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Microbial rhodopsins of *Halorubrum* species isolated from Ejinoor salt lake in Inner Mongolia of China

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Abstract

Microbial rhodopsins are photoactive proteins that use retinal molecule as the photoactive center. Because of structural simplicity and functional diversity, microbial rhodopsins have been an excellent model system for structural biology. In this study, a halophilic archaea that has three microbial rhodopsin-type genes in its genome was isolated from Ejinoor salt lake in Inner Mongolia of China. Sequence of 16S rRNA showed that the strain belongs to *Halorubrum* genus and named *Halorubrum* sp. ejinoor (*He*). Translated amino acid sequences of its microbial rhodopsin-type genes suggest that they are homologs of archaerhodopsin (*He*AR), halorhodopsin (*He*HR) and sensory rhodopsin II (*He*SRII). The mRNA of three types of genes were detected by RT-PCR and the amounts of them were investigated by Real-Time RT-PCR. The mRNA of *He*SRII was least and that of *He*AR and *He*HR were 30 times and 10 times more than that of *He*SRII, respectively. The results of light-induced pH changes suggested the presence of a light-driven proton pump and a light-driven chloride ion pump in the membrane vesicles of *He*. Flash induced absorbance changes of the *He* membrane fraction indicated that *He*AR and *He*HR are photoactive and undergo their own photocycle. This study revealed that three microbial rhodopsin-type genes are all expressed in the strain and at least two of them, *He*AR and *He*HR, are photochemically and physiologically active like BR and HR of

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Halobacterium salinarum, respectively. To our knowledge, this is the first report of physiological activity of HR-homolog of *Halorubrum* species.

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Introduction

Microbial rhodopsins form a large family of membrane proteins that use retinal molecule as the chromophore. Since the first discovery of bacteriorhodopsin (BR) more than four decades ago,[1] microbial rhodopsins have been found to be diversely distributed in the microbes of various ecological niches. The functions of microbial rhodopsins were also revealed to be diverse ranging from light-driven ion pumps, light-gated ion channels, and photoreceptors coupled to bacterial two component signaling system.[2]

Most frequently characterized microbial rhodopsins so far are those from halophilic archaea represented by four types of microbial rhodopsins in the cell membrane of *Halobacterium salinarum (Hs)*. The four types of microbial rhodopsins found in *Hs* are bacteriorhodopsin (BR),[1] halorhodopsin (HR),[3] sensory rhodopsin I (SRI) and sensory rhodopsin II (SRII).[4] The former two are light-driven ion pumps and later two are photosensors. BR is a light-driven proton pump. When activated by light, BR transports protons from the cytoplasmic side to the extracellular medium. The proton gradient created by BR can be utilized to synthesize ATP.[5] This kind of machinery enables the host organisms survive in the environment that lacks sufficient substrates. The machinery should be particularly important for the microbes in the saline environments, because such an environment is always densely populated by halophilic microbes and oxygen in such environment is prone to be depleted

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because of the densely populated microorganisms. HR is a light-driven chloride ion pump. When activated by light, HR transports chloride ion from the extracellular medium to the cytoplasmic medium to regain the intracellular chloride lost by membrane potential-driven extrusion.[6] SRI is a photosensor responsible for attractive and repellent phototactic behavior of the bacterium. SRI has two states that generate different signal upon light activation. One of the states, the dark state, whose absorption spectrum is largely overlapped with those of BR and HR, generates attractive signal when activated by light. Another one, a photo-intermediate of SRI dark state, whose absorption spectrum largely covers the harmful blue light region, generates repellent signal when activated by light. Thus, SRI mediates host cell swim toward optimum light harvested by BR and HR while avoiding the harmful blue green light.[7-9] SRII has been identified to be a mono-functional photoreceptor that is responsible for the negative phototaxis of the bacterium. SRII mainly absorbs blue-green light (480-500 nm).[9-11]

Microbial rhodopsin genes have been found in several genera of *Halobacteriaceae* family. The genera include *Halobacterium*, *Haloarcula*, *Halorubrum*, *Haloquadratum*, *Natronomonas*, *Haloterrigena.*[12, 13] Four types of microbial rhodopsins do not necessarily appear together in one strain. Some strain possesses as few as one microbial rhodopsin gene while some strain possesses as many as six microbial rhodopsin genes.[14] It was suggested that gene duplication,[13]

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gene lose and horizontal gene transfer[12, 15, 16] are responsible for the diverse and patchy distribution of microbial rhodopsin gene across *Halobacteriaceae* family.

Emerge of optogenetic research field elevates the research on microbial rhodosins to a new height. Optogenetics refers to a technique that, by expressing light sensitive protein in the target cell, uses light to control specific behavior of cell. The first microbial rhodopsin applied as optogenetic tool is channelrhodopsin (ChR). ChR, a light-gated cation channel, serves as a photoreceptor of unicellular alga that directs host organism toward or away from light to optimize photosynthetic growth.[17] When expressed in a mammalian excitable cell, ChR discharges membrane potential of the cell upon light excitation.[18-20] This feature made light controlled depolarization of excitable cell possible. After ChRs, several archaeal-type microbial rhodopsins were tested for optogenetic applications. HR from *Natronomonas pharaonis* (*Np*),[21, 22] archaealrhodopsin from *Halorubrum sodomense* (*Hrs*) and archaealrhodopsin from *Halorubrum* strain TP009[12, 13] were proven to be applicable as optogenetic tools. Later two are light-driven proton pumps. When these ion pumps were expressed in an excitable cell, light exposure causes hyperpolarization of the target cell. Some properties of microbial rhodopsins, such as cell toxicity, expression level and the absorption maximum wavelength are expected to be modified to enhance their utility in optogenetics. Modifications of the well characterized microbial rhodopsins by genetic engineering are mostly tried approach to achieve these goals.

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However, as the microbial rhodopsins in the natural environments are so diverse in both functionally and photochemically, screening of the microbial rhodopsins should also be one of good ways to achieve the goals.

