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Supporting Information

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

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1. Materials and Methods

***H. nivis* cell culture and membrane preparation.** *H. nivis* cells were cultured in R2A Broth "DAIGO" (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) by shaking at 4ºC (NR-30 shaker, TAITEC Corp., Saitama, Japan). For the measurements of the proton pump activity and the cellular ATP content, *H. nivis* cells were cultured under a fluorescent light. Under this condition, it takes about 20 days for reaching plateau. The cells were collected when the optical density at 660 nm (abbreviated as OD_{660}) reached to 0.8 – 0.9 (around 10 - 14 days after starting the culture; OD_{660} was measured using miniphoto 518R (TAITEC Corp.), which is the middle-late phase of the cell growth. For the spectroscopic measurements, *H. nivis* cells were cultured in 20 L of the R2A medium at 4ºC under a fluorescent light. The cultures were incubated under aerobic conditions for approximately one month by pumping sterile filtered air into the culture medium. *H. nivis* membrane was prepared using almost the same procedure as in the previous study applied for *Natronomonas pharaonis* halorhodopsin[1]. Briefly, *H. nivis* cells were disrupted by freeze-thaw method. The thawed cell suspension was incubated in the presence of 10 mg DNAse and 10 mM $MgCl_2$ for 1 day at 4ºC to digest genomic DNA. The suspension was ultracentrifuged (40,000 rpm; CP 90NX equipped with a P70AT rotor; Eppendorf Himac Technologies Co., Ltd., Ibaraki, Japan) at 4ºC for 60 min. The precipitated membrane was washed by MilliQ water (Merk-Millipore) for several times using the ultracentrifuge to almost completely remove impurities that have turned white and precipitated out. Finally, the purified precipitated *H. nivis* membrane was suspended in the 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. nivis* cells. The natural *H. nivis* cells were collected and then washed with 50 mM NaCl solution for 3 times by centrifuge (4ºC, 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 OD_{660} was adjusted to around 0.7 measured using UV-1800 spectrophotometer (Shimadzu Corp., Kyoto, Japan). Two-milliliter of the cell suspension was poured into a plastic cuvette with a magnetic stirrer bar and set into a cuvette holder connecting a circulated cooler. Temperature was kept at 5ºC. For the proton pump activity measurement, a glass pH electrode (micro ToupH; HORIBA Advanced Techno, Co., Ltd., Kyoto, Japan) was set into the cell suspension. The pH was recorded using 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, respectively. The light intensity was adjusted to around 10 mW/cm^2. The measurements were repeated independently for 3 times. When necessary, carbonyl cyanide m-chlorophenyl hydrazone (abbreviated as CCCP; Sigma-Aldrich, St. Louis, MO, U.S.A.) was added at 10 μM concentration. The proton pump activity was estimated by the initial slope for 10 s immediately after the light exposure.

Measurement of cellular ATP content in *H. nivis* cells. The natural *H. nivis* cells were collected and then washed with 50 mM NaCl solution for 3 times by centrifuge (4ºC, 8000 × g, 10 min; himac CF16RN equipped with a T9A31 rotor). The cells were re-suspended in the same solution and stored at 4ºC overnight in the presence of 10 μM CCCP to starve. In the next day, the cells were collected and then washed with 50 mM NaCl solution for 3 times by centrifuge again (4ºC, 8000 × g, 10 min) to wash out CCCP. Then, the cells were re-suspended in the same solution to adjust the OD_{660} to 0.1. All the steps up to this point were conducted in the dark. For the measurements under light conditions, blue (505 nm), green (530 nm), and orange (590 nm) LED lights were illuminated, respectively, for 5 min at the intensity of 10 mW/cm^2 on average on ice. For the measurements under dark condition, the samples prepared under the dark condition as described above were used as they were. When necessary, CCCP was added at 10 μM concentration. ATP extraction using Kinshiro ATP Luminescent kit ver. 2 (TOYO B-Net Co., Ltd., Tokyo, Japan) was performed by reference to the manufacturer's protocol. Luminescence was measured using 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 condition at 4ºC for 10 – 14 days in a glass test tube. The pre-culture suspension was transferred to 5 mL of 4-fold diluted R2A medium at the calculated OD_{660} of 0.01 in 24 glass test tubes (6 test tubes per condition). For the measurement of the cell growth, *H. nivis* cells were cultured under constant illumination of blue (505 nm), green (530 nm), and orange (590 nm) LED lights and under dark for 10 days. The light intensity was adjusted to around 14.5 mW/cm^2 on average. The OD_{660} was measured each day using miniphoto 518R (TAITEC Corp.). The growth curve was analyzed by the logistic growth model [2] (shown as Eq.1), which is used in general for the analysis of microbial cell growth, to estimate the number of days of growth (T, shown as Eq.2), which was defined in this study as the time it takes to grow to half of the change of OD (($OD_{\max} - OD_0$)/2).

