博士論文 (要約)

Functional analysis of new rhodopsins present in marine bacteria

(海洋細菌が保有する新規ロドプシンの機能解析)

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General Introduction

Biochemical energy production

Almost all organisms rely on adenosine triphosphate (ATP) synthesized by electrochemical gradients. Phototrophs and chemotrophs use different sources of energy: phototrophs absorb light and chemotrophs oxidize organic compounds. Autotrophs and heterotrophs use different sources of carbon: autotrophs use carbon dioxide and heterotrophs use organic compounds; mixotrophs switch between autotrophy and heterotrophy (e.g., Elter, 2006). Green plants and cyanobacteria are photoautotrophs and use light energy to synthesize organic matter from carbon dioxide. Anaerobic anoxygenic photosynthetic bacteria (AAP; Shiba et al., 1979) and bacteria that possess rhodopsin are photoheterotrophs; they use light energy to generate an ion gradient, and use organic compounds as a carbon source.

What are rhodopsins?

Rhodopsins are seven-transmembrane proteins that are widely distributed among animals, fungi, algae, and prokaryotes. Rhodopsins are classified into two groups: microbial and animal rhodopsin (Spudich et al., 2000). Each rhodopsin molecule comprises an opsin protein moiety covalently bound to the light-absorbing chromophore, retinal. Upon activation by light, isomerization of the retinal causes the rhodopsin to undergo a conformation change and begin to function as an ion pump, ion channel, or light sensor. For example, ion-pumping rhodopsins can function as light-driven outward

proton (H^+) pumps, outward sodium ion (Na^+) pumps, inward chloride ion (Cl^-) pumps, or inward H^+ pumps. This ion-pumping function is important because light-driven H^+ pumps drive protons out of cells, which generates an inward-directed proton motive force (*pmf*) that supplies energy for ATP synthesis (Oesterheit and Stoeckenius, 1973), flagella rotation (McCain et al., 1987), and other cellular functions such as the transport of substrates in and out of cells (Gómez-Consarnau et al., 2015).

Discovery of microbial rhodopsins

The first microbial rhodopsin, a light-driven outward H^+ pump called bacteriorhodopsin (BR) (Oesterheit and Stoeckenius, 1971), was discovered in halophilic archaea. Subsequently, a light-driven inward Cl^- pump called halorhodopsin (HR) and a light-sensing rhodopsin called sensory rhodopsin (SR) were also discovered in halophilic archaea (Matsuno-Yagi and Mukobata, 1980; Spudich, 1998). Initially, microbial rhodopsins were considered proteins specific to hypersaline environments and archaea; however, in 2000, a light-driven H^+ pump called proteorhodopsin (PR) was discovered via metagenomic analysis of marine bacterial genomic fragments (Béja et al., 2000).

Recent advances in sequencing technologies have resulted in the discovery of numerous rhodopsin-encoding genes. Marine bacteria have been found to possess an outward Na^+-pumping rhodopsin (NaR) (Inoue et al., 2013), an inward Cl^--pumping rhodopsin (ClR) (Yoshizawa et al., 2014), and an inward H^+-pumping rhodopsin (XeR) (Inoue et al., 2016). In addition, an outward H^+-pumping rhodopsin (xanthorhodopsin [XR]) (Balashov et al., 2005), two cyanobacterial halorhodopsins (MrHR and SyHR)

(Hasemi et al., 2015; Niho et al., 2017), and a group of DTG-motif rhodopsins (Sudo and Yoshizawa, 2016) have been discovered. Genetic analyses indicate that rhodopsin-encoding genes may have spread from organism to organism via lateral gene transfer (LGT) (Mongodin et al., 2005; Frigaard et al., 2006). Figure 1 shows a phylogenetic tree of rhodopsin proteins in bacteria and archaea. Rhodopsins with the same function are present in different phylogenetic clades, and bacteria possessing these rhodopsins inhabit various environments.

Genes encoding ion-pumping and sensory-like rhodopsins have been found in marine dinoflagellates (Slamovits et al., 2011), diatoms, haptophytes, cryptophytes, and chlorophytes (Marchetti et al., 2012; Marchetti et al., 2015). These eukaryotic rhodopsin genes have also been detected in metagenomic and metatranscriptomic analysis (Marchetti et al., 2012). Furthermore, recent metagenomic and genomic analyses indicate that genes encoding rhodopsin-like proteins are found not only in prokaryotes and eukaryotes but also in viruses (Yutin and Koonin, 2012; Philosof and Béjà, 2013). The rhodopsin-like genes in viruses which form specific clades are distinct from other microbial clades that include organisms possessing other rhodopsin-encoding genes. The functions of the viral rhodopsins remain unknown.

Measurement of rhodopsin ion-pump activity

The ion specificity and activity of a particular rhodopsin can be determined by assessing the change in pH of a suspension of the rhodopsin-possessing bacteria. A typical experimental setup for measuring rhodopsin ion-pump activity is shown in Figure 2.

When outward H^+-pumping rhodopsin drives protons out of the cells, the pH of the cell suspension decreases. In contrast, increases in pH indicate that cations other than H^+ are being driven out of the cells, causing H^+ to accumulate inside the cells. An increase in pH can also indicate inward anion pumping. Using ionic compounds, it is possible to determine which ion is being transported. For example, an increase in pH in a cell suspension containing NaCl, KCl, or $MgCl_2$ in the presence of Na_2SO_4 or $NaNO_3$ indicates that the rhodopsin is likely a Cl^--pumping rhodopsin.

The slope or rate of pH change depends on factors such as cell concentration, cellular physiological state, number of rhodopsin molecules per cell, pumping rate of each rhodopsin molecule, light intensity, light wavelength, and temperature. If these conditions are kept constant, the slope of pH change can be used to estimate the pumping activity of the rhodopsin. However, caution must be taken when extrapolating such data to natural environments because such data is obtained by measurement using limited a few strains (Yoshizawa et al., 2012).

Heterologous overexpression in *Escherichia coli* was used to confirm the ion-pump activity of PR (Béja et al., 2000). This approach can also be used when a strain possesses multiple rhodopsin genes (Yoshizawa et al., 2014) because the co-occurrence of pumps with different ion specificities makes it difficult to identify the function of the individual pumps. The activity of PR in native cells was first evaluated by Yoshizawa et al. (2012) and then by other groups (Wang et al., 2012; Feng et al., 2013).

The number of rhodopsin molecules per cell and the photochemical properties of a rhodopsin can be determined by spectroscopic analysis. The photochemical

properties of a rhodopsin indicate its ion affinities and structural changes upon activation by light, and these properties can differ even among rhodopsins that are functionally identical. To quantify the number of rhodopsin molecules per cell, extracted membrane fraction is treated with hydroxylamine, which bleaches the rhodopsin, and the absorption spectrum before and after adding hydroxylamine is measured. One rhodopsin molecule contains one retinal moiety; therefore, the amount of retinal oxime produced by the addition of hydroxylamine is used as an index of the number of rhodopsin molecules (Bogomolni and Spudich, 1982).

Retinal synthesis in rhodopsin-possessing bacterium

Sabehi et al. (2005) showed that co-expression of marine bacterial 15,15' β-carotene dioxygenase encoded by the *blh* gene and PR gene from the bacterial artificial chromosome libraries of the Mediterranean Sea and Red Sea metagenomes led to β-carotene cleavage and subsequent formation of retinal-bound PR. A set of six genes related to the retinal production pathway in rhodopsin-containing bacteria was subsequently reported by Martinez et al. (2007). In cyanobacteria, retinal is produced from β-*apo*-carotenal by apocarotenoid-15,15'-oxygenase, which is encoded by the *diox1* gene (Ruch et al., 2005). Therefore, bacteria need either *blh* or *diox1* for retinal synthesis.

However, recent genomic and metagenomic analyses have shown that some bacteria and archaea have rhodopsin but not the *blh* and *diox1* genes (Dupont et al., 2012; Pinhassi et al., 2016). These prokaryotes are likely numerous in terms of the number of species and the total number of cells in the marine environment. For example, SAR86

belonging to γ-proteobacteria, Chloroflexi CL500-11, and archaeal Marine Group II species are abundant in the natural environment (Morris et al., 2005; Mary et al., 2006; Iverson et al., 2012; Okazaki et al., 2012) and these organisms do not have the *blh* gene, despite possessing rhodopsin genes. It is reported that rhodopsin-encoding and carotenoid synthetic pathway genes have been spread by LGT (Frigaard et al., 2006; Klassen, 2010). Therefore, it is possible that the *blh* gene is also spread by LGT. Whether the function of rhodopsin is prevented in microbial strains lacking the *blh* gene and how retinal in such strains is produced or obtained remain to be determined.

Physiological study of rhodopsins in marine bacteria

The electrochemical gradient generated by ion-pumping rhodopsin provides the energy needed for ATP synthesis and other cellular functions. Therefore, the growth and survival of rhodopsin-possessing marine bacteria under starvation conditions may be promoted by exposure to light (Gómez-Consarnau et al., 2007; Gómez-Consarnau et al., 2010; Kimura et al., 2011; Akram et al., 2013; Feng et al., 2013; Yoshizawa et al., 2014). Although such growth stimulation of rhodopsin-possessing bacteria is yet to be fully confirmed (Giovannoni et al., 2005; Stingl et al., 2007; Gonzalez et al., 2008; Wang et al., 2012), it has been shown that while the growth of *Candidatus* Pelagibacter ubique HTCC1062, a PR-possessing SAR11 strain, is not stimulated by light, this strain does reduced oxygen consumption under light conditions (Steindler et al., 2011).

Transcriptomic and proteomic analyses can be used to examine the effects of light on cellular metabolism. By using *Dokdonia* sp. MED134, which possesses a PR

gene, Kimura et al. (2011) showed that the expression of a PR gene, retinal biosynthetic pathway genes, central metabolism genes, and Na^+-translocating NADH-quinone oxidoreductase (NQR)-linked respiratory chain genes is higher under light conditions under light than the dark. Because the expression of the Na^+/alanine symporter and Na^+/phosphate symporter genes under light is more than twice that in the dark, Kimura et al. (2011) hypothesized that it is important in terms of growth for bacteria to import these solutes by exporting Na^+ via NQR. Palovaara et al. (2014) showed that gene expression related to anaplerotic carbon fixation and glyoxylate shunt in *Dokdonia* sp. MED134 is much higher under light than in the dark in an oligotrophic medium. Additionally, Feng et al. (2015) reported that psychrophilic bacterium *Psychroflexus torquis* ATCC 700755[T] possesses more PR under high salinity than under low salinity. Gómez-Consarnau et al. (2015) used transcriptomic analysis to find genes functionally relevant to PR and found that the expression of TonB-dependent vitamin-B_1 transporter and PR genes was higher under light conditions than under dark conditions. Because TonB-dependent transporter needs a *pmf* to import a substance, Gómez-Consarnau et al. (2015) hypothesized that the *pmf* generated by PR is used for vitamin-B_1 uptake. Kwon et al. (2013) determined the relative expression of the NaR genes, and showed that the gene expression was higher under high salinity than under low salinity, suggesting that it is important for the bacterium possessing NaR gene to use sodium gradient not only *pmf* the in cells.

