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Halobacterial Rhodopsins[1]

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Following the discovery of the bacteriorhodopsin proton pump in *Halobacterium halobium* (*salinarum*), not only the halorhodopsin halide pump and two photosensor rhodopsins (sensory rhodopsin and phoborhodopsin) in the same species, but also homologs of these four rhodopsins in strains of other genera of *Halobacteriaceae* have been reported. Twenty-eight full (and partial) sequences of the genomic DNA of these rhodopsins have been analyzed. The deduced amino acid sequences have led to new strategies and tactics for understanding bacterial rhodopsins on a comparative basis, as summarized briefly in this article. The data discussed include (i) alignment of the sequences to qualify/characterize the conserved residues; (ii) assignment of residues that cause differences in function(s)/properties; and (iii) phylogeny of the halobacterial rhodopsins to suggest their evolutionary paths. The four kinds of rhodopsin in each strain are assumed, on the basis of their genera-specific distributions, to have arisen by at least two gene-duplication processes during evolution prior to generic speciation. The first duplication of the rhodopsin ancestor gene yielded two genes, each of which was duplicated again to give four genes in the ancestor halobacterium. The bacterium carrying four rhodopsin genes, after accumulating mutations, became ready for generic speciation and the delivery of four rhodopsins to each species. The original rhodopsin ancestor is speculated to be closest to the proton pump (bacteriorhodopsin).

Key words: bacterial rhodopsin, bacteriorhodopsin, evolution of bacterial rhodopsin, halobacteria, retinal protein.

Introduction: Historical aspects of halobacterial rhodopsins

In the early 1970s, unique patch structures were demonstrated in electron micrographs of the freeze-etched cell membranes of *Halobacterium halobium* (now *Hb. salinarum*) found to contaminate the south San Francisco Bay saltern (*1*). These patches were two-dimensional purple crystals (purple membrane) composed of only one 26 kDa protein and space-filling lipids (*2*). The protein held retinal as its chromophore similar to visual rhodopsins and was thus named bacteriorhodopsin (bR; *2*). Surprisingly, bR was found to pump protons out of the cell in the light (*3*). The discovery of this light-driven proton pump was revolutionary not only in biology, but also in chemistry and physics.

Intensive studies of this ion pump led to new strategies and tactics for sharper resolution of the structure and function of membrane proteins: three-dimensional structures by (cryo-)electron microscopy (*4-6*) as well known seven transmembrane helices (*4*); structural prediction by hydropathy plot (*7*) of possible transmembrane segments; molecular orientation by accessibility of proteolytic enzymes and antibodies (*8*); FTIR spectroscopy for functioning residues (*9, 10*); mutageneses for essential amino acid residues (*11, 12*). The functional convertibilities of bR to chloride pump were demonstrated with bR under specified conditions (*13*) and with bR point-mutated at Asp85 to Thr (D85TbR; *14, 15*), and that of sensory rhodopsin (sR) to proton pump with sR isolated from its accompanying regulatory protein, Htr (halobacterial transducer protein for rhodopsin) (*16, 17*). The accumulated knowledge led to the proposal of a common mechanism for the light-driven ion pump of bacterial rhodopsins (*18*). BR also contributed to the chemiosmotic theory as the driving machinery for F_oF_1 ATP-synthase in the reconstituted proteoliposome (*19*).

The pH of a suspension of reddish purple *Hb. salinarum* (wild type) cells upon actinic illumination shows a transient onset opposing the direction expected for bR (*3*) which had been thought to be the sole primary light-energy transducer in the bacterium. This strange onset led us to investigate and then isolate a "red" mutant of *Hb. salinarum* (*20, 21*) which contains no bR due to an infertile insertion in its gene (*22*). To our surprise, actinic illumination caused the pH of the red mutant suspension to increase and the cellular ATP

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Abbreviations: aR, archaerhodopsin; bR, bacteriorhodopsin; cR, cruxrhodopsin; dR, deltarhodopsin; *Ha.*, *Haloarcula*; *Hb.*, *Halobacterium*; *Hc.*, *Halococcus*; *Hf.*, *Haloferax*; *Hr.*, *Halorubrum*; hR, halorhodopsin; Htr, halobacterial transducer protein for rhodopsin; *Nm.*, *Natronomonas*; pR, phoborhodopsin (sensory rhodopsin II); sR, sensory rhodopsin (sensory rhodopsin I); 16SrDNA, DNA encoding 16S ribosomal RNA.

level also rose (*20, 23*) to a level as high as that in the illuminated wild cells (*24*). The action spectrum for the increase in both ATP synthesis and pH was clearly red-shifted from the absorption spectrum of bR. A retinal protein distinct from bR in its heat instability and NH_2OH liability was identified as responsible for the light-energy transduction (*20, 21*). This second retinal protein in *Hb. salinarum* was named halorhodopsin (hR) (*25, 26*), which upon actinic illumination makes the inside-negative membrane potential larger (*26-28*) by pumping in halide ions (*29*). The increased potential drives "A-type" H^+-ATP synthase (*30*), causes proton uptake and thus alkalization of the cell suspension which is enhanced by the protonophore (*21*). This transient onset is due to the difference in the time constant and the size of the pH increase (faster and smaller) by hR and the pH decrease by bR in wild type *Hb. salinarum*.

