The primary structures of halobacterial rhodopsins and light-sensing transducer proteins: An implication to the evolution of bacterial rhodopsins

高度好塩性古細菌に存在する細菌ロドプシンと光感覚情報変換タンパクの一次構造：細菌ロドプシンの進化についての一知見

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The primary structures of halobacterial rhodopsins and light-sensing transducer proteins: An implication to the evolution of bacterial rhodopsins

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ABSTRACT

New bacterial rhodopsins, a bacteriorhodopsin-like and a halorhodopsin-like protein, were identified in *Haloarcula vallismortis*. The genes encoding these ion pumps were cloned and sequenced. .The gene encoding the putative sensory rhodopsins were also cloned. The deduced amino acid sequence of the proton-pump protein was distant from the sequences of both bacteriorhodopsin in *Halobacterium* and archaerhodopsins in *Halorubrum*, but was very similar to cruxrhodopsin-1 (94% homology) in *Haloarcula*, and has thus been included in the cruxrhodopsin tribe as cruxrhodopsin-3. The deduced amino acid sequence of the anion-pump protein was also close to that of a putative anion pump, cruxhalorhodopsin-1, which forms a pair with cruxrhodopsin-1. This anion-pump protein was named cruxhalorhodopsin-3 and the putative sensor protein was named cruxsensory rhodopsin-3. The degree of conservation in the amino acid sequence was discussed for the individual helices of three each rhodopsin homologs, proton pumps, anion pumps and sensors in three different genera, with respect to their functional roles.

The genes of light-sensing transducer proteins were also identified from *Haloarcula vallismortis*, *Halorubrum sodomense* and *Halorubrum* sp. aus-1. These genes were all located upstream of the genes of corresponding sensory rhodopsins. The homology betweeen sensory rhodopsins and that between transducer proteins were well correlated. These

results strongly suggest that there were twice gene duplications of an ancestry rhodopsin gene in the course to develop into four different rhodopsin homologs in the present day species; an ancestry rhodopsin had diverged to be a pump and a sensor, and then the proton pump diverged to be two ion pumps while the sensor diverged after acquisition of a transducer gene into two sensor systems.

INTRODUCTION

Halobacteria are a group of microorganism in the urkingdom Archaea. They are facultatively anaerobic and live only in extremely salty environments as high as 4M. In the presence of oxygen the cells grow by respiration, whereas under anaerobic conditions they can produce ATP by fermentation of arginine (1). The capability for anaerobic growth is important since the oxygen tension in the natural habitat of brines and salt ponds can be very low.

In addition to respiration and fermentation, halobacteria of some genera possess a unique system of photophosphorylation not found in any other living organisms. This photophosphorylation utilizes retinal proteins (rhodopsins) which directly translocate ions across the cell membrane upon absorption of light. Bacteriorhodopsin (bR, (2)) is a light-driven proton pump and halorhodopsin (hR, (3)) is an anion pump. Furthermore, those halobacteria have another retinal proteins, sensory rhodopsin (sR; also known as sensory rhodopsin-I, (4, 5)) and phoborhodopsin (pR; also known as sensory rhodopsin-II, (6)). They are photoreceptors for phototaxis allowing them to move into more comfortable environment (sR) or move out from harmful environment (pR). These four rhodopsins have been studied best in the strain *Halobacterium salinarium* (*halobium*) and the primary structures of bR (7), hR (8) and sR (9) were determined. In these rhodopsins, common features

have been known that they carry (i) retinal as the chromophore which binds to the ε-amino group of the lysine residue conserved in the helix G (ii) seven α-helix structures similar to bR (10) as predicted by hydropathy analysis.

Mukohata et al. isolated new retinal proteins from *Halobacterium* sp. aus-1 and aus-2, collected in Western Australia (11). These proteins, archaerhodopsin-1 (aR-1) in sp. aus-1 (12) and archaerhodopsin-2 (aR-2) in sp. aus-2 (13), have an amino-acid sequence homology of 85% between them, but each has a lower homology (60%, 56%) to bR (12, 13). They pump protons to the same extent as bR, although they have some physicochemical properties different from bR (14). These second proton pumps suggested the nature-conserved residues essential for the structure and function of proton pumps. The strains hosting these new rhodopsins were characterized as in the genus different from those established ones and once described under "Halorubra" (15). The genus was recently established as *Halorubrum* (16). Mukohata et al. also isolated other new retinal proteins from halophiles collected in Argentina, and gave the names cruxrhodopsin-1 (cR-1) and cruxhalorhodopsin-1 (chR-1) (17) for the proton pump and the anion pump in strain sp. arg-1, respectively, and cruxrhodopsin-2 (cR-2) (18) for the proton pump in the strain sp. arg-2 (the corresponding anion pump is absent). The amino acid sequence homology between cR-1 and cR-2 was much higher than that between cR-1 (or cR-2) and bR, aR-1, or aR-2. The host halophiles, strains sp. arg-

1 and sp. arg-2, were considered to be classified in *Haloarcula* (Ihara et al., manuscript in preparation).

After aR-1 was found in *Halorubrum* sp. aus-1, numbers of rhodopsins have been found from various halophilic archaea and their primary structures were determined (Table 1). As described above, these new rhodopsins were classified into (at least) three groups, which we termed "tribes", by their amino-acid sequence homology. We proposed a correlation between the rhodopsin tribe and the halobacterial genus, that is, the species in the genus *Halobacterium* host the rhodopsins in "bR tribe", *Halorubrum* host "aR tribe" and *Haloarcula* host "cR tribe". In order to confirm this concept, a rhodopsin in a predicted tribe should be found in a type-strain. With a type strain *Haloarcula vallismortis* this concept was confirmed in this work.

In the type strain *Haloarcula vallismortis* new rhodopsins of about 26 kDa, a light-driven proton pump and an anion pump were found. Since the strain hosting cR-1 was *Haloarcula*, the rhodopsins in this type strain should be included in the cR tribe. The genes encoding these ion pumps were cloned and sequenced. As expected the amino acid sequence of the proton pump was close to those of cR-1 (94%) (17) and cR-2 (75%) (18), and that of the anion pump was close to that of chR-1 (17) (95%). The proton pump and the anion pump were named cruxrhodopsin-3 (cR-3) and cruxhalorhodopsin-3 (chR-3), respectively. In addition, the gene encoding sensory rhodopsin in *Haloarcula vallismortis*

Table 1. Halobacterial strains and their rhodopsins.

Rhodopsins whose primary structures are now known are shown here. The primary structures of the rhodopsins in bold letters and the HtrIs paired with the underlined sensory rhodopsins were determined in this work.

Table 1. Halobacterial strains and their rhodopsins

bacterial strains		proton pump	anion pump	sensor I	sensor II
Haloarcula	*vallismortis*	**cR-3**	**chR-3**	**csR-3**	vsR-II
	sp. arg-1	cR-1	chR-1		
	sp. arg-2	cR-2	-		
	sp. port	port bR	port hR		
	sp. shark	shark bR	shark hR		
Halobacterium	*salinarium*	bR	hR	sR	
Halorubrum	sp. aus-1 (SG)	aR-1 (SG bR)	SG hR	SG sR	
	sp. aus-2	aR-2			
	sodomense	aR-3		asR-3	
	sp. mex	mex bR	mex hR		
Natronobacterium	*pharaonis*	-	phR		psR-II

(csR-3) was cloned and sequenced. Therefore, with the reported data (19) for the gene encoding vsR-II (= cpR-3 in our notation), the putative primary structures of four different rhodopsin homologs were all determined for the first time in one species, *Haloarcula vallismortis*.

The photoreceptor rhodopsins for phototaxis, sR and pR, are paired with HtrI (halobacterial transducerI for rhodopsin) and HtrII, respectively, in the cell membrane. HtrI (and HtrII) receives light stimulus from sR (and pR) and transmits it into cytoplasm to control swimming behavior (20, 21). So far the primary structures have been known only for HtrI of *Halobacterium salinarium* (22), HtrII of *Haloarcula vallismortis* (19) and HtrII of *Natronobacterium pharaonis* (19). All three Htr genes are commonly flanked by the corresponding sensor genes, and Htr and sensor rhodopsin are known to be expressed as a single transcriptional unit (23). It is predicted by the hydropathy analysis that the Htrs have two putative transmembrane helices and a large cytoplasmic domain (22).

In this work, together with the sR gene, the HtrI gene paired with the sRI gene was also cloned and sequenced. The HtrI gene of *Haloarcula vallismortis* was located just upstream of its sRI gene and the HtrI protein was found to be much more homologous to HtrI of *Halobacterium salinarium* (22) than HtrII of *Haloarcula vallismortis* (19). The relatedness is similar to those between sR and pR; sRI of *Haloarcula vallismortis* is much closer to sRI of *Halobacterium salinarium* (9) than sRII of *Haloarcula*

vallismortis (19). That is, the homology between the same homologs in the different tribe is higher than that between the different homologs in the same tribe both in sR and Htr. In order to understand relatedness of halobacterial rhodopsins and Htrs, HtrI genes of *Halorubrum sodomense* and *Halorubrum* sp. aus-1 were also cloned and sequenced. Both genes also carried the HtrI-sR tandem gene structure. These HtrI-sR tandem gene and the relatedness of each homology index gave a hypothesis how bacterial rhodopsin have diverged into four different rhodopsin homologs in a single cell, this was also discussed in this article.

MATERIALS AND METHODS

1. Bacterial strains and growth conditions

Haloarcula vallismortis (NCMB 2082) was purchased from NCMB (The National Collection of Marine Bacteria, Ltd.). *Halorubrum sodomense* (IFO 14740) was puchased from Institute for Fermentation, Osaka. *Halorubrum* sp. aus-1 was collected from a nameless clay pan near Northampton, Western Australia and registered as in the genus *Halorubrum*. Cells were cultured in a medium composed of 25% NaCl, 2% $MgSO_4$, 0.2% KCl, 0.02% $CaCl_2$, 0.3% sodium citrate and 1% peptone, at pH 7.0 in tap water, under vigorous shaking at 40°C until the absorbance of the culture at 660 nm approached 1. *Escherichia coli* strains JM 83 and JM 109 was grown in LB medium (24) and used for gene manipulation.

2. Preparation of cell envelope vesicles of *Haloarcula vallismortis* and pH measurement

Haloarcula vallismortis cells were harvested by centrifugation and washed three times with 4M NaCl. The cell suspension in 4M NaCl was sonicated, and the cell envelope vesicles were collected by centrifugation (45,000 x *g* for 30 min). The vesicles suspended in 4M NaCl were layered on 15% sodium tartrate in 4M NaCl and centrifuged at 30,000 x *g* for 30min (25). Vesicles obtained in a layer just above the tartrate/NaCl were washed once with 4M NaCl. A part of these vesicles was dialyzed overnight against 1.5M Na_2SO_4 to

replace chloride with sulfate. pH changes in stirred vesicle suspensions (1 mg/ml protein) were measured at 30°C with a micro pH electrode under actinic illumination by a slide projector (about 10^5 lx).