$$OD = \frac{OD_{max} \times OD_0}{(OD_{max} - OD_0)exp(-kt) + OD_0} \qquad \text{(Eq. 1)}$$

where OD_{max} and OD_0 represent the maximum OD_{660} and the starting OD_{660}, k represents the rate constant calculated by data fitting, and t represents the time (days), respectively.

$$\frac{(OD_{max} - OD_0)}{2} = \frac{OD_{max} \times OD_0}{(OD_{max} - OD_0)exp(-kT) + OD_0}$$

$$T = -\frac{1}{k}\ln\frac{2OD_{max} \times OD_0 - (OD_{max} - OD_0) \times OD_0}{(OD_{max} - OD_0)^2} \qquad \text{(Eq. 2)}$$

Gene preparation for recombinant expression of HnPR. Gene information and amino acid sequence of HnPR was referred from Terashima et al[3]. An 8-histidine tag was attached to the C-terminus of HnPR (abbreviated as HnPR_His_8). The gene encoding HnPR_His_8 with codon optimization for expression in *Pichia pastoris* was purchased from GENEWIZ (South Plainfield, NJ, U.S.A.). The stop codon was attached directly at the end of the HnPR_His_8 gene. For incorporation into pPICZ B vector (Thermo Fischer Scientific, Waltham, MA, U.S.A.), EcoRI and NotI restriction sites were attached to the 5'- and 3'-ends of HnPR_His_8 gene, respectively. FastDigest EcoRI and FastDigest NotI enzymes (Thermo Fischer Scientific) were used for digesting HnPR_His8 gene and pPICZ B vector. The digested genes were ligated using DNA Ligation kit <Mighty Mix> (Takara Bio Inc., Shiga, Japan). HnPR-M124L and -M124Q mutants were prepared using PrimeSTAR Max DNA polymerase (Takara Bio Inc., Shiga, Japan). The correctness of all nucleotide sequences was verified by dideoxy sequencing (Eurofins Genomics, Tokyo Japan).

The methylotrophic yeast *Pichia pastoris* SMD1168H strain (Thermo Fischer Scientific) was used as the protein expression host. The procedure for the transformation of *P. pastoris* was the same as our previous report[4]. Briefly, HnPR_His_8 gene-incorporated pPICZ B vector was digested using PmeI enzyme (New England Biolabs Japan Inc., Tokyo, Japan). Transformation of *P. pastoris* SMD1168H cells were performed using electroporation method (MicroPulser, Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.). After incubation on YPDS agar plate (1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol, 2% agar) containing 100 μg/mL Zeocin (Thermo Fischer Scientific), the protein expression was checked for some selected colonies.

Protein expression and purification. The procedures for yeast cell culture, protein expression, and purification were the same as our previous reports[4–6]. Briefly, The transformed cells were pre-cultured in BMGY medium containing 100 μg/mL Zeocin (Thermo Fischer Scientific) for 1 day at 30ºC in a shaker incubator (BR-22FP, TAITEC Corp.). The medium was exchanged to BMMY medium containing 100 μg/mL Zeocin, 20 μM all-*trans*-retinal (Sigma-Aldrich), and 2% (v/v) methanol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and protein expression was induced for 1 day at 30ºC in a shaker incubator (BR-43FH, TAITEC Corp.). The cells were collected by centrifugation. After the cells were suspended in 20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl, the cells were disrupted enough at 100 MPa using French Press (Ohtake, Tokyo, Japan). Membrane fraction was collected by ultracentrifugation (40,000 rpm, 90 min, 4ºC; CP 90NX equipped with a P70AT rotor; Eppendorf Himac Technologies Co., Ltd.) and was solubilized in the presence of 1.5% (w/v) *n*-dodecyl-β-D-maltoside (abbreviated as DDM, Dojindo Laboratories, Kumamoto, Japan) at 4ºC. After the ultracentrifugation again, the solubilized fraction was loaded onto HisTrap FF pre-packed column (Cytiva, Tokyo, Japan). The column was washed using 50 mM Tris-HCl buffer (pH 8.0) containing 1 M NaCl, 70 mM imidazole, and 0.05% (w/v) DDM. Finally, the protein was eluted by 50 mM Tris-HCl buffer (pH 7.0) containing 1 M NaCl, 1 M imidazole, and 0.1% (w/v) DDM.