During the course of hR purification, one more rhodopsin was identified in the same cells by its slow photocycle and salt dependency (*31*). This third-rhodopsin-like-protein (*32*) or sensory rhodopsin (sR; *33*) seemed to be the expected photo-sensor for the reported cell movement under stimulant or repellant light (*34*). Later, one more rhodopsin, phoborhodopsin (pR) (*35*) or sensory rhodopsin II (*36*), was found as the second sensor for ultraviolet light. Therefore, four different kinds of rhodopsins, two pumps and two sensors, are now known in *Hb. salinarum* cells.

In a field survey in Western Australia in 1983 for other halobacterial strains possibly carrying new rhodopsins, several new strains were collected and isolated. One of these strains, *Halobacterium* (now *Halorubrum*) sp. aus-1, was found to contain another proton pump, archaerhodopsin(-1) (aR-1), which shows 60% identity to bR in its amino acid sequence (*37, 38*).

This second proton pump suggested a new comparative study of halobacterial rhodopsins; (i) alignment of amino acid sequences to find conserved amino acid residues that would/should be essential for structure and/or functions; (ii) assignment of the amino acid residue(s) responsible for the differences in properties and/or functions. More rhodopsins have been found from various sources (*39, 40*), and new species (*41*) collected in our second field work in Argentina in 1992 (*42, 43*). Research has resulted to date to descriptions of 28 halobacterial rhodopsins (Table I). Because of the unexpected number of rhodopsin homologs found in various species of halobacteria, our study was further extended to (iii) the phylogeny of halobacterial rhodopsins on the basis of their DNA/amino acid sequence to estimate the evolutionary path of rhodopsins (*44*). This study was also combined with studies of the phylogeny of halobacteria (16SrRNA) to find the genera-specific distribution of rhodopsin homologs.

2. Primary structures of two pumps and two sensors conserved residues. The primary structures of two pumps and two sensors are aligned in Fig. 1. The hydropathy plots (*7*) suggest that all these rhodopsins have seven helices (A to G in Fig. 1) similar to bR. The structural model of bR indicates 58 amino acid residues to be located in the intramolecular transmembrane space enclosed by the seven transmembrane helices (*5*). Of these, Tyr57, Arg82, Tyr83, Trp86, Thr90, Pro91, Gly122, Leu152, Leu174, Trp182, Tyr185, Pro186, Trp189, Gly195, Asp212, and Lys216 (shown in bold letters in Fig. 1; numbers refer to bR, hereafter) are common to all four rhodopsin homologs, and are essential for rhodopsin structure. Lys216 in helix G binds the chromophore retinal to its ε-amino residue, which, along with the seven-helix structure, is also conserved in visual rhodopsins suggesting that all these retinal proteins belong to the rhodopsin super-family. Trp86, Trp182, Pro186, and Trp189 help to fix the polyene chain of retinal in the proper position/orientation. Several residues are conserved in only the two pumps (Thr46, Leu94, and Leu223; blue letters) or in the two sensors (Leu46, Val94, and Phe138; green letters). Some residues such as Gly23, Ala53, Met60, Asp85, and Thr89, are common to the proton pump and the two sensors (red letters). Since the sensors carry latent proton pumping activity, these residues might be related to proton pumping. The following four residues in halide pumps are also conserved (Ser53, Leu60, Thr85, and Ser89; purple letters), suggesting that the replacement is also crucial for halide pumping. Met118, Ser141, and Ala215 are conserved in the two pumps and sensor I (pink letters) and would contribute to opsin shift

TABLE I. **Halobacterial rhodopsins identified/sequenced to date and the strains that host them.**

Ion pump		Sensor		Strain	Rhodopsin family/subfamily
Proton pump	Halide pump	Sensor I	Sensor II		
bR	hR	sR	pR (sRII)	*Hb. salinarum* (*halobium*)	bac
aR-1 (=SGbR)	ahR-1 (=SGhR)	asR-1 (=SGsR)		*Hr.* sp. aus-1 (=strain SG1)	rub/aR-1
aR-2	n.d.			*Hr.* sp. aus-2	/aR-2
aR-3	ahR-3	asR-3		*Hr. sodomense*	/aR-3
mex-bR	mex-hR			strain mex	
cR-1	chR-1			*Ha. argentinensis* (sp. arg-1)	arc/cR-1
cR-2				*Ha. mukohataei* (sp. arg-2)	/cR-2
cR-3	chR-3	csR-3	cpR-3 (=val-pR)	*Ha. vallismortis*	/cR-3
shark-bR	shark-hR			strain shark	
port-bR	port-hR			strain port	
dR-1	dhR-1			strain sp. arg-4	*new genus?*
n.d.	phR		ppR	*Nm. pharaonis* (*Natronobacterium phraonis*)	nam

Strains determined taxonomically are in italics. Strains mex, shark, port, and sp. arg-4 are grouped according to the phylogenies of rhodopsin and/or 16SrRNA. In order to avoid confusion, the names of rhodopsins in the established genera/species are shown in common nomenclature. SGbR in strain SG1 (*45*) is identical to aR-1 (*38*), and thus SGhR and SGsR are located in the aR-1 subfamily in *Hr.* sp. aus-1. Although bR, hR, sR, and pR are, under our nomenclature system, suffixed with -1 as members of the first subfamily found in the genus *Halobacterium*, the suffix is omitted here because of their historical significance. n.d., not detectable.