Ecological implications of rhodopsins in marine bacteria

Many bacterial communities in various aquatic environments have been analyzed by using sequencing technologies. It is estimated that more than half of bacteria inhabiting marine surface layers possess PR (Fuhrman et al., 2008). Furthermore, species in SAR11 and SAR86, the two dominant bacterial clusters in marine environments, have the PR gene (Morris et al., 2002; Morris et al., 2005).

Dokdonia sp. MED134, a PR-possessing bacterium, produces approximately 24% to 31% of its total carbon demand via light-induced anaplerotic carbon fixation (Palovaara et al., 2014). In the strain MED134, expression of a PR gene and a gene related to the glyoxylate shunt is induced by light. The notion of this induction is supported by metagenomic data showing that it occurs in the natural environment (Palovaara et al., 2014). Kumagai et al. (2018), however, showed that some PR-possessing strains belonging to class *Flavobacteriia* do not possess a complete anaplerotic carbon-fixation pathway or glyoxylate shunt–related genes, indicating that anaplerotic carbon fixation is not a common characteristic of PR-possessing bacteria. These strains have the *bicA* gene, which encodes a bicarbonate transporter, and a gene that encodes carbonic anhydrase. Although further quantitative investigations are needed, carbon fixation by rhodopsin-possessing bacteria may be ecologically important, especially with respect to carbon cycles in aquatic environments.

Several studies have quantitatively estimated the significance of PR, especially in comparison with photosynthetic activity. According to Yoshizawa et al. (2012), one *Flavobacteriia* cell contains $52{,}200 \pm 30{,}700$ PR molecules, which exclude 2×10^6 H⁺

per minute. This means that 0.4 fg of ATP per minute (3.4×10^{-14} kJ cell^{-1} day^{-1}) can be generated with the *pmf* produced by the activity of the PR molecules. Kirchman and Hanson (2013), assuming that one *Flavobacteriia* cell contains 10,000 PR molecules, estimated the amount of energy produced as 6×10^{-14} kJ cell^{-1} day^{-1}. Gómez-Consarnau et al. (2017) estimated a value of 1×10^{-13} to 2×10^{-12} kJ cell^{-1} day^{-1} by quantifying the number of retinal molecules bound to rhodopsin molecules in environmental samples from Mediterranean Sea and the Eastern Atlantic Ocean. Gómez-Consarnau et al. (2017) also calculated the contribution of photosynthesis to energy production in a marine environment and reported a value of 2×10^{-12} to 1.8×10^{-11} kJ cell^{-1} day^{-1}. According to that study, the number of heterotrophic bacterial cells was approximately 10 times that of chlorophyll *a*–containing cells; therefore, assuming that the number of PR-possessing bacteria is half the total number of heterotrophic bacterial cells, the energy amount produced may be equivalent between rhodopsin-based phototrophy and chlorophyll-based photosynthesis.

Current issues in rhodopsin research

Research on microbial rhodopsins has progressed rapidly due to advances in genetic, physiological, biochemical, and physicochemical analysis of environmental samples and cultured cells. For example, we are now able to generate metagenomic data from environmental samples, sequence particular genes of interest, and conduct whole genomic, phylogenetic, and transcriptomic analyses (Kimura et al., 2011; Yoshizawa et al., 2014; Gómez-Consarnau et al., 2015). Physiological techniques are mainly used with

cells cultured in the laboratory to clarify a physiological implication possessing PR and a condition expressing it (Gómez-Consarnau et al., 2010; Akram et al., 2013; Palovaara et al., 2014). Quantitative analysis of cellular components such as retinal is an example of a biochemical technique to discuss how many rhodopsin molecules function in the cells (Yoshizawa et al., 2012; Gómez-Consarnau et al., 2017). Finally, recent developments in physicochemical techniques have made it possible to clarify the 3D structure of rhodopsins (Kato et al., 2015; Hosaka et al., 2016).

There are many issues that currently need to be addressed in the field of microbial rhodopsins. Below they are categorized into environmental, physiological and biochemical, and physicochemical issues, although it should be noted that some of these issues belong to multiple categories.

Environmental issues:

・Clarification of the distribution of rhodopsins in different organisms and environments

・Quantification of retinal and rhodopsins in different organisms and environments

・Elucidation of the mechanisms of retinal synthesis and ecological implications of rhodopsin-possessing microbes that lack the *blh* gene

・Quantification of the solar energy captured in aquatic environments via rhodopsins

Physiological and biochemical issues:

・Clarification of the genetic diversity of rhodopsins

・Elucidation of the functions of rhodopsins, i.e., pump, channel, or sensor

・Identification of the ions transferred by rhodopsin pumps and channels

・Quantification of the pumping activity of rhodopsins

・Clarification of the contribution of rhodopsins to cellular energetics

・Clarification of the contribution of rhodopsins to cellular growth

・Clarification of the wavelength dependency of rhodopsins

・Clarification of the function of rhodopsins in cells lacking *blh* or other retinal synthesis
  genes

・Clarification of the physiological characteristics of rhodopsin-possessing cells

・Identification of the mechanisms underlying the activities of multiple rhodopsins in a
  single cell

・Clarification of the implications of having different types of rhodopsins in one cell

・Phylogenetic analysis of rhodopsin genes

Physicochemical issues:

・Clarification of the structures of rhodopsins

・Clarification of photocycle differences among rhodopsins that transport same ion

・Clarification of the ion-binding sites of rhodopsins

      Some of these issues, especially those pertaining to the ecological and phylogenetic distribution and diversity of rhodopsin genes in the environment, have already been examined through genomic and metagenomic analyses (e.g. Pinhassi et al., 2016; Gómez-Consarnau et al., 2017) and the distributions of different rhodopsins have been determined (Vollmers et al., 2013). However, culture-dependent studies using PR-possessing strains or strains with various "rhodopsin usages" are necessary to resolve the

physiological issues described above, with "rhodopsin usage" defined as the combination of the types and amounts of rhodopsins that are possessed, the process through which retinal is obtained, the conditions under which rhodopsin genes are expressed, and how rhodopsin function contributes to cellular energetics and growth. Thus, culture-dependent physiological studies will also help resolve the biochemical, physicochemical, and ecological issues listed above.

Objectives of this study

Genetic analyses have shown that rhodopsin genes are present in the environment and that rhodopsins may have novel functions (e.g. Inoue et al., 2013; Yoshizawa et al., 2014; Hasemi et al., 2015; Inoue et al., 2016). Genetic analyses have also been used to clarify the phylogenetic relationships among rhodopsin genes and infer their evolutionary development (Pinhassi et al., 2016). However, it is necessary to use cultured cells to confirm intracellular "rhodopsin usage". Using cultured cells will provide important information on the conditions required for rhodopsin gene expression and the effects of rhodopsins on cell physiology. Additionally, there are relatively few studies in the current literature on rhodopsin-possessing cells, probably because it is difficult to isolate cells until 2012, at the early stage of culture-depend rhodopsin research in marine bacteria (Yoshizawa et al., 2012). In this doctoral study, my first aim was to identify novel functions of rhodopsin. My second aim was to determine the functions of rhodopsin in cells lacking the *blh* gene or other retinal synthesis genes. My third aim was to examine the ion-pumping functions in cells containing three different rhodopsins.

Chapter 1 describes my genomic analysis of the marine bacterium *Rubricoccus marinus* SG-29^T, which possesses a rhodopsin gene that is separated from a closely related cluster by a different motif sequence and encodes a rhodopsin with an unknown function. In addition to conducting phylogenetic analyses of the genes encoding the rhodopsin and those involved in the retinal biosynthetic pathway, I determined the ion specificity of this rhodopsin by using an *E. coli* overexpression system that has been widely used in functional analyses of rhodopsins.

In Chapter 2, I describe my examination of the ultra-small bacterium *Aurantimicrobium minutum* KNC^T, which possesses a rhodopsin but lacks the *blh* gene. This gene encodes β-carotene 15,15'-dioxygenase, which splits β-carotene to produce retinal. Retinal is essential for the function of rhodopsin because without this chromophore rhodopsin cannot capture light. Although numerous bacteria, such as SAR86, lack the *blh* gene but possess a rhodopsin gene, it is unclear how SAR86 and other strains lacking *blh* obtain retinal. To determine whether *A. minutum* KNC^T obtains retinal from the environment or produces it itself, I examined the activity of the rhodopsin in native *A. minutum* KNC^T cells under light and dark conditions by using pigment analysis and transcriptome analyses.