In the present work we isolated a novel strain that possesses three types of microbial rhodopsin genes, AR-like, HR-like and SRII-like genes. Analysis of 16S rRNA gene sequence suggests that the strain belongs to *Halorubrum* genus. Expression level of these genes, ion-pumping activities as well as flash induced absorbance changes were studied. The results showed that these genes were expressed in the bacterium in different levels and that the AR-like and HR-like proteins are photoactive, undergo their own photocycle and have light-driven pump activities. Although the BR-like proteins of *Halorubrum* species were found and their photoreaction and proton pump activities were reported as archaerhodopsin (AR1-4), [23-27] the photoreaction and light-induced chloride ion-pump activity of HR-like proteins of *Halorubrum* species were first reported. As already stated, homologs of proton pump in *Halorubrum* species have already been applied in optogenetics because of its low cell toxicity and high expression level comparing to its homologs from the other species. Applicability of chloride ion pump from *Halorubrum* species as *He*HR in optogenetics may worth to be tested in the future.

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Experimental

Isolation of halophilc archaea from Ejinoor salt lake

The lake water was collected from a salt lake in Inner Mongolia of China named Ejinoor and filtered by 0.22 μm filter. The collected bacteria on the filter were suspended in a mineral salt medium (MS) containing: NaCl, 250; $MgSO_4$ $7H_2O$, 20; $FeSO_4$ $7H_2O$, 0.05; KCl, 2.0; Trisodium citrate, 3; Bacto Casamino Acids (Difco), 7.5; and yeast extract, 10 (g/liter) at pH 7.4 and then spread onto an agar plate containing MS. After 15 days` incubation at 37 ºC under white light illumination, red colonies grew on the plate. A red colony was picked up and streaked onto new plates for isolation. The procedure was repeated for three times.

DNA extraction, sequence analysis and construction of phylogenetic trees

Genomic DNA was isolated from the bacteria culture (3 ml) grown in MS under white light illumination until the absorbance at 600 nm reached to 1.0. The purified genomic DNA was used as template for PCR. For PCR amplification of 16S rRNA gene fragment, a forward primer (5'-TTC CGG TTG ATC CYG CCG GA-3') and a reverse primer (5'-GGT TAC CTT GTT ACG ACT T-3') were used.[28] The amplification was performed as following: 95 °C (3 min) followed by 30 cycles of 95 °C (1 min), 50 °C (1 min) and 72 °C (1 min) with a final 10 min extension step at 72 °C. PCR products were then separated by agarose gel electrophoresis and

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recovered for cloning into pT7blue vector. The 16S rRNA gene sequences were determined using ABI 3130/3130xl Genetic Analyzer (Applied Biosystems, Foster City, USA). Archaea 16S rRNA sequence data were obtained from the nucleotide database and blast searches at National Center for Biotechnology Information, NCBI. The accession numbers for 16S rRNA sequences are listed in the caption of Fig. 1. The full-length sequences of a AR-like, HR-like, SRII-like, and a transducer protein genes were obtained by genome DNA analysis performed in Center for Gene Research of Nagoya University. Amino acid sequences of the other microbial rhodopsins used for constructing the phylogenetic tree were obtained from NCBI and their accession numbers are listed in Table SA1. The sequences were aligned using GENETYX ver. 10.0.2 (Genetyx, Tokyo, Japan). The phylogenetic tree was constructed using the neighbor joining (NJ)[29] algorithms based on Kimura's two-parameter model[30] with 1,000 randomly selected bootstrap replicates.

The real-time polymerase chain reaction (RT-PCR)

Total RNA of our isolated bacterium and that of *Hs* were extracted from cultures grown to A_{600} of 1.0 using RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The total RNA was dissolved in RNase-free H_2O and the residual DNA was treated with the DNase I (Thermo, Lafayette, USA). The purified total RNA was used to check the expression of the genes by RT-PCR using QIAGEN OneStep RT-PCR Kit (Qiagen, Tokyo,

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Japan). The PCR amplicons were analyzed by agarose gel electrophoresis. The amount of the mRNA of the genes were measured by the real-time RT-PCR using the One Step SYBR® PrimeScript™ PLUS RT-PCR Kit (TaKaRa, Tokyo, Japan) analyzed by StepOnePlus™ Analyzer (Applied Biosystems, Foster City, USA). Quantitative data were presented as the mean ± standard deviation (SD). The data with P-value less than 0.02 were considered statistically significant. The primers used to amplify the three microbial rhodopsins or one transducer protein of *He* are listed in Table SA2 in the supplemental material.

Measurements of ion pump activity of membrane vesicles of *He*

The *He* cells were harvested by centrifugation at 8,000 *g* for 15 min, and the pellet was suspended in 4 M NaCl. The membrane vesicles of *He* were prepared according to the procedure for preparing membrane vesicles of halobacterial strain mex.[31] Pump activity of the membrane vesicles was measured as previously described.[6] Membrane vesicles were suspended in 3 M $NaNO_3$ or 3 M NaCl solution. The sample in the magnetically-stirred 10-mm optical cuvette was irradiated by orange light (590 ± 8.5 nm) from a high power LED (LXHL-LL3C, Philips Lumileds Lighting Co., San Jose, USA). The light-induced pH changes were also measured in the presence of carbonyl cyanide m-chlorophenylhydrazone (CCCP) or tetraphenylphosphonium (TPP^+).

Isolation of the membrane from *He* and *Hs*

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The purple membrane (*Hs*PM) was isolated from *Hs* cells by sucrose density step gradient according to the reported procedure.[32, 33] Isolation of the membrane fraction of *He* followed the same procedure utilized for preparation of *Hs*PM. After centrifuging the sample (80,000 rpm in the TLA-100.4 rotor of a Beckman UItracentrifuge for 20 h), a brown colored membrane was found in the 40 % sucrose layer. It was collected and washed for the sample of flash-photolysis spectroscopy. The collected membrane samples were suspended in 50 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) pH 7.0 at room temperature. Absorption spectra were measured by MPS 2000 recording spectrophotometer (Shimadzu, Kyoto, Japan).