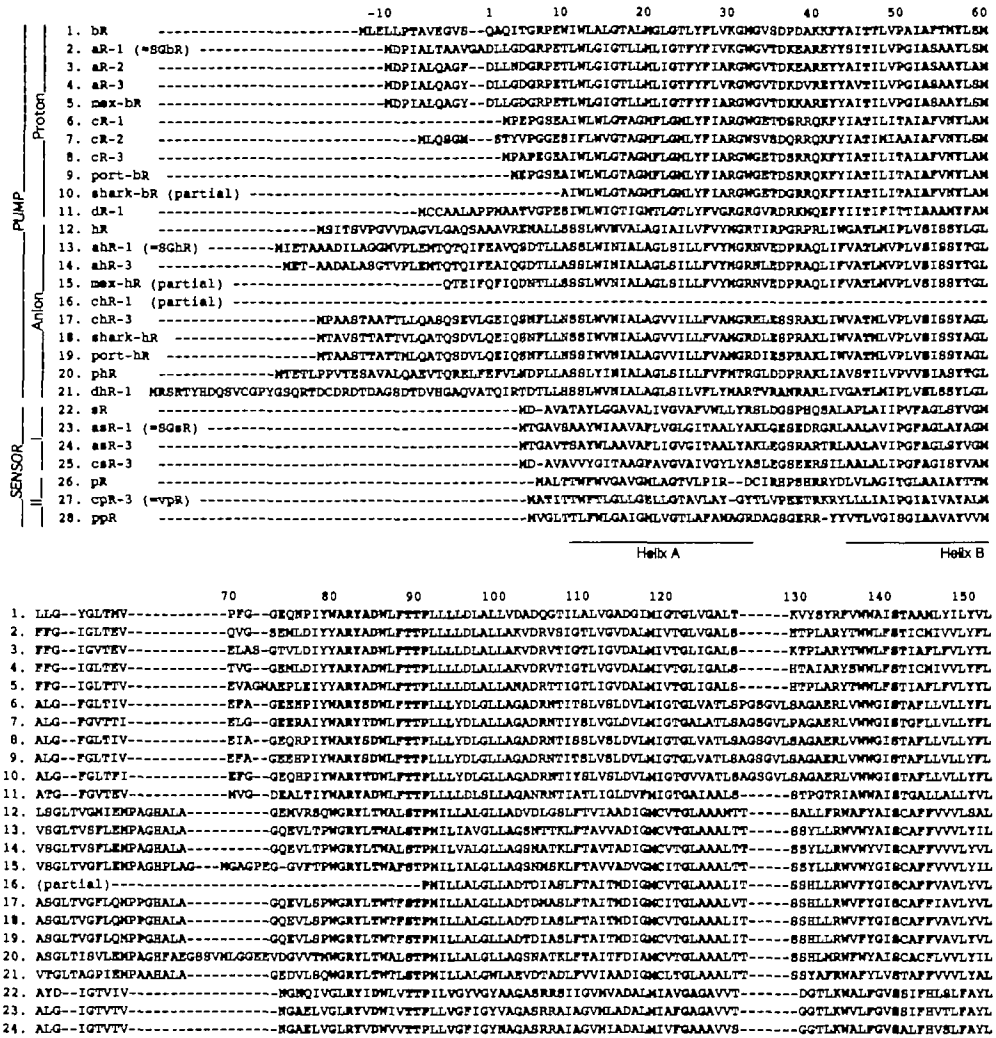

Fig. 1. **Amino acid sequences of halobacterial rhodopsins.** Twenty-five full and 3 partial sequences analyzed so far are shown. Since bR, hR, sR, and pR were the first members found in the genus *Halobacterium*, under our nomenclature system they should be suffixed as bR-1, hR-1, sR-1, and pR-1; the suffixes are ignored here, however, because of their historical significance. In order to avoid confusion, the names of rhodopsins in the established genera/species are shown in common nomenclature. The residues conserved throughout the known rhodopsins are in bold letters. Those conserved only within proton pumps or halide pumps are in blue or green, respectively. Residues ascribed to opsin shift are in pink. The residues are numbered referring to bR (*in situ*) and the segments of the seven transmembrane helices (A to G) suggested for bR are also indicated. For details see text.