In Chapter 3, I describe my examination of the bacterium *Nonlabens marinus* S1-08^T, which possesses genes encoding the rhodopsins PR, NaR, and ClR. To determine how these three rhodopsins function in this bacterium and how salinity affects bacterial growth and the expression and functions of these rhodopsins, I performed a growth experiment and transcriptome analysis.
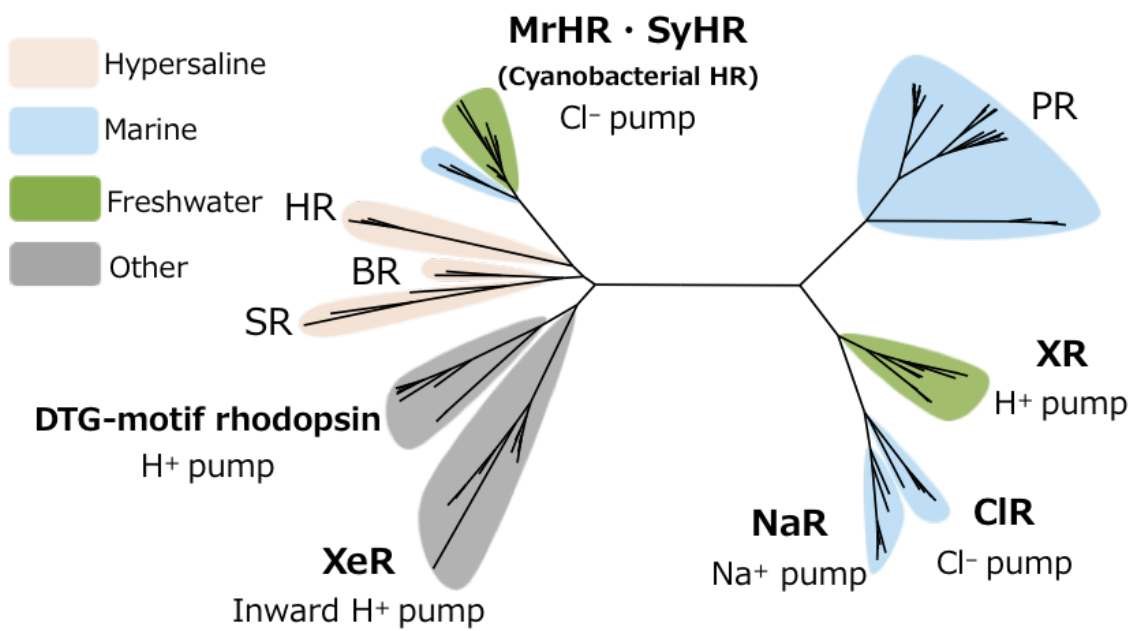
Fig. 1. Unrooted phylogenetic tree of microbial rhodopsin genes. HR: halorhodopsin; BR: bacteriorhodopsin; SR: sensory rhodopsin; XeR: xenorhodopsin; NaR: Na^+-pumping rhodopsin; ClR: Cl^--pumping rhodopsin; XR: xanthorhodopsin; PR: proteorhodopsin; MrHR: *Mastigocladopsis repens* halorhodopsin; SyHR: *Synechocystis* halorhodopsin
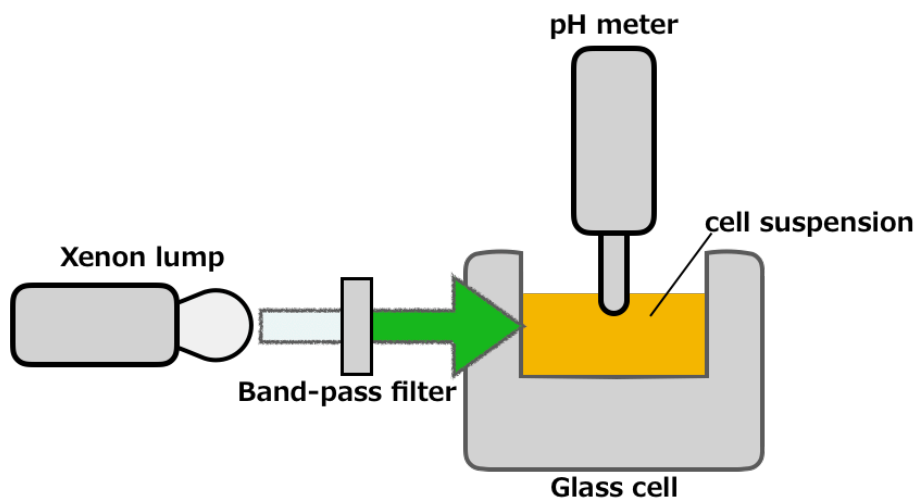

Fig. 2. Schematic of instrument for measuring the ion-pumping activity of microbial rhodopsins. A glass cell containing a microbial cell suspension is connected to a thermostat, and the temperature is kept at 4°C. In the glass cell, the cell suspension is mixed using a stirrer bar. A band-pass filter is positioned after the xenon light source.

Chapter 1. Ion specificity and phylogenetic analysis of rhodopsin in *Rubricoccus marinus* SG-29^T.

Introduction

Numerous rhodopsins, such as outward Na$^+$ pumping rhodopsin (NaR) (Inoue et al., 2013), inward Cl$^-$ pumping rhodopsin (ClR) (Yoshizawa et al., 2014), and inward H$^+$ pumping rhodopsin (XeR) (Inoue et al., 2016), have been discovered in marine bacteria. This suggests that other rhodopsins with novel functions or belonging to novel clusters may exist among marine bacteria.

The Family *Rhodothermaceae* belongs to the class *Cytophagia*, phylum Bacteroidetes, and includes seven genera: (*Longimonas* (Xia et al., 2015), *Longibacter* (Xia et al., 2016), *Rhodothermus* (Alfreddson et al., 1988), *Rubricoccus* (Park et al., 2011), *Rubrivirga* (Park et al., 2013a), *Salinibacter* (Antón et al., 2002), *Salisaeta* (Vaisman et al., 2009). These groups are distributed in diverse environments such as hydrothermal vents, hypersaline environments, and oceans. Previous studies have shown that *Salinibacter ruber* M31^T possesses multiple rhodopsin genes including xanthorhodopsin (XR), HR, and sensory rhodopsin (SR) (Mongodin et al., 2005). Additionally, they have shown that *S. ruber* contains not only the *HR* and *SR* genes, but also many other genes necessary for adaptation to highly saline conditions; these genes were acquired by *S. ruber* from halophilic archaea by lateral gene transfer (LGT). Both of these halophilic prokaryotes inhabit and predominate saline environments. Members of the genus

Salinibacter comprise 5 – 25 % (determined using fluorescence in-situ hybridization [Antón et al., 2000]) and 7 – 9 % (determined using metagenomic analysis [Ghai et al., 2011]) of prokaryotes in hypersaline environments.

Although *Salinibacter* and *Salisaeta* have rhodopsin genes, while *Rhodothermus* does not (Mongodin et al., 2005), it is still unknown whether *Rubricoccus* and *Rubrivirga* possess rhodopsin genes. Therefore, I next sought answers to the following questions: 1. Do these strains contain rhodopsin genes? 2. If they do, what types of ion pumps are involved? 3. Were any of these genes acquired via LGT, such as in the case of *S. ruber* and other genera belonging to family *Rhodothermaceae*.

Here, I analyzed three genomes in the genera *Rubricoccus* and *Rubrivirga*, and determined ion specificity of rhodopsins found in these strains. I also examined the phylogenetic relationship of rhodopsin and genes involved in the retinal biosynthetic pathway in marine *Rhodothermaceae* and halophilic archaea.

Materials and Methods

Strains and genomic sequencing

Rubricoccus marinus SG-29^T was isolated from surface seawater (30° 40′ N, 138° 00′ E; depth 50 m) during the KT-09-11 cruise of R/V *Tansei Maru* (Atmosphere and Ocean Research Institute, The University of Tokyo and Japan Agency for Marine-Earth Science and Technology [JAMSTEC]) from 2 to 6th, July 2009. (Park et al., 2011). *Rubrivirga marina* SAORIC-28^T was isolated from the depth of 3000 m in western North Pacific Ocean (32° 00′ N, 138° 13′ E) during the KT-10-12 cruise of R/V *Tansei maru* on

3 July 2010 (Park et al., 2013). *Rubrivirga profundi* SAORIC-476[T] was isolated from the depth of 3000 m in western North Pacific Ocean (32° 00′ N, 140° 00′ E) during the MR-11-05 cruise of R/V *Mirai* (JAMSTEC) in May 2011 (Song et al., 2016). Although these *Rubrivirga* strains were isolated from deep-sea water, genus *Rubrivirga* is phylogenetically close to the genus *Rubricoccus*. Therefore, it was used for comparative genomic analysis.

The genomic DNA of these three strains was extracted using phenol-chloroform and ethanol precipitation as described previously (Mamur, 1961). For strains SG-29[T] and SAORIC-28[T], an 800-bp paired-end library and an 8-kb mate-pair library were prepared using the Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA) and Nextera mate-pair sample preparation kit (Illumina), respectively. For strain SAORIC-476[T], KAPA HyperPlus Kit (Kapa Biosystems, Boston, MA, USA) was used for preparation of the library. Each 300-bp end of libraries of strains SG-29[T], SAORIC-28[T] and SAORIC-476[T] were sequenced on an MiSeq (Illumina) using MiSeq Reagent kit version 3 (Illumina). Genome sequencing was performed at Laboratory of Bacteriology, Department of Basic Medicine, Faculty of Medical Sciences, Kyushu University.

Phylogenetic analysis of *R. marinus* SG-29[T] and closely related species

Multiple alignments of sequences were performed using CLUSTALW in MEGA 6.0 (Tamura et al., 2013). The maximum-likelihood (ML) (Felsenstein et al., 1981) phylogenetic tree was constructed using bootstrap values based on 1000

replications; evolutionary analyses were conducted using MEGA 6.0. Bootstrap values >80% are indicated as a percentage of replicates determined. The nucleotide sequence of 16S rRNA and amino acid sequences of the genes encoding rhodopsin, 15,15'-β-carotene dioxygenase (*blh*), and lycopene cyclase (*crtY* and *crtYcd*) were collected from the NCBI database (http://www.ncbi.nlm.nih.gov/) and RAST annotated sequences (http://rast.nmpdr.org/rast.cgi) for genera *Rubricoccus* and *Rubrivirga*. Additionally, 16S rRNA sequences of the out-group of the family *Rhodothermaceae* were obtained from Park et al. (2011), Park et al. (2013), and Song et al. (2016).

Gene preparation and function analysis of rhodopsin from *R. marinus* SG-29[T].

The DNA fragment encoding the rhodopsin present in *R. marinus* SG-29[T] was chemically synthesized by Eurofins Genomics (Tokyo, Japan) with codon optimization for *E. coli*. This gene fragment was inserted into the NdeI and XhoI sites of pET21a vectors (Novagen, Madison, WI, USA); the plasmids encoded hexahistidines at the C terminus. These vectors were transformed into *E. coli* strain C41 (DE3) (Lucigen, Middleton, WI, USA). *E. coli* cells containing the plasmid were incubated at 37°C in Luria Bertani (LB) medium [Bacto Tryptone, Bacto Peptone, and Bacto Yeast extract were purchased from BD Bioscience (Tokyo Japan). NaCl was purchased from Guaranteed Reagent (WAKO, Osaka Japan).] (Table 1-1) supplemented with ampicillin (at the final concentration of 100 μg/mL). The cells possessing the rhodopsin gene were grown at 37°C in 200 mL of 2X YT medium (Table 1-1) supplemented with ampicillin (at the final concentration of 100 μg/mL) and induced at an OD_{660} of 0.4 – 0.6 with 0.2

mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 10 μM all-*trans*-retinal. The rhodopsin-expressing cells were collected by centrifugation (8000 × g for 3 min), washed three times with 100 mM NaCl, and then re-suspended in the solvent for measurements.