Flash-photolysis spectroscopy

The absorbance change of membrane fraction of *He* and *Hs*PM were monitored after photoexcitation with a Nd:YAG laser pulse (Nd:YAG, 532 nm, 7 ns, 5 mJ per pulse) as described previously.[34, 35] The membrane samples were suspended in 50 mM HEPES with 1 M NaCl or 50 mM HEPES with 1 M Na_2SO_4. Absorption kinetic signals were recorded at four wavelengths (410, 500, 570, and 670 nm) at room temperature.

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Results and Discussion

Isolation and identification of *Halorubrum* sp. ejinoor from Ejinoor salt lake

We isolated a strain of the haloarchaea from Ejinoor salt lake in Inner Mongolia of China. The isolated colony showed red color and was round shaped. Figure 1 shows the phylogenetic tree based on the 16S rRNA gene sequence of halophilc archaea. Constructed phylogenetic tree suggests that the isolated strain should belong to *Halorubrum* genus. Thus we tentatively named the strain as *Halorubrum* sp. ejinoor. Comparison with related sequences showed that the strain shares highest similarities with the following strains: *Halorubrum xinjiangense* AY510707 (99.0 % 16S rRNA gene sequence similarity), *Halorubrum chaoviator* NR042429 (99.0 %), *Halorubrum sodomense* X82169 (98 %). Figure 1 also showed the archaeal strains that possess microbial rhodopsin genes.

Microbial rhodopsin genes from *He*

Three microbial rhodopsin genes, BR-like, HR-like, and SRII-like genes, (Fig. 2) and a transducer protein gene were identified in genome sequence of *He*. The three microbial rhodopsin-type genes encode proteins of 258, 290 and 256 amino acids residues, respectively. The phylogenetic analysis showed that genes can be classified in BR family, HR family and SRII family, respectively (Fig. 2). The sequence of the BR-like gene was most similar to that of archaerhodopsin gene from *Halorubrum xinjiangense* (*Hx*AR).[36] Sequence identities of the gene

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with *Hx*AR, AR from *Halorubrum* sp. aus-1[26, 27] and *Hs*BR are 95 %, 94 % and 57 %, respectively. Because of the high sequence similarities to archaerhodopsin gene, it was tentetivily named *He*AR. All the key amino acids for the proton pumping are conserved in *He*AR as shown in Table 1. These amino acids are Asp95 (corresponding to Asp85 of *Hs*BR), Asp106 (Asp96 of *Hs*BR), Arg92 (Arg82 of *Hs*BR), Glu214 (Glu204 of *Hs*BR), and Asp222 (Asp212 of *Hs*BR).

The identity of the translated amino acid sequences between HR-like gene of *He* and *Hs*HR is 65 %, and most similar to that of HR-like gene of *H. sodomense* (*Hrs*HR)[13] with 84 % of sequence identity. The putative protein was named *He*HR accordingly. The conserved amino acids for the possible chloride pumping proteins are also conserved in *He*HR as shown in Table 1. SRII-like gene is followed by the gene of a cognate transducer protein. The sequence of SRII-like gene showed 60 % of identity with that of *Hs*SRII in deduced amino acids sequence, and most similar to that of SRII-like gene of *Halorubrum chaovitor* (80 %). The putative protein was named *He*SRII. It has been shown that Tyr174 and Thr204 of *Natronomonas pharaonis* SRII (*Np*SRII) are key amino acids for signal relay.[37, 38] Thr204 of *Np*SRII was replaced with Ser209 in *He*SRII as same as Ser201 of *Hs*SRII, although Tyr174 was conserved in *He*SRII.

RT-PCR analysis

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The clear amplified bands of *He*AR, *He*HR, *He*SRII and *He*HtrII were observed in RT-PCR (Fig. 3a) indicating that these genes are expressed in the strain. According to the quantitative real-time RT-PCR measurements, significant differences in expression level of *He*AR, *He*HR, *He*SRII were observed ($P < 0.02$) while the difference in the expression of *He*SRII and *He*HtrII genes was not significant (Fig. 3b). The amount of microbial rhodopsins in the cell membrane of *Hs* is estimated to be BR:HR:SRI:SRII = 1000:100:10:1.[39] However, the relative expression level of these genes in *He* was *He*AR:*He*HR:*He*SRII:*He*HtrII = 30:10:1:1 (Fig. 3b), though the ratio was estimated based on the amount of the mRNA not on that of the proteins. In *Hs*, expression levels of *Hs*BR and *Hs*HR are dependent on oxygen and light conditions.[40, 41] The light and oxygen dependency of expression levels of *He*BR, *He*HR and *He*SRII should be tested in the future.

Ion pump activity of *He* membrane vesicles

Figure 4 shows light-induced proton concentration change in the membrane vesicles of *He* suspensions. A transient alkalization was observed in membrane vesicles of *He* suspended in 4 M NaCl (Fig. 4a). The result was similar to that caused by *Hs*HR. As *Hs*HR is an inward directed chloride ion pump, light irradiation of *Hs*HR containing vesicles creates inside negative membrane potential.[6] Under the motive force, protons are taken up by vesicles that cause alkalization of the outer medium. CCCP is a proton ionophore that facilitates proton movement

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across membrane.[42] Because light induced alkalization observed in the suspension of HR containing membrane vesicles is resulted from passive cross-membrane movement of proton, addition of CCCP would further decrease the concentration of proton of the outer medium. As shown in Fig. 4a, light-induced alkalization of the suspension containing 10 μM CCCP was larger than that observed in the CCCP-free suspension. These results indicated the presence of a light-driven chloride ion pump in the cell membrane.

To test the presence of a light-driven proton pump, TPP^+ was applied to the suspension of *He* membrane vesicles. TPP^+ is a liposoluble cation that can pass through the membrane and eliminate the membrane potential rapidly.[42, 43] Thus, addition of TPP^+ should abolish the proton concentration changes caused by a light-driven chloride ion pump. As shown in Fig. 4b, a light-induced alkalization was abolished and a light-induced acidification was observed after the addition of TPP^+ indicating the presence of both ion pumps. To further confirm the presence of light-driven proton pump, the membrane vesicles of *He* was suspended in 3 M $NaNO_3$ solution to cancel the effect of chloride ion pump. As shown in Fig. 4c decrease of the proton concentration of the outer medium was no longer observed, and a transient acidification of the outer medium was observed with the light irradiation. This result suggests that there did exist a light-driven proton pump in the membrane that pumps proton towards the outside medium as *Hs*BR.[44]

Above experimental results confirmed the presence of light-driven proton pump and light-driven chloride ion pump in the cell membrane of *He*. Because the amino acids sequence of *He*AR and *He*HR are closely related with *Hs*BR and *Hs*HR, the putative proton pump and chloride ion pump are attributable to *He*AR and *He*HR, respectively.