(the large spectral red-shift of the chromophore retinal *in situ*; sensor II is not purple). More residues are conserved only within proton pumps or halide pumps, such as Asp96, one of the key residues in proton pumping. Some other residues are seen to be conserved in one of sensors, although the sequence data are insufficient for a detailed discussion. The functional and structural roles of these residues may be elucidated when the three dimensional structures of hR, sR, and pR are determined. A common insertion, MPXGH, between helices B and C is found only among the halide pump homologs, in which the His residue has been shown to be essential for halide pumping (*46*). Another insertion, GSGVL, between helices D and E, is found only in cruxrhodopsin (cR) homologs, although its functional role has not been determined.

Homology correlation. The homology (identity) index of the amino-acid sequences between any two of the four homolog groups (six pairs; proton pump *vs.* halide pump, proton pump *vs.* sensor I, proton pump *vs.* sensor II, halide pump *vs.* sensor I, halide pump *vs.* sensor II, or sensor I *vs.* sensor II) are in the range of 20-30% (Table II). Within the same group (*e.g.*, proton pump group; aR/bR/cR/dR), the index is in the range of 50-60% or higher. Note that there are distinct subgroups with homology indices as high as 90%, *e.g.* aR-1/aR-2/aR-3. Archaerhodopsins (*37, 38, 47*) are thus named separately from bR and numbered in the order of their identification. Cruxrhodopsins (cRs) and deltarhodopsin-1 (dR-1) were also found to be separate subgroups and so are differently named and numbered (see Tables I and II). Similar homology relationships were found in the accompanying halide pumps and sensors identified in the individual strains. These are prefixed ahR or csR, and numbered similarly. Subgroups with higher indices are also clearly present in each homolog group; *e.g.*, ahR-1/ahR-3, chR-1/chR-3, asR-1/asR-3. Therefore, *Halorubrum* (formerly *Halobacterium*) sp. aus-1 contains aR-1 [=SGbR (*45*)], ahR-1 (=SGhR), asR-1 (=SGsR), and apR-1 (=SGsRII), which are grouped together as an (aR-1) subfamily [a set of one each of (at most) four kinds of rhodopsin homolog present in one species]. The subfamilies of rhodopsin in *Halorubrum* form a family (the rub-family of rhodopsin), and those in *Haloarcula* and *Halobacterium* form the arc-family and the bac-family of rhodopsin, respectively.

3. Assignment of the key residue(s) from the primary structure

When bR is kept in the dark, the chromophore all-*trans* retinal in the retinal pocket spontaneously isomerizes to 13-*cis* C=N *syn* retinal and reaches an equilibrium with an isomer ratio (all-*trans*:13-*cis*) of about 1:2. In aR-2, this equilibrium reaches an isomer ratio of only about 3:1 (*48*). The amino acid residues composing the retinal pocket (*4*) of aR-2 differ in only one residue, Met145 in bR, which is replaced by Phe in aR-2. The bR with a point-mutation at this assigned residue, M145FbR, was expressed in halobacteria and found to give an isomer ratio of 3:1 the same as in aR-2 (*49*). This result indicates that the size of the amino acid residue in the retinal pocket influences the dark isomerization equilibrium of retinal. However, an isomer ratio of 1:1 was found for aR-1, although the amino acid composition of the retinal pocket is identical to that of bR. This suggests that the relative positioning of the retinal pocket residues may not be identical, even between bR and

TABLE II. **Homology indices between halobacterial rhodopsins.**

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1. bR																											
2. aR-1	56																										
3. aR-2	54	**85**																									
4. aR-3	57	**92**	**85**																								
5. mex-bR	54	**83**	**89**	**84**																							
6. cR-1	50	47	47	47	48																						
7. cR-2	47	45	46	46	47	**72**																					
8. cR-3	51	46	47	46	46	**94**	**73**																				
9. shark-bR	52	49	47	47	47	**97**	**73**	**95**																			
10. port-bR	54	48	49	49	49	**93**	**79**	**94**	**94**																		
11. dR-1	50	50	50	49	49	52	54	52	52	55																	
12. hR	24	28	28	28	28	27	29	27	26	29	24																
13. ahR-1	25	24	25	23	25	30	28	28	28	30	26	62															
14. ahR-3	25	25	25	24	25	28	27	28	29	30	27	61	**88**														
15. mex-hR	28	27	26	24	25	28	30	28	28	30	25	62	**86**	**85**													
16. chR-1	34	34	33	33	34	35	34	35	35	35	29	71	70	74	64												
17. chR-3	25	25	26	24	26	31	27	28	31	30	25	61	66	68	69	**94**											
18. shark-hR	25	25	25	23	24	30	26	28	29	30	24	60	67	69	68	**99**	**92**										
19. port-hR	28	23	25	23	24	31	29	30	30	30	24	60	66	68	68	**99**	**92**	**98**									
20. phR	25	26	27	27	26	28	23	26	26	29	28	53	64	66	68	72	60	60	60								
21. dhR-1	26	26	27	26	27	26	27	26	26	29	25	66	57	58	61	70	59	59	59	48							
22. sR	24	23	23	24	24	25	23	25	25	24	23	18	17	17	18	23	20	17	19	20	15						
23. asR-1	25	24	25	23	25	26	25	26	26	26	23	19	19	19	17	24	21	20	20	22	17	64					
24. asR-3	23	23	25	23	25	26	22	26	27	28	22	20	19	19	19	25	22	22	21	18	19	63	**82**				
25. csR-3	23	22	24	23	24	27	28	28	29	29	22	22	22	21	21	26	22	22	21	21	21	65	64	64			
26. pR	29	28	29	27	29	27	28	28	27	30	33	24	21	21	20	30	22	22	23	23	25	28	32	30	27		
27. ppR	30	33	32	31	32	35	33	34	33	34	29	24	22	23	20	26	20	20	20	22	21	26	30	29	25	36	
28. cpR-3	26	26	28	26	28	30	32	32	31	31	31	22	18	20	17	30	26	27	26	22	21	25	28	29	28	37	40

