Microbial rhodopsins, a diverse group of photoactive proteins found in Archaea, Bacteria, and Eukarya, function in photosensing and photon energy harvesting and may have been present in the resource-limited early global environment. Four different physiological functions have been identified and characterized for nearly 5,000 retinal-binding photoreceptors, these being ion transporters that transport proton or chloride and sensory rhodopsins that mediate light-attractant and/or -repellent responses. The greatest number of rhodopsins previously observed in a single archaeon had been four. Here, we report a newly discovered six-rhodopsin system in a single archaeon, *Haloarcula marismortui*, which shows a more diverse absorbance spectral distribution than any previously known rhodopsin system, and, for the first time, two light-driven proton transporters that respond to the same wavelength. All six rhodopsins, the greatest number ever identified in a single archaeon, were first shown to be expressed in *H. marismortui*, and these were then overexpressed in *Escherichia coli*. The proteins were purified for absorption spectra and photocycle determination, followed by measurement of ion transportation and phototaxis. The results clearly indicate the existence of a proton transporter system with two isochromatic rhodopsins and a new type of sensory rhodopsin-like transducer in *H. marismortui*.
phens represent the protein sequence for the gene of interest and the underlined amino acids represent the introduced HindIII site. The accuracy of all constructs was confirmed using nucleotide sequencing. *H. marismortui* (ATCC 43049) cells were grown in medium designed for *H. marismortui* and herein designated halomedium (0.28 M NaCl, 51 mM MgSO4, 1.02 mM Na3C6H5O7, 27 mM KCl, 1% [wt/vol] Oxoid peptone LP0034, 2.30 mM ZnSO4, 1.01 mM MnSO4, 1.40 mM Fe(III)SO4, 0.28 mM Co(NH3)6 SO4 (23) at 42°C. Different illumination conditions and culture periods were selected for different experiments.

Protein expression and purification. The protein sample was prepared as described previously and optimized with some modifications (14, 27). Briefly, both rhodopsins and transducers were expressed in E. coli C43(DE3). Freshly transformed E. coli C43(DE3) cells were grown in an LB medium containing 50 mg/liter ampicillin at 37°C. Overexpression of the protein was induced using IPTG (isopropyl-[β-D-thiogalactopyranoside) plus 100 ng/ml all-trans retinal for rhodopsin expression). Cells were harvested by centrifugation (6,000 × g) and resuspended in buffer A (50 mM Tris, 4 mM NaCl, pH 7.8, 0.2 mM phenylmethysulfonyl fluoride [PMSF], 14.7 mM β-mercaptoethanol). Cell disruption was performed with sonication. Membranes were sedimented at 100,000 × g for 1 h at 4°C and solubilized in buffer A containing 1.5% [wt/vol] n-dodecyl-β-D-maltoside (DDM) (Anatrace) for 16 h at 4°C. After centrifugation of the solubilized membranes (50,000 × g, 30 min, 4°C), the supernatant was treated with Nitritoltriatic acid (NTA) agarose, which had been preequilibrated with buffer B (50 mM Tris, 4 mM NaCl, 0.05% DDM, pH 7.8) containing 20 mM imidazole for 6 to 8 h at 4°C with slow rotation. The loaded resin was filled onto a chromatography column and washed extensively with buffer C (buffer B plus 50 mM imidazole). Target proteins were then eluted from the column with buffer D (buffer B plus 250 mM imidazole). Imidazole was removed from these fractions using a Sephadex G-25 spin column against buffer E (50 mM MES [morpholineethanesulfonic acid], 100 mM NaCl, pH 5.8, 0.05% DDM) for further assay and long-term storage. The typical yield of purified protein was a 2-mg/liter culture, with minor variations among six rhodopsins.

RT-PCR of rhodopsins and transducers transcript. For isolation of *H. marismortui* total RNA, approximately 50 ml of *H. marismortui* culture (optical density at 600 nm [OD600] = 0.5) were centrifuged at 7,500 × g and 4°C for 15 min. The cell pellet was lysed in 1.2 ml of TRI reagent (Sigma, St. Louis, MO), and the total RNA was isolated by following manufacturer-suggested procedures. Finally, the RNA pellet was resuspended in RNase-free H2O and stored at −80°C.

Phototaxis measurements. Phototaxis measurements were performed according to a previously described motion analysis algorithm (17).

RESULTS

Primary sequence analysis and biological expression of six opsin genes and transducers in *H. marismortui*. Three photosensory rhodopsin candidates (Fig. 1A), hinted at in the original *H. marismortui* genomic study, were first identified and analyzed from the whole-genome map (see Table SA1 in the supplemental material). Protein domains and structural-features analysis (Fig. 1B) using BLAST (2, 12, 18) and SMART (16, 29) revealed that the encoded protein of the first htr (GenBank locus tag rnaAC0013), HtrII of *H. marismortui* (HmHtrII), is a sensory rhodopsin transducer, conjugated upstream of sop1, as with HtrII from *H. salinarum* (HsHtrII) (34).

