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Contribution of proteorhodopsin to light-dependent biological responses in *Hymenobacter nivis* P3^T isolated from red snow in Antarctica

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ABSTRACT

Proteorhodopsin (PR) is a major family of microbial rhodopsins that function as light-driven outward proton pumps. PR is now widely recognized for its ecological importance as a molecule responsible for solar energy flow in various ecosystems on the earth. However, few concrete examples of the actual use of light by natural microorganisms via PR have been demonstrated experimentally. This study reveals one example of that in a cryophilic bacterium *Hymenobacter nivalis* P3^T isolated from red snow in Antarctica. The results demonstrate light-dependent biochemical and biological responses in *H. nivalis* cells, such as the proton pump activity of *H. nivalis* PR (HnPR), which leads to the production of proton motive force, cellular ATP production and cell growth. In addition, the results of this study demonstrate the photochemical properties of a PR, namely HnPR, in the membrane of a natural host bacterium. The photocycle of HnPR was much faster than other PRs even at 5°C, indicating that the proton pump function of HnPR has adapted to the low-temperature environment of Antarctica. Although it is well known that PR helps natural host microorganisms to use light energy, this study provides another concrete example for understanding the biological role of PR by demonstrating the link between the molecular functions of PR and the light-dependent biochemical and biological responses of a PR-bearing host.

INTRODUCTION

Sunlight is critically important to the viability of most organisms on earth. The most representative ways of using sunlight are for information and for energy. In the former case, sunlight is converted to information for vision in animals, for regulating gene expression and morphogenesis in plants, and for determining tactic behavior in microbes. On the other hand, in the latter case, sunlight is converted to energy via a photosynthetic machinery in plants and in photosynthetic microbes. In recent years, it has become evident that microbial rhodopsins, retinal-based photoreceptor proteins, significantly contribute to the solar energy flow into various ecosystems on the planet ^{1,2}.

Microbial rhodopsins are commonly composed of a seven-transmembrane α -helical protein moiety and a chromophore all-*trans* retinal that is covalently attached to a conserved Lys residue in the middle of the 7th transmembrane helix. In addition, the light activation process of rhodopsins is always triggered by photoisomerization of the retinal from the all-*trans* to the 13-*cis* configuration. On the other hand, rhodopsins have diverse molecular functions, acting as ion pumps, ion channels, light sensors, enzymes, and so on ³. Among them, light-driven outward proton pumps comprise a major group. Proton pump rhodopsins pump protons from the inside to the outside against the inside-negative membrane potential of the cell membrane. As a result, they create a proton motive force (abbreviated as PMF), which is an electrochemical potential gradient for protons and is used as a driving force for several membrane proteins, such as adenosine triphosphate (abbreviated as ATP) synthases, drug transporters, flagellar motor proteins, among others ⁴.

Proteorhodopsin (abbreviated as PR) is the major group of proton pump rhodopsins. In 2000, the first PR gene was discovered from an uncultivated member of the marine *γ -Proteobacteria* SAR86 through a metagenomic analysis ⁵. Now, more than 2 decades later, many research papers have shown that PR contributes significantly to the solar energy flow in the global ecosystem ^{1,2}. Many previous studies of PR have been conducted using a recombinant expression system. In *E. coli* cells, several light-induced biological responses connected to the function of PR have been observed, such as the outward proton pump activity to create the PMF, the increase in cellular ATP level, and the increase in flagellar responses ⁵⁻⁸. It is conceivable that these responses are similar in natural hosts expressing PR. Indeed, although there are fewer reported cases compared to the *E. coli* recombinant expression system, some previous studies have experimentally demonstrated the biological contribution of PR using natural hosts, such as the light-induced proton pump activity of PR, the light-induced increase in cellular ATP concentration, and the light-enhanced cell growth of PR-bearing natural hosts ⁹⁻¹⁸. In many of those cases, the light-dependent responses have been concluded to be connected

to the function of PR based on the results of genetic analysis, such as knockout of the PR gene, quantitative evaluation of the PR gene expression by qPCR, and so on. Those experiments provided concrete evidence for the biological contributions of PR. However, it may also be possible to link the light-dependent responses to the function of PR without modifying the genes of natural hosts by alternative methods. In this study, we demonstrate the link between the light-dependent responses of a PR-bearing natural host and the molecular function of PR by examining their light wavelength dependences.

For this purpose, we needed a PR-bearing natural host that could be maintained in culture. In 2019, Terashima and co-workers reported the entire genomic sequence of a cryophilic bacterium *Hymenobacter nivalis* P3^T strain¹⁹, which was isolated from red snow in the Yatude Valley of Antarctica^{20,21}. They identified only a single PR gene in the strain together with genes encoding carotenoid biosynthetic proteins and a putative β -carotene 15,15'-monooxygenase located downstream of the PR gene, which produces retinal from β -carotene. This indicates that the PR is in an active form in the microbe in nature. In addition, cell growth was enhanced under light illumination although the strain had no genes encoding photosynthetic proteins. This suggested that the PR contributed to the enhancement of cell growth possibly by supporting the production of ATP under light conditions. However, direct evidence that the PMF produced by PR enhances ATP production and cell growth by light needs to be elucidated.

As described above, it is well known that PR helps natural host microbes to use light energy^{1,2,9-18}, however there are few studies that clearly observed light-dependent responses. Because Antarctica is a nutrient-poor environment, the biological contribution of the PR found in that region might be much greater than in other regions of the world, such as the oceans. In the future, it is expected that quantitative comparisons can be made to characterize the region-specific energy flows through PRs into ecosystems. As a first step towards this, this study attempted to demonstrate the light-induced proton pump activity of *H. nivalis* PR (abbreviated as HnPR) to produce the PMF, the increase in ATP production, and the acceleration of cell growth under light and dark conditions using the natural *H. nivalis* strain. In this study, we aimed to link the molecular functions of HnPR to the biological responses of *H. nivalis* by examining light wavelength dependences and to elucidate some aspects of the biological role of HnPR in Antarctica. Thus, we characterized the molecular function of HnPR using several spectroscopic techniques. In this study, photochemical properties were analyzed using natural HnPR as a sample by reference to a previous study of PR²². From the spectroscopic analyses, we identified the cold-adapted functional features of HnPR.