3. Preparation of cR-3 and chR-3

Haloarcula vallismortis cells in 4M NaCl were frozen in liquid nitrogen then thawed in water. After DNase I (Sigma Chemical) digestion, membranes were collected by centrifugation at 40,000 x *g* at 4°C for 60 min and washed. The membranes were solubilized with 1.5% sodium cholate in 4M NaCl, then centrifuged (40,000 x *g*, 60 min) to remove insoluble debris. The supernatant was loaded onto a 2.6 ϕ x 10 cm column of Phenyl-Sepharose CL-4B (Pharmacia LKB Biotechnology) pre-equilibrated with buffer A (4M NaCl, 25mM Tris-HCl, pH 7.2, 0.4% sodium cholate). After the column was washed with buffer A, a purple-colored fraction was eluted with buffer B (4M NaCl, 25mM Tris-HCl, pH 7.2, 0.5% nonanoyl-N-methylglucamide (MEGA-9)). The eluted fraction containing cR-3 and chR-3 was dissolved in buffer C (1M NaCl, 20% saturated $(NH_4)_2SO_4$, 25mM Tris-HCl, pH 7.2, 1.5% sodium cholate), and loaded onto a 1.6 ϕ x 10 cm column of Octyl-Sephrose CL-4B (Pharmacia LKB Biotechnology) pre-equilibrated with buffer C. When the column was eluted with buffer D (1M NaCl, 20% $(NH_4)_2SO_4$, 25mM Tris-HCl, pH 7.2, 0.1% sodium cholate, 0.35% MEGA-9), two purple protein fractions were separated. The front fraction was found to be cR-3 and the rear was chR-3. The obtained cR-3 protein was

dialyzed against 0.5% sodium dodecyl sulfate (SDS), 10mM Tris-HCl, pH 7.2 and separated on SDS-polyacrylamide gel electrophoresis (1% SDS, 12.5% gel) (26) from trace contaminants. The protein was transferred to a Polyvinylidene-difluoride membrane (Immobilon-P, Millipore) by electroblotting, and then stained by Coomassie Brilliant Blue. A stained protein was cut out and analyzed by a gas-phase amino acid sequencer.

4. PCR amplification of retinal proteins

Parts of the genes coding for cR-3, chR-3 and csR-3 were amplified from genomic DNA of *Haloarcula vallismortis* by PCR method (27). The gene coding for partial asR-3 was amplified from genomic DNA of *Halorubrum sodomense*. Genomic DNA of *Haloarcula vallismortis* and *Halorubrum sodomense* was prepared from 300 ml of full-growth culture . For the cR-3 gene, a sense primer was designed for the N-terminal 6 amino acids of cR-3 protein and an antisense primer was designed for the region of helix G highly conserved among 7 proton pumps (bR, aR-1, aR-2, cR-1, mexbR, portbR, sharkbR) (Fig. 1). For the gene of chR-3, two primers encoding respectively the highly conserved regions of helix C and helix G between hR (8) and pharaonis halorhodopsin (phR, (28)) were used (Fig. 1). For the gene of csR-3 and asR-3, two primers encoding respectively the highly conserved regions of helix E and helix G between sR and SG-sR (29) were used (Fig. 1). PCR of these four genes was performed (DNA Thermal Cycler, Perkin-Elmer Cetus) by 30 cycles of denaturation at 94°C for

1. 5'-ATGCC(GC)GC(AGCT)CC(AG)GA(AG)GG-3'

2. 5'-GA(GT)(AGC)CCGAA(GC)CCGACCTT-3'

3. 5'-CCGATGATACTGCTGGCGCT-3'

4. 5'-GGATGAACGCGAAGACGTAC-3'

5. 5'-AAGTGGG(CT)GCTGTTCGGCGT-3'

6. 5'-TA(GC)ACGTACGG(GC)ACCTTCGC-3'

7. 5'-ATGGCAACGATAACAACCTGGTTC-3'

8. 5'-CTAGTCCCCTGCAACCGCTGT-3'

9. 5'-CCACAGCAG(GC)CCGACGTGGTTCTTCAG(CT)AG-3'

10. 5'-ACGTCGAGGAAGGCGTACGTCAGCGCGGCG-3'

Figure 1. Primers for PCR amplification

Primers were used; 1, 2 for the proton pump gene; 3, 4 for the anion pump gene; 5, 6 for the sensor I gene and 7, 8 for the transducer gene.

30 sec, annealing at 37°C for 30 sec, and chain extension at 72°C for 3 min in a 100μl reaction mixture containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, 200μM each dNTPs, 2.5U *Taq* polymerase, 100pmol primers (DNA synthesizer 392, Applied Biosystems), and 1μg genomic DNA. For the probes in the following Southern hybridization, the DNA fragment amplified by PCR was isolated by polyacrylamidegel electrophoresis (5% gel) and labeled with [α-^{32}P]dCTP (DuPont/New England Nuclear) by the random primer method (30). After genomic DNA digestion with different restriction endnucleases (Toyobo, Nippon Gene), the fragment was separated on 0.8% agarose gel electrophoresis and transferred to nylon membranes by capillary blotting (24). The membranes were hybridized with each probe and washed in 6 x SSC at 60°C.

5. Cloning and sequencing of retinal protein genes

Standard methods were used for cloning and sequencing (24) of the genes of cRs and asR-3. Each ^{32}P-labeled PCR product was shown to hybridize with a restriction fragment of *Haloarcula vallismortis* genomic DNA digested by *Sac* I (cR-3, chR-3) or *Sal* I (csR-3). Part of the asR-3 gene was shown to hybridize with a restriction fragment of the *Sac* I digested genomic DNA of *Halorubrum sodomense*. Then 10μg of genomic DNA was digested by *Sac* I or *Sal* I and ligated into the vector pUC18 (Takara Shuzo) which was *a priori* dephosphorylated by calf intestine alkaline phosphatase (Boehringer Mannheim). The recombinant DNA was transformed

into *Escherichia coli* JM 83 then processed to determine the nucleotide sequences which encode cR-3, chR-3 and csR-3 by the dideoxy chain termination method (31). The sequence of asR-3 was determined by an ALF*red* DNA sequencer (Pharmacia). Both strands of these genes were sequenced.

6. Cloning and sequencing of transducer protein genes

The *Sal* I fragment containing csR-3 contained 71 base of the HtrI gene upstream of the sR gene. Southern analysis showed that the 3.5 kb *Kpn* I fragment hybridized with the *Sal* I-*Kpn* I fragment of pVSR3 (Fig. 2) containing csR-3 as a probe. Using this *Sal* I-*Kpn* I fragment as a probe, the positive clone containing the HtrI gene was screened from the genomic *Kpn* I library of *Haloarcula vallismortis*. The HtrI gene of *Halorubrum sodomense* was obtained in the *Bam*H I fragment containing asR-3.

7. PCR amplification of transducer proteins in *Halorubrum* sp. aus-1

From the DNA sequence of SG-sR (asR-1), the HtrI gene of aus-1 was amplified *in vitro* by LA PCR (32). The PCR was performed with the two antisense primer (Fig. 1) using the *Bam*H I digested fragment of the *Halorubrum* sp. aus-1 genomic DNA as template. After cloning of the amplified fragment into the vector pUC 18, the HtrI gene of *Halorubrum* sp. aus-1 was sequenced.

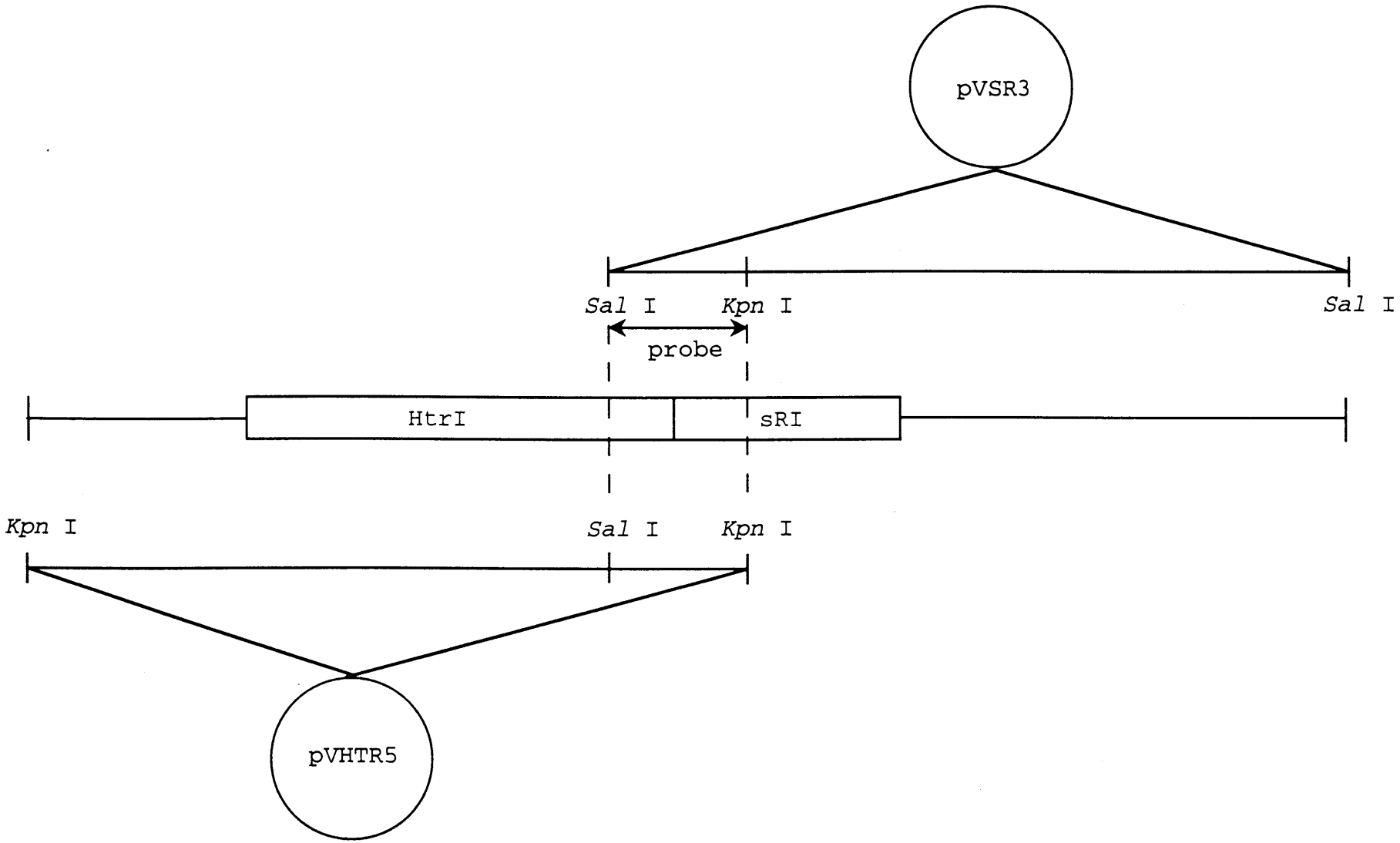

Figure 2. Probe for the HtrI gene of *Haloarcula vallismortis*

Figure 2. Probe for the HtrI gene of *Haloarcula vallismortis*.