HmHtrII contained a two-transmembrane region connected by a large extracellular loop, followed by a histidine kinase-binding domain extruding to the cytoplasmic side in the C terminus (15, 37). The protein encoded by the second transducer candidate (htr, rnaAC3281), HmHtrI, conjugated upstream of sop1, was first determined to share 53.8% identity with the cytoplasmic region in the C terminus of HsHtrI (25), but it lacks the two-transmembrane region (Fig. 1B). Later sequence analysis recognized that the two-transmembrane region existed if an upstream alternative start codon, GTG (~291), was adopted, making it a 528-residue protein with 57.3% identity with full-length HsHtrI, designated reHmHtrI (interchangeable with HmHtrI for the rest of this article). For the third transducer gene candidate (rnaAC0558) conjugated downstream of sop1, no known analogue was identified using the same analysis, but the theoretically translated product, HmHtrM, clearly showed a two-transmembrane region with an unusual shortened C terminus containing only a HAMP (stands for histidine kinases, adenylyl cyclases, methyl-binding proteins, and phosphatases) domain and extruded to the cytoplasmic side, a conserved structural pattern for known sensory rhodopsin cognate transducers (Fig. 1B). No matching cognate transducer genes were found for *bop, sop1*, and *hop*.

Together with the six annotated opsin genes, a six-rhodopsin three-transducer system, including protein names, was proposed (Fig. 2) (protein names and maximum absorbance values for each rhodopsin were determined and are explained below).

Experiments were performed to examine the biological expression of all six rhodopsins and the three proposed transducers, including the restored two-transmembrane htr for sop1 and the unusual short transducer for sop2 (Fig. 3), in *H. marismortui*. Total RNA was prepared from cells grown in the presence or absence of white-light illumination for reverse transcription-PCR, RT-PCR, and analysis, and despite minor differences in expression levels under different illumination conditions, mRNAs coding for all rhodopsins and transducers were found to be detectable throughout the early-log, mid-log, and stationary phases in both the presence and absence of (k = x, where Vmax is maximum velocity, k is the Michaelis constant, and n is the number of cooperative sites. The k value was considered a dissociation constant.

Motile-cell culture. *H. marismortui* cells were selected for motility, with swarm plates containing 0.4% (wt/vol) agar in halomedium. Cells at the edge of diffuse cell masses were picked and inoculated into halomedium and shaken at 42°C for at least 5 days to reach saturation. Saturated culture was diluted 1:100 in halo-medium and subcultured at 42°C for 48 h and then diluted 1:3 with halomedium immediately preceding microscopic observation.

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Phototaxis measurements. Phototaxis measurements were performed according to a previously described motion analysis algorithm (17).
white light (Fig. 3), showing that these genes are indeed expressed and physiologically active independent of environmental illumination, and these results offer further direct proof of the existence of a six-rhodopsin system in a single archaeon.

Cloning and purification of all six rhodopsins from genomic DNA. Expression plasmids were prepared by obtaining each gene fragment from the genomic DNA of *H. marismortui* by PCR, and the gene fragments were further cloned into the pET-21b(+) vector before the vector was transformed into *E. coli* for expression as described in Materials and Methods to first observe the responding light wavelength for each protein encoded by the six opsin genes.

The purified proteins (Fig. 4A) from six genes all showed visually distinguishable colors, and their maximum absorptions were determined (Fig. 4B) with a UV/Vis spectrophotometer. All six proteins were found to absorb within the visible light range from 483 nm to 578 nm and were named (Fig. 2) according to their \( \lambda_{\text{max}} \), sequence analysis, and functional assays (described later).

HmSRII and HmSRM absorbed at 483 nm and 503 nm,
respectively, and appeared to fall into the range of wavelengths known to trigger repellent responses based on the results of previous studies (32). Further toward the red, both of the products of two rhodopsin genes that were annotated as “bacteriorhodopsin” and “bacteriorhodopsin precursor” in the genome project (3) absorbed at the same wavelength of 552 nm and were named HmBRI and HmBRII, respectively. Toward the longer wavelengths, the protein absorbing at 578 nm was named HmSRI, while HmHR (explained later) was named for the protein maximally absorbed at 576 nm (32).