MATERIALS AND METHODS

For each procedure, details are described in the Supporting Information.

***H. nivalis* cell culture and membrane preparation.** *H. nivalis* cells were cultured in R2A Broth “DAIGO” (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) by shaking at 4°C (NR-30 shaker, TAITEC Corp., Saitama, Japan). For measurements of proton pump activity and cellular ATP content, *H. nivalis* cells were cultured under a fluorescent light. The cells were collected when the optical density at 660 nm (abbreviated as OD₆₆₀) reached 0.8 – 0.9, which corresponds to the middle-late phase of cell growth in this condition. For spectroscopic measurements, *H. nivalis* cells were cultured in 20 L of R2A medium at 4°C under a fluorescent light. *H. nivalis* membranes were prepared using almost the same procedure described in a previous study of *Natronomonas pharaonis* halorhodopsin²³. The purified *H. nivalis* membranes were suspended in a 6-mix buffer (0.89 mM citrate, 0.89 mM MES, 1.1 mM TES, 0.78 mM TAPS, 1.1 mM CHES, 0.33 mM CAPS; pH 7.0) containing 1 M NaCl.

Measurement of proton pump activity of HnPR using *H. nivalis* cells. *H. nivalis* cells were collected and then washed 3 times with 50 mM NaCl solution by centrifugation (4°C, 8000 × g, 10 min; himac CF16RN equipped with a T9A31 rotor, Eppendorf Himac Technologies Co., Ltd.). The cells were re-suspended in the same solution and the OD₆₆₀ was adjusted to around 0.7. The cell suspension was poured into a plastic cuvette with a magnetic stirrer bar and set into a cuvette holder connected a circulating cooler with the temperature kept at 5°C. A glass pH electrode (micro ToupH; HORIBA Advanced Techno, Co., Ltd., Kyoto, Japan) was placed in the cell suspension and the pH was recorded using a LAQUA pH meter (HORIBA Advanced Techno, Co., Ltd.). For activation, blue (peak wavelength is 505 nm), green (peak wavelength is 530 nm) and orange (peak wavelength is 590 nm) LED lights were illuminated to the suspension for 3 min. The light intensity was adjusted to 10 mW/cm² on average. The measurements were repeated 3 times independently. When necessary, carbonyl cyanide *m*-chlorophenyl hydrazone (abbreviated as CCCP; Sigma-Aldrich, St. Louis, MO, U.S.A.) was added at a 10 μM concentration. The proton pump activity was estimated by the initial slope for 10 s after the light was turned on.

Measurement of cellular ATP content in *H. nivalis* cells. *H. nivalis* cells were collected and then washed 3 times with 50 mM NaCl solution by centrifugation (4°C, 8000 × g, 10 min; himac CF16RN equipped with a T9A31 rotor). The cells were re-suspended in the same solution and stored at 4°C overnight in the presence of 10 μM CCCP to inhibit oxidative phosphorylation and starve the cells of ATP. The next day, the

cells were collected and then washed again 3 times with 50 mM NaCl by centrifugation (4°C, 8000 × g, 10 min) to wash out the CCCP. The cells were then re-suspended in the same solution to adjust the OD₆₆₀ to 0.1. All steps up to this point were conducted in the dark. For measurements under light conditions, blue (505 nm), green (530 nm) and orange (590 nm) LED lights were illuminated for 5 min at an intensity of 10 mW/cm² on average on ice. For measurements under dark conditions, the samples prepared under the dark conditions described above were used as they were without any illumination. When necessary, CCCP was added at a 10 μM concentration. ATP extraction using a Kinshiro ATP Luminescent kit ver. 2 (TOYO B-Net Co., Ltd., Tokyo, Japan) was performed according to the manufacturer's protocol. Luminescence was measured using an AB-2250 luminometer (ATTO Corporation, Tokyo, Japan). More than 4 independent measurements were averaged.

Measurement of *H. nivis* cell growth. *H. nivis* cells were pre-cultured under dark conditions at 4°C for 10 – 14 days in a glass test tube. The pre-culture suspension was transferred to 5 mL 4-fold diluted R2A medium at the calculated OD₆₆₀ of 0.01 in 24 glass test tubes (6 test tubes per condition). For the measurement of cell growth, *H. nivis* cells were cultured under a constant illumination of blue (505 nm), green (530 nm) and orange (590 nm) LED light or in the dark for 10 days. The light intensity was adjusted to 14.5 mW/cm² on average. The OD₆₆₀ was measured each day using a miniphoto 518R (TAITEC Corp.). The growth curve was analyzed using the logistic growth model ²⁴, which is used in general for the analysis of microbial cell growth, to estimate the number of days of growth, which was defined in this study as the time it takes to grow to half of the change of OD.

Gene preparation, expression and purification of recombinant HnPR. Gene information and the amino acid sequence of HnPR was obtained from Terashima et al. ¹⁹. An 8-histidine tag was attached to the C-terminus of HnPR (abbreviated as HnPR_His₈). The gene encoding HnPR_His₈ with codon optimization for expression in *Pichia pastoris* was purchased from GENEWIZ (South Plainfield, NJ, U.S.A.). A stop codon was attached directly at the end of the HnPR_His₈ gene. HnPR-M124L and -M124Q mutants were prepared using PrimeSTAR Max DNA polymerase (Takara Bio Inc., Shiga, Japan). The methylotrophic yeast *Pichia pastoris* SMD1168H strain (Thermo Fisher Scientific, Waltham, MA, U.S.A.) was used as the protein expression host. Protein expression was induced by the addition of 2% (v/v) methanol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and 20 μM all-*trans*-retinal (Sigma-Aldrich). The recombinant HnPR proteins were purified in the presence of a detergent *n*-dodecyl-β-D-maltoside (abbreviated as DDM, Dojindo Laboratories, Kumamoto, Japan) using a

HisTrap FF pre-packed column (Cytiva, Tokyo, Japan).