The plasmid pVSR3 contained the csR-3 gene and 71 bases of the HtrI gene of *Haloarcula vallismortis*. The *Sal* I-*Kpn* I fragment of pVSR3 was used as probe for cloning of pVHTR5 containing the HtrI gene.

8. Computer programs

For analysis of the relationship among the resolved sequences, the program GENETYX 6.2.0 (Software Development Co., Ltd) was used. The protein sequences were initially aligned using the program CLUSTAL W (33) and then further edited manually. The percentage of identical amino acids or nucleotides between two sequences was calculated using the program Homology 2.2.2 (Software Development Co., Ltd). The divergence of the individual helices was determined as follows. The amino acid sequences of each helix were aligned and then the fraction of identical amino acids between each pair of helix was calculated.

RESULTS

I. Rhodopsins in halobacteria

I-1. Light-induced pH changes

When cell envelope vesicles of *Haloarcula vallismortis* in 4M NaCl were illuminated, the vesicle suspension was monophasically acidified (Fig. 3). In the presence of 10μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP), a protonophore, the suspension was monophasically alkalized in the light. With the further addition of triphenylmethylphosphonium cation ($TPMP^+$) to 1mM, light-induced pH changes were almost vanished. $TPMP^+$ alone induced larger light-induced acidification. The vesicles dialyzed overnight against 1.5M Na_2SO_4 (to inactivate the anion pump), in the presence of 10μM CCCP (to blind the proton pump), showed only slight alkalization in the light. By adding NaCl, the light-induced alkalization increased with the chloride concentration (Fig. 4). Bromide induced much greater alkalization while nitrate induced less.

I-2. The proton pump gene of *Haloarcula vallismortis*

After PCR amplification with sets of probes, the products were electrophoresed on 1.5% agarose gel and selected for the expected sizes of about 700 bp from the N-terminus to helix G. This 700 bp PCR fragment was inserted into plasmid pUC 18 and sequenced. The deduced amino acid

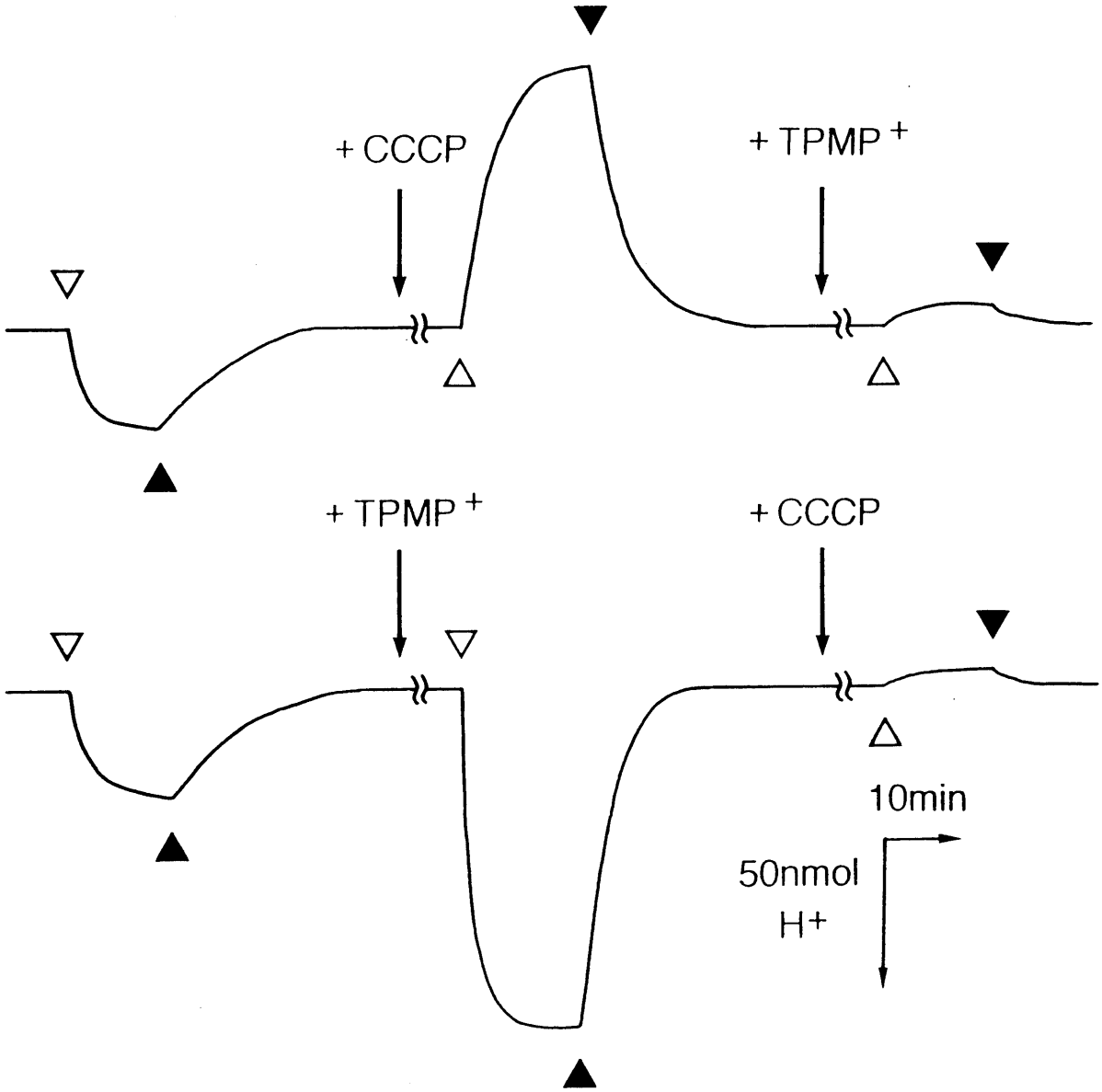

Figure 3. Light-induced pH changes of the cell vesicles of *Haloarcula vallismortis*

pH measurements were carried out in 1 mg/ml protein in 4M NaCl at 30°C with an actinic light on (△) and off (▲), before and after sequential addition of CCCP and $TPMP^+$ or vice versa.

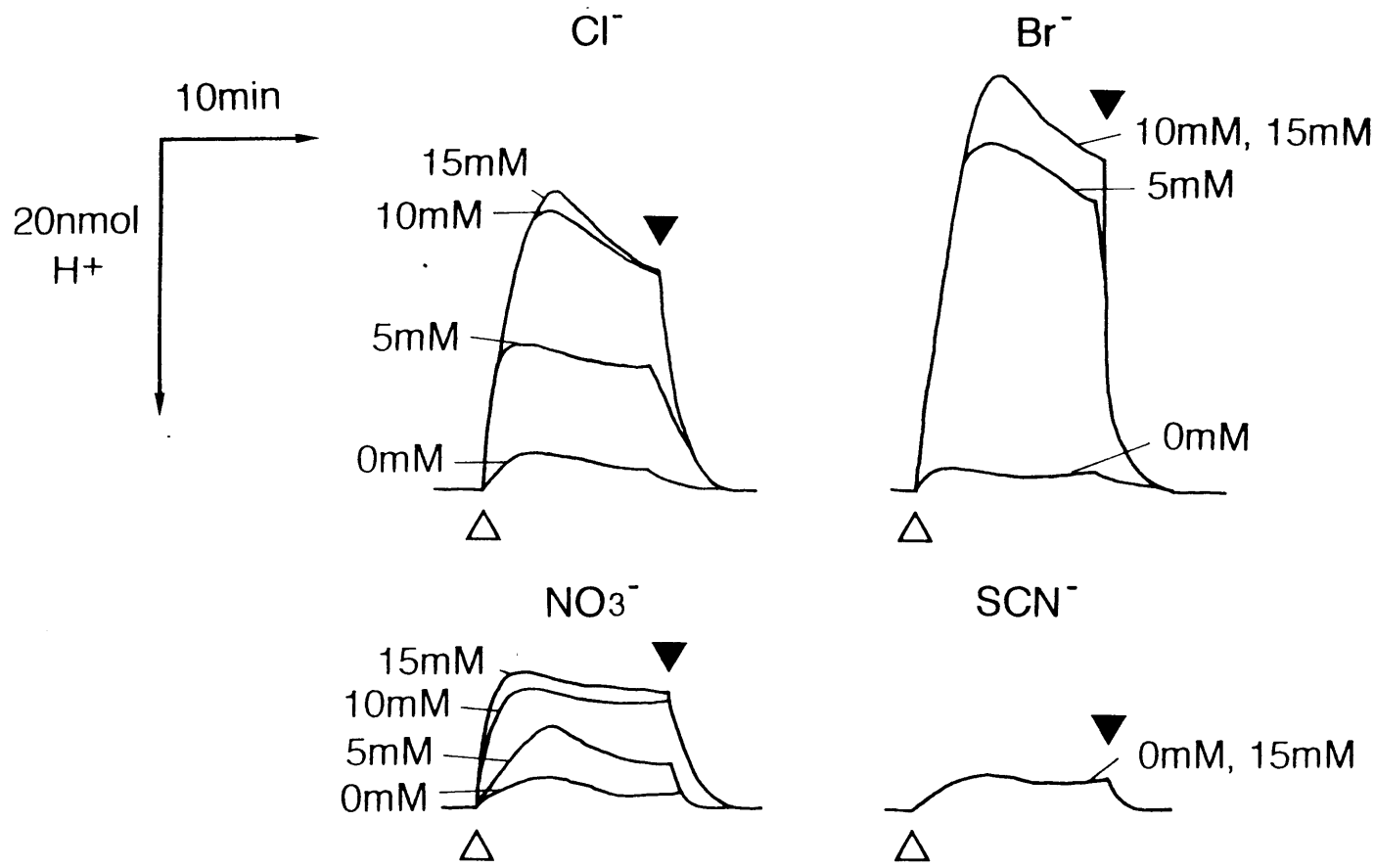

Figure 4. Dependence on anion concentration of the anion pump activity

pH measurements were carried out in 1 mg/ml protein in 1.5M Na_2SO_4 and 10μM CCCP at 30°C with an actinic light on (△) and off (▲) after each adddition of NaCl or $NaNO_3$ or NaBr or NaSCN at given final concentrations.

sequence of the insert DNA showed high homology to bR, suggesting the partial genes for the bR-like proteins of *Haloarcula vallismortis*. Southern analysis showed that this PCR amplified 700 bp partial genes hybridized with a single band in 4.4 kbp *Sac* I fragment of *Haloarcula vallismortis* genomic DNA digestion. Then, from the genomic *Sac* I library of *Haloarcula vallismortis*, the positive clones were screened with the amplified 700bp fragments as probes by colony hybridization. Using the standard procedure, a DNA fragment including an open reading frame of cR-3 was sequenced from these clones. The cR-3 gene consisted of 753 bp nucleotides coding for 250 amino acids with a molecular mass of 26852 Da (Table 2). The N-terminal sequence of the cR-3 protein, MPAPEGEAI, determined by amino acid sequencer was exactly found in this deduced amino acid sequence.

