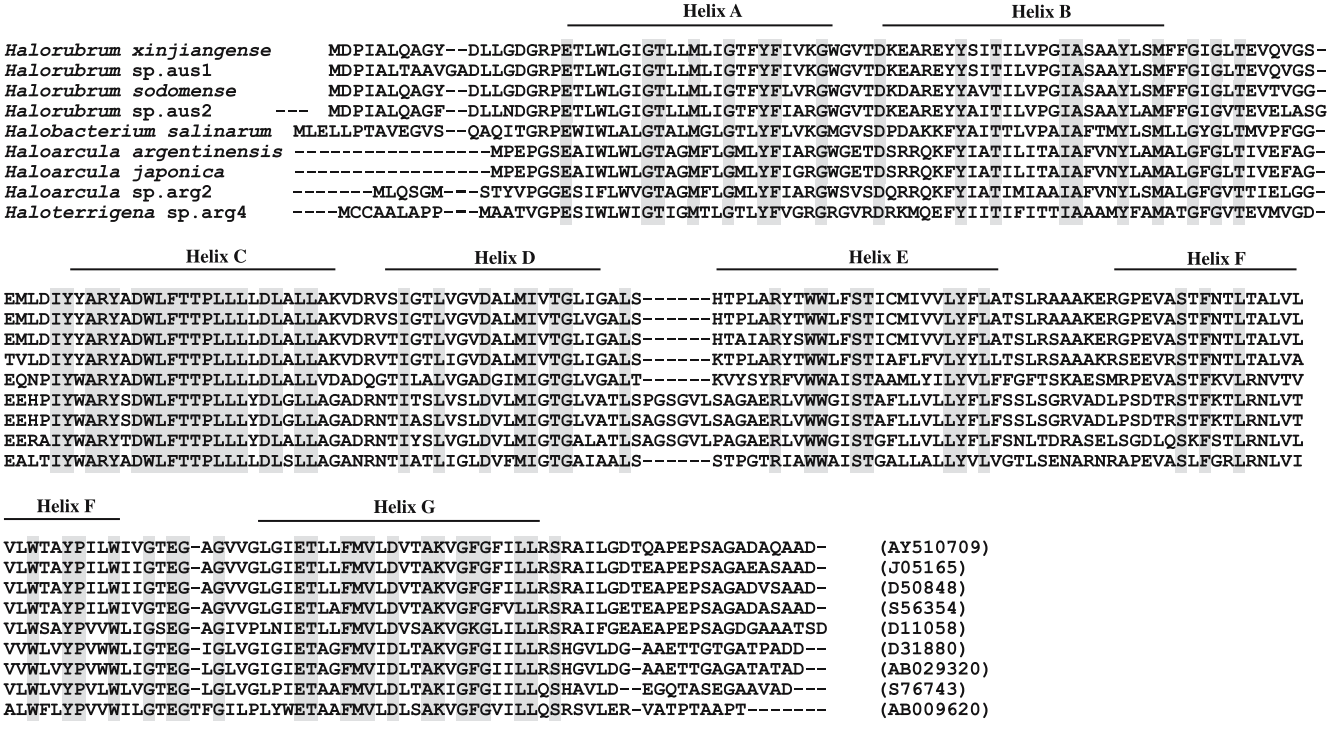

Fig. 1 The alignment of nine archaerhodopsin and bacteriorhodopsin proteins that function as proton pump. The names of the bacterial species that the archaerhodopsin and bacteriorhodopsin isolated are given at the *beginning* and Genbank accession numbers of these proteins are provided at the *end*. All conserved amino acid residues are shaded in *gray*