Spectroscopic measurements. UV-Vis absorption spectra were recorded using a UV-1800 spectrophotometer (Shimadzu Corp., Kyoto, Japan). The sample buffer was exchanged into the 6-mix buffer containing 1 M NaCl and 0.05% (w/v) DDM for recombinant HnPR, and into the same buffer without DDM for *H. nivalis* membranes. For spectroscopic pH titration experiments for recombinant HnPR, the pH was adjusted to the desired value by adding small aliquots of 0.1 M HCl or 0.1 M NaOH. After spectral baseline correction, normalization, and preparing difference spectra, the pH-dependent absorption changes were analyzed using the Henderson-Hasselbalch equation with a single pK_a . For flash photolysis measurements, the recombinant HnPR was reconstituted into a lipid membrane composed of phosphatidylcholine from egg yolk (abbreviated as EggPC; purchased from Avanti Polar Lipids, Inc., Alabaster, AL, U.S.A.) at a molar ratio of 1:20. The reconstituted HnPR was encapsulated into a 15% (v/v) polyacrylamide gel to prevent precipitation of the sample during measurements. Buffer was exchanged by soaking gels in the 6-mix buffer at pH 7.0. For *H. nivalis* membranes, the sample buffer was exchanged into the 6-mix buffer (pH 7.0) containing 1 M NaCl. Flash photolysis measurements were performed using a homemade computer-controlled apparatus equipped with an Nd-YAG laser as reported previously ²⁵. The laser pulse energy was adjusted to 5 mJ/pulse on average and the temperature was kept at 5°C on average. To reduce noise, a SVD-filter was applied to the transient absorption data. The data was analyzed by a sequential irreversible model as used previously ²⁵. The fluorescence excitation spectra were recorded using a F-2000 fluorescence spectrophotometer (Hitachi High-Tech Corp., Tokyo, Japan). The sample buffer was exchanged into the 6-mix buffer (pH 7.0) containing 1 M NaCl and 0.05% DDM for recombinant HnPR, and into the same buffer without DDM for *H. nivalis* membranes. The emission wavelength was set at 720 nm, which detects emission from the retinal chromophore ²⁶. The excitation spectra were recorded from 450 nm to 650 nm. Vertical and horizontal polarizers were set into the excitation and emission beamlines, respectively ²⁶.

Retinal isomer composition analysis. Retinal isomer composition was analyzed by normal-phase high-performance liquid chromatography (abbreviated as HPLC). For measurements under dark conditions, the recombinant HnPR and natural *H. nivalis* membranes were kept in the dark for more than 1 week at 4°C. For measurements under light conditions, green (530 nm) LED light was illuminated for 5 min before retinal oxime extraction. The monitor light wavelength was 360 nm. For peak assignment, the retinal oximes extracted from *Halobacterium salinarum* bacteriorhodopsin were used.

RESULTS

HnPR is expressed in the natural *H. nivos* membrane. Initially, we performed spectral measurements to confirm that HnPR is present in the natural *H. nivos* membrane, as a previous study had suggested¹⁹. For this purpose, we generated a recombinant HnPR sample to compare the UV-Vis absorption spectra of the *H. nivos* membrane to the recombinant HnPR.

Figure 1

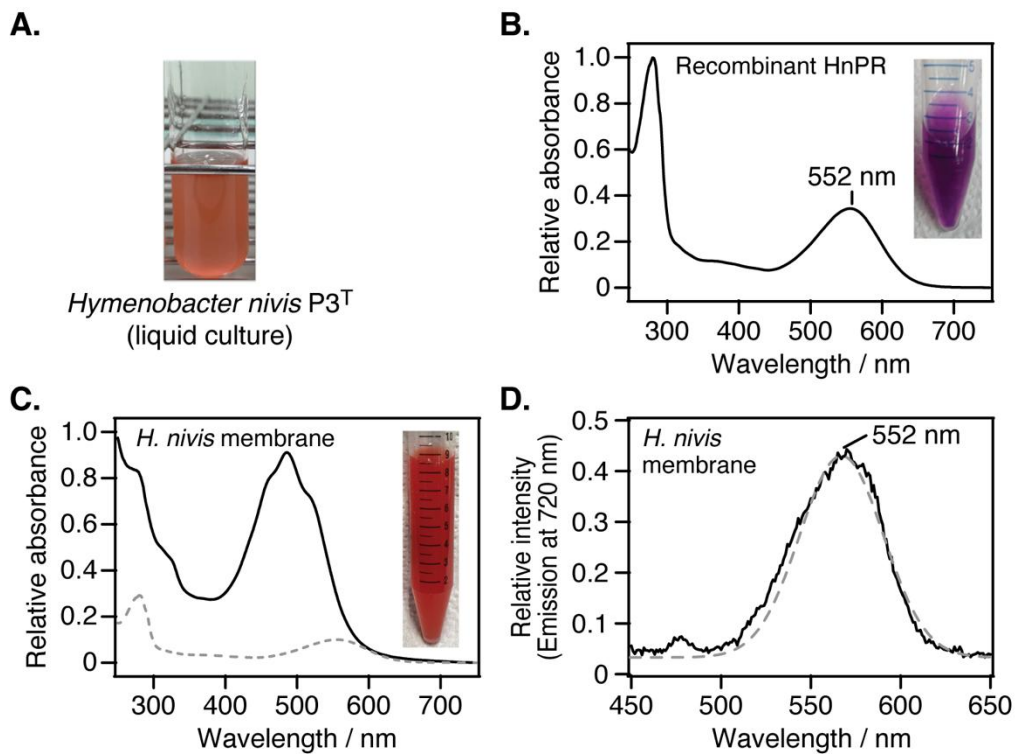


Figure 1. Sample properties of *H. nivos* membranes and recombinant HnPR. (A) *H. nivos* cell culture in R2A liquid medium. (B and C) UV-Vis absorption spectra of recombinant HnPR in the presence of DDM (B) and a *H. nivos* membrane suspension (C). Images of each sample are shown in the insets. The gray-dashed line in panel C indicates the absorption spectrum of recombinant HnPR in panel B adjusted by any multiplier. (D) Fluorescence excitation spectrum of the *H. nivos* membrane suspension. Gray dotted line represents Gaussian fitting curve.

