9 Light-driven ion-translocating rhodopsins in marine bacteria

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22

23 **Abstract**

24 Microbial rhodopsins are the photo-receptive membrane proteins found in diverse
25 microorganisms from within archaea, eubacteria and eukaryotes. They have a
26 heptahelical transmembrane structure, which binds to an all-*trans* retinal chromophore.
27 Since 2000, thousands of proteorhodopsins, genes of light-driven proton pump
28 rhodopsins, have been identified from various species of marine bacteria. This
29 suggests that they are used for the conversion of light into chemical energy, presumably
30 contributing to carbon circulation related to ATP synthesis in the ocean. Furthermore,
31 novel types of rhodopsins (sodium and chloride pumps) have been recently discovered.
32 Here we review the recent progress on ion-transporting rhodopsins of marine bacteria,
33 mainly based on biophysical and biochemical research.

34

35 **Microbial rhodopsins**

36 The word “rhodopsin” originates from the Greek words “rhodo” and “opsis”, which
37 indicate rose and sight, respectively. Thus, the classical meaning of rhodopsin is the
38 red-colored pigment in the retinal rods of eyes. The chromophore molecule that
39 absorbs light is retinal, which is the origin of red color. Then, similar colored
40 retinal-binding proteins were found in microbes, largely expanding the definition of the

41 word rhodopsin. The modern meaning of rhodopsin encompasses photoactive proteins
42 containing a retinal chromophore in animals and microbes [1]. Rhodopsins are now
43 found in all domains of life and are classified into two groups, animal and microbial
44 rhodopsins. Animal rhodopsins contain an 11-*cis* retinylidene chromophore via
45 protonated Schiff-base linkage to a conserved lysine residue in the seventh helix [1, 2].
46 Animal rhodopsins carry out light-signal transduction by interaction with G-protein
47 coupled receptor (GPCR) [3-7], where photo-activated animal rhodopsins induce a
48 GDP/GTP-exchange reaction of transducin (G_t).

49 Microbial rhodopsins, mainly bind to an all-*trans* type chromophore, and are found
50 in various unicellular microorganisms being classified to archaea, eubacteria and
51 eukaryotes. Unlike animal rhodopsins, the functions of microbial rhodopsins are
52 highly diverse [1, 8, 9]. The first microbial rhodopsin, bacteriorhodopsin (BR), was
53 discovered from the cytoplasmic membrane of the haloarchaeon *Halobacterium*
54 *salinarum* in 1971 [10, 11]. BR pumps protons from the cytoplasmic to the
55 extracellular side of the cell by using light energy [12, 13]. After the discovery of BR,
56 the rhodopsins functioning as light-driven inward Cl^- pumps (halorhodopsin, HR), with
57 both positive and negative phototactic sensors (sensory rhodopsin I and II) were found
58 from the same species [8, 14-19]. All of these microbial rhodopsins bind a common

59 chromophore, all-*trans* retinal (Figure 1A), which isomerizes to the 13-*cis* form if the
60 retinal absorbs light. The isomerization triggers subsequent conformational changes of
61 the protein, leading to various functions. Although several similar microbial
62 rhodopsins were found, they were mainly restricted to the haloarchaea before 2000.
63 However, metagenomic research identified a new species of microbial rhodopsin from
64 the gene of an uncultured γ -Proteobacteria from Monterey Bay [20]. A cell suspension
65 of *Escherichia coli* expressing this protein showed acidification upon illumination,
66 suggesting that this rhodopsin functions as a light-driven outward H^+ pump similar to
67 BR (Figure 1B). The new rhodopsin was named proteorhodopsin (PR) from the name
68 of the hosting bacterium [20]. After the first report of PR, thousands of PR genes have
69 been identified from various oceans to date [21-26]. It is estimated that 50% of
70 microbes in the photic zone possesses PR genes [27]. Interestingly, new PR-like genes
71 have also been found in some viruses [28, 29].