Although ratio of mRNA level of *He*AR and *He*HR was estimated to be 3:1, a prominent *He*HR-induced proton decrease in the outer medium was observed in the membrane vesicles of *He*. It should be noted that similar results were also observed when the native cell suspension of *He* was used as the sample (Supporting Information Fig. SA1). There are several possibilities that might explain these results. One of the possibilities is that the proton conductance of *He* cell membrane was high so that the *He*HR-induced pH change was increased and *He*AR-induced pH change was decreased. The proton conductance of *He* or *Hs* cell membrane was estimated by fitting the pH recovery curve of the membrane vesicle suspensions (3M of $NaNO_3$) after the light illumination with a single exponential equation. Proton conductances of *He* and *Hs* cell membrane vesicles were estimated to be the same within the experimental error. Another possibility is that the ion-pumping efficiency of *He*AR is much lower than that of *He*HR. To test the possibility, we compared the proton-pumping efficiencies of *He*AR and *Hs*BR using the membrane vesicles of *He* and *Hs* suspended in 3M $NaNO_3$. The difference in the proton-pumping efficiency between *Hs*BR and *He*AR was not so large (*Hs*BR:*He*AR = 1:0.86).

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It is difficult to estimate the ion-pumping efficiency of *He*HR separately at present. Heterogeneous expressions of both proteins are under progress in our lab., that would finally explain the apparent discrepancy between expression levels and the ion-pumping activities.

The BR-type retinal protein in *Halorubrum* species was first reported by Mukohata *et al.* as light-driven proton pump, archaerhodopsin-1 (AR1)[26, 27] and studied intensively to elucidate the crystal structure of them (AR1 and AR2).[25, 45] The new light-driven proton pump (AR4) was found in Tibetan salt lake and the pump properties of it was investigated in details.[24, 46] The AR proteins form a patch called a "claret membrane" in the cell membrane of the bacterium. The membrane fraction of *He* seems not to form a "membrane patch" and was not separated by a sucrose step gradient usually used for separation of the purple membrane (Fig. 5a) and even by a linear sucrose gradient centrifugation. An "abnormal" proton pump properties of AR was reported and the possible origin of the differences was presented.[46] The detailed studies on *He*AR pump activity will give a deeper insight of understanding of the issue.

The reports of HR-type protein in *Halorubrum* species, however, were less and limited only about the finding of gene homolog, *Hrs*HR.[13] We clearly showed in the present study that the expression of the *He*HR gene, relative amount of the mRNA (nearly 1/3 of *He*AR), and physiological and photochemical activities (a light-driven chloride pump and chloride ion-dependent photocycle, mentioned below).

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Isolation of the membrane fragment containing *He* rhodopsins

As shown in Fig. 5a, the red membrane and the purple membrane of *Hs* were well separated (layer 1 and 2 in right lane, respectively). But the membrane fragments of *He* were not separated and formed a single layer (layer 1 in left lane) showing brown color. The absorption spectra of the membrane fragment from *He* (Layer 1) and the purple membrane (*Hs*PM) of *Hs* were shown in Fig. 5b. The maximum wavelength of the absorbance was 570 nm in *Hs*PM (*Hs*BR). But in the case of the membrane fraction of *He*, absorption peaks of the carotenoid pigment were prominent (Fig. 5b) suggesting the presence of considerable amount of carotenoid in the membrane fraction of *He*. In *Hs,* BR forms two dimensional crystal that exclude carotenoid molecule. Thus the purple membrane (composed of BR) and red membrane (composed of carotenoid and the other membrane proteins) of *Hs* can be separated by sucrose density gradient.[32, 33, 47] The present results indicate that the distribution of carotenoid and *He*AR in the cell membrane of *He* was different from that of *Hs.* Distribution of *He*AR in its cell membrane may also be different from other ARs that form so called the claret membrane in their cell membrane. It was reported that SDS page of the claret membrane showed mainly the band of ARs and the bands of the other proteins were negligible.[45, 48] However SDS page of the isolated membrane fraction of *He* showed several dominant bands that were not attributable to *He*AR or *He*HR. These results suggest that ARs in the claret membrane formed tight complex

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that exclude the other proteins from the claret membrane while *He*AR may not form such a complex. The CD spectrum of the membrane fraction of *He* showed carotenoid-like bands. As free carotenoid does not represent the CD bands, this result suggests that *He*AR and carotenoid may form a complex in the cell membrane. However, the intensity of the CD bands normalized by the absorbance intensity due to the carotenoids was ten times smaller than that of the claret membrane[49], suggesting the stoichiometry of ARs and carotenoid in the membrane fraction of *He* and in the claret membrane should be different.

Detection of photoreaction intermediates of *He*AR and *He*HR

Upon light activation, both BR and HR undergo cyclic photoreaction with several spectroscopically distinctive intermediates. The photocycle of *Hs*BR composed of K (600), L (540), M (410), N (550) and O (640) intermediates.[50, 51] The photocycle of *Hs*HR composed of K (600), L1 (520), L2 (520), and O (640) intermediates.[52, 53] The numbers in the brackets showed the maximum absorption wavelengths of those intermediates. Figure 6 shows the flash-induced absorbance change at four typical wavelengths, 410, 500, 670 and 570 nm that track the formation and decay of M, L, O and the initial state, respectively.

In *Hs*BR, absorbance change around 410 nm mainly arises from M intermediate. Decay half time of M intermediate of *Hs* is estimated to be 10 ms. The short lived M intermediate is a

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unique character of BR, as HR does not have M intermediate and the lifetime of M intermediate of SRI and SRII is two order longer than that of BR.[9, 37, 54, 55] The absorbance change at 410 nm in Fig. 6a and b clearly indicates the presence of M-like intermediate of *He*AR in the membrane fraction of *He*.