I-3. The anion pump gene of *Haloarcula vallismortis*

In a similar procedure described above for proton pump, the PCR products were selected for the expected size of 400 bp between helix C and G. Southern analysis showed that 400 bp partial genes hybridized with a single band in the 5.0 kbp *Sac* I digested fragment. From the genomic *Sac* I library, the positive clones were screened with the amplified 400 bp fragment as probe. A DNA fragment including an open reading frame of possible chR-3 was sequenced from these clones. The gene encoding chR-3 consists of 831 bp nucleotides for 276 amino acids with a molecular mass of 29116 Da (Table 2). The

Table 2. Molecular weights of rhodopsins and transducers

The calculated molecular weights of the rhodopsins and transducers found in this work are shown. The molecular weights are from the deduced sequence, but not of expressed proteins. The HtrI of *Halorubrum* sp. aus-1 is a partial sequence.

strain	pump	sensor and transducer	
Haloarcula vallismortis	cR-3 26852 chR-3 29116	csR-3 25127	HtrI 55808
Halorubrum sodomense		asR-3 26281	HtrI 54773
Halorubrum sp. aus-1		asR-1 (=SG-sR) 25748	HtrI (45196)

N-terminal sequence of the chR-3 protein could not be found by an N-terminal analysis.

I-4. The sensor I and sensor II genes of *Haloarcula vallismortis*

The PCR products were selected for the expected sizes of 260 bp between helix E and G. Southern analysis showed that 260 bp partial genes hybridized with a single band in the 1.9 kbp *Sal* I fragment. From the genomic *Sal* I library, the positive clones were screened with the amplified 260 bp fragments as probes. A DNA fragment including an open reading frame of csR-3 was sequenced from these clones. The gene encoding possible csR-3 consists of 711 bp nucleotides for 236 amino acids with a molecular mass of 25127 Da (Table 2).

The gene of vsR-II (sensor II; cpR-3 in our notation) in *Haloarcula vallismortis* was sequenced by Seidel et. al (19). To confirm that the strain NCMB 2082 in our laboratory has the same vsR-II gene as in the reference (19), the gene corresponding to sensor II rhodopsin (pR) in *Haloarcula vallismortis* NCMB 2082 was amplified by PCR with the primers described for vsR-II. The PCR product was sequenced and found to be identical to the reported vsR-II gene. The amplified fragment also hybridized with a single band in the 3 kbp *Sma* I digested genomic DNA as in the reference (19).

I-5. The sensor I gene of *Halorubrum sodomense*

The PCR products were selected for the expected sizes of 260 bp between helix E and G. Southern analysis showed that 260 bp partial genes hybridized with a single band in the 5.0 kbp *Sac* I fragment. From the genomic *Sac* I library of *Halorubrum sodomense*, the positive clones were screened with the amplified 260 bp fragments as probes. A DNA fragment including an open reading frame of asR-3 was sequenced from these clones. The gene encoding possibly asR-3 consists of 765 bp nucleotides for 254 amino acids with a molecular mass of 26281 Da (Table 2).

I-6. The sensor I gene of *Halorubrum* sp. aus-1

Since the proton pump gene of *Halorubrum* sp. aus-1 was identical to that of the strain SG (29), the sensor I gene of sp. aus-1 was predicted to be identical to that of the strain SG (= SG-sR). The PCR products were selected for the expected sizes of 260 bp between helix E and G. This fragment was inserted into plasmid pUC 18 and sequenced. The DNA sequence was identical to the SG-sR gene (Table 2).

I-7. Alignment of four rhodopsins of *Haloarcula vallismortis*

The amino acid sequence of three rhodopsins of *Haloarcula vallismortis* determined in this work and vsR-II (19) were all aligned (Fig. 5). 21 amino acids were conserved in these four rhodopsins, most of which were localized in helix C and helix F. 17 out of these conserved

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                                                    --------helix A--------
cR1                                          MPEPGSE AIWLWLGTAGMFLGMLYFIARGW
cR2                                    MLQSGMSTYVPGGE SIFLWVGTAGMFLGMLYFIARGW
cR3                                          MPAPEGE AIWLWLGTAGMFLGMLYFIARGW
chR3                 MPAASTAATTLLQASQSEVLGEIQSNFLL NSSLWVNIALAGVVILLFVAMGR
csR3                                           MDAV AVVYGITAAGFAVGVAIVGYLYA
vsRII                                          MATI TTWFTLGLLGELLGTAVLAYGYT

                 ---------helix B---------                                -----
cR1      GETDS RRQKFYIATILITAIAFVNYLAMAL GFGLTIVEFA......GEEHPIY WARYS
cR2      SVSDQ RRQKFYIATIMIAAIAFVNYLSMAL GFGVTTIELG......GEERAIY WARYT
cR3      GETDS RRQKFYIATILITAIAFVNYLAMAL GFGLTIVEIA......GEQRPIY WARYS
chR3     ELESS RAKLIWVATMLVPLVSISSYAGLAS GLTVGFLQMPPGHALAGQEVLSP WGRYL
csR3     SLEGS EERSILAALALIPGFAGISYVAMAF GIGTVTIGET..........TLV GFRYL
vsRII    LVP.E ETRKRYLLLIAIPGIAIVAYALMAL GFGSIQSEG..........HAVY VVRYV

         helix C----------          ------helix D-------
cR1      DWLFTTPLLLYDLGLLA GADRNT ITSLVSLDVLMIGTGLVATL SPGSGVLSAGAERL
cR2      DWLFTTPLLLYDLALLA GADRNT IYSLVGLDVLMIGTGALATL SAGSGVLPAGAERL
cR3      DWLFTTPLLLYDLGLLA GADRNT ISSLVSLDVLMIGTGLVATL SAGSGVLSAGAERL
chR3     TWTFSTPMILLALGLLA DTDMAS LFTAITMDIGMCITGLAAAL VTSS.....HLLRW
csR3     DWVVTTPLLVGFVGYAA GASRRA IFGVMVADALMILTGVGAVV ADGT.......LKW
vsRII    DWLLTTPLNVWFLALLA GASRED TVKLVVLQALTIVFGFAGAV TPSP.......VSY

         -------helix E--------                ----------helix F----------
cR1      VWWGISTAFLLVLLYFLFSSLS GRVADLPSD. TRSTFKTLRNLVTVVWLVYPVWWLIGT
cR2      VWWGISTGFLLVLLYFLFSNLT DRASELSGD. LQSKFSTLRNLVLVLWLVYPVLWLVGT
cR3      VWWGISTAFLLVLLYFLFSSLS GRVADLPSD. TRSTFKTLRNLVTVVWLVYPVWWLVGT
chR3     VFYGISCAFFIAVLYVLLVEWP ADAEAAG... TSEIFGTLKLLTVVLWLGYPILWALGS
csR3     VLFGVSTVFHVSLFAYLYLVFP RSVPDDPQ.. RIGLFSLLKNHIGLLWIAYPLVWLAGP
vsRII    ALFAVGGALFGGVIYLLYRNIA VAAKSTLSDI EVSLYRTLRNFVVVLWLVYPVVWLLGA