72 While many PR genes were identified as acting as H^+ pumps from various
73 oceans [22, 30-33], other types of functions were not discovered. This is in contrast to
74 non-oceanic microbial rhodopsins whose functions are very diverse including: H^+ pump,
75 Cl^- pump, cation channel, positive phototaxis sensor, negative phototaxis sensor, gene
76 regulation and so on [1, 2, 19]. However, a second type of function was reported for a

77 rhodopsin of marine bacteria in 2013. It is an outward sodium (Na^+) pump rhodopsin
78 (NaR) [34]. The first NaR was identified from the gene of the flavobacterium
79 *Krokinobacter eikastus*. *K. eikastus* has two rhodopsin genes *Krokinobacter* rhodopsin
80 1 (KR1) and 2 (KR2). KR1 is homologous by sequence to PR and acidification
81 occurred upon illumination of *E. coli* cells expressing KR1 (Figure 1B). However, a
82 pH increase was observed when light was illuminated onto *E. coli* cells expressing KR2.
83 The pH change was not due to the active transport of H^+ inward, because it was
84 enhanced by the addition of a protonophore, carbonylcyanide m-chlorophenylhydrazone
85 (CCCP). This means that KR2 transports ions other than H^+ that generate membrane
86 potential leading to inward H^+ movement. Similarly, a light-induced pH increase was
87 reported for HR [15]. Because HR transports anions, the pump efficiency depends on
88 the size of anions present in the environment (Figure 1B, right) [15, 35, 36]. In
89 contrast, even if anions were exchanged to larger ones, Br^- or even SO_4^{2-} , KR2 showed
90 identical alkalization. However, the direction of the light-induced pH change was
91 inverted in the solvent containing the salt of larger cations such as KCl, RbCl, and CsCl
92 [34]. The cation-dependence of the pump function indicates that KR2 functions as an
93 outward Na^+ pump in physiological sea water containing ~400 mM NaCl, while it
94 pumps H^+ only in the presence of cation(s) larger than Na^+ (Figure 1B, middle).

95 Following NaR, the inward chloride pump rhodopsin (CIR) was discovered
96 from the flavobacterium *Nonlabens marinus* S1-08^T [9, 37]. This rhodopsin also
97 shows the increase of pH upon illumination similar to NaR. However, the dependence
98 on ions is opposite to KR2. While pH increases even in solvents containing larger
99 cations such as K⁺, it is abolished in the presence of larger anions such as SO₄²⁻. This
100 indicates that CIR inwardly pumps Cl⁻ as well as HR does, and the molecular
101 mechanism of another light-driven chloride pump from the marine bacteria *Fulvimarina*
102 *pelagi* (FR) is similar to that of HR, though FR is evolutionally closer to NaR than HR
103 [38] .

104 We now know that marine bacteria use three different ion pump rhodopsins (H⁺,
105 Na⁺, and Cl⁻ pumps) [9, 37]. Each type of marine bacterial rhodopsin forms an
106 independent clade from each other and archaeal rhodopsins (Figure 1C) [37, 39].
107 Furthermore, they can be distinguished by their sequences. BR has two aspartic acid
108 residues, D85 and D96, in helix C which function as the proton acceptor and donor,
109 respectively, for the retinal Schiff base during its proton pumping photocycle. In
110 addition, the former forms a hydrogen bond with T89 [40]. These three residues are
111 important for the function of BR. Their conserved alignment in BR is called the DTD
112 motif (Figure 1D) [9, 34]. At the corresponding position, most of PRs have a DTE

113 motif in which the proton donor is glutamic acid instead of D96 in BR. Furthermore, a
114 new type of H^+ pump was recently found from *Exiguobacterium sibiricum* (ESR) whose
115 H^+ donor is lysine [41]. The extremely halophilic eubacterium, *Salinibacter ruber*, has
116 a proton pump rhodopsin with DTE motif as PR. It is called xanthorhodopsin, XR
117 [42]. XR binds not only the chromophore retinal, but also the carotenoid salinixanthin.
118 The absorbed light energy by salinixanthin was transferred to the retinal leading to the
119 isomerization. Thus, the salinixanthin works as a light antenna increasing the spectral
120 range of light which can be used for retinal isomerization [42]. NaR and CIR have
121 NDQ and NTQ motifs at the same position, respectively (Figure 1D) [9, 34, 37]. They
122 are also called NQ-rhodopsins [39, 43]. Thus, these three residues corresponding to
123 position 85, 89, and 96 of BR are important for categorizing the ion pump rhodopsins of
124 marine bacteria. Here, we review the recent insights on the molecular mechanism of
125 ion-transport function for marine bacterial rhodopsins. As studies on the molecular
126 level of the Cl^- pump (FR) only appeared recently [38], here we focus on the H^+ pump
127 (PR) and Na^+ pump (KR2). To understand the ion-transport mechanism, spectroscopic
128 study is highly useful, as the ion-transport process proceeds through several
129 photointermediates.
130