Rhodopsins 1-11 are proton pumps, 12-21 are halide pumps, 22-25 and 26-28 are in the sR and the pR group, respectively. High homology indices in bold letters suggest subgroups within the individual ion pump and sensor groups.

aR-1, because of the additional influence of adjacent residues or helices. The three dimensional structure of aR-1 would resolve these issues at atomic resolution.

When Met145 in bR was replaced with Ala, a much smaller side chain, the M145AbR chromophore, which was expressed in *Escherichia coli* and refolded *in vitro*, showed a salt-dependent reversible conversion from the 470 nm to the 550 nm form (*50*). The M-like photointermediate of M145AbR is blue-shifted by 20 nm compared with that of the wild-type and its decay kinetics are 20-fold slower than those of the wild-type. Furthermore, M145AbR exhibits only 10% of the wild-type proton pumping activity. Thus the size of the amino acid residue at position 145 also appears to be important for energy transduction. M145AbR expressed in *Hb. salinarum* showed a similar temperature-dependent and salt-dependent interconversion (N. Yamada *et al.*, unpublished results).

When Asp85 of bR was replaced with Thr, D85TbR expressed in *Hb. salinarum* transported Cl^- into the cells (*14*). Interestingly, the same D85TbR pumped protons outward, like the wild type, under appropriate conditions (*18*). This is thus an example in which the same protein pumps either protons or chloride. A similar situation has been observed for hR. Based on these observations, the isomerization/switch/transfer model for ion translocation was recently proposed despite the uncertainty of the structural basis of the switch and the thermodynamic driving force of ion transfer (*18*). In addition to the interconversion of ion pumps, it is known that the sensor protein itself possesses proton pumping (translocating) activity as expected from the fact that Asp85 is conserved in the sR homolog (*51*).

4. Halobacterial genera and the rhodopsin families

Halobacterial taxonomy. The homology indices between homologs within the same group (*e.g.*, bR/aR-1) are, in general, around 60% (Table II). As more proton pumps were identified, much higher indices made it possible to classify the homologs into subgroups (*e.g.*, aR/cR). In order to explain these two index ranges, the new strains carrying these new homologs were examined taxonomically.

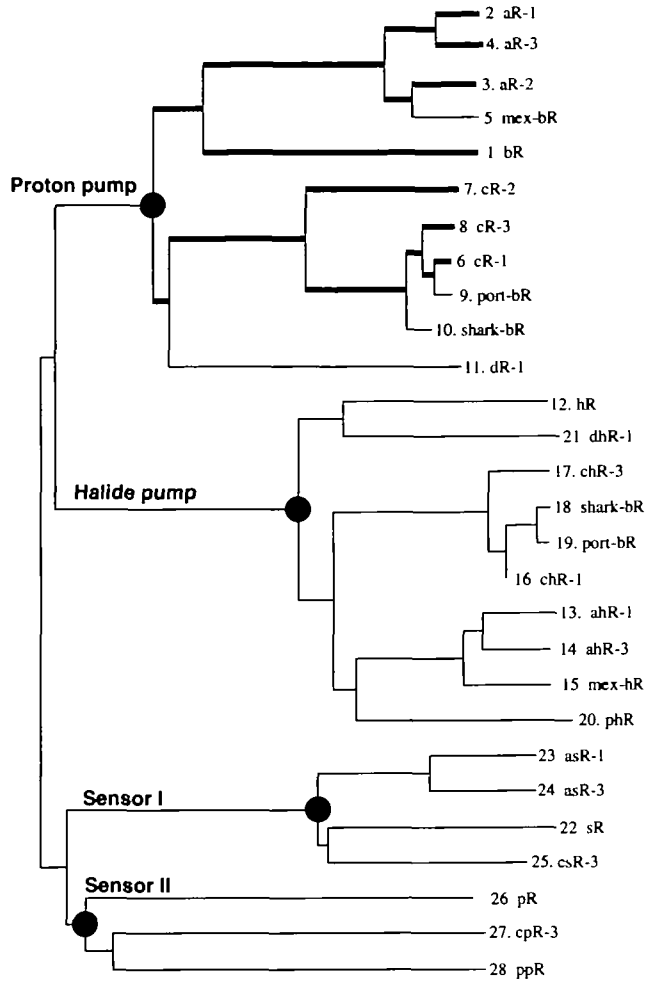

Fig. 2. **Phylogenetic tree of four groups of halobacterial rhodopsins.** The tree was constructed by the Neighbor-Joining method from the amino acid sequences in Fig. 1. The proton pump bough is shown in thick lines especially for comparison with the 16SrRNA tree (Fig. 3). The closed circles indicate the point of simultaneous diversion when generic speciation took place in the single ancestral halobacterium.