                  ---------helix G----------
cR1       EGIGLVGIG IETAGFMVIDLTAKVGFGIILLRSHG VLDG....AAETTGTGATPADD
cR2       EGLGLVGLP IETAAFMVLDLTAKIGFGIILLQSHA VLD.....EGQTASEGAAVAD
cR3       EGIGLVGIG IETAGFMVIDLVAKVGFGIILLRSHG VLDG....AAETTGAGATATAD
chR3      EGVALLSVG VTSWGYSGLDILAKYVFAFLLLRWVA ANEDTVTQAGMSLGSGGAAPADD
csR3      EGLGLATYV GVSITYAFLDLLAKVPYVYFFYARRQ VFAT...KLLRDSGEVTATPAD
vsRII     AGVGLMDVE TATLVVVYLDVVTKVGFGVIALLAMI DLGS....AGETAEEPTAVAGD
```

Figure 5. Alignment of four kind of homologs of *Haloarcula vallismortis* and proton pumps in cR tribe

Figure 5. Alignment of four kind of homologs of *Haloarcula vallismortis* and the reported proton pumps in the cR tribe.

The vsR-II (cpR-3 in our notation) sequence is cited from (19). The helix sequences are shown with broken lines. The red color shows the conserved amino acids among all known rhodopsins. The blue color shows the conserved amino acids only among the cR tribe rhodopsins.

21 residues were commonly conserved throughout the known pumps and sensors. The remaining four residues were conserved only in the cR tribe (cR-1, cR-2, cR-3, chR-1, chR-3, csR-3 and vsR-II). The insertion sequence GVLS/PA between helix D and helix E which was conserved only in the proton pumps of the cR tribe was also found in cR-3. There was no conserved amino acid at N-terminal region and in helix A in four kinds of rhodopsins, therefore the sequences of these regions could not be aligned in one way.

II. Halobacterial transducer proteins for rhodopsin

II-1. The genes of transducer protein

The HtrI gene of *Haloarcula vallismortis* consists of 1587 bp nucleotides for 528 amino acids with a molecular mass of 55808 Da. The HtrI gene of *Halorubrum sodomense* consists of 1545 bp nucleotides for 514 amino acids with a molecular mass of 54773 Da. The HtrI gene of *Halorubrum* sp. aus-1 was partial gene (Table 2). All the genes of transducer protein HtrI were located just upstream of the genes of the corresponding photoreceptor sR in *Haloarcula vallismortis*, *Halorubrum sodomense* and *Halorubrum* sp. aus-1. The Htr gene and the paired sensor gene overlapped by several bases so as to locate the stop codon of HtrI after the start codon of sR (Fig. 6). In contrast, the HtrII genes were located upstream of sRII genes, the two genes were separated by two bases in *Haloarcula vallismortis* (19) and *Natronobacterium pharaonis* (19). Although only a few examples were available, the same homolog (sR-HtrI and pR-HtrII) seems to have the same way to arrange two genes.

II-2. The putative secondary structure of HtrI

The Fig. 7 shows the hydropathy plots of HtrI of *Haloarcula vallismortis*. There are two hydrophobic regions near the N-terminal, which have been predicted to form two transmembrane helices (helix 1 and 2, in Fig. 8). The rest part of the protein is hydrophilic, which has been

Figure 6. Operons of the halobacterial photo-sensing system

Figure 6. Operons of the halobacterial photo-sensing systems.

Regions contain the stop codon of Htrs and the start codon of sRs/pRs are shown. The yellow color indicates the Htr genes and the blue color indicates the sR/pR genes.

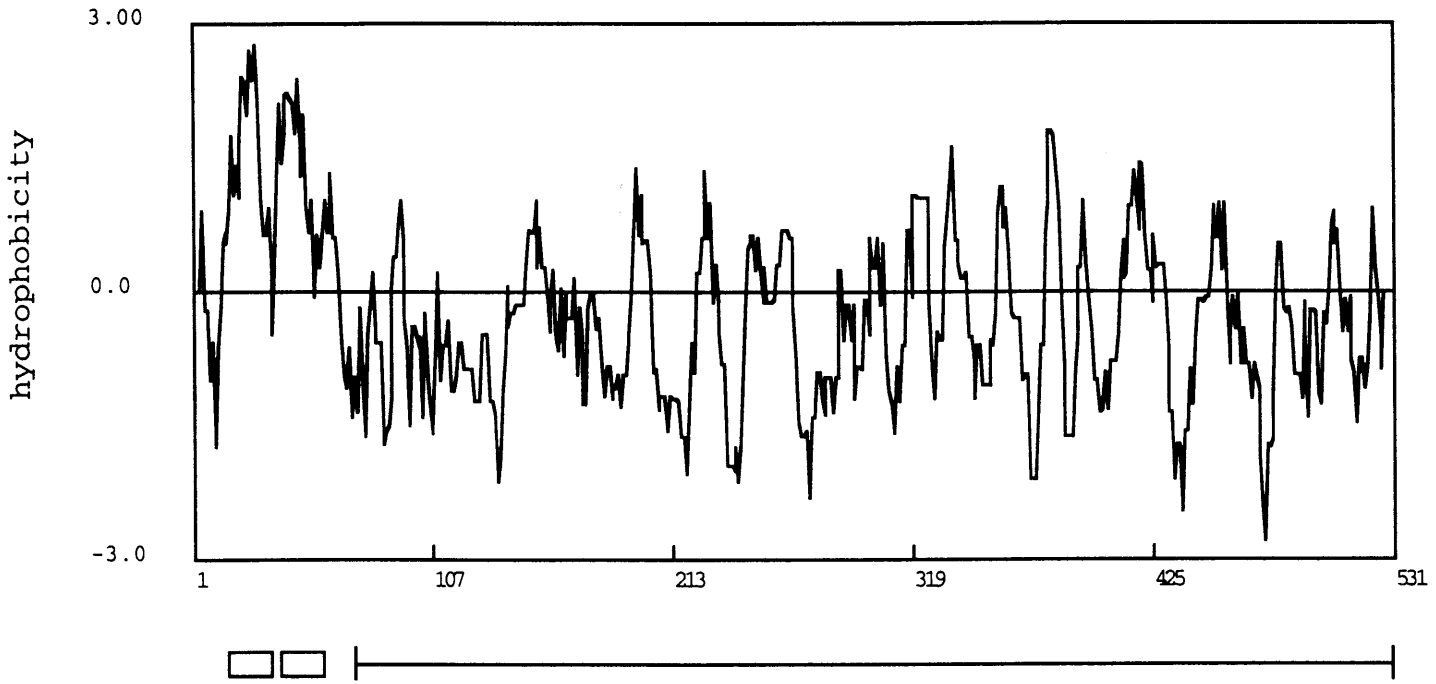

Figure 7. The hydropathy plots of the HtrI of *Haloarcula vallismortis*

The plots were obtained by the Kyte and Doolittle method with an amino acid span of 7.

considered to be a cytoplasmic domain (22). This entire structure with two transmembrane helices and the following large hydrophilic domain was commonly found in all the known Htrs.

II-3. Alignment of the primary structures of Htrs

The primary structures of all known Htr were aligned in Fig. 8. Mainly two regions were highly conserved in the cytoplasmic domain. In HtrI, the two transmembrane helices were also conserved as clearly seen in the hydropathy profile (Fig. 7).

II-4. Homology indices between Htrs and that between sRs.

Including newly isolated sRs and HtrIs, the percentage of the identical amino acids or nucleotides between sRs (Table 3) and that between Htrs are shown (Table 4). The HtrI of *Haloarcula vallismortis* has high homology to the HtrI of the different species *Halobacterium salinarium* and *Halorubrum sodomense*, but much lower homology to the HtrII of the same species *Haloarcula vallismortis*. SRs are divided into two groups (homologs), sR (sRI) and pR (sRII). Similarly, Htrs are divided into two groups (homologs), HtrI paired with sR and HtrII paired with pR. HtrI and sR in *Halorubrum sodomense* are very close to those in the same genus *Halorubrum* sp. aus-1 as shown proton pump rhodopsins, aR-1 and aR-3. SR and pR independently showed high homology (~60%) within the same homologs (Table 3). This relatedness was also found in Htrs. The homology between the same

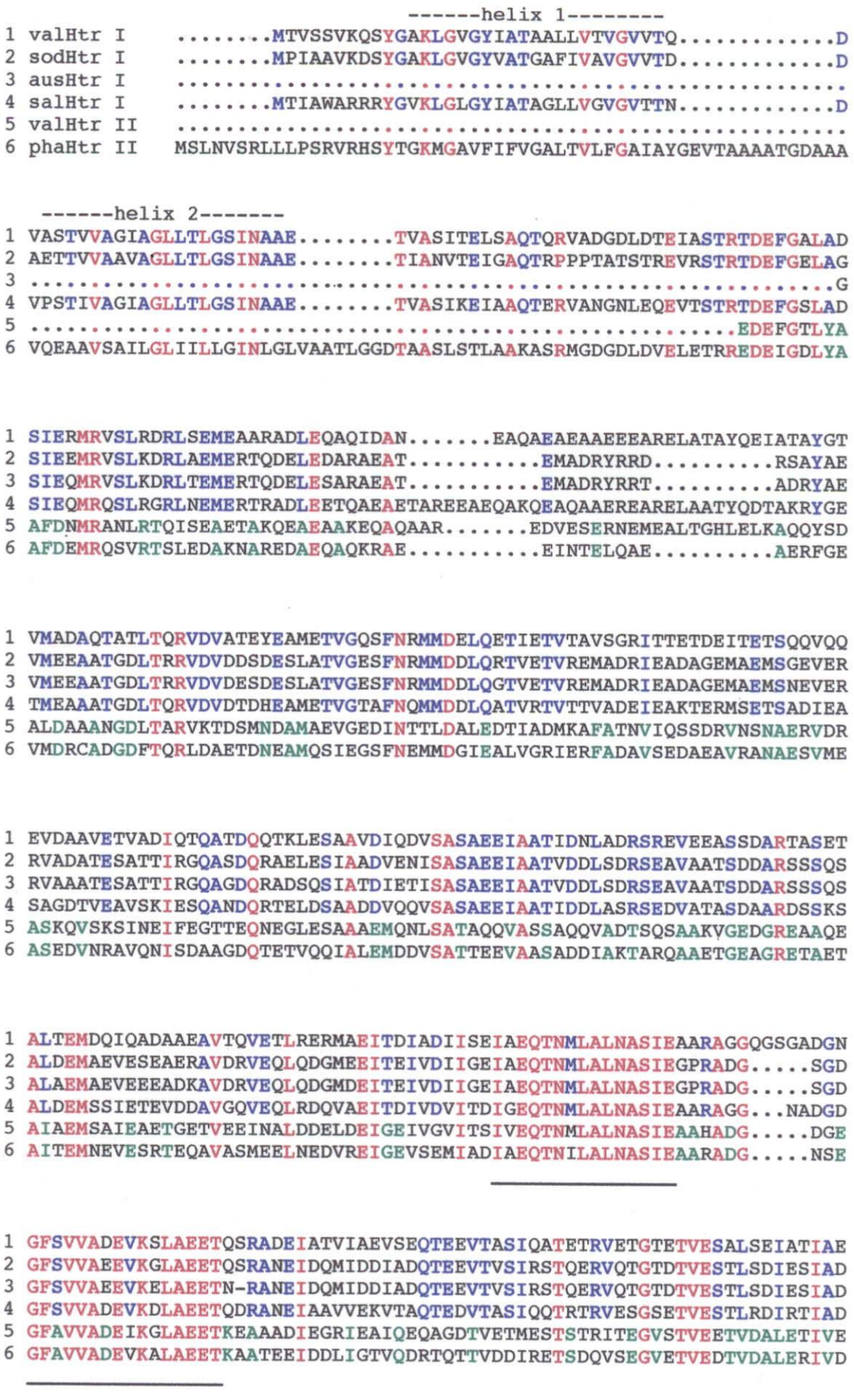
------helix 1--------
1 valHtr I .....MTVSSVKQSYGAKLGVGYIATAALLVTVGVVTQ.....D
2 sodHtr I .....MPIAAVKDSYGAKLGVGYVATGAFIVAVGVVTD.....D
3 ausHtr I .....
4 salHtr I .....MTIAWARRRYGVKLGLGYIATAGLLVGVGVTTN.....D
5 valHtr II .....