131 **Proteorhodopsin**

132 The first PR gene was isolated from an uncultured γ -Proteobacterium classified
133 to SAR86 [20]. PR could be heterologously expressed in *E. coli* cells yielding a
134 reddish color. The cell suspension showed acidification upon illumination, indicating
135 that PR is a light-driven outward proton pump. Although the amino-acid sequence of
136 PR has less than 30% identity to BR, D85 and T89 of BR are conserved in PR (D97 and
137 T101). In contrast, the proton donor of BR (D96) is replaced by glutamic acid and the
138 proton release group (BR E194 and E204) is not conserved in PR [20]. Another
139 characteristic feature of PR is its color variation. There are two known types of PR.
140 The green-proteorhodopsins (GPRs) show absorption maxima ($\lambda_{\max}$) at ca. 525 nm,
141 while the blue-proteorhodopsins (BPRs) has $\lambda_{\max}$ at around 490 nm (Figure 2A) [23].
142 This spectral tuning is achieved by the difference of only one residue at the position of
143 L105 of GPR which is replaced by a glutamine residue in BPR (Figure 2B) [24].
144 Furthermore, L-to-Q mutation of GPR makes its spectrum similar to BPR and *vice*
145 *versa* [24, , 44]. As leucine and glutamine are hydrophobic and hydrophilic amino
146 acids, respectively, one might expect that hydrophobicity at position 105 is the
147 determinant of color tuning. However, a recent comprehensive mutation study of GPR
148 revealed that absorption does not correlate with the hydropathy index of residues at all,

149 but does correlate with volume of residues, suggesting a more complex color tuning
150 mechanism than expected [45].

151 It is suggested that the color-tuning between GPR and BPR represents the
152 Darwinian selection for the sun-light spectrum sensed by each bacterial species living at
153 different depths of the ocean [46]. That is, because green light is more abundant near
154 the surface, GPR-possessing bacteria would be more adapted to the environment there.
155 However, in the deep region of ocean, only blue-light can be used and BPR is more
156 suitable to generate energy. In fact, while GPR possessing strains are present near the
157 sea surface, the BPR gene was found from the deeper region in Monterey Bay [23].
158 Furthermore, a metagenomic survey in the Mediterranean Sea revealed that GPR is
159 more abundant in shallow water and is BPR exclusively present in deep water [30].
160 However, only BPR was found from both of shallow and deep waters in Sargasso sea
161 [30] which suggests that natural selection for the sun-light spectrum does not always
162 play a key role to determine the color of PRs. In order to solve this problem, further
163 research is required.

164 HPLC, Raman and NMR studies revealed that GPR contains >95% all-*trans*
165 retinal [47-49]. The main population was all-*trans* retinal even in the light condition,
166 although a small increase of 13-*cis* composition was observed [47]. This is

167 significantly different from BR. The dark-adapted BR has an approximately equal
168 amount of all-*trans*- and 13-*cis* retinal. Upon light adaptation, the all-*trans* form of
169 BR increased to almost 100% [50, 51]. Although PR and BR show a difference of
170 retinal composition in the dark-adapted state, the functional photocycle occurs upon
171 photo-excitation of the all-*trans* form for both of them.

172 The photocycle of BR has been studied in detail [1, 52-58]. It mainly
173 contains the six intermediates (K, L, M₁, M₂, N, and O) as follows [55, 57]:

174

175

176 BR (light activation) → K → L → M₁ → M₂ → N → O → BR

177

178 In the dark state, the extracellular surface of BR is open. The retinal isomerizes to the
179 13-*cis* form upon the absorption of light. This affects the pK_a of the retinal
180 Schiff-base and the counterion (D85). As the result, proton transfer occurs from the
181 Schiff-base to the counterion [59, 60]. Then, another proton is released from the
182 proton releasing group (PRG) of BR consisting of E194 and E204 upon the first half of
183 the photocycle (K-to-M₂) (Figure 2C). Next, a switching of the accessibility occurs by
184 a tilting of helix F and an exposure of intracellular part of the protein to bulk solvent