A 300-W xenon lump (MAX-303, Asahi Spectra, Tokyo, Japan) was used as light source. Six milliliters of each cell suspension were first incubated in the dark at 4 ºC and then radiated through a 520 ± 10 nm band pass filter (MX0520, Asahi Spectra, Tokyo, Japan) for 3 minutes. pH was measured using a pH meter (LAQUA F-72, HORIBA, Kyoto, Japan). Measurements were repeated under the same conditions after the addition of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP, used as the final concentration of 30 μM). All measurements were performed at 4°C. Different solutes (100 mM NaCl, KCl, $MgCl_2$, NaBr, NaI, $NaNO_3$, Na_2SO_4) were used for ion selectivity analysis of *Rubricoccus marinus* rhodopsin (RmR).

Evaluating the LGT potential of strains belonging to the family *Rhodothermaceae* and of halophilic archaea

To compare gene synteny around the Cl⁻ pumping rhodopsin gene and *blh* gene, the genomes of *Halobacterium salinarum* R1, *S. ruber* M31^T, *Synechocystis* sp. PCC 7509, *Mastigocladopsis repens* PCC 10914, *Salisaeta longa* DSM 21114^T, and *Rhodothermus marinus* DSM 4252^T were collected from the NCBI RefSeq database. Ortholog clustering of the coding sequences (CDSs) of each genome was performed using eggNOG-mapper (Huerta-Cepas et al., 2015) and bactNOG dataset in the eggNOG database version 4.5 (Huerta-Cepas et al., 2017). Operon structure was visualized based

on each eggNOG ortholog cluster using R software kindly provided by Dr. Yohei Kumagai, AORI, The University of Tokyo.

Basic Local Alignment Search Tool (BLAST) analysis was performed on the genomes of *H. salinarum* R1 to evaluate whether LGT occurred in this strain. Genes below e-value 1e-10 were counted, and a threshold for sequence identity >50% was established. I obtained the genomes of the family *Rhodothermaceae*, *Cytophaga hutchinsonii* ATCC 33406[T] (which is the type species of class *Cytophagia)*, *Indibacter alkaliphilus* LW1[T], *Spirosoma linguale* DSM 74[T], *N. marinus* S1-08[T], and *Flavobacterium aquatile* LMG 4008[T] *I. alkaliphilus* and *S. linguale* belong to the class *Cytophagia* and contain a rhodopsin gene; therefore, the genomic data of these organisms were used for this assay. *N. marinus* is a marine Flavobacterium containing the ClR gene, and *F. aquatile* is a type species of the class *Flavobacteriia*; therefore, the genomic data of these organisms were also used. Dr. Yohei Kumagai performed these data analyses.

Results and Discussion

Genomic analysis of *R. marinus* SG-29[T], *R. marina* SAORIC-28[T], and *R. profundi* SAORIC-476[T]

Genomic analyses of *R. marinus* SG-29[T], *R. marina* SAORIC-28[T], and *R. profundi* SAORIC-476[T] revealed that these strains have 4.43-Mbp, 4.98-Mbp, and 4.48-Mbp chromosomes that encode 3847, 4267, and 3842 CDS, respectively (Table 1-2). Among the genomes available for class *Cytophagia* in NCBI database, *R. marinus* SG-29[T], *R. marina* SAORIC-28[T], and *R. profundi* SAORIC-476[T] contained the G+C content

of 69.0, 72.5, and 71.3%, respectively. Genomic analyses showed the presence of five rhodopsin genes. The genome of *R. marinus* SG-29^T encodes two different rhodopsin genes: RmR1 [WP_094549673] and RmR2 [WP_094550238]. The genome of *R. marina* SAORIC-28^T encodes three rhodopsin genes: R28R1 (*Rubrivirga marina* SAORIC-28^T rhodopsin) [WP_095509440], R28R2 [WP_095509924], and R28R3 [WP_095512583]). No rhodopsin genes were found in the genome of *R. profundi* SAORIC-476^T (Fig. 1-1).

Phylogenetic and functional analysis of new rhodopsin genes

Figs. 1-2 and 1-3 show a phylogenetic tree of the amino acid sequence of rhodopsin; table 1-3 shows residues in the motif sequence of rhodopsins. RmR1 and R28R1 were included in the XeR cluster, and RmR1 has previously been identified as a light-driven inward H$^+$ pumping rhodopsin by functional analysis (Inoue et al., 2018). Therefore, RmR1 and R28R1 were named RmXeR and R28XeR, respectively. RmR2, R28R2, and R28R3 formed a new cluster closely related to cyanobacterial halorhodopsin (e.g., MrHR and SyHR) and HR in halophiles. Although the phylogenetic position and identity of the RmR2 cluster were close to those of the SyHR cluster, RmR2 had the same TSA motif sequence as that of HR (Table 1-3). Conversely, cyanobacterial HR contained TSD, TSV, or TSL motif sequences. These motifs indicate amino-acid residues 85, 89, and 96 in BR numbering.

A light-induced alkalization was observed in 100 mM NaCl; this light-induced alkalization was not abolished by the addition of CCCP (Fig. 1-4A). CCCP is a protonophore; therefore, I determined that this alkalization was caused by passive proton

influx stemming from negative membrane potential, which was created by outward cation or inward anion translocation. Next, I performed pump activity measurements in different salt solutions to identify ion selectivity of RmR2 (Fig. 1-4B). In 100 mM NaI and Na_2SO_4, the signal was nearly undetectable, suggesting that RmHR did not transport Na^+. In contrast, strong signals were detected in 100 mM KCl, NaBr, and $MgCl_2$, and a slight pH change was observed in 100 mM $NaNO_3$. These anion-dependent transport properties are very similar to those of HRs in halophilic archaea, confirming that RmR2 functions as a light-driven inward anion (Cl^-, Br^-, and NO_3^-) pump; therefore, RmR2 was named RmHR. Because light-induced pH changes in R28R2 were smaller than those in RmHR, R28R2 was named R28HR1. Although light-induced pH changes in R28R3 were not observed in this study, R28R3 was named R28HR2 because this gene was closely related to the R28HR1 gene.

Phylogenetic analyses of genes involved in the retinal biosynthesis pathway

Functional analyses revealed that RmHR was a light-driven inward Cl^- pumping rhodopsin similar to HR in halophilic archaea. Phylogenetic analysis indicated that RmHR was more closely related to HR than to ClR of marine flavobacteria (Fig. 1-2). To further examine the evolutionary history of *Rubricoccus* RmHR, which is closely related to the rhodopsin gene in halophilic archaea, I performed phylogenetic analyses of retinal biosynthesis pathway genes (*crtY* and *blh*) related to the function of rhodopsin. The *crtY* and *blh* genes encode enzymes that catalyze terminal cyclization of all-*trans*-lycopene to all-*trans*-β-carotene and cleavage of β-carotene, producing two molecules of

all-*trans*-retinal, respectively.

The phylogenetic trees of *crtY* and *blh* genes are shown in Figs. 1-5 and 1-6, respectively. Because the strain SAORIC-476[T] does not contain rhodopsin or *blh* genes, only the *crtY* gene was used. The phylogenetic tree of *crtY* shows that three clusters, Bacteroidetes *crtY*, Proteobacteria *crtY* and *crtYcd*, were formed (Fig. 1-5). The genes of the strains SG-29[T], SAORIC-28[T], and SAORIC-476[T] fell into the *crtYcd* cluster. The carotenoid cyclase is diversified by LGT or gene duplication (Klassen, 2010); several Actinobacteria (Krubasik et al., 2000), archaea (Hemmi et al., 2003), and Bacteroidetes (Klassen 2010) contain *crtYcd*. Although the similarity between *crtYcd* and *crtY* genes is not high, the *crtYcd* gene was found in a carotenoid biosynthesis gene cluster; similar to the function of the *crtY* gene, *crtYcd* encodes an enzyme that also functions as lycopene cyclase. Halophilic archaea, genera *Natronomonas*, *Halobacterium* and *Haloarcula*, and halophilic bacteria *S. ruber* M31[T] and *S. longa* DSM 21114[T] possess *crtYcd*, but not the *crtY*, gene. Although rhodopsin-possessing Bacteroidetes species, shown in Fig. 1-3, contain the *crtY* gene, lycopene cyclase genes of the genera *Rubricoccus* and *Rubrivirga* were also closely related to the *crtYcd* cluster of halophilic archaea.

In the phylogenetic tree of the *blh* gene, *blh* genes of the strains SG-29[T] and SAORIC-28[T] fell into the lineage containing halophilic archaea and bacteria (Fig. 1-6). Although the family *Rhodothermaceae* belongs to phylum *Bacteroidetes*, *blh* and lycopene cyclase genes showed phylogenetic lineage that was distinct from that of Bacteroidetes (Table 1-4). This indicates that a common ancestor received *blh* and *crtY* genes, as well as rhodopsin genes, by LGT.

Visualizing genomic flanking regions and evaluating potential of LGT

Next, I compared the genomic flanking regions of the rhodopsin and *blh* genes in the genera *Rubricoccus* and *Rubrivirga* with those of closely related strains to determine whether *Rubricoccus* and *Rubrivirga* acquired the rhodopsin and *blh* genes via LGT from halophilic archaea (Fig. 1-7). The analysis showed that there was scant similarity around the rhodopsin genes (Fig. 1-7A), suggesting that only the Cl⁻ pumping rhodopsin genes were acquired by LGT. Although there was a conserved region between *S. ruber* M31^T and *S. longa* DSM 21114^T, analysis of the *blh* gene also indicated no notable similarities among these strains. Interestingly, multiple conserved genes were found in the flanking regions of both SAORIC-28^T and *R. profundi* SAORIC-476^T; *R. profundi* SAORIC-476^T lacks the rhodopsin and *blh* genes (Fig. 1-7B). This suggests that although these two strains are closely related species, only the *blh* gene was deleted in SAORIC-476^T, or that SAORIC-28^T acquired the *blh* gene.