6 phaHtr II MSLNVSRLLLPSRVRHSYTGKMGAVFIFVGALTVLFGAIAYGEVTAAAATGDAAA
------helix 2-------
1 VASTVVAGIAGLLTLGSINAAE.....TVASITELSAQTQRVADGDLDTEIASTRTDEFGALAD
2 AETTVVAAVAGLLTLGSINAAE.....TIANVTEIGAQTRPPPTATSTREVRSTRTDEFGELAG
3 .....G
4 VPSTIVAGIAGLLTLGSINAAE.....TVASIKEIAAQTERVANGNLEQEVTSTRTDEFGSLAD
5 .....EDEFGTLYA
6 VQEAAVSAILGLIILLGINLGLVAATLGGDTAASLSTLAAKASRMGDGDLDVELETRREDEIGDLYA
1 SIERMRVSLRDRLSEMEAARADLEQAQIDAN.....EAQAEAEAAEEEARELATAYQEIATAYGT
2 SIEEMRVSLKDRLAEMERTQDELEDARAEAT.....EMADRYRRD.....RSAYAE
3 SIEQMRVSLKDRLTEMERTQDELESARAEAT.....EMADRYRRT.....ADRYAE
4 SIEQMRQSLRGRLNEMERTRADLEETQAEAETAREEAEQAKQEAQAAEREARELAATYQDTAKRYGE
5 AFDNMRANLRTQISEAETAKQEAEAAKEQAQAAR.....EDVESERNEMEALTGHLELKAQQYSD
6 AFDEMRQSVRTSLEDAKNAREDAEQAQKRAE.....EINTELQAE.....AERFGE
1 VMADAQTATLTQRVDVATEYEAMETVGQSFNRMMDELQETIETVTAVSGRITTETDEITETSQQVQQ
2 VMEEAATGDLTRRVDVDDSDESLATVGESFNRMMDDLQRTVETVREMADRIEADAGEMAEMSGEVER
3 VMEEAATGDLTRRVDVDESDESLATVGESFNRMMDDLQGTVETVREMADRIEADAGEMAEMSNEVER
4 TMEAAATGDLTQRVDVDTDHEAMETVGTAFNQMMDDLQATVRTVTTVADEIEAKTERMSETSADIEA
5 ALDAAANGDLTARVKTDSMNDAMAEVGEDINTTLDALEDTIADMKAFATNVIQSSDRVNSNAERVDR
6 VMDRCADGDFTQRLDAETDNEAMQSIEGSFNEMMDGIEALVGRIERFADAVSEDAEAVRANAESVME
1 EVDAAVETVADIQTQATDQQTKLESAAVDIQDVSASAEEIAATIDNLADRSREVEEASSDARTASET
2 RVADATESATTIRGQASDQRAELESIAADVENISASAEEIAATVDDLSDRSEAVAATSDDARSSSQS
3 RVAAATESATTIRGQAGDQRADSQSIATDIETISASAEEIAATVDDLSDRSEAVAATSDDARSSSQS
4 SAGDTVEAVSKIESQANDQRTELDSAADDVQQVSASAEEIAATIDDLASRSEDVATASDAARDSSKS
5 ASKQVSKSINEIFEGTTEQNEGLESAAAEMQNLSATAQQVASSAQQVADTSQSAAKVGEDGREAAQE
6 ASEDVNRAVQNISDAAGDQTETVQQIALEMDDVSATTEEVAASADDIAKTARQAAETGEAGRETAET
1 ALTEMDQIQADAAEAVTQVETLRERMAEITDIADIISEIAEQTNMLALNASIEAARAGGQGSGADGN
2 ALDEMAEVESEAERAVDRVEQLQDGMEEITEIVDIIGEIAEQTNMLALNASIEGPRADG.....SGD
3 ALAEMAEVEEEADKAVDRVEQLQDGMDEITEIVDIIGEIAEQTNMLALNASIEGPRADG.....SGD
4 ALDEMSSIETEVDDAVGQVEQLRDQVAEITDIVDVITDIGEQTNMLALNASIEAARAGG...NADGD
5 AIAEMSAIEAETGETVEEINALDDELDEIGEIVGVITSIVEQTNMLALNASIEAAHADG.....DGE
6 AITEMNEVESRTEQAVASMEELNEDVREIGEVSEMIADIAEQTNILALNASIEAARADG.....NSE
1 GFSVVADEVKSLAEETQSRADEIATVIAEVSEQTEEVTASIQATETRVETGTETVESALSEIATIAE
2 GFSVVAEEVKGLAEETQSRANEIDQMIDDIADQTEEVTVSIRSTQERVQTGTDTVESTLSDIESIAD
3 GFSVVAEEVKELAEETN-RANEIDQMIDDIADQTEEVTVSIRSTQERVQTGTDTVESTLSDIESIAD
4 GFSVVADEVKDLAEETQDRANEIAAVVEKVTAQTEDVTASIQQTRTRVESGSETVESTLRDIRTIAD
5 GFAVVADEIKGLAEETKEAAADIEGRIEAIQEQAGDTVETMESTSTRITEGVSTVEETVDALETIVE
6 GFAVVADEVKALAEETKAATEEIDDLIGTVQDRTQTTVDDIRETSDQVSEGVETVEDTVDALERIVD

Figure 8. Alignment of the amino acid sequences of HtrI and HtrII

The red color indicates the conserved amino acids throughout Htrs, except for the N-terminal part of HtrI of *Halorubrum* sp. aus-1 and the that of HtrII of *Haloarcula vallismortis* which have not been known. The blue color indicates the conserved amino acids between HtrIs, and the green color between HtrIIs. The highly conserved regions common to all the four known Htrs are underlined.

Table 3. Homology indices of halobacterial sensory rhodopsins

Percentages of the identical amino acids or nucleotides between sRs were calculated.

DNA / Protein	sR	csR3	asR3	SGsR	vsRII	psRII
sR		67	73	73	52	54
csR3	70		66	68	52	51
asR3	65	69		83	54	54
SGsR3	65	70	83		55	54
vsRII	31	30	36	38		56
psRII	31	32	30	28	44	

Table 4. Homology indices of halobacterial transducer proteins

Percentages of the identical amino acids or nucleotides between Htrs were calculated.

DNA / Protein	Hb.sal HtrI	Ha.val HtrI	Hr.sod HtrI	Hr.aus HtrI	Ha.val HtrII	Nb.pha HtrII
Hb.sal HtrI		63	62	61	57	54
Ha.val HtrI	55		59	60	53	48
Hr.sod HtrI	51	50		88	56	57
Hr.aus HtrI	44	48	86		56	58
Ha.val HtrII	33	33	35	32		58
Nb.pha HtrII	35	34	35	30	39	

homologs, HtrIs, was higher (~50%) than that between the different homologs, HtrI and HtrII (~30%) (Table 4). The homology between the same tribe was considerably high (~80%).

DISCUSSION

1. The rhodopsin of *Haloarcula vallismortis*

In the cell envelope vesicles of *Haloarcula vallismortis*, activities of both proton pump and anion pump were detected (Figs. 3 and 4). The CCCP-sensitive and $TPMP^+$-enhanced acidification suggested the presence of a light-driven primary proton pump. The $TPMP^+$-sensitive and CCCP-enhanced alkalization suggested a light-driven primary anion pump in the same cell envelope vesicles. The sensitivity of the light-induced alkalization of the Na_2SO_4-dialyzed vesicles to anions, $Br^- > Cl^- > NO_3^-$ and $SCN^- = 0$ is similar to that known for hR (34). Purple proteins corresponding to those pumps were purified by repeating hydrophobic column chromatography. Using flash photolysis, the purified proton pump protein (cR-3) showed properties analogous to bR (such as a 410 nm-(M)intermediate and a 640 nm-(O)intermediate in the bR photocycle), whereas the anion pump protein (chR-3) showed properties analogous to hR (520 nm- and 640 nm-intermediates). Therefore, *Haloarcula vallismortis* have a proton pump (= cR-3) and an anion pump (= chR-3) that function. The sensory rhodopsin has not been found as protein. Although this strain is motile, its phototaxis was hardly detected. Even so, the identified gene for sensor I seems to be the csR-3 gene of *Haloarcula vallismortis* because the deduced amino acid sequence of csR-3 was reasonably homologous to other sRs.