185 (Figure 2C) [61, 62]. At this stage, H^+ is transferred from D96 to the retinal Schiff
186 base (M_2 -to-N) [63]. Then, D96 is reprotonated from the bulk solvent (N-to-O) [64].
187 Retinal recovers to the all-*trans* form upon formation of the O-intermediate [65].
188 Finally, H^+ transfer from D85 to the PRG occurs to recover the initial state. The
189 photocycle of PR has many similarities to that of BR [66]. By laser flash photolysis
190 experiment, five intermediates [K ($\lambda_{\max} = 555$ nm), M_1 ($\lambda_{\max} = 405$ nm), M_2 ($\lambda_{\max} = 405$
191 nm), N ($\lambda_{\max} = 560$ nm), and PR' ($\lambda_{\max} = 525$ nm)] have been identified in the
192 photocycle of PR [66]. Here, PR' represents the transient intermediate with the
193 identical absorption spectra to the initial state. Unlike BR, no significant accumulation
194 of the L-intermediate is observed in the photocycle of PR, probably because of kinetic
195 reasons. Another difference from BR is the red-shifted absorption of N. One may
196 consider that the red-shifted intermediates should be called O in analogy to BR.
197 However, time-resolved FTIR spectroscopy showed that the late red-shifted
198 intermediate of PR has a 13-*cis* form similar to N of BR [48]. On the other hand, the
199 following PR' is in the all-*trans* form and it must represent the corresponding state to O
200 of BR [48, 66].

201 The photocycle of BPR also shows high accumulation of a red-shifted late
202 intermediate (called O, but it is expected to be identical to the red-shifted N of GPR) as

203 GPR [67]. However, its turnover rate is >10-times slower than that of GPR [67].
204 The slow photocycling rate seems to be a disadvantage for the efficient H⁺ pump.
205 Therefore, it is suggested that BPR could function as a photo-sensory machinery.

206 PR has a highly conserved histidine in helix B (H75 in GPR) which is not
207 observed in BR [68]. In the case of GPR, it contributes to adjusting the pKa of the
208 counterion [69]. The counterion (D97) of GPR has a relatively higher pKa (= 7.5)
209 compared with BR (pKa = ~2.5) [13], and mutation of H75 lowers it 0.8-2.2. However,
210 in the case of a variant of homologous histidine of *Gloeobacter* rhodopsin (GR) (GR
211 H87M) from the cyanobacterium *Gloeobacter violaceus* PCC 7421, no significant
212 difference of pKa of the counterion (D121) was observed from wild type (pKa = 4.5)
213 [70]. Similarly, the homologous variant of ESR (H57M) showed similar pKa (= 6.3)
214 to the wild type (pKa = 6.0) [71]. Therefore, the elevation of the pKa of the
215 counterion and its higher value observed for GPR are not true for all of eubacterial
216 proton pumps. Time-resolved FTIR spectroscopy revealed that H75 is fully protonated
217 in the initial state and it is deprotonated in the photocycle [68]. This suggests that H75
218 participates in the proton transfer pathway in PR. This is supported by the highly
219 reduced charge movement for the H75 mutated variants [68].

220 In the crystal structure of BPR, H75 seems to interact with D97 and W34 of the

221 neighboring protomer (Figure 2D) [72]. In the case of GR, its trimeric structure is
222 disrupted upon the mutation of the corresponding histidine (H87M) [70]. It implies
223 the importance of this conserved histidine for oligomerization. However, the
224 corresponding residue to W34 of BPR is replaced by a serine in GR. This suggests
225 that there is a difference between PR and GR in how oligomerization occurs. In fact,
226 atomic force microscopy and X-ray crystallographic structure showed hexameric
227 association of PR in contrast to the trimeric structure of GR [70, 72, 73].

228 Because thousands of PR genes were identified from diverse bacteria, their
229 contribution to the carbon circulation related to ATP synthesis in the ocean must be
230 much higher than that previously expected [32]. Their absorption spectra and pump
231 efficiency are optimized for efficient proton transport in oceanic environment. The
232 spectroscopic and biological research reviewed in this section provide various insight
233 about the mechanism of how PR adapted to the environment at molecular level.