H. nivos is a bacterium that can be maintained in culture in a laboratory^{19,21}. As shown in Figure 1A, *H. nivos* grew well in liquid medium in a glass test tube, and the culture was reddish. On the other hand, the recombinant HnPR appeared to be reddish-

purple rather than red (Figure 1B). The absorption spectrum of recombinant HnPR revealed a maximum absorption wavelength of 552 nm at pH 7.0, which was red-shifted by 32 nm compared with the first-reported PR from *γ-Proteobacteria* SAR86 (referred to as PR hereafter) ⁵. We then purified the *H. nivalis* membrane to measure its absorption spectrum (Figure 1C). As a result, the 552 nm absorption peak of HnPR was not visible, hidden by a larger absorption that may have originated from some carotenoids. To find the 552 nm absorption peak, we measured the excitation spectrum for the *H. nivalis* membrane sample. The emission wavelength was set at 720 nm, which detects emission from the retinal chromophore ²⁶ in HnPR. Under this condition, the excitation spectrum of the *H. nivalis* membrane sample is expected to overlap with the absorption spectrum of the recombinant HnPR sample. As a result, the excitation spectrum showed a peak at around 552 nm (Figure 1D), which indicated that HnPR existed in the natural *H. nivalis* membrane.

If the native *H. nivalis* membranes contain carotenoids, which seems certain because relevant genes for carotenoid synthesis have been found, HnPR could interact with carotenoids and the energy transfer from carotenoids to the retinal chromophore could occur as has been reported in some other proton pump rhodopsins ^{26–28}. This would broaden the wavelength range in which HnPR can be activated. However, there was no peak that originated from carotenoids in the excitation spectrum (Figure 1D). This result indicates that carotenoids in native *H. nivalis* membranes are used for processes that are not intended to cause energy transfer, e.g. for protection against short-wavelength light or for their antioxidant properties ¹⁹.

Light-dependent biological responses of *H. nivalis* originate from HnPR function. A previous study revealed that *H. nivalis* lacks genes encoding photosynthetic proteins in its genome, however, cell growth is enhanced under light conditions ¹⁹. From these facts and previous studies ^{9–18}, we hypothesized a mechanism for the light-dependent biological response of *H. nivalis*, i.e. the enhanced growth after exposure to light, as follows: (1) Light activates HnPR to pump out protons and to create the PMF; (2) The PMF is consumed to synthesize ATP inside the cells by F₀F₁-ATP synthetase ¹⁹; and (3) The cellular ATP is utilized for cell growth.

To test that hypothesis, we measured the proton pump activity of natural HnPR in *H. nivalis* cells using a pH electrode method at 5°C. Figure 2A shows the results of the proton pump activity measurements. When green light was illuminated, a pH decrease was observed. On the other hand, the pH decrease disappeared in the presence of the proton selective ionophore CCCP. These results indicate that protons were pumped out

from inside to outside the cells upon the light illumination, which resulted in the PMF production. In addition to the green light, the activity was also measured using blue and orange light, which resulted in light-wavelength dependent changes in the proton pump activities (Figure 2A). As shown in Figure 2B, the initial slope amplitudes, meaning the proton pump activities, were in good agreement with the absorption spectrum of the recombinant HnPR. This result confirms that the proton pump activity is derived from HnPR in *H. nivis* cells.

Figure 2.