2. Genus-specific divergence of rhodopsins

After the second proton pump aR-1 (11) was discovered, varieties of rhodopsins have been found in strains of halophilic archaea. We proposed that those new rhodopsins can be grouped as "tribe" according to the percentage of identical amino acids in the primary structures (Fig. 9). We found that those strains hosting the proton pumps in the aR tribe, aR-1 and aR-2, should be separated as an independent genus (described under "Halorubra" (15), now established as *Halorubrum* (16)), from the established genus, and those hosting cR-1 and cR-2 should be included in *Haloarcula* (17, 18). We also proposed that the strains of one genus host the rhodopsins of its own tribe (Table 1). Along this line, the rhodopsins in the type strain of *Haloarcula* were cloned and sequenced in this work. As expected, the rhodopsins in *Haloarcula vallismortis* were grouped in the cR-tribe (Table 1). Furthermore, the proton pump and sensor I rhodopsins in *Halorubrum sodomense* were grouped in the aR tribe (35). These data would provide further evidence supporting genus-specific divergence of rhodopsins.

3. Differential conservativity among helices and rhodopsin homologs

Although 23 sequences of halobacterial rhodopsins are now known, there are only three strains in which the sequences of the three different homologs of rhodopsins (proton pump, anion pump and sensor I) are available;

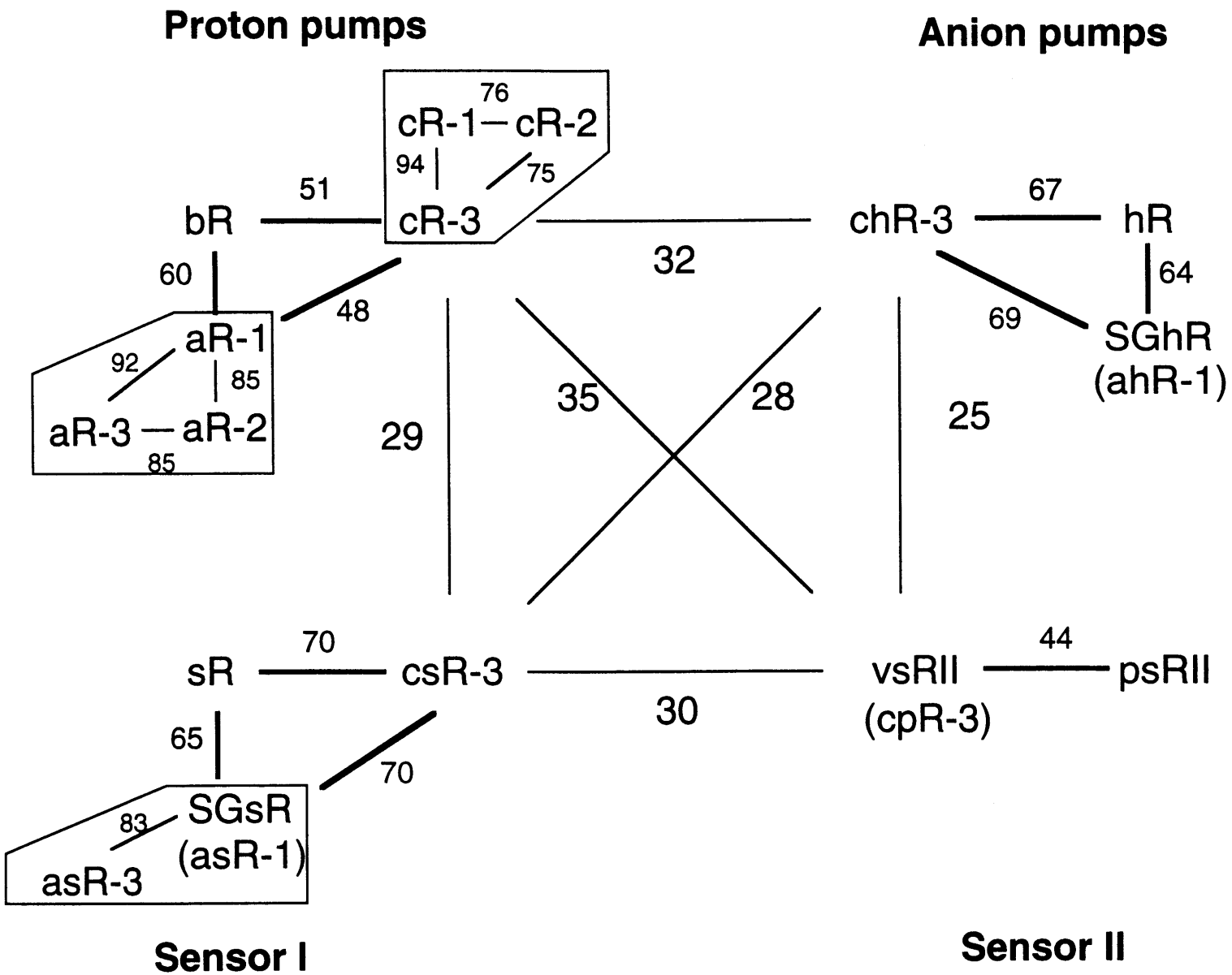

Figure 9. The homology indices in amino acid sequence between bacterial rhodopsins

Figure 9. The homology indices in the amino acid sequence between bacterial rhodopsins.

The numbers show percentages of identical amino acids between the given two rhodopsins. The rhodopsins in the same tribe are enclosed.

Halobacterium salinarium, *Halorubrum* sp. aus-1 (= SG) (29) and *Haloarcula vallismortis*. The percentage of the identical amino acids in each helix was calculated among three each proton pumps (aR-1, bR, cR-3), anion pumps (SG-hR, hR, chR-3) and sensor I (SG-sR, sR, csR-3) (Fig. 10). In proton pumps, helix C is well conserved and helix B and helix E are largely diverged. Although the average divergency of the anion pumps is smaller than that of the proton pumps, the pattern of the graph is similar to that of the proton pump. This "W" pattern was little modified after the homologies were re-calculated with additional sequences of proton pumps (13, 17, 18, 36), or anion pumps (28, 37). Therefore, in ion pumps, it would be concluded that helix C is conserved and helix B and helix E are diverged relatively largely.

The helix C in proton pumps holds Asp-85 and Asp-96 which are the acceptor and donor of proton from and to the Schiff base on the helix G, respectively (38), and forms a part of the proton channel (10). In anion pumps Asp-85 is commonly replaced with Thr. Since the point mutation of Asp-85 to Thr in bR resulted in giving it a chloride pump activity (39), and a common mechanism is proposed for proton and anion pumping, the helix C should also be important for anion pump. Similarly in the helix C of sR, Asp-85 is conserved, while Asp-96 is replaced with Phe so that no net proton pumping takes place. Dissociation and re-association of a proton between Asp-85 and the Schiff base on the helix G (40) results in charge displacement and is thought to be one of the step of signal transduction. These would explain

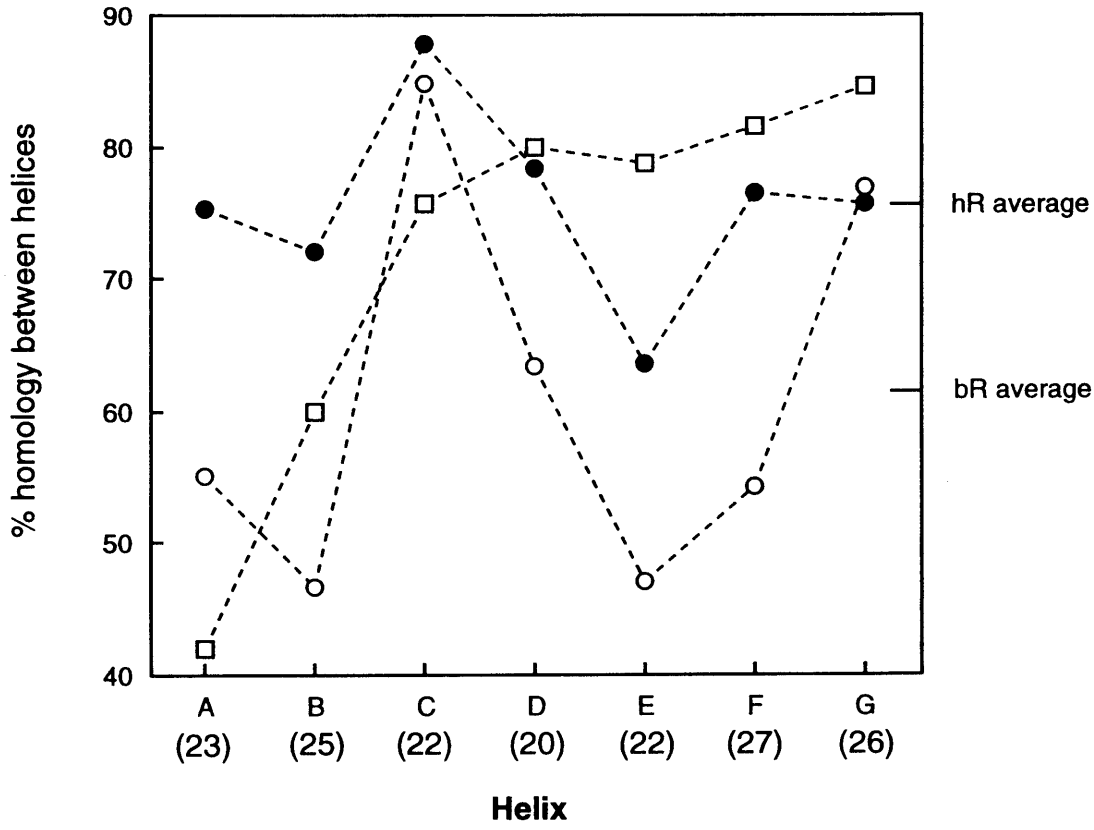

Figure 10. Percentage of identical amino acids in each helix of three rhodopsin homologs

Three rhodopsin homologs (bR (○), hR (●), sR (□)) in three species (Halobacterium salinarium, Haloarcula vallismortis, Halorubrum sp. aus-1) were used. Within the same homolog, the identical amino acid residues in every transmembrane helix portion (number of amino acids in the parenthesis) were calculated and shown as the fractional numbers. Three percentage values for each homolog were averaged and plotted.

necessary conservation of the helices C and G in sR as well as in bR and hR.

The pattern of the homologies of each helix in sensor I was distinguished from those of ion pumps: helix A and B were considerably diverged, whereas helix C, D, E, F and G were rather well conserved, suggesting their relative importance in sensor I (Fig. 10). In the halobacterial signal transduction system, HtrI (halobacterial transducer I for rhodopsin) is thought to transmit light stimuli from sR into cytoplasm to control swimming behavior (41). This HtrI is considered to have two transmembrane helices and a cytoplasmic domain (Fig. 7). Although the mechanism of interaction between sR and HtrI has not been elucidated, the two transmembrane helices and the first ~80 residues of the cytoplasmic domain are known to be sufficient to control the sR photocycle (42). One possible explanation is that these two transmembrane helices of HtrI interact with the helices DEF of sR so as to hold the cytoplasmic domain of HtrI in the crucial position of sR in order to transduce the "charge displacement" signal from sR into the defined conformation change in the cytoplasmic domain (Fig. 11). These would explain the higher conservation of helices DEF in sR, but the photosensing system is still left to be investigated.