BD-1^T. This fragment showed 93% identity to a part of the *aR* gene of *Halorubrum* aus-1, indicating that an *aR* gene existed in strain BD-1^T. A second pair of primers (Primers 3 and 4, Table 1) according to the *aR* gene sequence of *Halorubrum* aus-1 was synthesized and used for amplification of the entire *aR* gene from strain BD-1^T. The amplified entire *aR* gene of strain BD-1^T was 777 bp in length, and encoded a protein of 258 amino acids. When this protein was aligned to other known aR or bR proteins, all the amino acid residues that were previously revealed to be essential for proton transport and linkage to retinal were completely conserved (Fig. 1). The aR protein from strain BD-1^T showed high identity (86–95%) to the aRs of *Halorubrum* aus-1, aus-2, and *Halorubrum sodomense*, and showed relatively low identities to the bacteriorhodopsin (55%) of *Halobacterium salinarum*(Dunn et al. 1981), to the cruxrhodopsins of *Haloarcula* spp. (38–47%, Yatsunami et al. 1997; Kitajima et al. 1996; Tateno et al. 1994; Otomo et al. 1992), and to the archaerhodopsin of *Haloterrigena* (49%, Ihara et al. 1999).

Expression of *aR* gene and absorption spectra of aR-membranes from *E. coli* cells

The entire *aR* gene was PCR-amplified with primers 5 and 6 (Table 1) from strain BD-1^T and was ligated to pET28a. The resulting plasmid, pET28aR, was electroporated into *E. coli* cells. Depending on the presence of all- *trans* retinal or 3,4-dihydroretinal, recombinant *E. coli* cells that harbored pET28aR became purple (with all- *trans* retinal) or grayish blue (with 3,4-dihydroretinal) during cultivation in LB broth and induction with IPTG. The membrane fractions were isolated from the recombinant *E. coli* cells and their absorption spectra were determined (Fig. 2). The maximal absorption of the purple membrane obtained with all- *trans* retinal was at 555 nm and of the blue membrane with 3,4-dihydroretinal was 588 nm (Fig. 2), which were different from the maximal absorption at 568 nm of purple membrane from halophilic archaea (Fig. 2 and also Lukashev et al. 1994). The difference in maximal absorption of purple membrane from recombinant *E. coli* and from *Halorubrum* sp. was attributed to the monomer state of archaerhodopsin in *E. coli* and the trimer state in wild membrane, as revealed by Corcelli et al. (2002).

Photoelectric response of film made from wild and recombinant purple membranes

To demonstrate that the aR proteins synthesized in recombinant *E. coli* are active for proton transport, the purple membranes from both wild strain BD-1 and recombinant *E. coli* were used to fabricate thin films (Chu et al. 2003). Upon illumination, the film generated electric currents due to proton movement across the film. Figure 3 shows the photoelectric response profiles of the films that were made of purple membranes from either strain BD-1^T (Fig. 3a) or recombinant *E. coli* (Fig. 3b). Positive and anodic responses were corresponding to light-on and light-off photocurrents that were caused by proton release and uptake, respectively. This provided further evidence that the aR proteins in the recombinant *E. coli* membrane were correctly folded and functionally active.

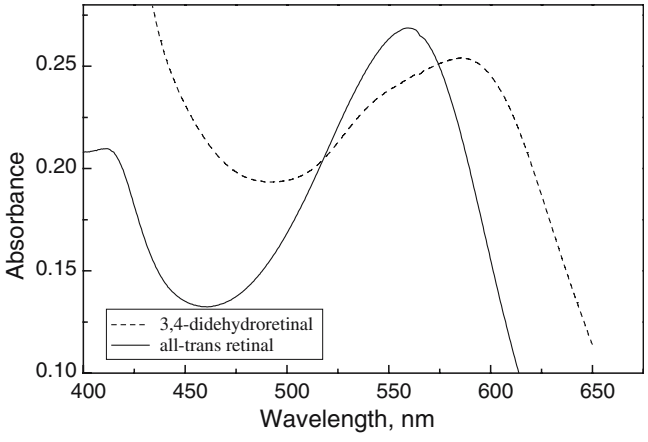

Fig. 2 Absorption spectra of purple (*solid curve*) and blue (*broken curve*) membranes from recombinant *E. coli*