Spectroscopic measurements. The UV-Vis absorption spectra were recorded for the purified recombinant HnPR and the purified natural *H. nivis* membrane, respectively, using UV-1800 spectrophotometer (Shimadzu Corp.). The sample buffer was exchanged into the 6-mix buffer containing 1 M NaCl and 0.05% DDM for the purified HnPR, and into the same buffer without DDM for the purified *H. nivis* membrane, respectively. For spectroscopic pH titration experiments for the purified HnPR, pH was adjusted to the desired value by adding small aliquot of 0.1 M HCl or 0.1 M NaOH. After spectral baseline correction, normalization, and preparing difference spectra, the pH-dependent absorption changes (*ΔAbs.*) were analyzed by Henderson-Hasselbalch equation with a single pK_a;

$$\Delta Abs.(acidic\ side) = \frac{1}{1 + 10^{(pH - pK_a)}}$$

$$\Delta Abs.(alkaline\ side) = \frac{1}{1 + 10^{(pK_a - pH)}}$$

For flash photolysis measurements, the purified HnPR was reconstituted into 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 in the presence of BioBeads SM-2 resin (Bio-Rad Laboratories, Inc.). The EggPC-reconstituted proteins were collected by the ultracentrifugation and then washed more than 5 times using MilliQ water. The reconstituted HnPR was homogenized and then encapsulated into 15% (v/v) polyacrylamide gel to prevent precipitation of the sample during measurements. Buffer was exchanged more than 5 times by soaking gels into the 6-mix buffer at pH 7.0. For the purified *H. nivis* membrane, the sample buffer was exchanged into the 6-mix buffer (pH 7.0) containing 1 M NaCl and not encapsulated into the gel. Flash photolysis measurements were performed using a homemade computer-controlled apparatus equipped with an Nd-YAG laser as reported previously[7]. The laser pulse energy was adjusted to 5 mJ/pulse, which was measured by a laser energy meter (Maestro, Gentec Electro Optics, Inc., Quebec city, QC, Canada). Temperature was kept at 5°C using a circulated water bath. To analyze the photocycle of HnPR, data for the transient absorption changes from 380 nm to 700 nm every 10 nm were obtained. The number of data acquisition was 200 for 380 – 400 nm and 50 for the rest of the measured wavelengths. To reduce noise, the SVD-filter was applied to the transient absorption data. The data was simultaneously fitted with the exponential decay function with four exponents;

$$\Delta Absorbance\ (t) = \sum_{n=1}^{4} A_n \exp\left(-\frac{t}{\tau_n}\right)$$

where A_n, t, and τ_n stand for the amplitude of absorbance change for the component n, the time, and the time constant for the component n, respectively. Then, the data was analyzed by a sequential irreversible model as used previously [7];

$$P_0 \rightarrow P_1 \rightarrow P_2 \rightarrow P_3 \rightarrow P_4 \rightarrow P_0$$

where P_0 represents the unphotolyzed original pigment and P_n (n = 1 – 4) represents the n-th kinetically defined states. In this model, the P_n states are allowed to contain a few physically defined photointermediates such as K – O with different absorption maxima when the quasi-equilibrium state exists between them.

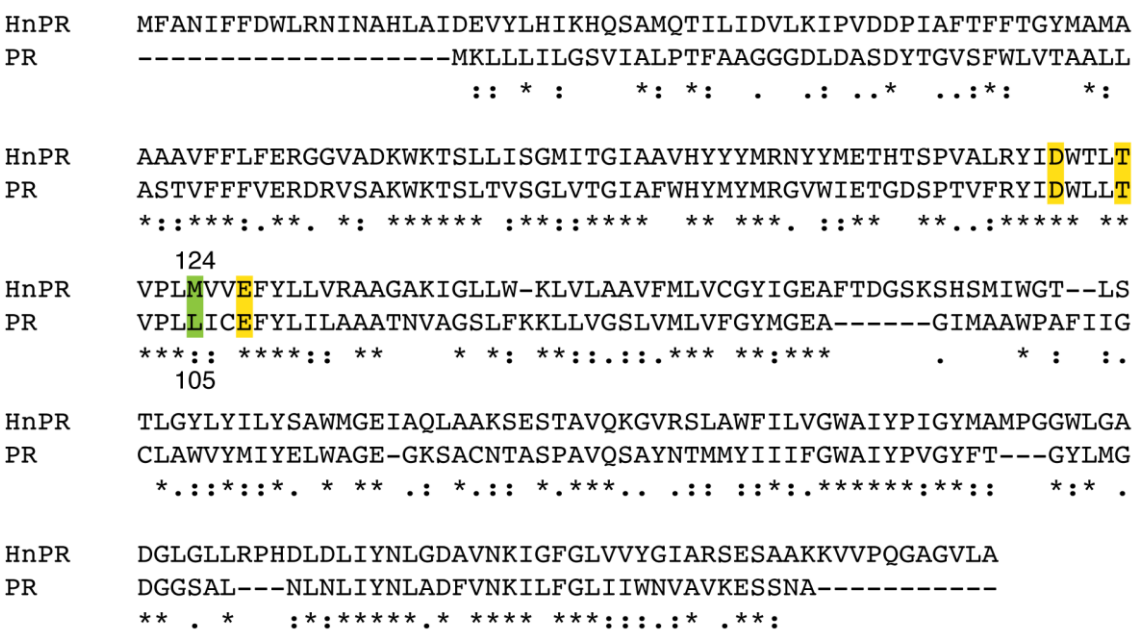
The fluorescence excitation spectra were recorded for the purified recombinant HnPR and the purified natural *H. nivis* membrane, respectively, using F-2000 fluorescence spectrophotometer (Hitachi High-Tech Corp., Tokyo, Japan). The sample buffer was exchanged into the 6-mix buffer (pH 7.0) containing 1 M NaCl and 0.05% DDM for the purified HnPR, and into the same buffer without DDM for the purified *H.*