Fig. 1-8, generated using protein BLAST, shows the number of potential genes transferred from the halophilic archaea *H. salinarum*. Among the type species of the classes *Flavobacteriia* and *Cytophagia* and other strains belonging to the family *Rhodothermaceae*, *S. ruber* M31^T received the maximal number of genes from halophilic archaea via LGT (Mongodin et al., 2005). The number of LGT-transferred genes was lower in the strains SG-29^T, SAORIC-28^T, and SAORIC-476^T than that in *S. ruber* M31^T, but approximately 2- to 4-times higher than those in the type species *N. marinus* S1-08^T, *I. alkaliphilus*, and *S. linguale*. This suggests that a common ancestor of this family inhabited a hypersaline environment, and that several genes in the strains SG-29^T,

SAORIC-28^T, and SAORIC-476^T were derived from *H. salinarum*. The direction of LGT among *R. marinus* SG-29^T, *R. marina* SAORIC-28^T, and halophilic archaea could not be determined using these data. Further analysis, such as comparative genomic analysis and ancestor estimation by phylogenetic analysis, (Mongodin et al., 2005) need to be conducted to answer this question.

Conclusion

In this chapter, I investigated the function and phylogenetic characteristics of rhodopsin RmHR, present in the genus *Rubricoccus*. I also examined with the function and phylogenetic characteristics of the genes *blh*, 15,15' β-carotene dioxygenase, *crtY*, and lycopene cyclase. RmHR was identified as a light-driven inward Cl⁻ pump and its gene was closely related to the *HR* genes of halophilic bacteria and archaea. The *blh* and lycopene cyclase genes of the genera *Rubricoccus* and *Rubrivirga* were closely related to those of halophilic archaea.

This is the first study reporting on the inward Cl⁻ pump that does not belong to ClR cluster in marine bacteria. These results show that lateral gene transfer of rhodopsin and retinal synthesis genes may have impacted the distribution and diversification of these genes during the course of evolution. A considerable number of prokaryotic species possess rhodopsin genes but lack the *blh* gene. Therefore, I focused on the function of rhodopsin and possible source of retinal in such strains. This is described in Chapter 2.

Table 1-1. Chemical component of LB and 2X YT medium

	LB	2X YT
Bacto Tryptone	10 g	16 g
Bacto Yeast extract	5 g	10 g
NaCl	5 g	5 g
DW	1000 mL	1000 mL

pH 7.0 ~ 7.5 in LB medium and pH 7.0 in 2xYT medium adjusted with 1N NaOH.

Table 1-2. General genomic characteristics of *Rubricoccus marinus* SG-29^T, *Rubrivirga marina* SAORIC-28^T and *Rubrivirga profundi* SAORIC-476^T.

Name	Isolation source (depth)	Genome size (Mbp)	Cove rage	G+C content (%mol)	CDS number	N$_{50}$ (bp)	Scaffold	Putative Rhodopsin gene
Rubricoccus marinus SG-29^T	Euphotic Sea water (50 m)	4.43	58	69.0	3,847	167,811	15	2
Rubrivirga marina SAORIC-28^T	Deep sea water (3000 m)	4.98	81	72.5	4,267	326,174	11	3
Rubrivirga profundi SAORIC-476^T	Deep sea water (3000 m)	4.48	86	71.3	3,842	447,061	19	0

CDS: coding sequence;

N$_{50}$: Defined as the shortest sequence length at 50% of a genome;

Scaffold was created by bridging the gaps between each contig.

Table 1-3. Residues in the motifs of ion pumps.

			Residue number in BR (above) and PR (below)											
			85	86	87	88	89	90	91	92	93	94	95	96
Rhodopsin type	Strain	Motif	97	98	99	100	101	102	103	104	105	106	107	108
BR	*Halobacterium salinarum* R1	DTD	D	W	L	F	T	L	P	L	L	L	L	D
PR	*Ca.* Pelagibacter ubique HTCC 1062	DTE	D	W	L	I	T	V	P	L	L	M	L	E
ClR	*Nonlabens marinus* S1-08^{T}	NTQ	N	W	M	A	T	I	P	C	L	L	L	Q
SyHR	*Synechocystis* sp. PCC 7509	TSD	T	W	F	L	S	T	P	L	L	L	L	D
HR	*Salinibacter ruber* M31^{T}	TSA	T	W	A	F	S	T	P	F	I	L	L	A
HR	*Halobacterium salinarum* R1	TSA	T	W	A	L	S	T	P	M	I	L	L	A
RmXeR (RmR1)	*Rubricoccus marinus* SG-29^{T}	DTA	D	W	V	V	T	T	P	L	L	L	T	A
RmHR (RmR2)	*Rubricoccus marinus* SG-29^{T}	TSA	T	W	F	T	S	T	P	L	L	L	L	A
R28HR2 (R28R3)	*Rubrivirga marina* SAORIC-28^{T}	TTD	T	W	F	L	T	T	P	L	L	L	L	D

BR: Bacteriorhodopsin; PR: Proteorhodopsin

Table 1-4. Phylogenetic characteristics of carotenoid biosynthetic pathway genes.

	Family *Rhodothermaceae*				Halophilic archaea *1	Other Bacteroidetes *2
	Rubricoccus	*Rubrivirga*	*Salinibacter*	*Salisaeta*		
Habitat	Marine	Marine	Hypersaline	Hypersaline	Hypersaline	Several environment
blh	Haliphilic archaea cluster	Haliphilic archaea cluster	Haliphilic archaea cluster	Haliphilic archaea cluster	Haliphilic archaea cluster	*Flavobacteriia* or *Cytophagia* cluster
lycopene cyalase	*crtYcd*	*crtYcd*	*crtYcd*	*crtYcd*	*crtYcd*	*crtY*

*1: Genera *Natronomonas*, *Halobacterium*, and *Haloarcula*; *2: Rhodopsin-possessing Bacteroidetes described in Fig. 1-3.
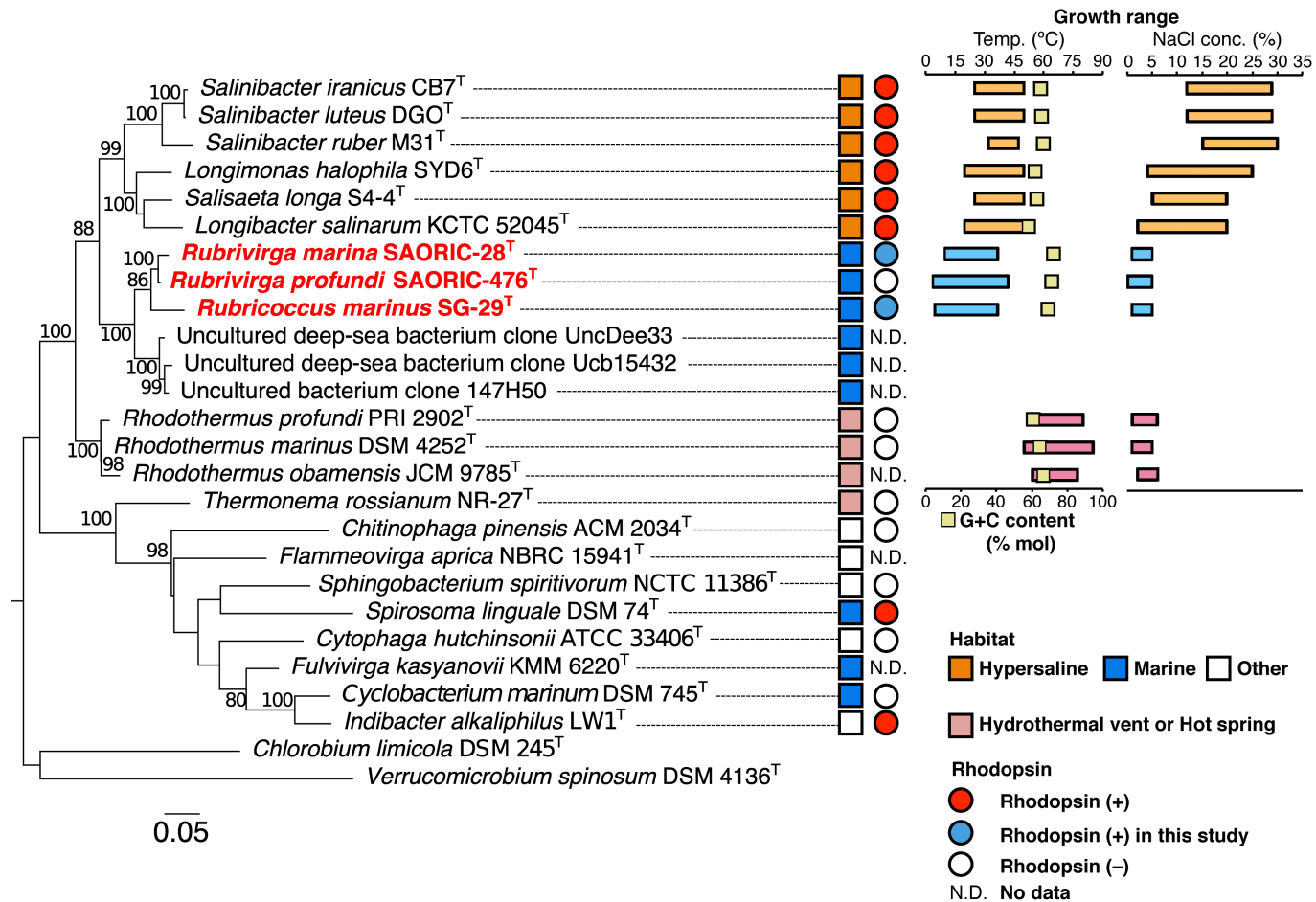
Fig. 1-1. Phylogenetic tree, growth range, and GC content of family *Rhodothermaceae* and related members determined using 16S rRNA gene sequences. Maximum likelihood phylogenetic tree of 16S rRNA shows the taxonomic position of strains SG-29[T], SAORIC-28[T],

and SAORIC-476^T among related species of the family *Rhodothermaceae*. The growth range at different temperatures and NaCl

concentrations, as well as G+C contents, are shown as gray bar and small black circle. The red and open circles indicate strains possessing