Flash-laser-induced photolysis measurement of the six rhodopsins. Photocycle time represents the basic definition for a light-inducible photoreceptor and the kinetic properties of its functionality (32). For each of those six rhodopsins (Fig. 5), the photocycle period, representing the decay and regeneration of the ground state, was measured and found to fall into two distinct groups; one, including HmBRI, HmBRII, and HmHR, was completed within ~6 ms, while HmSRI, HmSRII, and HmSRM showed a slower recovery time of 4 to 6 s. Faster photocycle kinetics in the millisecond range has been proposed to indicate, but not determine, an ion transporter, while slower kinetics in the seconds range suggests sensory rhodopsin (7–8). HmSRM was thus classified as sensory-like based on the kinetics of the photocycle and the biologically expressed HmHtrM with which it associated.

Measurements of light-driven ion transportation. A unique light-driven proton transporter system composed of isochromatic rhodopsins HmBRI and HmBRII was proposed from the results of sequence alignments with bacteriorhodopsin, HmBRI and HmBRII having identical maximum absorbances at 552 nm and kinetics of photocycle in the millisecond range. To further confirm this speculation, the two genes were first expressed in E. coli cells and then suspended in nonbuffer as described in Materials and Methods for light-driven proton transportation measurements (Fig. 6A). Upon light activation, HmBRI and HmBRII were observed to indeed undergo light-dependent outward proton transportation, directly supporting the existence of a light-driven proton transportation system with two isochromatic rhodopsins.

HmHR was analyzed to mediate light-triggered inward chloride transportation and was examined through proton cotransport associated with a chloride affinity assay (24). Passive proton transportation (Fig. 6A) showed that HmHR performed light-driven chloride transportation. The further experiment was performed to determine the chloride binding affinity (26). Within the chloride concentration decrease from 1 M to 0.24 mM (Fig. 6B), the spectrum exhibited a red-shift pattern and the determined $K_d$ was 9.66 mM, a value close to those for both HsHR and Natronobacterium pharaonis HR (NpHR). HmSRI and HmSRII mediated phototaxis for H. marismortui cells. H. marismortui cells were cultured at 42°C for 2 days and then were observed under infrared light (770 nm) under a phase-contrast light microscope. A motion observation and

![FIG. 3. RT-PCR analysis of the rhodopsins and signal transducers. The mRNAs coding for all rhodopsins (bop, hop, sop1, sop2, xop1, and xop2) and transducers (htr1, htr2, and htrM) were detectable in the cDNA of H. marismortui grown with and without white-light illumination. The expression profiles of these genes in early-, mid-, and late-log-phase cultures (i.e., OD$_{600}$ of 0.5, 1.0, and 1.5, respectively) were analyzed by RT-PCR with gene-specific primers. The 16s rRNA transcript was used as an internal reference for relative quantification (see Fig. SA1 in the supplemental material).](http://jb.asm.org/)

![FIG. 4. Spectrum determination of the six individual purified rhodopsins from H. marismortui. (A) Group photo of all six rhodopsins purified from E. coli dissolved in MES buffer containing 0.05% n-dodecyl-β-D-maltoside, pH 5.8. From left to right, HmBRI, HmHR, HmSRI, HmSRII, HmBRII, and HmSRM. (B) Absorbance spectrum and absorbance peak of each rhodopsin. AU, arbitrary units.](http://jb.asm.org/)
recording system with motion-analyzing software (17) developed in our laboratory were used to study the swimming responses to various wavelengths of light. When the swimming cells were illuminated with 465-nm light, a rapid reverse swimming response was clearly observed (17), which led us to conclude that HmSRII mediated the photorepellent response. When light with a wavelength greater than 600 nm, designed to stimulate only HmSRI, was applied, the reverse-swimming rates decreased to a level below 50% of the control level, indicating that HmSRI is a photoattractant rhodopsin. HmSRM, on the other hand, showed no phototactic response upon light illumination (Fig. 7). The whole six-rhodopsin system is summarized in Fig. 2.

**DISCUSSION**

In this study, a six-rhodopsin system containing the greatest number of rhodopsins so far identified in any single archaeon...
(3, 10, 32) was confirmed in *H. marismortui*. Three features not previously observed in any other archaea were unveiled, including the more diverse \( \lambda_{\text{max}} \) distributions of the expressed rhodopsins, the light-driven proton transporter system with two isochromatic rhodopsins, and a new type of sensory rhodopsin-like protein with a transducer containing only the short cytoplasmic region without the usual histidine kinase binding domain.

First, *H. marismortui* has a more diverse photosensing system. Based on analysis of the \( \lambda_{\text{max}} \) distribution pattern (Fig. SA2 in the supplemental material), it displayed a more finely tuned rhodopsin system than any currently known microbes with rhodopsins (32). In the known range of wavelength for sensory, i.e., 470 nm to 540 nm, the *H. marismortui* system has two dedicated rhodopsins, HmSRII (483 nm) and HmSRM (503 nm), instead of one, as do NpSRII (498 nm) and HsSRII (487 nm) for *Natronobacterium pharaonis* and *H. salinarum*, respectively (see Fig. SA2 in the supplemental material).