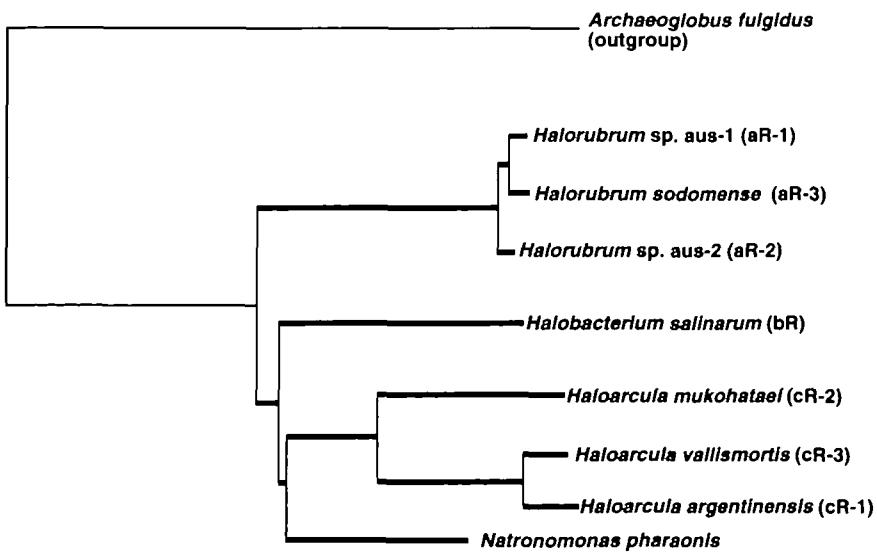

Fig. 3. **Phylogenetic tree of 16SrRNA in halobacteria carrying rhodopsins.** The tree was constructed by the Neighbor-Joining method with the 16SrRNA of *Archaeoglobus fulgidus* as the outgroup. *Natronomonas* does not contain a proton pump.

Halobacterium sp. aus-1 and sp. aus-2 were shown not to be included in any of the established genera. We proposed a new genus "Halorubra" (*44*), which is now defined as *Halorubrum* (*52*) and includes the former *Halobacterium sodomense* and some others. Our finding that a new rhodopsin subfamily (*e.g.*, the aR-1 subfamily, which includes aR-1, ahR-1, asR-1, and apR-1; a member of the rub family) exists in a new species (*Halorubrum* sp. aus-1) suggested to us to examine those strains carrying new rhodopsins taxonomically and identify new rhodopsin (sub)-family members in the already established genus/species. As expected, we found that strains that carry the cR-1 and cR-2 subfamilies (*i.e.*, the arc family) are new species, *Haloarcula argentinensis* and *Ha. mukohataei*, respectively (*41*). In the established species *Halorubrum sodomense*, we identified members of the aR-3 subfamily (to be published) and members of the cR-3 subfamily in *Haloarcula vallismortis* (*53*) (Table I).

Starting with the bR subfamily in *Halobacterium salinarum*, we now know that each halobacterial genus/species carries its own rhodopsin families/subfamilies, although strains in some genera, such as *Halococcus* and *Haloferax*, do not have any rhodopsin. The 16SrRNA sequences of rhodopsin-carrying species were thus analyzed to find any phylogenetic relationship to rhodopsin.

Phylogeny of halobacterial rhodopsin and 16SrRNA. The phylogenetic tree of rhodopsins constructed from their amino acid sequences by the Neighbor-Joining method (Fig. 2), is composed of four discrete boughs for four rhodopsin homologs, each of which branches into families and further into the corresponding members of subfamilies. The branching pattern of the proton pump bough resembles that of the halide pump bough when the overall lengths of the two boughs are set roughly equal and the earlier branching order of low certainty is ignored. The sensor branches are needed to furnish more members for discussion. A phylogenetic tree of rhodopsin-carrying halobacteria was also constructed with the 16SrRNA sequences by the Neighbor-Joining method (Fig. 3). Five boughs for genera that carry individual rhodopsin families, *Halorubrum* for the rub family, *Halobacterium* for the bac family, *Haloarcula* for the arc family, *Natronomonas* (formerly *Natronobacterium*; does not possess a proton pump) for the nam family, and a fifth unidentified genus carrying the dR-1 family, are clearly separated. The branching pattern of 16SrRNA corresponds fairly well to that of the proton pump bough (and thus the halide pump bough). The early branching of cR-2 (Fig. 2) and its carrier, *Haloarcula mukohataei* (Fig. 3), is especially remarkable.