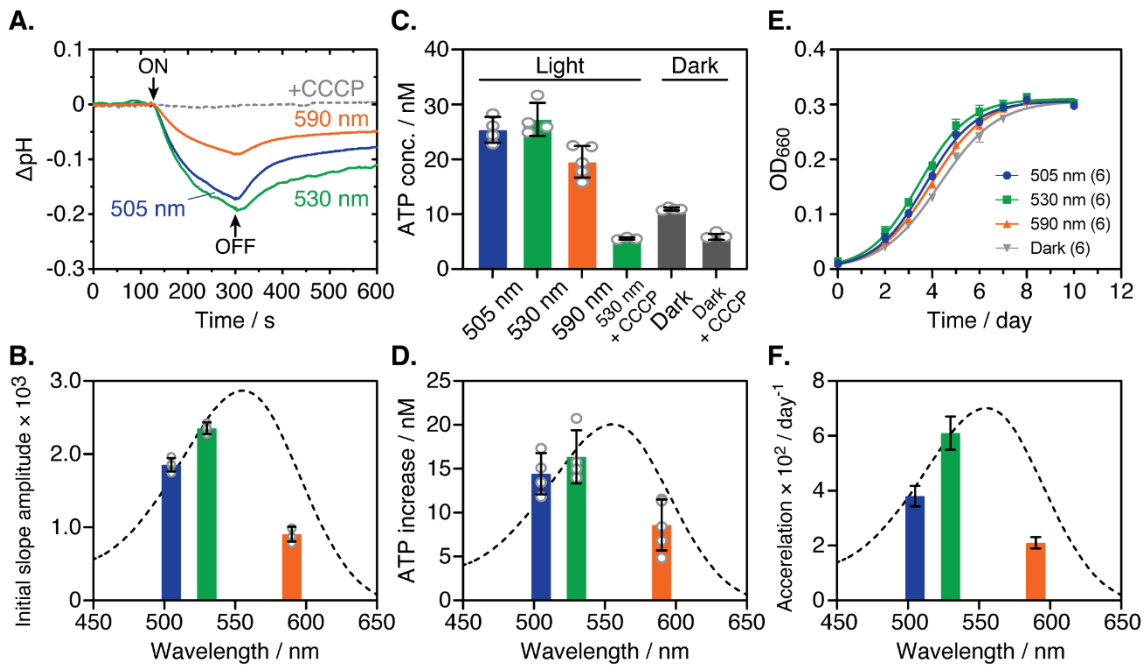


Figure 2. Light-dependent biochemical and biological responses of *H. nivis*. (A, C, E) Time-dependent pH changes of a *H. nivis* cell suspension (A), ATP concentrations in *H. nivis* cells (B), and time-dependent *H. nivis* cell growth (C) measured under light (505 nm (blue), 530 nm (green), 590 nm (orange)) and dark (gray) conditions. (B, D, F) The action spectra of the initial slope amplitudes of pH changes (B), the increase in cellular ATP concentration compared to dark conditions (D), and the acceleration of *H. nivis* cell growth compared to dark conditions (F). The black dashed line represents the UV-Vis absorption spectrum of recombinant HnPR. Individual data (white circles) are shown as a scatter plot together with the means (bar graphs) and standard deviations (error bars). Only for panel E, the means and the standard deviations are shown. For panel A, gray dots represent data obtained in the presence of CCCP. For panel E, the numbers in parentheses represents the number of individual experiments. For panel F, the error bars represent the fitting errors at each condition.

We then performed assays to measure ATP production levels in *H. nivalis* cells under light and dark conditions. A previous study revealed the presence of F_oF₁-ATPase¹⁹ that is driven by the PMF. As a result, the ATP production level was significantly increased in light conditions (Figure 2C). Moreover, the ATP production level under green light in the presence of CCCP resulted in similar values as that in the dark state. This was caused by the loss of the PMF even under the light illumination due to the presence of CCCP. We then measured the light-wavelength dependence on the ATP production and found that ATP production was highest under green light, followed by blue and then orange light conditions, which was the same tendency as the proton pump activity. Moreover, as shown in Figure 2D, the increase in ATP production at each light condition was in good agreement with the absorption spectrum of the recombinant HnPR. This result confirms that the PMF created by the function of HnPR is used to increase the ATP concentration in natural *H. nivalis* cells.

Figure 3.

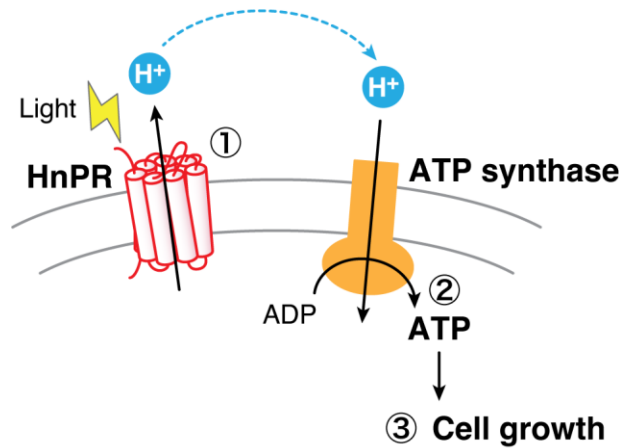


Figure 3. Biological contribution of HnPR in *H. nivalis* cells. (1) Light activates HnPR to pump out protons and create the PMF; (2) the PMF is consumed to synthesize ATP inside the cells by F_oF₁-ATP synthetase; and (3) the cellular ATP is utilized for cell growth.

Finally, we investigated the light-dependent growth of natural *H. nivalis* cells. As a result, the green light was the most effective to enhance cell growth followed by the blue light, with the orange light being the least effective among those three colors (Figure 2E). Then, we calculated the number of days of growth by analyzing the data using a logistic growth model²⁴. As a result, we obtained 3.4 ± 0.34 (mean $\pm$ standard deviation) days for the green light, 3.6 ± 0.38 days for the blue light, and 3.9 ± 0.30 days for the

orange light. The number of days of growth under dark conditions was 4.2 ± 0.44 days. Moreover, we calculated the reciprocal of the number of days of growth as the growth rate and superimposed it on the absorption spectrum of recombinant HnPR (Figure 2F), which gave results in good agreement. These results confirmed that the growth of *H. nivis* cells is accelerated by the function of HnPR.

In summary (Figure 3), light-dependent biological responses, such as PMF production (Figure 2B), cellular ATP production (Figure 2D) and cell growth (Figure 2F), can be attributed to the function of HnPR, supporting our hypothesis above.

Molecular characteristics of HnPR. The next focus of this study was to characterize the molecular properties of HnPR. Previous studies have intensively investigated the first PR, which absorbs green light and thus has also been called green PR. Conversely, the molecular properties of other PRs have hardly been investigated. Especially in the present case, HnPR is derived from a cryophilic bacterium and thus its cold-adapted function is an interesting research subject.

As characteristics in its amino acid sequence (Supporting Figure S1), HnPR is composed of 289 amino acid residues, which is 40 residues larger than PR (249 amino acid residues). HnPR conserves DTE motif residues in the 3rd transmembrane α -helix as in PR. However, a color-determinant residue Leu105 in PR²⁹ is replaced by Met in HnPR (Met124). Supporting Figure S2 shows the phylogenetic relationships among proton pump rhodopsins. HnPR belongs to a group of bacterial proton pumps. However, a closer look reveals that HnPR belongs to a sub-group that coexists with sodium-ion pump rhodopsins (abbreviated as NaRs), despite the fact that *H. nivis* has only a single PR gene but not one for NaR.

The retinal isomer compositions of HnPR were analyzed in dark and in light conditions (Figure 4A). The results show that HnPR predominantly possessed all-*trans* retinal both in dark and in light conditions. Therefore, as expected, no light-dark adaptation of the retinal isomer composition was observed for HnPR, which is the same property as PR^{30,31}.