234

235 **Sodium pump rhodopsin**

236 It took more than 40 years after the first report of BR for a Na⁺-pumping rhodopsin to
237 be discovered. The initial lack of discovery was thought to be likely because the
238 protonated Schiff-base, a positive charge, exists on the ion conductive pathway and

239 must inhibit the transport of non-proton cations. However, a light-driven Na^+ pump
240 rhodopsin (NaR) was found in the flavobacterium *K. eikastus* in 2013 [34]. *K. eikastus*
241 has two rhodopsin genes (KR1 and KR2). The former has the typical PR-like
242 sequence with DTE-motif, and shows outward H^+ pump function. On the other hand,
243 KR2 was a new outward Na^+ pump rhodopsin from its light-induced alkalization of cell
244 suspension (Figure1B) [34].

245 The amino acid sequence of KR2 shows a distinct difference in helix C from
246 other microbial rhodopsins. In fact, KR2 has an asparagine and a glutamine at the
247 corresponding position of the proton acceptor (D85 of BR) and donor (D96 of BR) of
248 H^+ pump rhodopsins, while an aspartic-acid residue exists between them at the position
249 of T89 of BR (Figure 1D). Because the residues in helix C forms an ion conductive
250 pathway, these three residues (NDQ-motif) are important for the Na^+ transport. More
251 than 10 rhodopsin genes with NDQ-motif have been identified to date [38], indicating
252 that Na^+ pump rhodopsins are diversely used among various species in nature.

253 The main configuration of retinal of KR2 is the all-*trans* form with >90% in
254 this form in the dark similar to other marine bacterial rhodopsins [34]. Light
255 absorption generates the red-shifted K-intermediate with 13-*cis* retinal leading to the
256 photocyclic reaction which recovers to the initial state in 100 ms [34, 74]. Following

257 the K-intermediate, the $L \rightleftharpoons M$ and O intermediates appear in the order of
 258 accumulation. The nomenclature of these intermediates is according to that of BR
 259 based on their absorption spectra and the time-scale in which each intermediate appears
 260 (Figure 3A). While the K and O intermediates have red-shifted absorption, the
 261 spectrum of $L \rightleftharpoons M$ is blue-shifted compared with the initial state. In particular, M
 262 ($\lambda_{\text{max}} = 400 \text{ nm}$) has a more than 100-nm shorter absorption wavelength compared to
 263 KR2 ($\lambda_{\text{max}} = 526 \text{ nm}$), as the retinal Schiff base is deprotonated in this state [34]. The
 264 proton acceptor of KR2 is D116 constituting a NDQ-motif, supported by the inhibition
 265 of M-accumulation by the D-to-N mutation of this residue. However, the rate of
 266 O-accumulation is accelerated by the increase of Na^+ concentration [34]. This
 267 suggests that Na^+ uptake occurs upon $L \rightleftharpoons M$ -to-O process. This finding explains
 268 how Na^+ is transported to the extracellular region across the protonated Schiff base of
 269 retinal. In the case of proton transport by H^+ pump rhodopsin, the proton bound to the
 270 Schiff base itself is transported (Figure 3B, left). In contrast, the H^+ transfer to the
 271 counterion (D116) upon M formation of KR2 transiently eliminates the positive charge
 272 in the Schiff base region. This enables the conduction of Na^+ beside the Schiff base
 273 upon the $L \rightleftharpoons M$ -to-O process (Figure 3B, right). Because the red-shifted absorption
 274 of O indicates that the retinal is reprotonated at this state, H^+ goes back from D116 to

275 the retinal Schiff base. This would be important for efficient pumping function by
276 inhibiting the backward flow of transported Na^+ .

277 H^+ and Cl^- pump rhodopsins bind the substrate ions near the active center in the
278 dark. In the case of H^+ pumps (BR, PR, and so on), the substrate proton is bound to
279 the retinal Schiff base and acidic amino acid residues constitute the H^+ -transfer pathway
280 [1]. The binding site of Cl^- in HR was identified by X-ray crystallography in the
281 vicinity of the protonated Schiff base (PSB) [75, 76]. While the binding of ions affects
282 the colors of H^+ pumps and HRs, KR2 does not show any color change between the
283 presence and absence of Na^+ [34]. This implies that the Na^+ binding site of KR2 is
284 distant from retinal. Thus, the Na^+ binding to KR2 cannot be studied by conventional
285 UV-visible absorption spectroscopy. However, the conformational change of KR2
286 upon Na^+ -binding was clearly observed by using attenuated total reflection-Fourier
287 transform infrared (ATR-FTIR) spectroscopy [34]. ATR-FTIR revealed that KR2
288 binds Na^+ with a K_d of 11.4 mM. The position of the binding site was studied by
289 ATR-FTIR with various mutants of KR2. The results showed that Na^+ binding was lost
290 for the H30A, R109A, and N112D mutants, indicating that substituted residues are
291 prerequisite for Na^+ binding. However, all of the variants whose acidic residues on
292 each loop (AB, BC, DE, EF and FG loops) were removed by D-to-N and E-to-Q