Comparison of 500 nm absorbance changes in the presence (Fig. 6a) and absence of chloride ion (Fig. 6b) suggested the photoactivity of *He*HR. The L intermediate of HR (with the absorption maximum at 500 nm) only appears in the presence of chloride ion and did not appear under the chloride ion free conditions.[34, 52, 56 35, 53, 57 36, 53, 57] Chloride ion has little effect on the absorbance changes of BR at 500 nm. The large difference between the absorbance changes at 500 nm in Fig. 6a and b would be explained by the photoactivity of *He*HR with or without chrolide ion. Absorbance changes at 670 nm reflect mainly the change in O intermediate of BR or HR. In the present work, we could not determine the source of the absorbance change from *He*AR and/or *He*HR.

We could not found the absorbance change arose from the photo-intermediate(s) of *He*SRII. The small expression levels of the protein may be one reason for it.

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Conclusions

We successively isolated a new strain of *Halorubrum* species from a salt lake in Inner Mongolia and named *Halorubrum* sp. ejinoor. Three microbial rhodopsin genes, AR-like (*He*AR), HR-like (*He*HR) and SRII-like (*He*SRII) genes were obtained. The expression of the genes was confirmed by RT-PCR and the relative expression level was determined by the quantitative real-time RT-PCR as *He*AR:*He*HR:*He*SRII:*He*HtrII = 30:10:1:1. The pH change of the membrane vesicle caused by light showed the presence of "proton pump activity" and "chloride ion pump activity". The kinetics of the light-induced absorbance changes of the membrane fraction of *He* suggested the presence of M-like intermediate (*He*AR) and L-like intermediate (*He*HR).

The studies on retinal proteins of *Halorubrum* species were not so much and mainly about the light-driven proton pumps (AR1-4). This new strain and the new retinal proteins, especially *He*HR and *He*SRII provides a challenge to investigate in more detail and to get deeper understanding of the molecular mechanisms of the retinal proteins.

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Figure captions

Figure 1. Phylogenetic tree based on the neighbour-joining (NJ) algorithm for the archaeal 16S rRNA of *Halorubrum* sp. ejinoor and members of the other archaea. The numbers on the nodes indicate the bootstrap values. Archaea from which microbial rhodopsin genes have been found are marked with red color. The sequence data for constructing the tree were obtained from the NCBI database. The accession numbers are given in parentheses.

Figure 2. Phylogenetic tree of microbial rhodopsins. *Halorubrum* sp. ejinoor (*He*) rhodopsins (*He*AR, *He*HR and *He*SRII), can be classified to their respective archaeal rhodopsin groups. Four types of microbial rhodopsins (proton pump, chloride ion pump, sensory rhodopsin I and sensory rhodopsin II) were marked with different color. '*Hc*', '*Ha*', '*Hrs*', '*Hh*', '*Hx*', '*Hs*', '*Hw* I (and *Hw* II)', '*Hm* I (and *Hm* II)', '*Hj*', '*Hv*', and '*Np*' represent microbial rhodopsins from *Halorubrum chaoviator* (AR1), *Halorubrum* sp. aus 2 (AR2), *Halorubrum sodomense* (AR3), *Halobacterium halobium* XZ515 (AR4), *Halorubrum xinjiangense*, *Halobacterium salinarum*, *Haloquadratum walsbyi*, *Haloarcula marismortui*, *Haloarcula japonica*, *Haloarcula vallismortis*, and *Natronomonas pharaonis*, respectively. The scale bar represents 0.1 expected changes per site.

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Figure 3. Comparison of relative mRNA expression level for *He* rhodopsins and transducer protein genes. (a) RT-PCR products were run on an agarose gel (1 %) and stained with ethidium bromide. (b) Relative expression levels of *He*AR, *He*HR, *He*SRII and *He*HtrII genes investigated by the quantitative real-time RT-PCR. Vertical axis was given in logarithmic scale. Error bars represent the mean ± standard deviation (SD). * indicates $P < 0.02$.

Figure 4. Measurements of light-induced proton movement of the cell membrane vesicles of *He* at 20 °C. The red bar indicates the period of illumination by orange light (590 ± 8.5 nm). (a) The cell membrane vesicles were suspended in unbuffered 4 M NaCl solution. After the first light-induced proton concentration changes recovered to the base line, 10 μM CCCP was added and the second light illumination was performed. (b) The cell membrane vesicles were suspended in unbuffered 4 M NaCl solution. After the first light-induced proton concentration changes recovered to base line, 50 μM TPP^+ was added and the second light illumination was performed. (c) The cell membrane vesicles were suspended in unbuffered 3 M $NaNO_3$ solution.

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Figure 5. The membrane fraction of *He* and the purple membrane of *Hs* (*Hs*PM). (a) Separation of membrane fraction by the sucrose density step gradient. Left lane: membrane from *He*; layer 1, the membrane fraction of *He*. Right lane: the membrane of *Hs*; layer 1, the red membrane; layer 2, the purple membrane. (b) Absorption spectra of the membrane fraction of *He* (red) and *Hs*PM (black) suspended in 50 mM HEPES, pH 7.0 at 20 °C.

Figure 6. Comparison of the flash-induced absorbance changes of the membrane fraction of *He* and *Hs*PM. Light-induced absorbance changes of membrane fraction of *He* and *Hs*PM at 20 °C were measured at four wavelengths, 570, 410, 500, and 670 nm. (a) membrane fraction of *He* suspended in 50 mM HEPES, 1 M Na_2SO_4 (under Cl^- free conditions) at pH 7.0. (b) membrane fraction of *He* suspended in 50 mM HEPES, 1 M NaCl (under Cl^- conditions) at pH 7.0.

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Table 1. Comparison of key amino acids of microbial rhodopsins from *He* and *Hs*.