We then performed a pH titration experiment for absorption spectra to estimate the pK_a values of functionally important residues in HnPR. Figures 4B and 4C represent the relative absorbance difference at certain wavelengths when the pH is changed from alkaline to acidic and from neutral to alkaline, respectively. From the analysis using the Henderson-Hasselbalch equation with a single pK_a , we obtained apparent pK_a values of 7.6 (Figure 4B) and of 9.7 (Figure 4C). In analogy with PR, the former pK_a of 7.6 was estimated as that of Asp116 (Asp97 in PR with a pK_a of 7.1 – 7.7^{32–34}), and the latter pK_a

of 9.7 was estimated as that of the Schiff base (the pK_a is reported to be 11.3³²). These results indicate that HnPR is active at an alkaline pH as is PR. Therefore, according to Figure 4B, it is preferable to perform experiments above pH 9. However, we were concerned about the deprotonation of the Schiff base at pH 9 and the consequent loss of structural stability of the protein and we used pH 7.0 as the standard condition in subsequent experiments.

Figure 4.

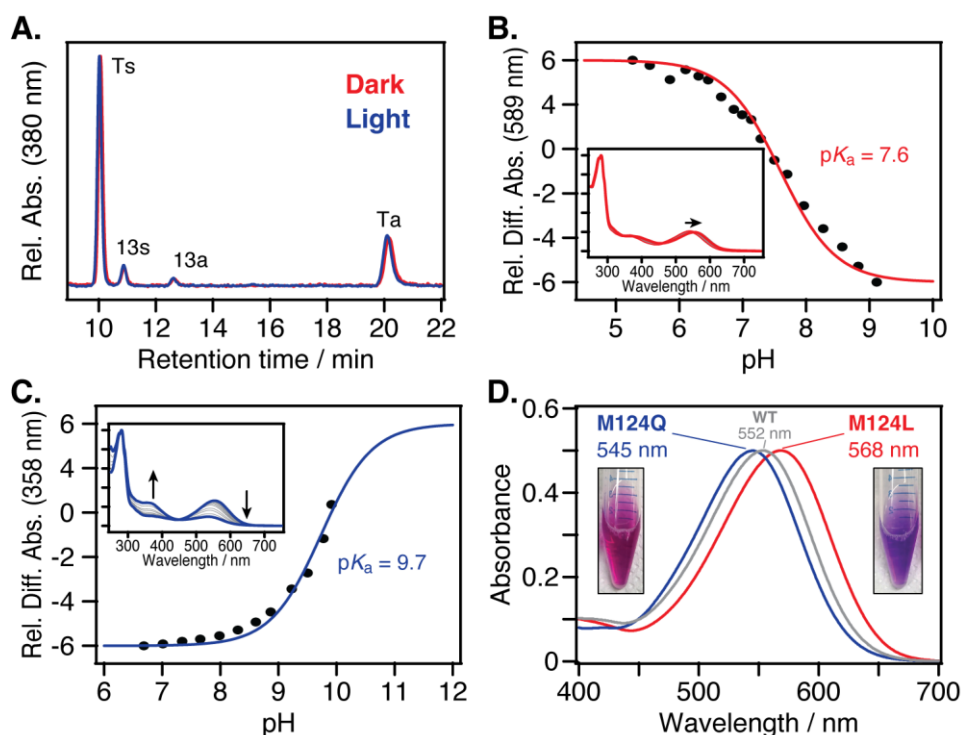
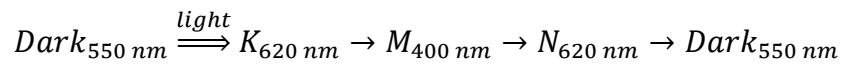


Figure 4. Basic photochemical properties of HnPR. (A) Retinal isomer compositions analyzed under dark (red) and light (blue) conditions. Ts, Ta, 13s and 13a represent all-*trans* and 15-*syn*, all-*trans* and 15-*anti*, 13-*cis* and 15-*syn*, and 13-*cis* and 15-*anti*, respectively³⁸. (B and C) Spectroscopic pH titration experiments from neutral to acidic pH (B) and from neutral to alkaline pH (C). Relative difference absorbance at 589 nm and 358 nm were plotted and analyzed using the Henderson-Hasselbalch equation with a single pK_a value. The insets show the absorption spectra measured at each condition. (D) Visible absorption spectra of wild-type HnPR (gray), M124L (red) and M124Q (blue) mutants measured at pH 7.0. The insets show images of each sample.

As described above, HnPR has Met124, which corresponds to Leu105 in PR. In blue-light absorbing PR, that residue is substituted by Gln. A previous study reported that

one of the mechanisms of color tuning in green PR (λ_{max} is 525 nm) and in blue PR (λ_{max} is 490 nm) originated from the difference between Leu and Gln, respectively. This mechanism is known as the L/Q-switch²⁹. A recent study revealed that several variations were found at this position other than Leu and Gln, for example Thr, and Met as found in HnPR. Here, we compared the absorption spectra of wild-type HnPR (abbreviated as WT), with the M124L and M124Q mutants to estimate the effect on color tuning. As shown in Figure 4D, a spectral blue-shift was observed for the M124Q mutant (λ_{max} is 545 nm at pH 7.0) and a spectral red-shift was observed for the M124L mutant (λ_{max} is 568 nm at pH 7.0), compared with WT (λ_{max} is 552 nm at pH 7.0). In summary, according to the rule of the L/Q-switch²⁹, the replacement of Met by Leu and Gln made HnPR the blue- and red-absorbing forms, respectively. In both mutations, the shift width was small, and even if shifted, the absorption wavelength of HnPR was within the range of the visible light spectrum of sunlight. Therefore, it seems unlikely that the presence of Met at the 124th position is important for HnPR to function in a physiological environment.