Second, in a previous study, the two isochromatic, light-driven proton transporter genes for both HmBRI and HmBRII were proposed to be two BR-like light-driven transporters with different \( \lambda_{\text{max}} \)s, extending the light-harvesting range (3). The results of this study revealed an unexpected isochromatic feature: both proteins absorb in the same range of light with the same \( \lambda_{\text{max}} \). In addition, functional measurements showed that both proteins transport protons out of the cell upon being illuminated, and these two proteins were also shown to be expressed under both dark and light conditions with similar photocycle kinetics. Further research into the differences between HmBRI and HmBRII will likely discover properties that have not yet been identified.

Third, the newly discovered type of sensory-like rhodopsin and transducer system, HmSRM-HmHtrM, was unexpected. The transducer associated with HmSRM, HmHtrM, appears to have an unusual short cytoplasmic region containing only a
HAMP domain instead of only a HAMP domain and a histidine kinase binding domain, as have been found in all other known photosensory transducers. This configuration of HmHtrM therefore hints that direct controlling of flagellar proteins is unlikely. Our motion analysis experiments directly support this speculation, since cells under light designed to activate HmSRM showed no phototactic response.

The existence of an HmSRM-HmHtrM system was supported by other results as well. In addition to (i) HmHtrM’s lack of phototactic function, (ii) information from the htrM gene locus, (iii) translated-protein sequence analysis, and (iv) the fact it was shown to be biologically expressed, HmHtrM is yet to be directly shown to be a transducer for HmSRM. HmSRM-HmHtrM and HmSRM-HmHtrI fusion proteins were therefore constructed. The HmSRM-HmHtrM protein showed an increased duration time for M-intermediate formation upon activation with a 532-nm laser (Fig. 8). In contrast, the HmSRM-HmHtrI combination resulted in an extremely unstable protein complex which tended to denature immediately after purification and under any dim-light illumination (data not shown).

The lack of a histidine kinase binding domain to directly contact flagellar proteins while still responding to a specific wavelength, 503 nm, suggests a plausible conclusion: the HmSRM-HmHtrM system must exert a function or mediate a mechanism which is new to our knowledge of the photosensory system. The possible physiological role of HmSRM-HmHtrM is still to be determined, but HmSRM-HmHtrM shows the appropriate configuration to mediate light-dependent interactions for other proteins. This speculation arises from the observation that HmHtrM features a unique short C terminus extruded into the cytosol with only one HAMP domain, which is known to initialize mainly protein-protein interactions (11), while lacking the whole histidine kinase binding domain that is universally observed in all other microbial transducers associated with sensory rhodopsins. Several possibilities can thus be proposed. Perhaps the HmSRM-HmHtrM complex modulates the availability of CheB or CheR to HmSRI or HmSRII by interacting with CheB or CheR and consequently regulates the sensitivity of HmSRI or HmSRII, or it may interact with other proteins for various illumination-related functions, like photoadaptation or circadian rhythm.

Two rhodopsin phototaxis receptors found in *Chlamydomonas reinhardtii* have the same function in vivo, namely, mediating light-gated cation channel activity (31). These receptors, called channelrhodopsins-1 and -2 differ by their absorption maxima, 460 nm versus 500 nm, respectively, as well as in their levels of light saturation, which extend the wavelength and intensity ranges for phototaxis by *C. reinhardtii* (30). Yet-undiscovered differences in properties of the two HmBR proteins are likely to provide an advantage to the organism, but our results rule out the possibility that they expand the wavelength range since they have identical absorption spectra. Another recently completed genomic project on another archaeon, *Haloquadratum walsbyi*, hinted at, but did not confirm, the presence of two annotated bacteriorhodopsin-like genes (5). Here, *H. marismortui* has two BRs simultaneously absorbing the same wavelength of light and both functioning as light-driven proton transporters. We further found that those two proteins exhibit different tolerances to pH changes, as the pH-dependent $\lambda_{\text{max}}$ shift measurements showed HmBR II to leave unchanged the $\lambda_{\text{max}}$ in buffers with pHs of 3.0 to 9.0, while a >10-nm blue-shift for $\lambda_{\text{max}}$ in pH 9.0 was observed in HmBRI.
Retinal-binding proteins represent one of the most efficient signal-capturing and signal transduction systems that relay activation information to G-protein systems, histidine kinase systems, and channels. Exploring the physiological roles and significance of this six-rhodopsin system will surely expand our understanding of the already-complex rhodopsin system and will unveil more of the survival strategies which supported some life forms in the early global environment.

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