nivis membrane, respectively. The emission wavelength was set at 720 nm, which detects the emission from the retinal chromophore. The excitation spectra were recorded from 450 nm to 650 nm. Vertical and horizontal polarizers were set into the excitation and emission beamlines, respectively.

Retinal isomer composition analysis. Retinal isomer composition was analyzed by normal-phase high-performance liquid chromatography (abbreviated as HPLC). The procedures were the same as our previous report[4]. The normal-phase HPLC system was composed of a pump module LC-10AT and a UV-visible detector module SPD-10AV (Shimadzu Corp.), and a column YMC-Pack SIL (particle size was 3 μm; YMC Co., Ltd., Kyoto, Japan). The composition of development solvent was 88% (v/v) *n*-hexane, 12% (v/v) ethyl acetate, and 0.12% (v/v) ethanol. For measurements under dark conditions, HnPR was 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 was used as a standard.

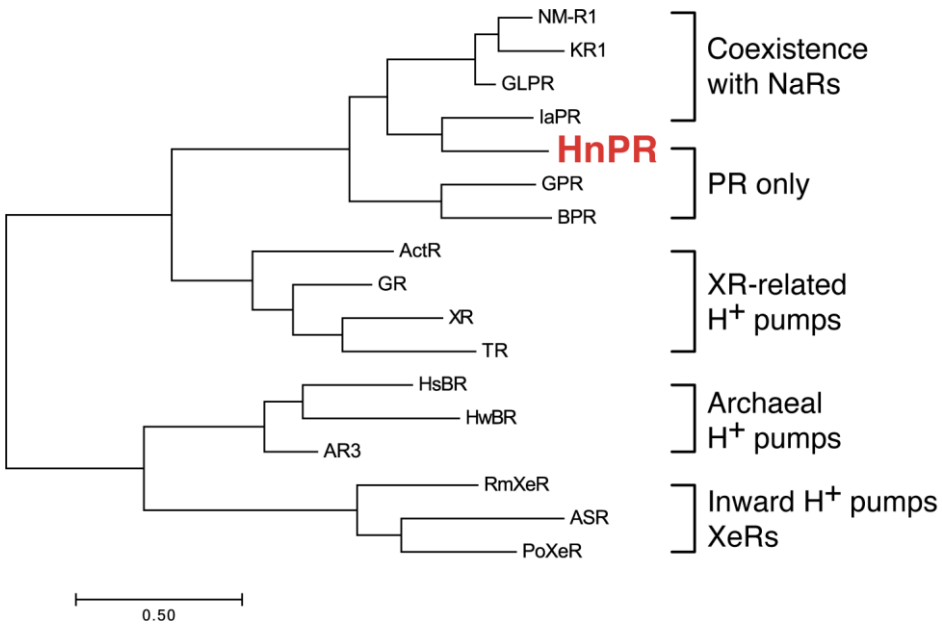
2. Supporting Figures

Supporting Figure S1.



Supporting Figure S1. Amino acid sequence alignment of HnPR and PR. The amino acids composed of the DTE motif are highlighted in yellow. Met124 in HnPR and Leu105 in PR, corresponding to the L/Q switch [8], are highlighted in green.

Supporting Figure S2.



Supporting Figure S2. Phylogenetic tree of PRs and other proton pump rhodopsins. The phylogenetic tree was constructed using MEGA11 software. The scale bar indicates the phylogenetic distance.

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