293 mutations showed normal Na^+ -binding except for the BC-loop mutant
294 (E90Q/E91Q/D98N/D102N). Loss of Na^+ binding suggests that extracellular residues
295 including H30, R109, N112 and carboxylates in BC-loop constitute the Na^+ -binding site
296 (Figure 3C). Interestingly, despite these residues being highly conserved among most
297 of the putative NaRs with a NDQ-motif, the Na^+ -transport efficiencies of H30- or
298 BC-loop mutants are identical to that of wild-type KR2 without the ability to bind Na^+ .
299 This indicates Na^+ binding is not a prerequisite for pump function. We believe that the
300 role of Na^+ binding of NaR probably contributes to increasing the stability of the
301 protein.

302 NaR is a new class of ion pump rhodopsin and the presence suggests further
303 physiological role of rhodopsins in nature. The molecular mechanism of the transport
304 still has many mysteries. How does Na^+ bind to the protein, how are Na^+ and H^+
305 transported by the same protein, and which residue(s) is related to the transport? In
306 particular, structural study including X-ray crystallography is strongly required for more
307 precise understanding of these issues.

308

309 **Concluding remarks**

310 Here, we reviewed recent research on ion-pumping rhodopsins from marine bacteria,

311 particularly for H⁺ pump (PR) and Na⁺ pump (KR2). Because metagenomic analysis
312 of the ocean environment is still ongoing and identifying rhodopsin genes day by day, it
313 is expected that additional functional rhodopsins will be found in future [21-23, 30].
314 As H⁺ and Cl⁻ pump rhodopsins are now used as optogenetic tools for optical silencing
315 of neurons [77, 78], we expect new rhodopsins from marine bacteria to provide a
316 molecular basis for developing novel optogenetic tools (Box 1). In particular, NaR has
317 potential to be a useful tool, because its outward Na⁺-transport evokes hyperpolarization
318 of membrane potential without unphysiological intracellular pH change and Cl⁻
319 accumulation. On the other hand, the physiological roles of Na⁺ and Cl⁻ pumps still
320 have not been revealed. The Na⁺ pump could provide sodium motive force to drive
321 sodium coupled transporters or control the intracellular sodium concentration.
322 Furthermore, the Cl⁻ pump could control osmotic pressure of the cell for both of them.
323 Further physiological research on marine bacteria is required to answer this question.

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330

331 **Box 1. Outstanding questions**

332 • Why can Na^+ be transported through rhodopsin?

333 • Can metagenomic study reveal further new classes of rhodopsins?

334 • Can NaR be applied as a new optogenetic tool?

335 • What are the physiological roles of Na^+ and Cl^- pumps?

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573

574

575 **Figure Legends**

576 **Figure 1.** The diversity of microbial rhodopsins of marine bacteria. **(A)** The structure
577 of all-*trans* retinal and 13-*cis* retinal bound to rhodopsin via the protonated Schiff base.
578 All-*trans* retinal isomerizes to 13-*cis* form upon the absorption of light.
579 Reisomerization from 13-*cis* is the thermal process which occurs at the end of
580 photocycle. **(B)** The ion-transport rhodopsins (outward H⁺ pump, outward Na⁺ pump
581 and inward Cl⁻ pump) found in marine bacteria. Their pumping ability upon
582 illumination of light is assayed by the pH monitoring system with various solvent
583 conditions (see text) [34, 37]. **(C)** Phylogenic tree of microbial rhodopsins. Unrooted
584 CLUSTALW guide tree of selected microbial rhodopsins from public genome databases
585 (<http://www.ncbi.nlm.nih.gov/protein>). The scale bar represents number of substitutions
586 per site (0.1 indicates 10 nucleotides substitutions per 100 nucleotides). H⁺ (PR), Na⁺
587 (NaR), and Cl⁻ (CIR) pumps of marine bacteria are indicated by yellow, orange, and
588 cyan circles, respectively. BR, HR, and sensor indicate H⁺ (BR), Cl⁻ (HR) pumps and
589 sensory rhodopsin from halophilic archaea and eubacteria. **(D)** The amino acid sequence
590 alignment of microbial rhodopsins for the residues highly conserved in each type of