the rhodopsin gene or no rhodopsin gene, respectively. ND: No data.
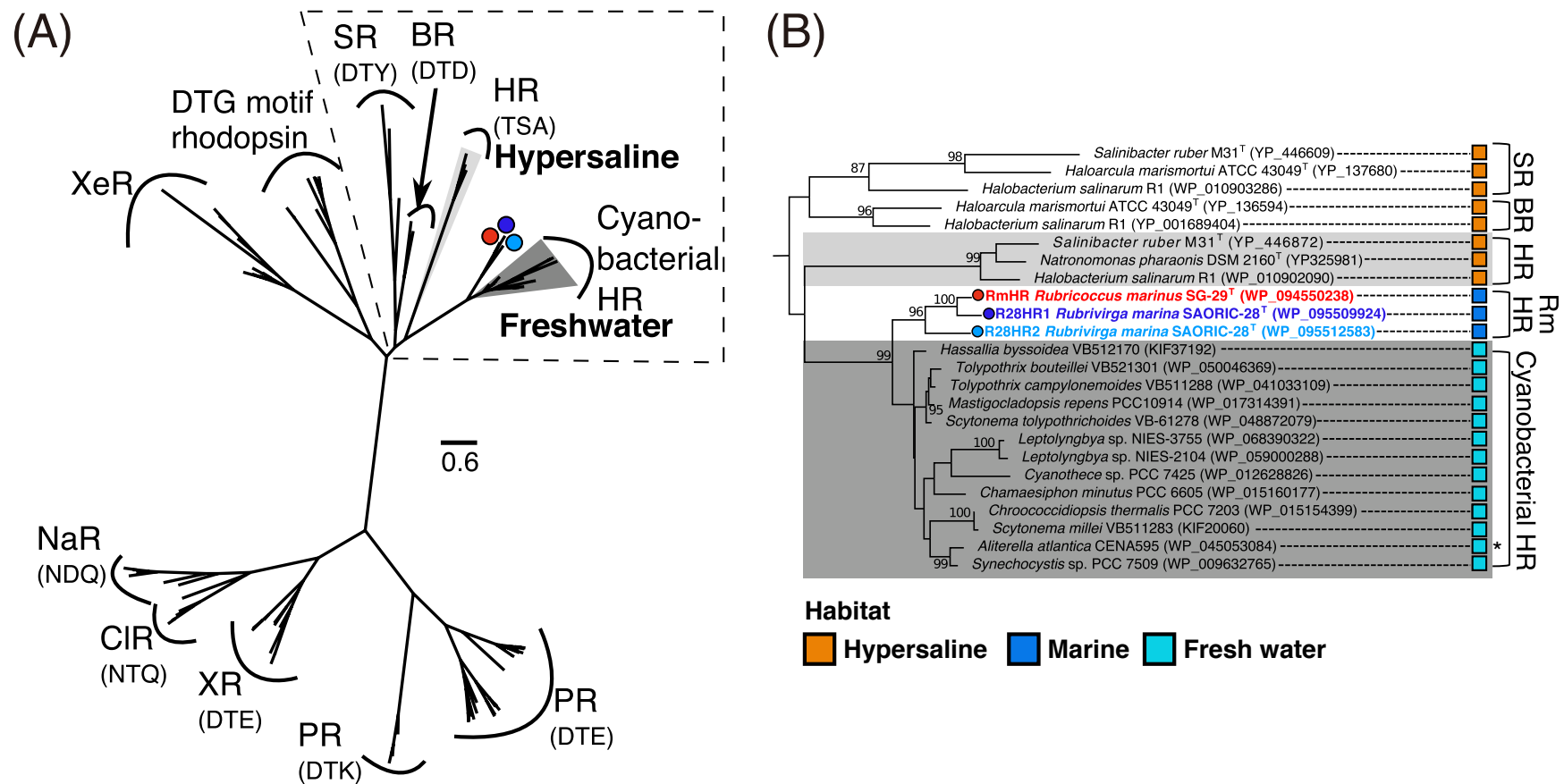
Fig. 1-2. Phylogenetic tree of microbial rhodopsin genes. (A) Unrooted maximum-likelihood phylogenetic tree of whole microbial rhodopsin genes. Dark gray indicates clade composed of freshwater cyanobacterial rhodopsins, and light gray indicates halorhodopsin. Open

circle and closed gray circle indicate the positions of strains SG-29[T] and SAORIC-28[T], respectively. NaR: Sodium ion pump rhodopsin; ClR: chloride ion pump rhodopsin; XLR: xanthorhodopsin-like rhodopsin; PR: proteorhodopsin; XeR: xenorhodopsin; SR: sensory rhodopsin; BR: bacteriorhodopsin; HR: halorhodopsin. Motif sequence (amino acid residues at 85, 89, and 96 in BR numbering) is shown under the name of each type of rhodopsin. (B) Detailed tree of SR, BR, HR, cyanobacterial HR, and RmHR homologues. The habitats of each strain are indicated by differently colored squares. Asterisk represents the strain that was isolated from seawater using a medium for freshwater cyanobacteria.
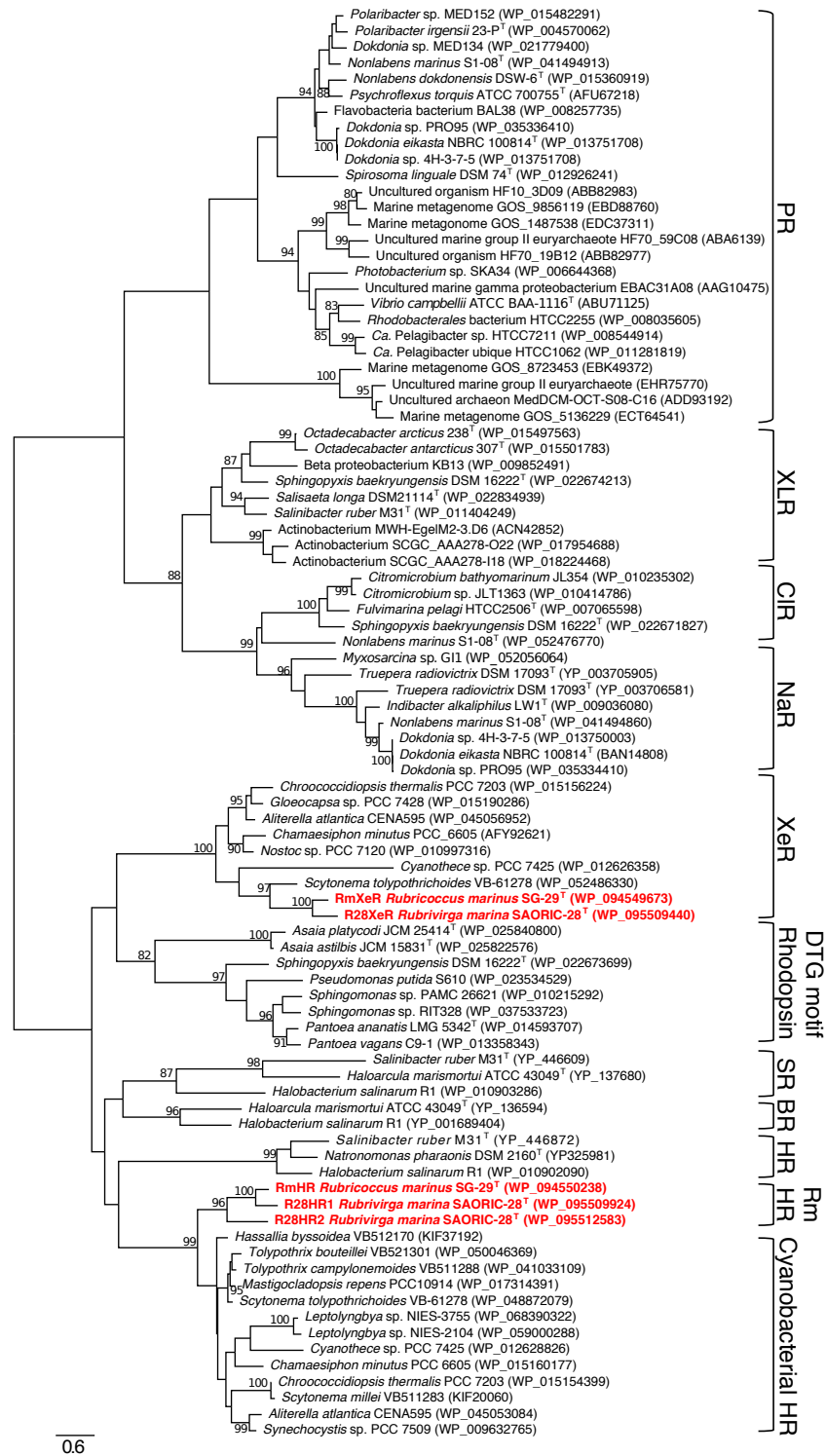
Fig. 1-3. Phylogenetic tree of microbial rhodopsin genes. The names of bacteria are shown with their accession numbers, used in Fig. 1-2A. The method of construction is the same as that used to construct Fig. 1-2.
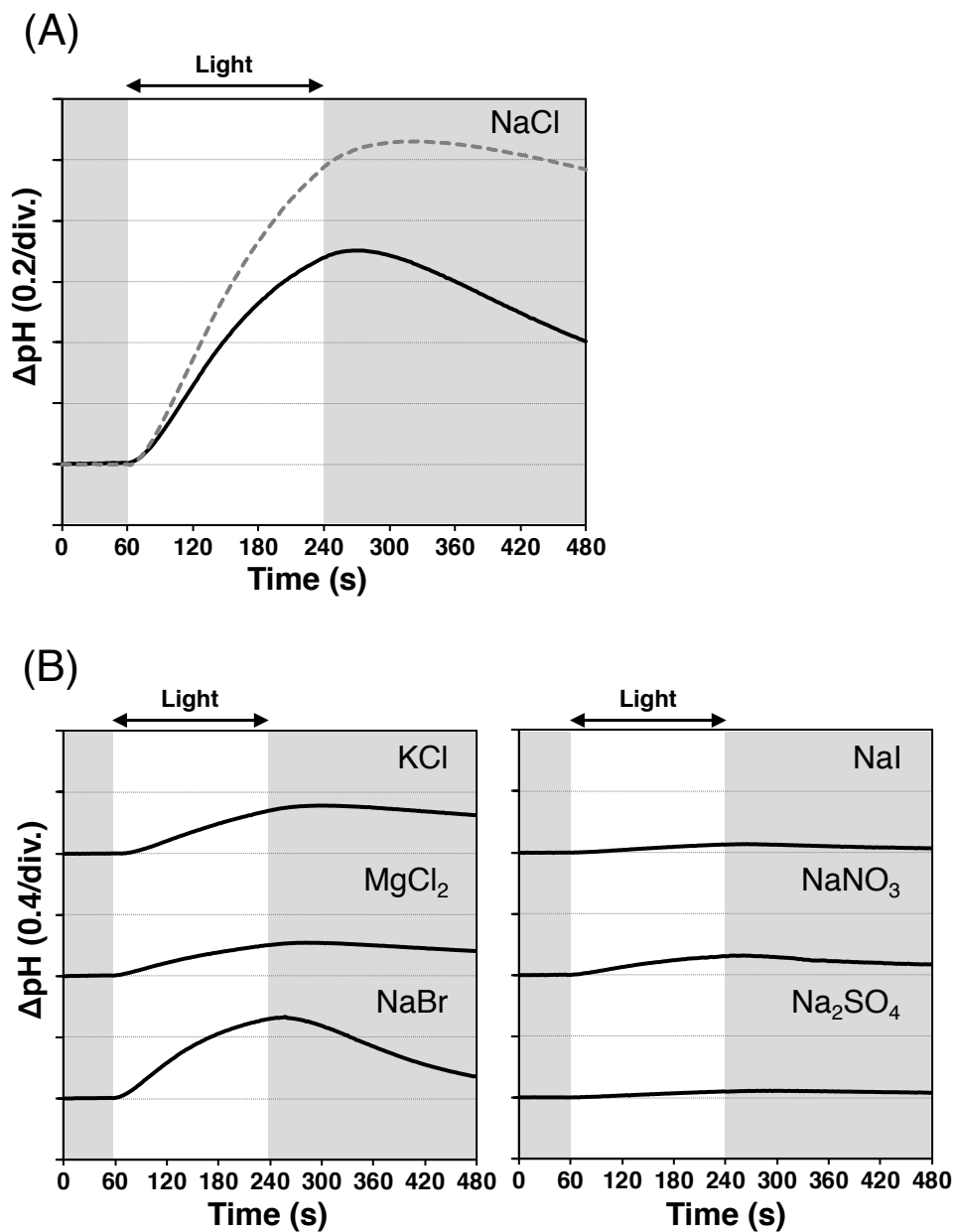
Fig. 1-4. Light-induced pH change in RmHR in *E. coli*. In solutions containing (A) 100 mM NaCl with (gray broken line) or without (black solid line) 30 μM CCCP, and (B) 100 mM KCl, $MgCl_2$, NaBr, NaI, $NaNO_3$, or Na_2SO_4 without CCCP. The cell suspension was illuminated with green light (520 nm) passed through a 300-W xenon lump. The temperature was maintained at 4°C. The abbreviation "div." represents division.
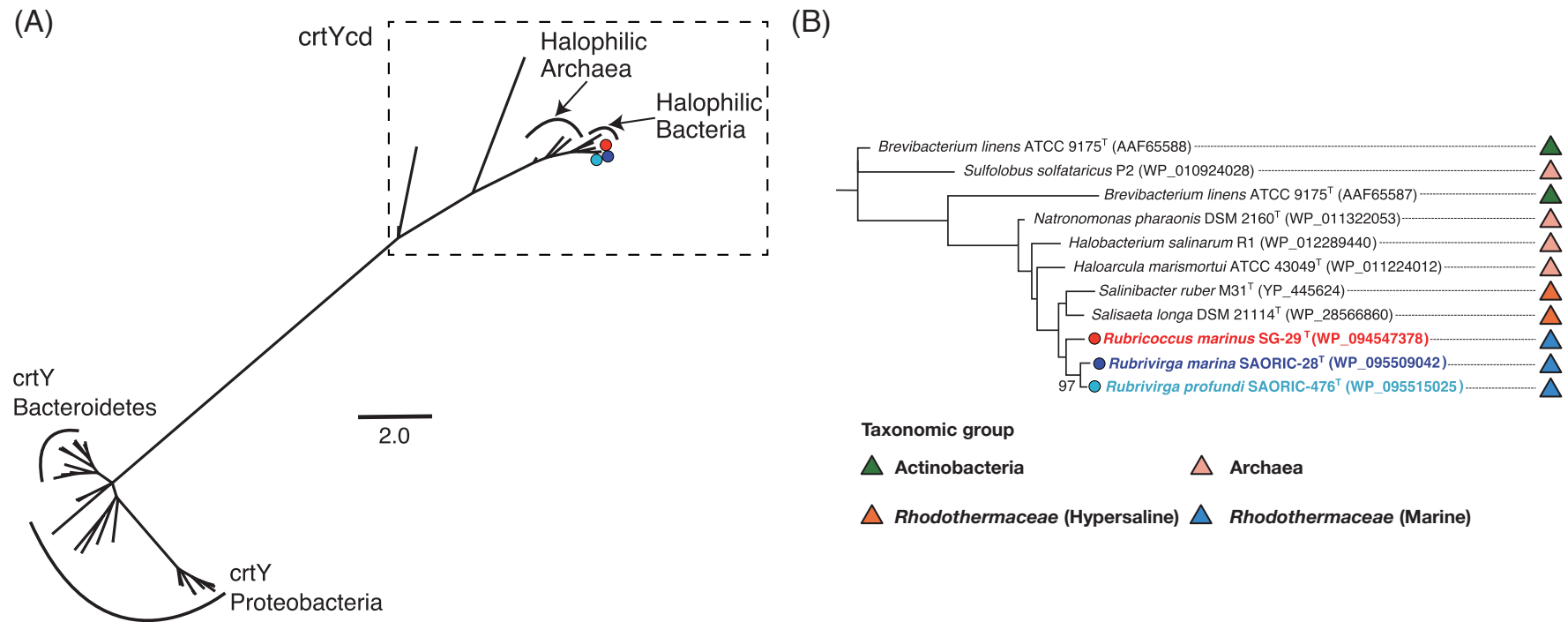
Fig. 1-5. Phylogenetic tree of lycopene cyclase genes (*crtY* and *crtYcd*). Amino acid sequences were aligned using CLUSTALW, and evolutionary distances were estimated using LG with Freg model. The unrooted maximum-likelihood tree was constructed using bootstrap values based on 1000 replications; evolutionary analyses were conducted using MEGA 6.0. (A) Phylogenetic tree of the lycopene cyclase gene in rhodopsin-possessing prokaryotes is shown in Fig. 1-3. Open circle, and closed gray and black circles, indicate the positions of strains SG-29[T], SAORIC-28[T], and SAORIC-476[T], respectively. Box with broken line was used for detailed phylogenetic tree. (B) Detailed