Protein	amino acids and number						
*Hs*BR	**R (82)**	**D (85)**	**D (96)**	**E (194)**	**E (204)**	**D (212)**	**K (216)**
*He*AR	R (92)	D (95)	D (106)	E (204)	E (214)	D (222)	K (226)
*Hs*HR	R (108)	T (111)	A (122)	E (219)	T (230)	D (238)	K (242)
*He*HR	R (119)	T (122)	A (133)	E (230)	T (240)	D (248)	K (252)
*Hs*SRII	R (70)	D (73)	Y (85)	A (180)	Y (190)	D (198)	K (202)
*He*SRII	R (77)	D (80)	Y (91)	T (188)	E (198)	D (206)	K (210)

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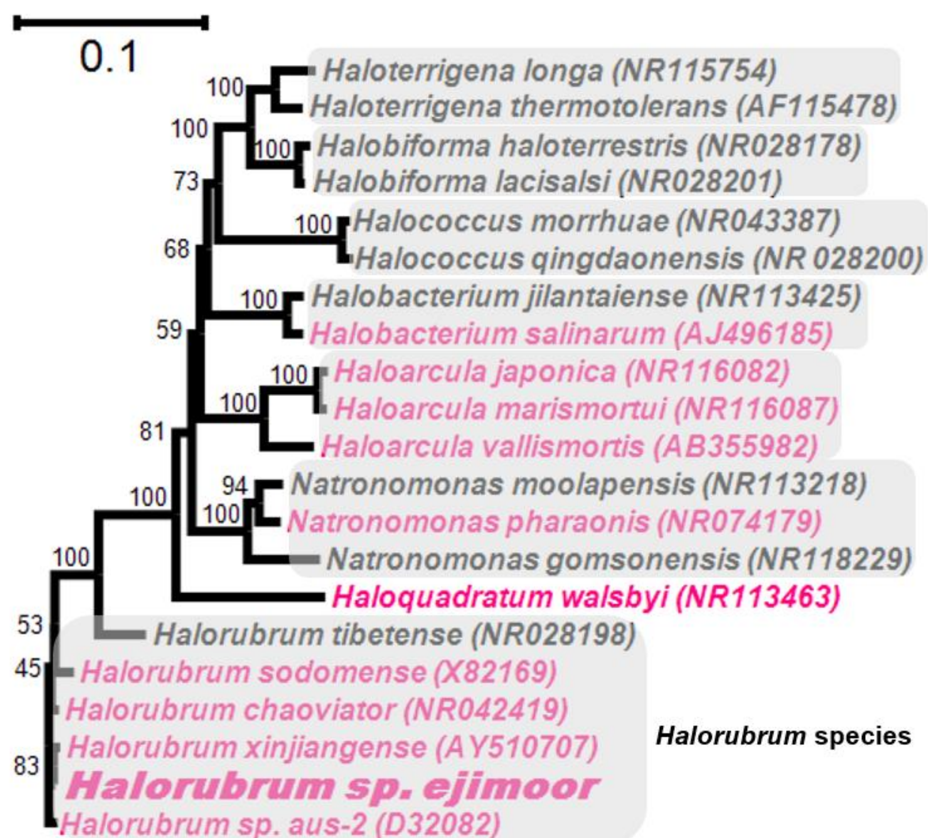

Figure 1. Chaoluomeng *et al*.

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Figure 2. Chaoluomeng *et al*.

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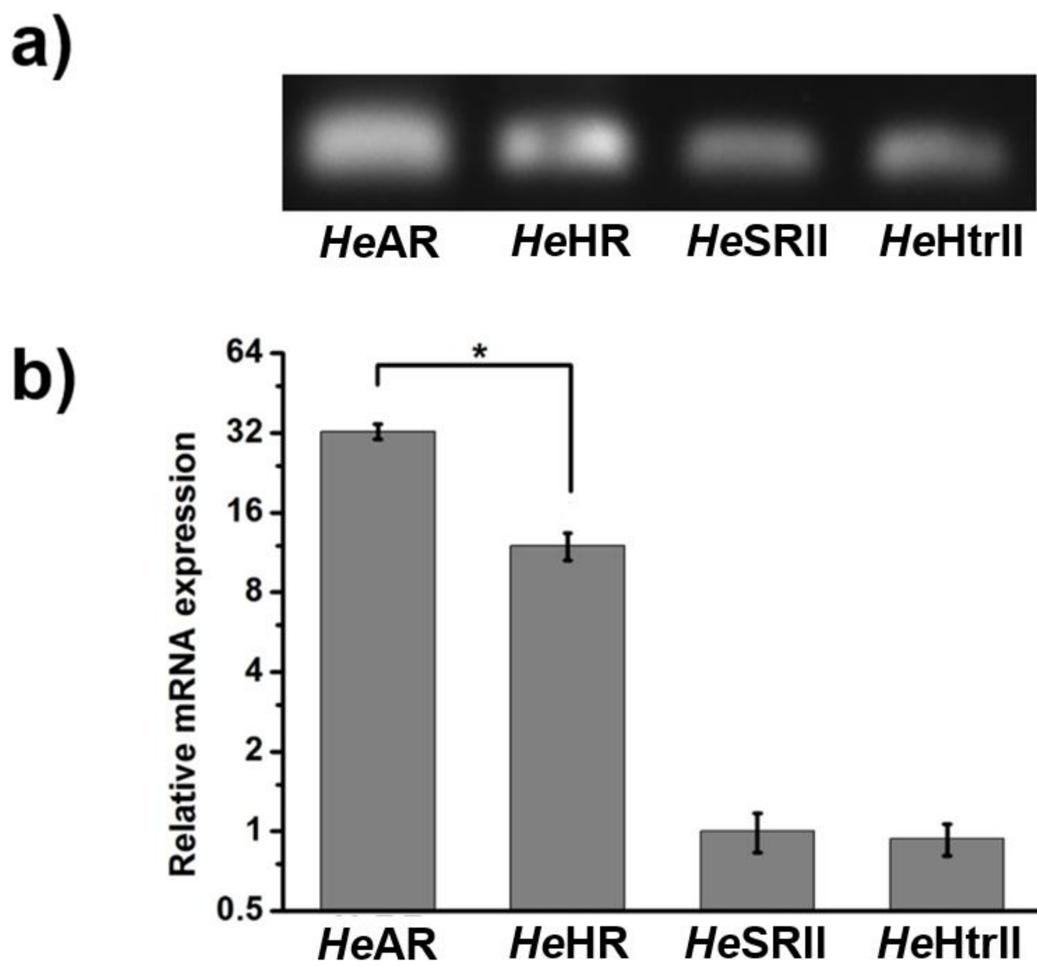

Figure 3. Chaoluomeng *et al*.