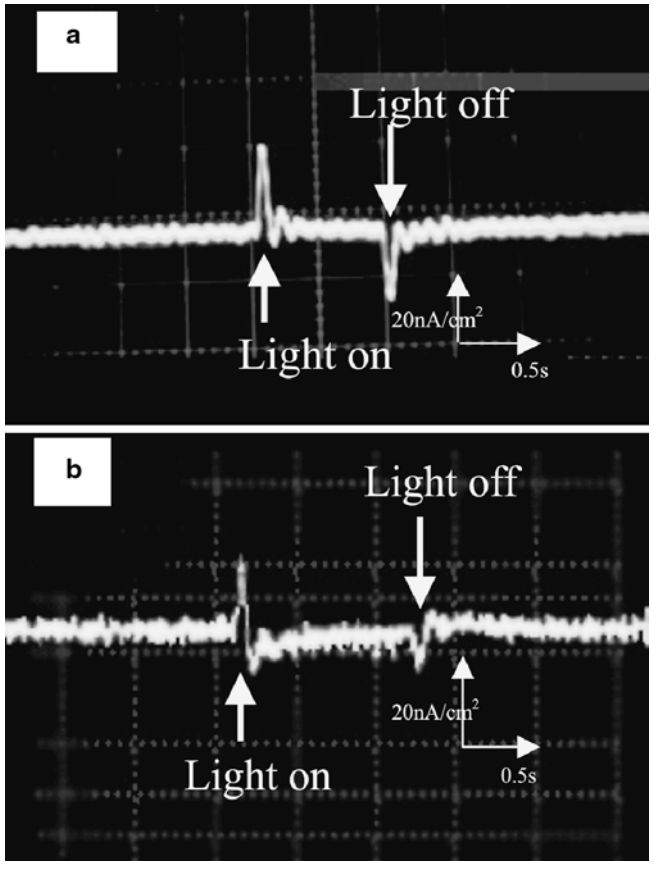

Fig. 3 Photoelectric response of films made from purple membranes of strain BD-1^T (**a**) and from recombinant *E. coli* (**b**)

Discussion

Heterologous expression of *bR* proteins in *E. coli* had been previously studied, e.g., Dunn et al. (1987) reported the synthesis and purification of retinal-free bacteriorhodopsin and Hohenfeld et al. (1999) reported the purification of histidine-tagged bR from recombinant *E. coli*. But to obtain active aR protein or functional membrane, it was necessary to refold the bR protein, in the presence of retinal, and reconstitute with polar lipid to form purple membrane (Dunn et al. 1987; Hohenfeld et al. 1999). Active expression of proteorhodopsin gene from uncultured proteobacteria and phoborhodopsin (a photosensory protein) gene from *Natronobacterium pharaonis*(NCIMB 2191) was reported (Béjà et al. 2000; Shimono et al. 1998). In this study, we had succeeded in the construction of an *E. coli* system that produces in one-step the active aR and purple membrane. Moreover, with this system, one aR with all- *trans* retinal and one aR analog with 3,4-dihydroretinal were synthesized in *E. coli*. Purple and blue membranes were obtained. We believe that preparation of other aR analogs with different retinal analogs, such as 3-hydroxyretinal and 4-ketoretinal, with this system is possible.

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References

Béjà O, Aravind L, Koonin EV, Suzuki MT, Hadd A, Nguyen LP, Jovanovich SB, Gates CM, Feldman RA, Spudich JL, Spudich EN, DeLong EF (2000) Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. Science 289:1902–1906

Chu JF, Li XC, Zhang JP, Tang JA (2003) Fabrication and photoelectric response of ploly(allylamine hydrochloride)/PM thin films by layer-by-layer deposition technique. Biochem Biophys Res Commum 305:116–121

Corcelli A, Lattanzio VMT, Mascolo G, Papadia P, Fanizzi F (2002) Lipid-protein stoichiometries in a crystalline biological membrane: NMR quantitative analysis of the lipid extract of the purple membrane. J Lipid Res 43:132–140

Drachev LA, Drachev A, Chekulaeva LN, Evstigneeva RP, Kaulen AD, Khitrina LV, Khodonov AA, Lazarova ZR, Mitsner BI (1989) An investigation of the electrochemical cycle of bacteriorhodopsin analogs with the modified ring. Arch Biochem Biophys 270:184–197

Druzhko AB, Chamorovsky SK (1995) The cycle of photochromic reactions of a bacteriorhodopsin analog with 4-keto-retinal. Biosystems 35:133–136