phylogenetic relationship of lycopene cyclase among prokaryotes possessing the *crtYcd* gene. Bootstrap values >80% are indicated as a

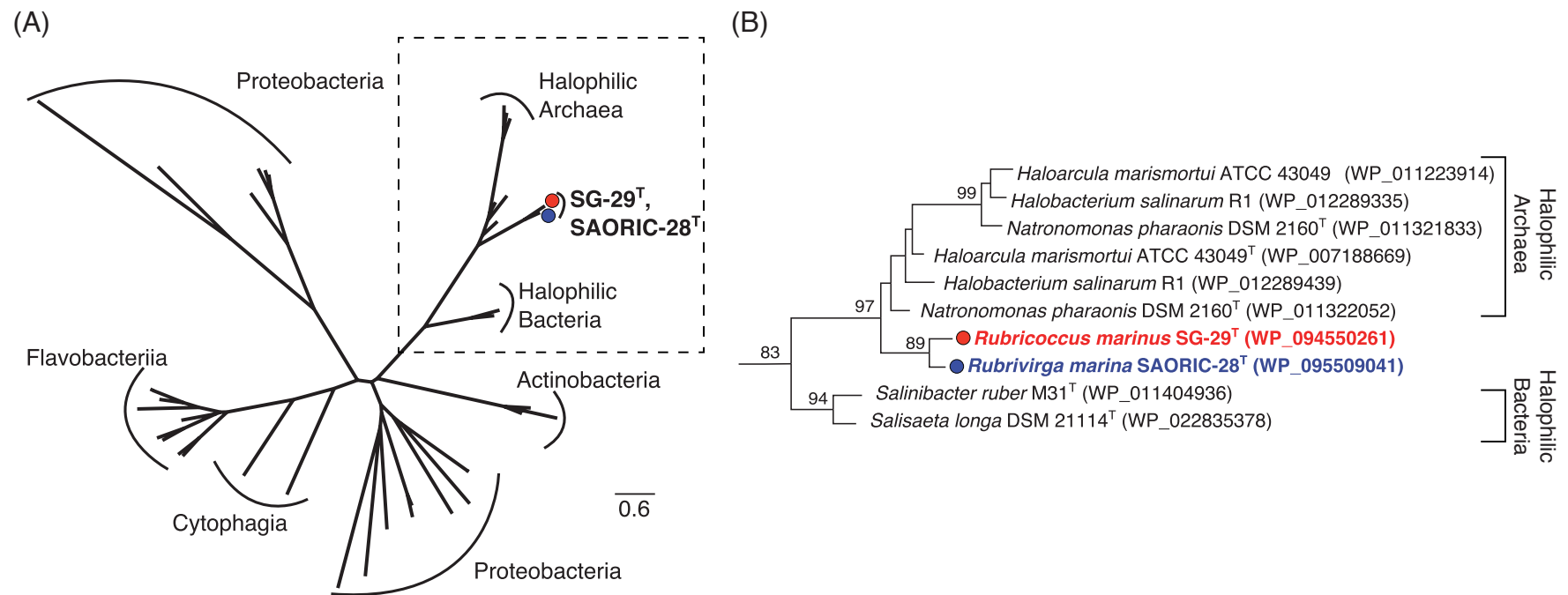percentage of replicates determined.