To observe the proton pump photocycle of HnPR, we performed flash photolysis measurements using not only the recombinant HnPR but also the natural HnPR in *H. nivalis* membranes. We reconstituted the recombinant HnPR into an EggPC membrane as described in the Materials and Methods section. The measurements were performed at 5°C, which was 2 or 3 °C higher than the daily maximum temperature at Showa Station in the Antarctic summer season (from December to January)³⁵. The time constants for the photocycle determined by global fitting was summarized in Table 1. For the EggPC-reconstituted recombinant HnPR (Figure 5A), soon after the laser flash activation, photo-intermediates that absorb longer (620 nm) and shorter (400 nm) wavelengths of light were sequentially generated together with the disappearance of the initial dark state (550 nm). In analogy with PR, the longer-wavelength intermediates were assigned as K and N, and the shorter-wavelength one as M³⁰. Figure 5B shows the transient absorption changes of K, M, N, and the dark state. From these results, the approximate photocycle of HnPR was found to proceed as follows:



The photocycle above is common with the natural HnPR in *H. nivalis* cell membrane. As shown in Figures 5C and 5D, the types of intermediates and their formation and decay behaviors were similar to the recombinant HnPR (Figures 5A and 5B). The only difference was the length of the photocycle duration. In the case of HnPR in the *H. nivalis* membrane, the time constant for the recovery of the dark state was 65.7 ms even at 5°C, whereas in the case of the EggPC-reconstituted HnPR it was 182 ms (Table 1), which was

almost 3-times slower than HnPR in the native membrane although still faster than PR in the *E. coli* membrane at 22°C (230 ms)³⁰. Such a difference must be related to the difference in lipid composition. In summary, the photocycle of HnPR is sufficiently fast even at a low temperature, which enables it to work efficiently as a proton pump in the cold environment of Antarctica.

Figure 5.

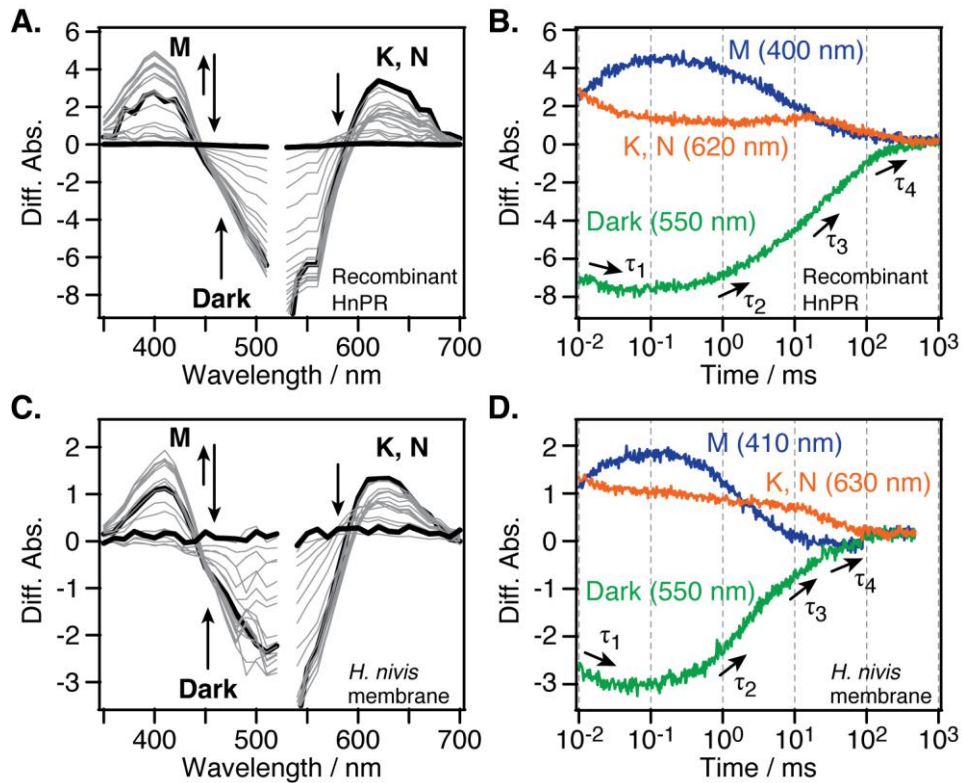


Figure 5. Comparison of the photoreactions of recombinant HnPR in an EggPC membrane and natural HnPR in a *H. nivalis* membrane. (A and C) Light-minus-dark difference absorption spectra of recombinant HnPR (A) and natural HnPR in *H. nivalis* membrane (C) at any given time. Bold black lines represent the spectra at start (0.01 ms) and end times. (B, D) Transient absorption changes at 400 nm (blue, M-intermediate), 550 nm (green, initial dark state) and 620 nm (orange, K- and N-intermediates) of recombinant HnPR (B) and natural HnPR in a *H. nivalis* membrane (D). Arrows indicate fitting time constants $\tau_1 - \tau_4$.

Table 1. Summary of the time constants and errors determined by global fitting.

Time constant	Native HnPR (<i>H. nivalis</i> membrane)	Recombinant HnPR (EggPC membrane)
τ_1 / μ s	24.8 ± 0.952	35.4 ± 6.42
τ_2 / ms	1.59 ± 0.0299	1.86 ± 0.272
τ_3 / ms	13.9 ± 0.659	38.3 ± 4.81
τ_4 / ms	65.7 ± 2.44	182 ± 26.8

DISCUSSION

Biological contributions of HnPR in *H. nivalis* cells. In general, the biological role of PR is expected to create a PMF by pumping out protons upon light illumination. The PMF drives many biochemical reactions, one example being ATP synthesis. Moreover, since ATP is referred to as the energy currency of living organisms, it is also involved in a range of biological processes, including cell growth. As described at the beginning of this paper, previous studies have experimentally demonstrated the link between the molecular function of PR and subsequent light-dependent biochemical and biological responses in natural hosts^{9–18}. The present study attempted to demonstrate this linkage from an alternative viewpoint, namely, from the effect of different wavelengths of light on the light-dependent responses of a natural PR-bearing microbial host. Together with previous studies^{9–18}, this study also provides a local but concrete example of energy flow via PR in the global ecosystem.