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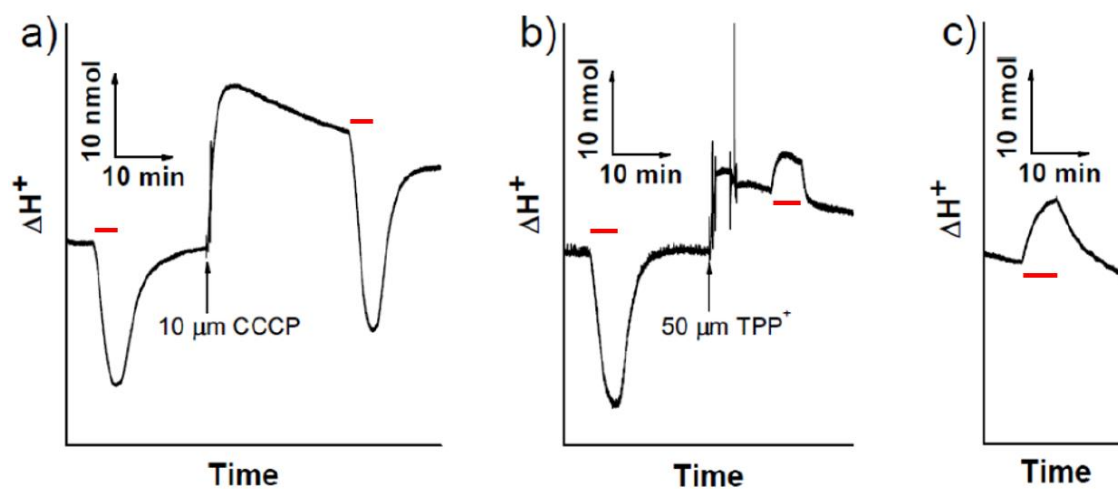

Figure 4. Chaoluomeng *et al*.

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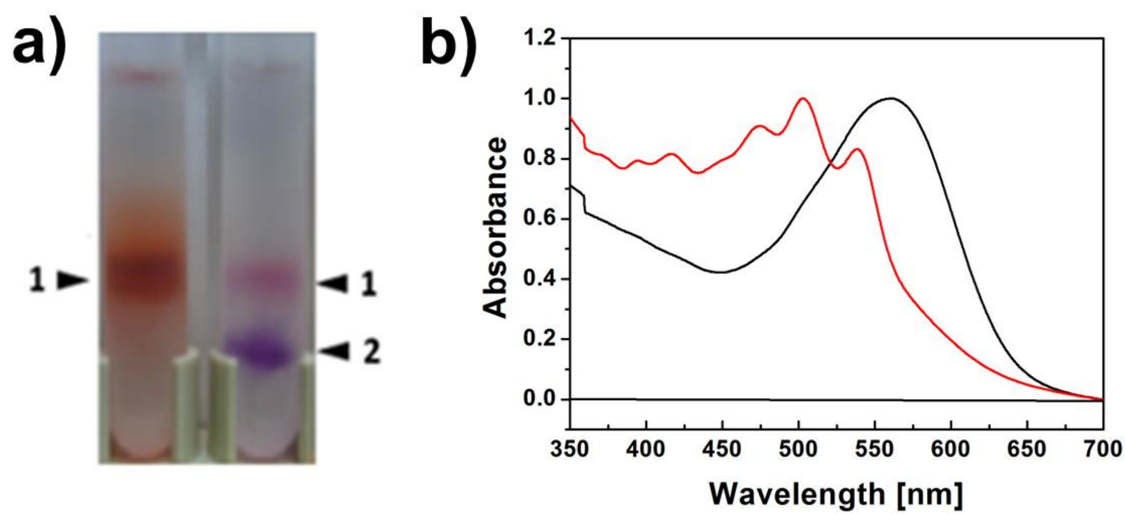

Figure 5. Chaoluomeng *et al*.

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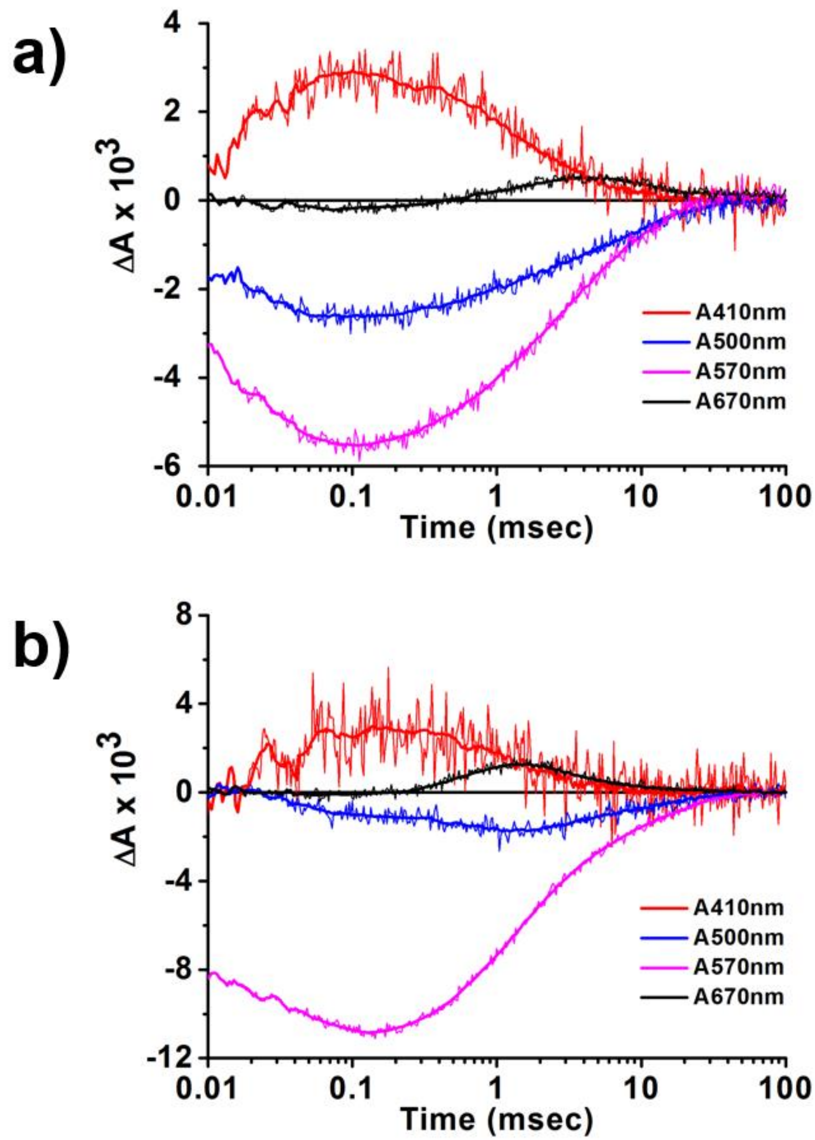