(A)

Proteobacteria

Halophilic Archaea

SG-29^T, SAORIC-28^T

Halophilic Bacteria

Actinobacteria

Flavobacteriia

Proteobacteria

Cytophagia

0.6

(B)

Haloarcula marismortui ATCC 43049 (WP_011223914)

99

Halobacterium salinarum R1 (WP_012289335)

Natronomonas pharaonis DSM 2160^T (WP_011321833)

Haloarcula marismortui ATCC 43049^T (WP_007188669)

Halobacterium salinarum R1 (WP_012289439)

97

Natronomonas pharaonis DSM 2160^T (WP_011322052)

Halophilic Archaea

83

89

Rubricoccus marinus SG-29^T (WP_094550261)

Rubrivirga marina SAORIC-28^T (WP_095509041)

94

Salinibacter ruber M31^T (WP_011404936)

Salisaeta longa DSM 21114^T (WP_022835378)

Halophilic Bacteria

Fig. 1-6. Unrooted maximum-likelihood phylogenetic tree of *blh* gene. Phylogenetic tree of *blh* gene. Amino acid sequences were aligned using CLUSTALW, and evolutionary distances were estimated using LG with Freg model. The unrooted maximum likelihood tree was constructed using bootstrap values based on 1000 replications; evolutionary analyses were conducted using MEGA 6.0. (A) Phylogenetic tree of the *blh* gene in rhodopsin-possessing prokaryotes is shown in Fig. 1-3. Open and closed gray circles indicate the positions of strains

SG-29^T and strain SAORIC-28^T, respectively. Box with broken line is used to show detailed phylogenetic tree. (B) Detailed phylogenetic

relationship of the *blh* gene among halophilic bacteria, halophilic archaea, strain SG-29^T, and strain SAORIC-28^T. Bootstrap values >80%
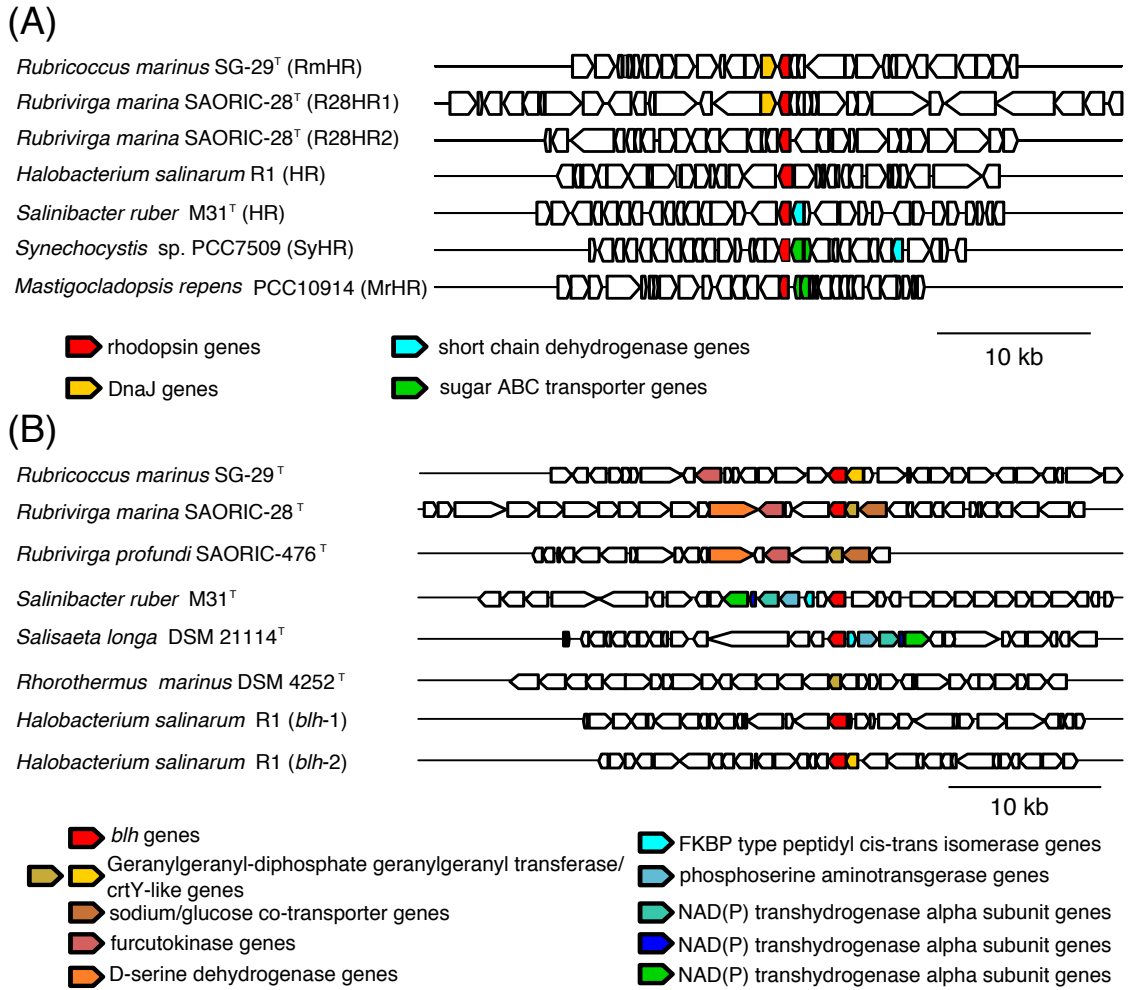
are indicated as a percentage of replicates determined.

(A)

Rubricoccus marinus SG-29[T] (RmHR)

Rubrivirga marina SAORIC-28[T] (R28HR1)

Rubrivirga marina SAORIC-28[T] (R28HR2)

Halobacterium salinarum R1 (HR)

Salinibacter ruber M31[T] (HR)

Synechocystis sp. PCC7509 (SyHR)

Mastigocladopsis repens PCC10914 (MrHR)

rhodopsin genes

DnaJ genes

short chain dehydrogenase genes

sugar ABC transporter genes

10 kb

(B)

Rubricoccus marinus SG-29[T]

Rubrivirga marina SAORIC-28[T]

Rubrivirga profundi SAORIC-476[T]

Salinibacter ruber M31[T]

Salisaeta longa DSM 21114[T]

Rhorothermus marinus DSM 4252[T]

Halobacterium salinarum R1 (*blh*-1)

Halobacterium salinarum R1 (*blh*-2)

10 kb

blh genes

Geranylgeranyl-diphosphate geranylgeranyl transferase/crtY-like genes

sodium/glucose co-transporter genes

furcutokinase genes

D-serine dehydrogenase genes

FKBP type peptidyl cis-trans isomerase genes

phosphoserine aminotransgerase genes

NAD(P) transhydrogenase alpha subunit genes

NAD(P) transhydrogenase alpha subunit genes

NAD(P) transhydrogenase alpha subunit genes

Fig. 1-7. Genomic flanking regions of chloride ion pumping rhodopsin (A) and *blh*

(B) genes. The positions of each rhodopsin and *blh* gene are indicated in red.

.
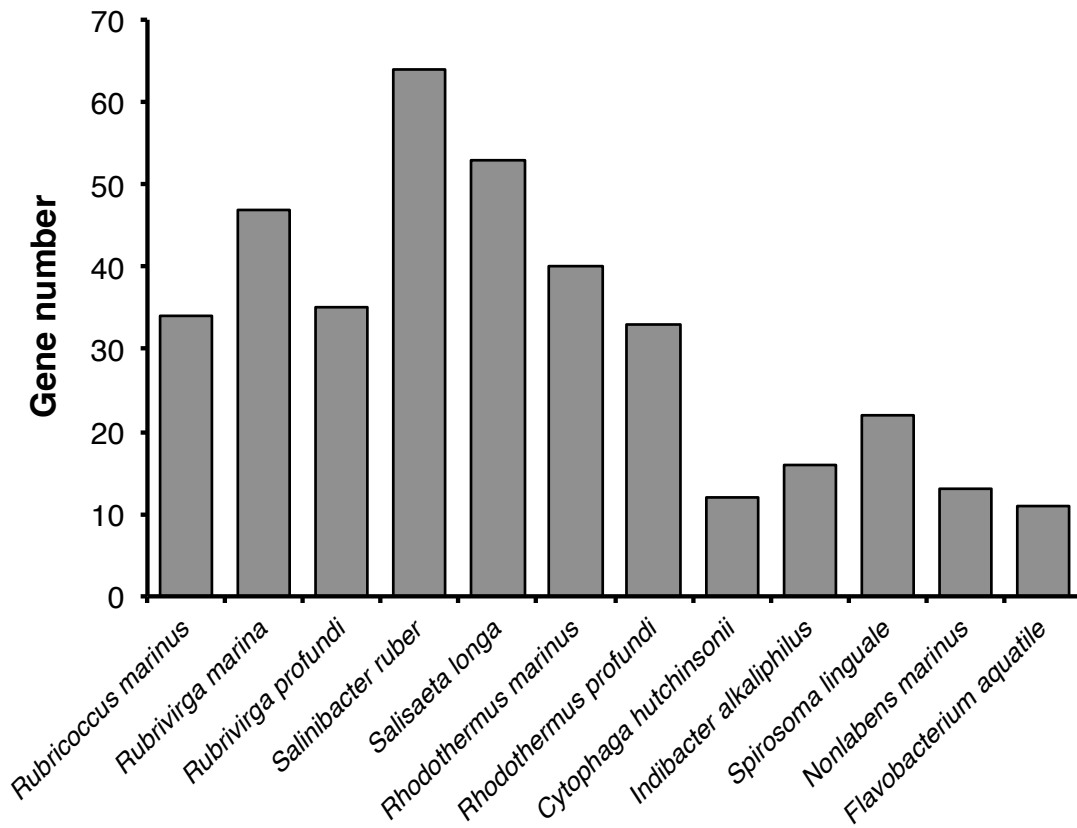
Fig. 1-8. The number of genes potentially transferred from the genome of *H. salinarum* R1. Each bar represents the number of genes exceeding the threshold with BLAST hits to the genome of *H. salinarum* R1.

Chapter 2. Retinal acquired condition in rhodopsin possessing ultra-small Actinobacteria, *Aurantimicrobium minutum* KNC^T.

第 2 章の内容は学術雑誌への投稿予定のため 5 年間は非公開です。

Chapter 3. Expression of multiple rhodopsin genes in *Nonlabens marinus* S1-08[T].

第 3 章の内容は学術雑誌への投稿予定のため 5 年間は非公開です。

General Discussion

General Discussion の内容は学術雑誌への投稿予定の内容を含むため

5年間は非公開です。

Table 1 Rhodopsin which transports H^+, Na^+, or Cl^- discovered from different salinity environment.

	Fresh water	Marine	Hypersaline
H^+ pump	XLR (Sharma et al., 2008)	PR (Béja et al., 2000)	BR (Oesterheit and Stoeckenius, 1971)
Na^+ pump	NaR (Thiel et al., 2017)	NaR (Inoue et al., 2013)	
Cl^- pump	SyHR (Niho et al., 2017)	RmHR, ClR (Yohiszawa et al., 2014; This study)	HR (Matsuno-Yagi and Mukobata, 1980)

Acknowledgements

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