Htr and its gene. Meanwhile, Htr (*54*) was found to act

TABLE III. **Homology indices between Htr's for sR and pR.**

			1	2	3	4	5	6
1.	HtrI	for sR						
2.	HtrI	for asR-1	50					
3.	HtrI	for asR-3	52	**90**				
4.	HtrI	for csR-3	57	51	53			
5.	HtrII	for pR	34	32	30	34		
6.	HtrII	for cpR-3	34	33	35	34	40	
7.	HtrII	for ppR	32	35	33	34	37	38

The bold value indicates high homology between Htr's accompanying sensor rhodopsins in the same subgroup.

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Sensor I [HtrI+sR] system
Hb.salinarum          ···GACGGGGGTGGTGCGTGATGGACGCCGTCGCAACCGCCTACCTC···(sR)
Hr.sp. aus-1          ···GACGGAGGTGCGCGATGACGGGCGCGGTCAGCGCCGCGTACTGG···(asR-1)
Hr.sodomense          ···GACGGGGGTGCGCGATGACCGGTGCCGTCACCAGTGCGTACTGG···(asR-3)
Ha.vallismortis       ···GTTGGGGGTGACGACTGATGGACGCCGTTGCAGTCGTGTACGGC···(csR-3)
Sensor II [HtrII+pR] system
Hb.salinarum          ···GAACCGGAGGACTACTGATATGGCACTCACGACATGGTTTTGGG···(pR)
Ha.vallismortis       ···GCTGTGGGGGATGACTAACAATGGCAACGATAACAACCTGGTTC···(cpR-3)
Nm.pharaonis          ···GCGGCGGGGGATGATTAACGATGGTGGGACTTACGACCCTCTTT···(ppR)
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Fig. 4. **Connecting regions of the tandem genes encoding sensor rhodopsins (sR's and pR's) and their Htr's.** The termination codon (TGA or TAA) of the Htr gene (upstream) and the initiation codon (ATG) of the sR/pR gene are shown in bold letters. In the sensor I system the two codons overlap, whereas in the sensor II system the two codons are separated by one or two nucleotides.

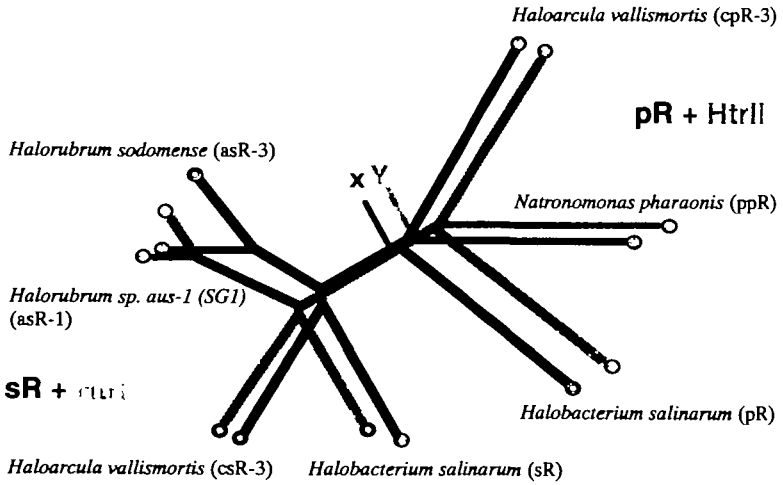

Fig. 5. **Unrooted trees suggesting the co-evolution of sensor rhodopsins (sR's and pR's) and their Htr's.** The trees were constructed for the two sensor systems (seven known members at present) by the Neighbor-Joining method, and are depicted so as to emphasize their similarities, *i.e.*, possible co-evolution. Thick lines are for rhodopsins and shadow lines for the corresponding Htr's. X denotes the branch to the ion pump cluster (*cf.* Fig. 2) and Y the branch to the outgroup.

as a transducer of light-stimuli from sensor rhodopsin to a CheA/CheY-like protein (*55*), similar to Tsr in the eubacterial chemo-sensor system (*56*); HtrI is paired with sR homolog and HtrII with pR homolog. Gene analyses have revealed that (i) the Htr gene locates in tandem upstream of the sensor rhodopsin gene, with one overlapping nucleotide (the HtrI-sR case) (*54*) or one nucleotide space (the HtrII-pR case) (*57*). When more Htr's were sequenced, this was found to be more-or-less common feature of the sensor systems (Fig. 4). (ii) The Htr gene/protein and its paired rhodopsin gene/protein show similar degrees of mutation in their overall frames; note the high indices between the Htr's for asR-1 and asR-3 (Tables II and III). (iii) Although the overall homology (identity) indices between HtrI and HtrII are in the range of 30%, both proteins contain highly conserved regions (*57*), *e.g.*, the region in the two helices for anchoring the protein to the membrane and the region for CheA interaction where 27 out of 40 residues are conserved (details to be published). Phylogenetic trees for sensor rhodopsins (sR+pR) and their Htr's are almost superimposable on each other (Fig. 5). These results strongly suggest that the HtrI-sR and HtrII-pR gene pairs were formed by gene duplication after the Htr and sensor genes became tandemly arranged in the ancestral halobacterium. The genes that encode the bR and hR homologs are also assumed to have been formed by gene duplication.