Figure 6. Chaoluomeng *et al*.

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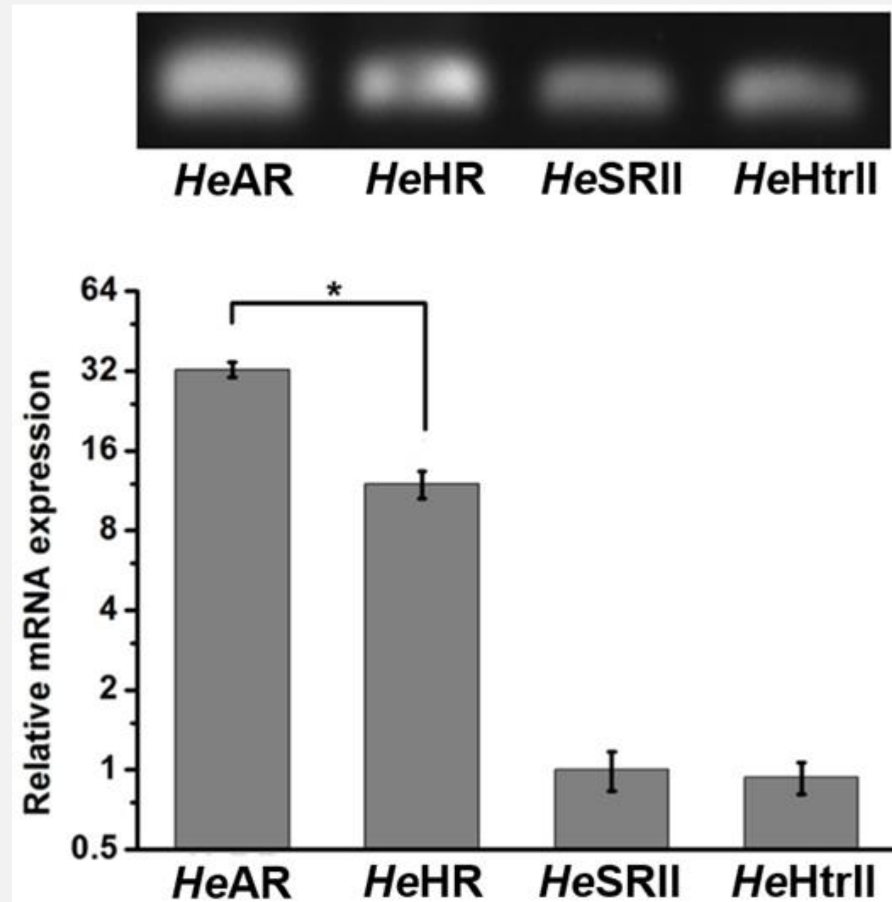
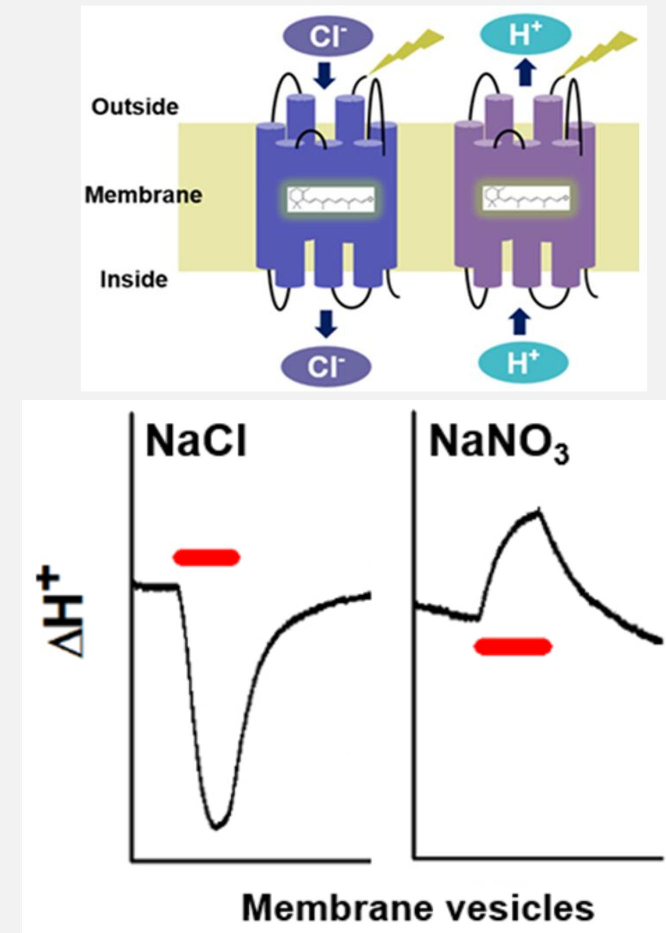
Microbial rhodopsins from *Halorubrum* sp ejinoor



RT-PCR analysis



Ion pump assay

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