We focused on a cryophilic bacterium *Hymenobacter nivalis* P3^T, which has only a single PR gene, named HnPR, and is easy and stable to culture in the laboratory¹⁹. As shown in Figure 2, we successfully confirmed experimentally that light-dependent PMF production, light-dependent ATP synthesis and light-dependent *H. nivalis* cell growth, are all connected to the molecular function of HnPR. Therefore, HnPR is certain to contribute to the light-dependent responses of *H. nivalis* (Figure 3), which lacks genes of the light-energy conversion system other than HnPR¹⁹.

Antarctica is covered with snow and ice all year round and is an extreme environment with very low temperatures, poor nutrients and strong sunlight. However, there are several microbes living under such extreme conditions. *H. nivalis* was found in the red snow where many microbes, including not only bacteria but also algae, live together to make colonies^{19–21}. *H. nivalis* is a heterotrophic bacterium and thus basically

acquires nutrients to grow by ingesting organic compounds produced by other organisms¹⁹. In this sense, HnPR can be considered an auxiliary device for maintenance of the vital activity of *H. nivalis*, especially during periods when sunlight hits the snow surface. Indeed, *H. nivalis* cells can grow in dark conditions (Figure 2E), where HnPR is inactive, which supports the idea stated above. Incidentally, no previous studies have reported that PR is a protein that is essential for life^{9–15}. As described in the Introduction, it is expected that future quantitative comparisons of the biological contributions of PRs distributed in several regions of the world will be made to characterize the region-specific energy flows through PRs into different ecosystems. On the other hand, as shown in Figure 1, although it was revealed that *H. nivalis* produces some carotenoids, they were not used as antennae molecules to expand the range of light wavelengths that activate HnPR. In this sense, the carotenoids in *H. nivalis* are likely used to protect the cell from strong light and UV irradiation¹⁹, which is the same strategy used by snow algae found in the same environment.

In addition to ATP production, we examined the possibility that the PMF created by HnPR induced other PMF-driven cellular responses. We focused on a resistance/nodulation/division (abbreviated as RND) multidrug efflux transporter, which is located directly upstream of the HnPR gene in the genome of *H. nivalis*¹⁹. The RND transporter was revealed to be expressed at the protein level in *H. nivalis* cells by a previous proteomics study. However, our experimental system was unable to detect the light-induced efflux of substrates, ethidium and acriflavine, using a fluorescence spectrometer. The reasons for the failure of that trial are unknown at present and further research is needed.

Cold-adapted photoreaction of HnPR. There are few previous molecular-level studies on microbial rhodopsins found in polar regions, such as Antarctica and the Arctic. Regarding PRs, their basic characteristics, such as the DTE sequential motif and pK_a values for the primary counterion and Schiff base, are well conserved, and are shared with the initially reported, well-studied PR^{5,32–34}. In this study, as shown in Figure 4, we examined the retinal compositions, the pK_a values, and the contribution of Met124, which corresponds to the L/Q-switch²⁹, to the color tuning. One of the notable features of HnPR identified in this study is the remarkably fast photocycle at low temperature. As shown in Figures 5B and D, the single photocycle reaction of HnPR was completed in less than 1 s at 5°C. Moreover, the single photocycle reaction of HnPR in the natural membrane was accelerated 3-times compared to the recombinant HnPR in an EggPC membrane. A previous study similarly reported the photocycle reaction at 5°C for a PR identified in the

Antarctic Ocean by metagenomics analysis³⁶, however the reaction was not as fast as the photocycle of HnPR investigated in this study, but rather took several seconds. Another previous study reported the photocycle reactions of PRs identified in the Arctic Ocean³⁷, however the photocycle reactions seemed to be slower than that of HnPR in this study, although the measured temperature was not reported. A fast photocycle at a low temperature is one of the functional characteristics of HnPR and may be part of a molecular strategy to adapt to the low-temperature environment of Antarctica. Taking this into account, it can be speculated that HnPR, although it is an auxiliary device as described above, makes a significant contribution to the light utilization of *H. nivis*, which results in a significant acceleration of growth even at low temperatures. The molecular mechanisms of the low temperature adaptation in HnPR will be reported in our future research paper.

CONCLUSION

Since the discovery of the first PR in 2000⁵, there has been a flurry of molecular exploration research, which could be termed rhodopsin mining. In contrast, there has been a clear decline in studies using natural hosts to reveal the biological roles of microbial rhodopsins, as was done in earlier eras. In the present study, we focused on HnPR to elucidate some aspects of its role in the light-dependent biological response of its natural host, *H. nivis* P3^T. In addition, we revealed the light-responsive properties of HnPR adapted to the low-temperature environment of Antarctica. PR is now estimated to play an important role in the energy flow of ecosystems comparable to that of photosynthesis². We believe that our study has contributed to demonstrating the link between the molecular function of HnPR and the light-dependent biological response of *H. nivis* experimentally and provides a concrete example of energy flow. Although more difficult to work with than recombinant systems, there are still many findings that can only be obtained using natural hosts.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at XXX.

Detailed materials and methods; amino acid sequence alignment, the phylogenetic relationships among proton pump rhodopsins

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Author Contributions

[‡] K.K., R.O., and S.N. contributed equally to this research. K.K., R.O., S.N., and T.T. constructed experimental systems, performed experiments, and analyzed data. M.D. and T.K. provided technical assistance. M.T., H.K., and M.F. provided the *H. nivis* P3^T strain. K.K., R.O., S.N., and T.T. wrote the paper. T.T. designed the research. All authors

discussed and confirmed the results of the paper.

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Notes

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