591 rhodopsin (red, acidic; blue, basic; grey, aromatic; green, -OH bearing; magenta,
 592 asparagine and glutamic residues). PR, NaR, and CIR have DTE-, NDQ-, and NTQ-
 593 motifs at the corresponding position to D85, T89, and D96 of BR (DTD), respectively.
 594 Abbreviations: AR1, archaerhodopsin-1; AR2, archaerhodopsin-2; AR3,
 595 archaerhodopsin-3; *HwBR*, BR from *Haloquadratum walsbyi*; MR, middle rhodopsin;
 596 *NpHR*, *HsHR*, *SrHR*, HR from *Natronomonas pharaonis*, *H. salinarum*, and
 597 *Salinibacter ruber*; *HsSRI*, *HvSRI*, *SrSRI*, sensory rhodopsin I from *H. salinarum*,
 598 *Haloarcula vallismortis*, and *S. ruber*; *HsSRI*, *NpSRI*, sensory rhodopsin I from *H.*
 599 *salinarum* and *N. pharaonis*; *VsPR*, *GlPR*, *NdR1*, proteorhodopsins from *Vibrio* sp.
 600 AND4, *Gillisia limnaea* DSM 15749, and *Nonlabens dokdonensis* DSW-6; XR,
 601 xanthorhodopsin; TR, proteorhodopsin from *Thermus thermophilus*; *CbCIR*, *CsCIR*,
 602 *SpCIR*, and *NmCIR*, CIR from *Citromicrobium bathyomarinum*, *Citromicrobium* sp.
 603 JLT1363, *Sphingopyxis baekryungensis* DSM 16222, and *N. marinus*; *GlNaR*, *NdNaR*,
 604 *IaNaR*, *TrNaR1*, and *TrNaR2*, NaR from *G. limnaea*, *Nonlabens dokdonensis*,
 605 *Indibacter alkaliphilus*, and two NaR from *Truepera radiovictrix*, respectively.

606

607 **Figure 2.** Proteorhodopsin (PR): light-driven outward H⁺ pump of marine bacteria. **(A)**
 608 UV-visible absorption spectra of GPR and BPR [24]. **(B)** The position of the L/Q

609 switch (L105 and Q105 of GPR and BPR, respectively). The structures are based on
 610 the crystal structure of BPR D97N/Q105L (green, PDB code: 4KNF) (upper) and BPR
 611 (blue, PDB code: 4JQ6) (lower) [72], respectively. The retinal is shown in yellow.
 612 **(C)** H^+ transport process of BR among retinal Schiff base and acidic residues. The
 613 protein is outwardly opened in its initial state and first half of the photocycle. Then,
 614 structural switching opens intracellular side of the protein in the latter half of the
 615 photocycle. **(D)** The conserved histidine (H75) of PR in helix B interacts with the
 616 Schiff-base counterion (D97) and W34 of the neighboring protomer. Yellow lines and
 617 numbers indicate the putative hydrogen bonds and the lengths between them. The
 618 numbering of the residue is according to that of GPR and the structure is based on the
 619 crystal structure of BPR (PDB code: 4JQ6) [72].

620

621 **Figure 3.** Na^+ transport and binding by sodium pumping rhodopsin, KR2. **(A)** The
 622 photocycle of KR2 upon Na^+ transport. The spectra of dark-state of KR2, L/M, O were
 623 shown by filled green, yellow and blue spectra. The dotted green line also represents
 624 the spectrum of dark-state of KR2. τ indicates the lifetime of each intermediate.
 625 Although K has red-shifted spectra compared with dark-state, the precise spectrum of K
 626 was not reported and the probable spectrum is shown by blue dotted line which is the

627 duplicate of O. (B) The Na⁺ transport mechanism of KR2 (right) compared with H⁺
628 transport by PR (left). (C) The position of the residues prerequisite for Na⁺ binding in
629 the dark state of KR2 (yellow spheres). The protein structures shown in (B) and (C) are
630 modeled based on the crystal structure of XR [79].
631

Figure 1

Figure 2

(A)

(B)

(C)

(D)

Figure 3