Dunn RJ, Mccoy JM, Simsek M, Majumdar A, Chang SH, Rajbhandary UL, Khorana HG (1981) The bacteriorhodopsin gene. Proc Natl Acad Sci USA 78:6744–6748

Dunn RJ, Hackett NR, McCoy JM, Chao BH, Kimura K, Khorana HG (1987) Structure-function studies on bacteriorhodopsin. I. Expression of the bacterio-opsin gene in *Escherichia coli*. J Biol Chem 262(19):9246–9254

Feng J, Zhou P, Liu SJ (2004) *Halorubrum xinjiangense*, a novel halophile from saline lakes of China. Int J Syst Evol Microbiol 54:1789–1791

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

Hohenfeld IP, Wegener AA, Engelhard M (1999) Purification of histidine tagged bacteriorhodopsin, *pharaonis* halorhodopsin and *pharaonis* sensory rhodopsin II functionally expressed in *Escherichia coli*. FEBS Lett 442:198–202

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

Jussila T, Tkachenko NV, Parkkinen S, Lemmetyinen H (2001) Kinetics of photo-active bacteriorhodopsin analog 3,4-didehydroretinal. J Photochem Photobiol B 62:128–132

Khodonov AA, Demina OV, Khitrina LV, Kaulen AD, Silfsten P, Parkkinen S, Parkkinen J, Jaaskelainen T (1997) Modified bacteriorhodopsin as a basis for new optical devices. Sens Actuators B 38–39:218–221

Kitajima T, Hirayama J, Ihara K, Sugiyama Y, Kamo N, Mukohata Y (1996) Novel bacterial rhodopsins from *Haloarcula vallismortis*. Biochem Biophys Res Commum 220:341–345

Lukashev EP, Govindjee R, Kono M, Ebrey TG, Sugiyama Y, Mukohata Y (1994) pH dependence of the absorption spectra and photochemical transformations of the archaerhodopsins. Photochem Photobiol 60:69–75

Mukohata Y, Sugiyama Y, Ihara K, Yoshida M (1988) An Australian halobacterium contains a novel proton pump retinal protein: archaerhodopsin. Biochem Biophys Res Commu 151:1339–1345

Oesterhelt D, Stoeckenius W (1974) Isolation of the cell membrane of *Halobacterium halobium* and its function into red and purple membrane. In: Fleischer S, Packer L (eds) Methods in enzymology, vol 31, pp 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

Sambrook J, Russell DW (2001) Molecular cloning, 3rd edn. CSHL Press, Cold Spring Harbor, New York

Shimono K, Iwamoto M, Sumi M, Kamo N (1997) Functional expression of *pharaonis* phoborhodopsin in *Escherichia coli*. FEBS Lett 420:54–56

Sugiyama Y, Maeda M, Futai M, Mukohata Y (1989) Isolation of a gene that encodes a new retinal protein, archaerhodopsin, from *Halobacterium* sp. aus-1. J Biol Chem 264:20859–20862

Tateno M, Ihara K, Mukohata Y (1994) The novel ion pump rhodopsins from *Haloarcula* form a family independent from both the bacteriorhodopsin and archaerhodopsin families/tribes. Arch Biochem Biophys 315:127–132

Uegaki K, Sugiyama Y, Mukohata Y (1991) Archaerhodopsin-2, from *Halobacterium* sp. aus-2 futher reveals essential amino acid residues for light-driven proton pumps. Arch Biochem Biophys 286:107–110

Weetall HH, Druzhko A, Lera AR, Alvarez R, Robertson B (2000) Measurement of proton release and uptake by analogs of bacteriorhodopsin. Bioelectrochemistry 51:27–33

Wise KJ, Gillespie NB, Stuart JA, Krebs MP, Birge RR (2002) Optimization of bacteriorhodopsin for bioelectronic devices. Trends Biotechnol 20:387–394

Yatsunami R, Kawakami T, Ohtani H, Nakamura S (1997) Primary structure of the novel bacterial rhodopsin from extremely halophilic archaeon *Haloarcula japonica* strain TR-1. Nucleic Acids Symp Ser 37:111–112