4. A possible structure of a signal transduction system in halobateria

The two putative transmembrane helices near the N-terminus in Htrs have more than 80% homology within the

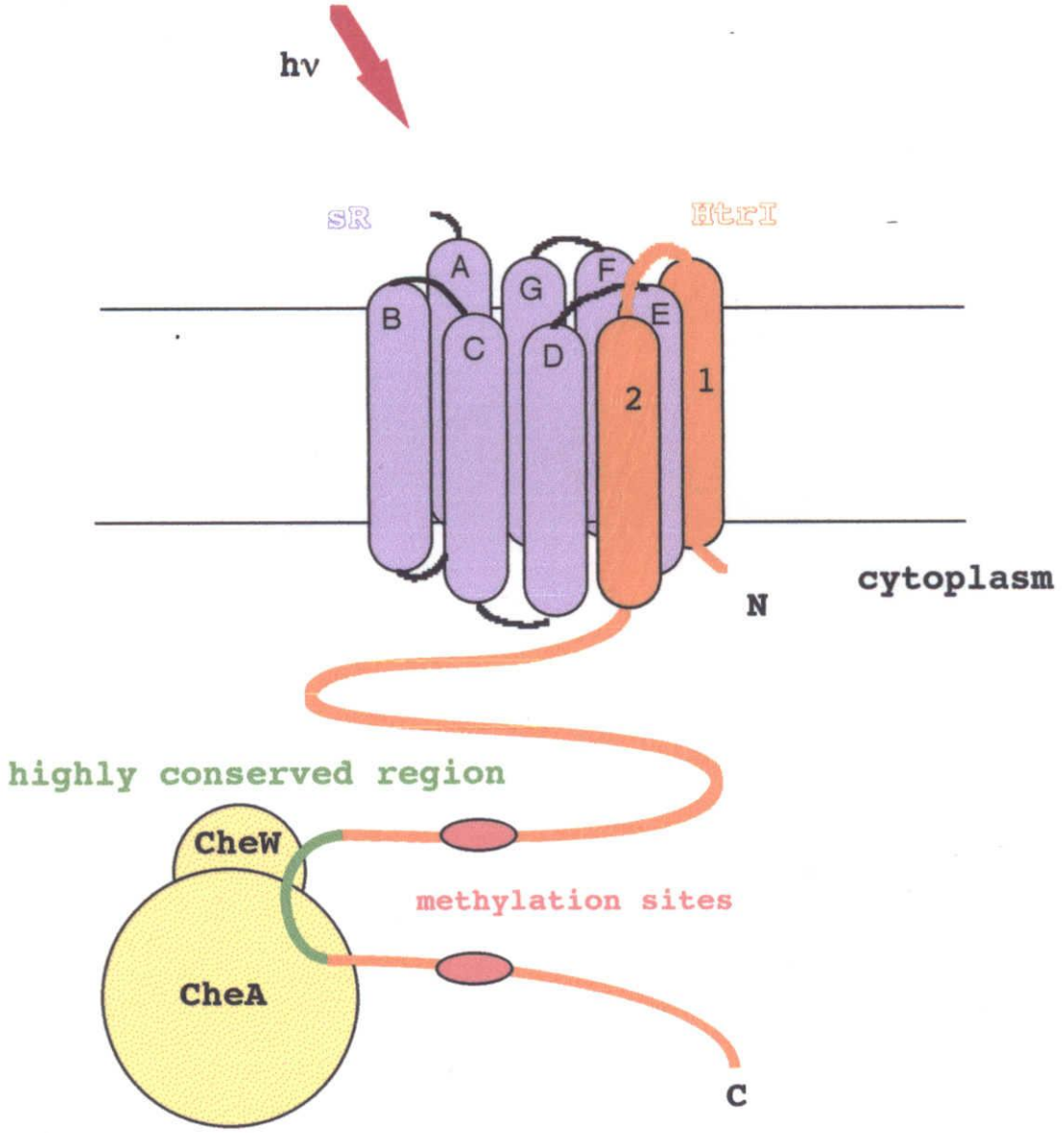

Figure 11. A model for the halobacterial photosensing system

Figure 11. A model for the halobacterial photosensing system.

HtrI forms a complex with helices DEF of sR at their transmembrane helices. HtrI has a large cytoplasmic domain which contains

1) the conserved regions throughout known Htrs which will bind the next protein in the signal transduction system,
2) a region which interacts with sR (about 80 residues following the helices) and
3) the regions which suffer reversible methylation.

HtrIs. These significantly conserved helices suggest that these regions will anchor Htr to the membrane so as to compose the photosensor system with sR (possibly with helices DEF of sR as discussed above).

Alignment of all the known HtrI and HtrII revealed that the highly conseved region at the cytoplasmic domain in Htr. A section of the cytoplasmic domain of HtrI (shown in Fig. 8) is known to be highly homologous to a part of the cytoplasmic domain of Tsr (43), one of the chemoreceptors in the eubacterial signal transduction system. In analogy to the eubacterial system, this highly conserved region would be the CheW/CheA binding site (Fig. 11), because the proteins analogous to CheA and CheY, and a gene analogous to the gene coding for CheB, signal transduction proteins in *E. coli* (43), have been found in *Halobacterium salinarium* (44, 45). Halobacteria would thus have similar signal transduction systems in photo/chemotaxis. CheW, next to the receptor/transducer protein in *E. coli*, would also be found in halobacteria. If this is the case, sRI-HtrI for the favorite-light sensing system and sRII-HtrII for the harmful-light sensing system would bind the same protein (CheW). Even so, they should transduce the opposite signal depending on the wavelength of stimulus light. Such the opposite signal would be transmitted through the rest part of the cytoplasmic domain of Htr and CheW.

5. The evolution of rhodopsins

The increasing numbers of bacterial rhodopsins are classified into four functionally different homologs. Since new genes of proton pump, anion pump and sensory rhodopsin were isolated from *Haloarcula vallismortis*, the primary structures of four different homologs of bacterial rhodopsins in one halobacterial strain are now all available. Fig. 9 shows the amino acid sequence homology (identity) between given pairs of rhodopsins. The homology among the rhodopsins in the same homolog is high (~60%) even in the different genus/species, and the homology among different homologs is ~30% even in the same genus/species. This suggests that four rhodopsin homologs keep almost even distance (mutation frequency) from each other and that those four homologs were derived from the one common ancestor.

All the known genes of HtrI located upstream the genes of sR. All the known genes of HtrII also located upstream the genes of pR (Fig. 6). These results can be explained by the following process of rhodopsin divergence (Fig. 12); An ancestry rhodopsin (its function is unknown) existed in a primitive halobacterium. Gene duplication of the rhodopsin resulted in two functionally different proteins, a pump and a sensor. The pump gene duplicated and then diverged to be a proton pump and an anion pump. The sensor gene first acquired a transducer gene and formed the Htr-sR tandem gene, which then concomitantly duplicated to form two sensor systems. The single primitive halobacterium which thus

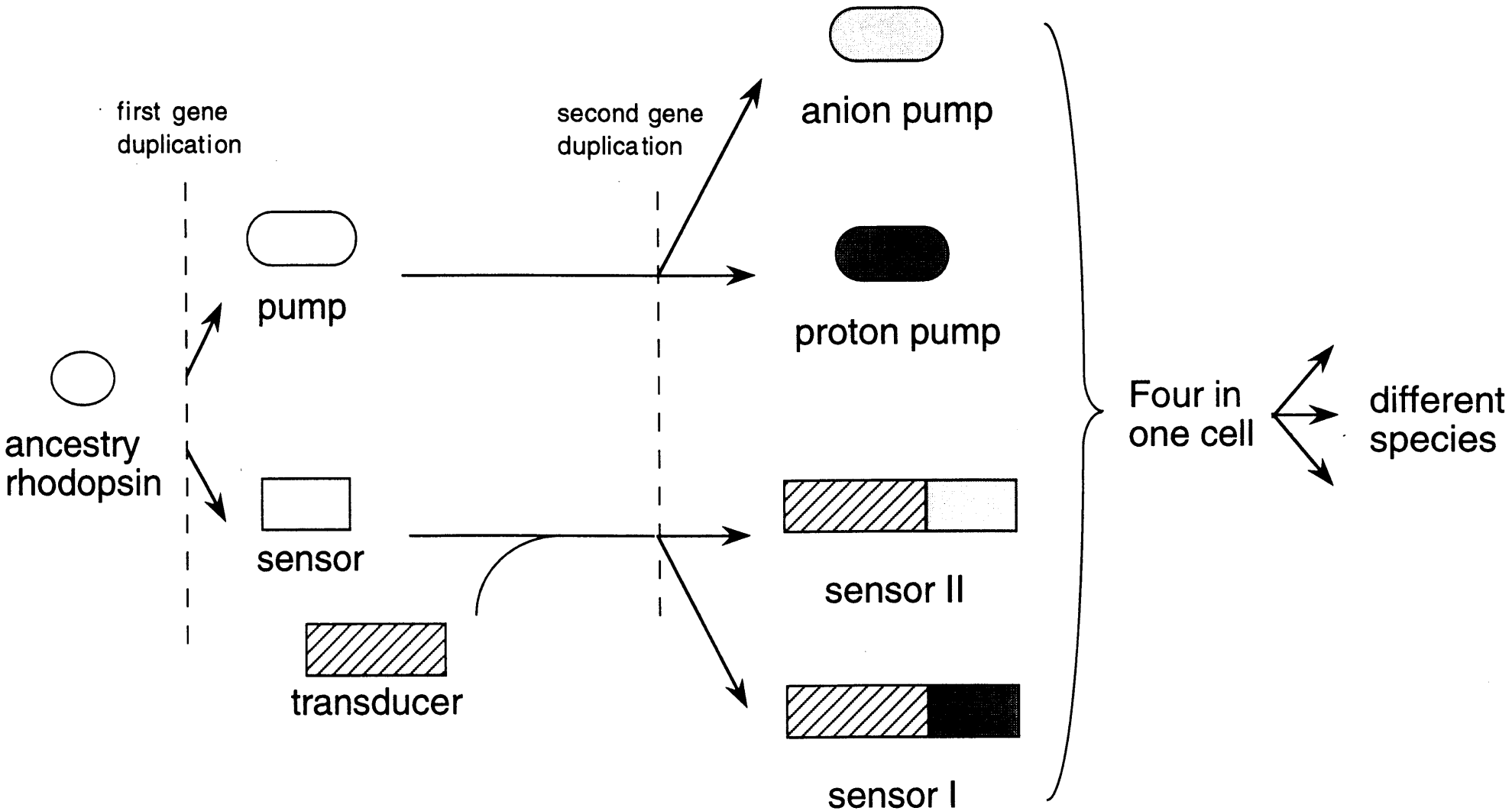

Figure 12. A possible evolutionary process of four halobacterial rhodopsin homologs

Figure 12. A possible evolutionary process of four halobacterial rhodopsin homologs.

An ancient halobacterium acquired four rhodopsins then diverged into various species.

posessed four kinds of rhodopsin homologs diverged into different genera/species. Fig. 13 shows the correlation in DNA homology of Htr and the paired sR. The ratio of Htr homology to sR homology is almost unity. This result strongly supports that *htr-sop* gene duplicated together in a primitive halobacterium, then two sets of transducer-sensor have evolved in the bacterium and then in each species under generic divergence.

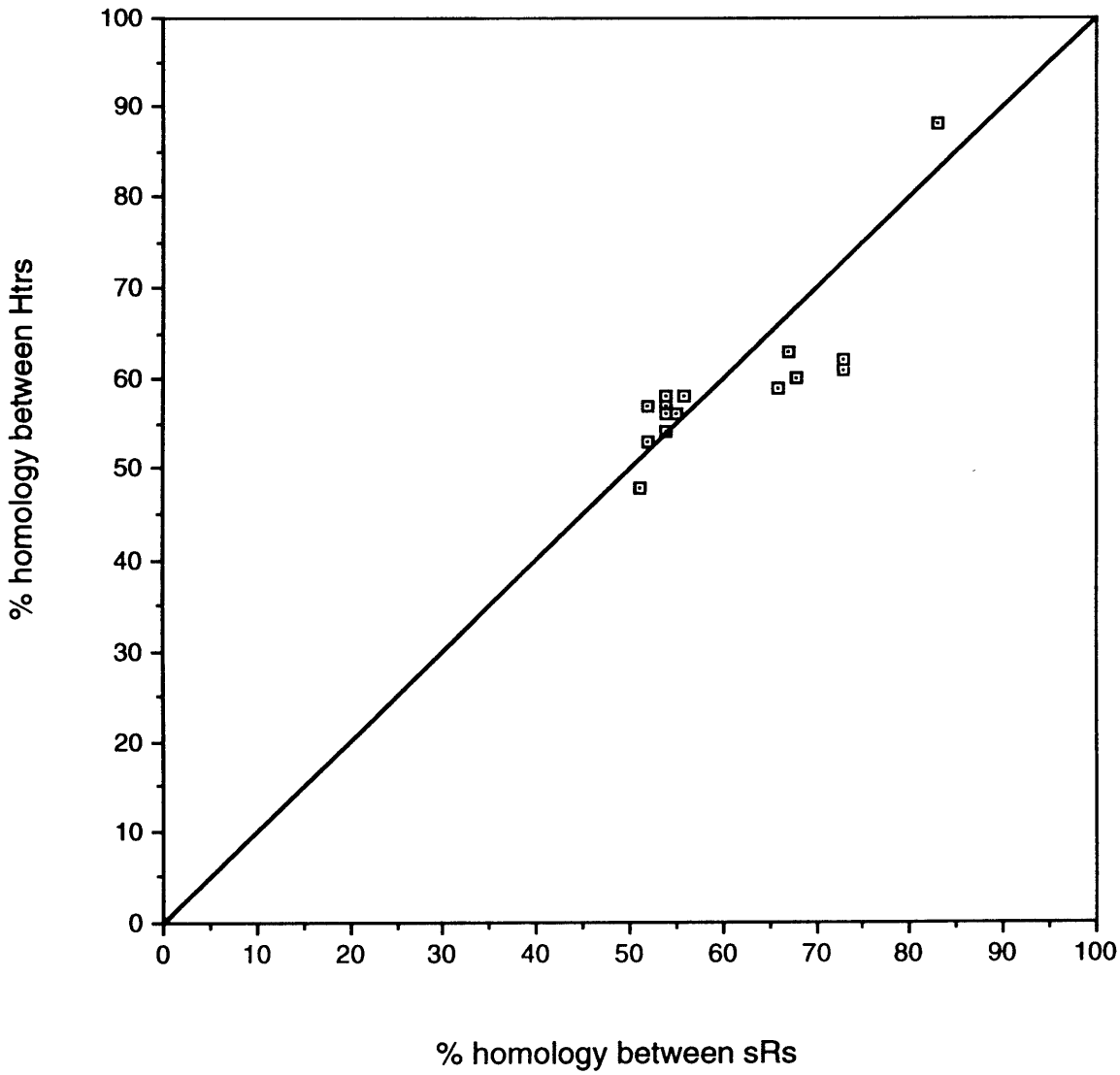

Figure 13. Correlation in homologs of the Htr gene and the sR gene

Percentages of identical nucleotides between sRs (Table 3) vs. those between Htrs (Table 4) in the same species were plotted.

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