Evolutionary aspects of rhodopsins. The observed similarity between the phylogenetic trees of rhodopsin homologs and 16SrRNA strengthens our hypothesis that the common ancestral halobacterium at the time of its initial divergence into genera already possessed genes encoding the four different rhodopsin homologs. When this ancestral halobacterium evolved into genera and then species, the four rhodopsin genes were inherited by the descendants, where they were subsequently modified in a genus-specific manner to be grouped as in the present families/subfamilies. This process gave rise to the genus-specific amino-acid insertions or deletions, such as in the proton pumps of the arc family (Fig. 1).

Concerning the original family of four rhodopsins in the ancestral halobacterium before generic speciation, we can speculate as most probable that these four families were formed by three (or two if the second duplication took place simultaneously) gene duplications in the single gene encoding the rhodopsin ancestor. In this ancestral halobacterium, the single gene was first duplicated to give the genes for the proto-type ion pump and the proto-type sensor. The proto-sensor gene then acquired an Htr gene (CheA gene?) which was fixed in tandem to the proto-sensor gene, and survived as a photo-sensor system (or was extinguished by random mutation). Later the proto-pump gene and the proto-sensor system (proto-Htr-sR/pR) gene were duplicated again to give two pump genes and two sensor-system genes. The two pump genes have diverged and been refined to express the proton and halide pumps. The two sensor-system genes diverged to express the systems sensing different (one favorable and one harmful) wavelengths (Fig. 6). Four functional rhodopsins as the original rhodopsin family were thus present in the single ancestral halobacterium, which then became ready for generic speciation (at the closed circles in Fig. 2; the abscissa of the N-J tree is not a time scale) and concomitant inheritance of the rhodopsin family into the evolving genera/species as families/subfamilies.

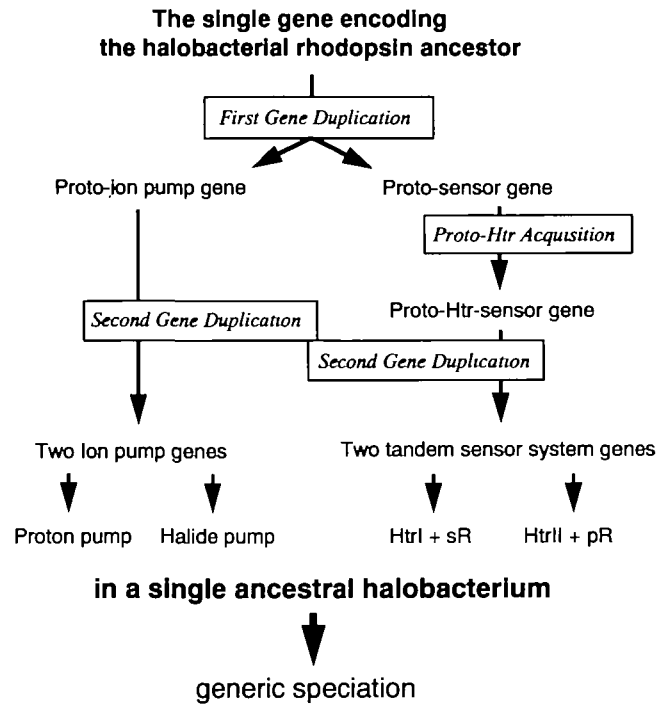

Fig. 6. **A possible pathway for the acquisition of four halobacterial rhodopsins in the ancestral halobacterium.** This is only the most readily imaginable pathway based on present knowledge. The second gene duplications may have taken place simultaneously.

Since the halide pump gene and the sensor system I gene seem to be needed to accumulate more mutations (longer length from the second duplication to generic speciation in the tree, Fig. 2) so as to yield the present hR homologs and the Htr-sR systems (Fig. 2), the proto-types of bR and pR would be the products of the first gene duplication. The initial single gene would have coded for the proto-type of proton pump simply because bR is a single molecular pump whereas pR is needed to get Htr to function as a sensor (evolutionary aspects of halobacterial rhodopsins are discussed in detail elsewhere; *58*).

In some genera, such as *Haloferax* and *Halococcus*, no rhodopsins have been found. The genes encoding the original rhodopsin family may have been inherited by the ancestor cells of these genera, then lost before any further speciation occurred. Nevertheless those genera survived in the absence of rhodopsin, which were not always necessary or helpful in their habitats.

Halobacterium halobium (*salinarium*), from which the red $bR^{-}hR^{+}$ mutant was isolated, was a gift of the late Prof. Masamiki Masui, Osaka City University Medical School, originated from Dr. M. Kates, University of Ottawa.

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