Bacterial rhodopsins of newly isolated halobacteria

JUN OTOMO,* HIROAKI TOMIOKA and HIROYUKI SASABE

Frontier Research Program, RIKEN Institute, Wako, Saitama 351-01, Japan

(Received 8 October 1991; revised 18 December 1991; accepted 6 January 1992)

Several new halobacterial strains were isolated from crude solar salts commercially produced in Mexico and Australia. The presence of bacteriorhodopsin (BR)- and halorhodopsin (HR)-like pigment in their total membrane fraction was measured by flash spectroscopy and light-induced ion pumping activity. Two of these strains contained both BR- and HR-like pigments; the others contained only BR-like pigment. DNA hybridization analysis with probes from bacteriorhodopsin and haloopsin genes revealed that the genes encoding the BR- and HR-like pigments were not homologous to those found in *Halobacterium halobium* R1. In addition, the presence of sensory rhodopsin (SR)- and phoborhodopsin (PR)-like pigment in these membranes was detected by flash spectroscopy. The kinetics of the cyclic photoreaction of the SR-like pigment was more than 15 times slower than that of *H. halobium*. A PR-like pigment existed in two strains, and the kinetics of the cyclic photoreaction of the PR-like pigment was similar to that found in *H. halobium*.

Introduction

Halobacteria, members of the domain Archaea (Woese *et al.*, 1990), live under extremely halophilic conditions. They are found in strongly saline lakes (such as the Great Salt Lake, the Dead Sea, or the alkaline, saline lakes of East Africa) and also in the worldwide salters where salt is commercially produced from seawater by evaporation.

The first bacterial retinal-containing protein, the light-driven proton pump bacteriorhodopsin (BR) was found in *Halobacterium halobium*. Since then, halorhodopsin (HR) with a light-driven chloride pump and two pigments with sensory functions, sensory rhodopsin (SR) and phoborhodopsin (PR) (or sensory rhodopsin-II) have also been found in *H. halobium* (Oesterhelt & Stoekenius, 1971; Matsuno-Yagi & Mukohata, 1977; Bogomolni & Spudich, 1982; Tomioka *et al.*, 1986*a*). Recently, these bacterial rhodopsins have been found in other halobacterial species, such as archaerhodopsin in halobacteria from Australia, and halorhodopsin- and phoborhodopsin-like pigments in the haloalkalophile *Natronobacterium pharaonis* (Mukohata *et al.*, 1988; Bivin & Stoekenius, 1986; Duschl *et al.*, 1990). The bacterial rhodopsin family is continuing to grow.

The amino acid sequences of bacteriorhodopsin, halorhodopsin and sensory rhodopsin in *H. halobium* are known (Dunn *et al.*, 1981; Blanck & Oesterhelt, 1987; Blanck *et al.*, 1989). Because BR exists in the purple membrane as highly ordered two-dimensional crystals, its three-dimensional structure has been obtained to a resolution of about 3.5 Å using electron diffraction imaging (Henderson *et al.*, 1990). Comparison of the amino acid sequences of these three bacterial rhodopsins reveals common residues enclosing the chromophore retinal (Blanck *et al.*, 1989).

BR has been extensively studied as a model for the elucidation of structure and function using biochemical and biophysical techniques (Stoekenius *et al.*, 1979). In addition, site-specific mutagenesis has identified several active-site residues for proton transport (Khorana, 1988). Three aspartic acids (positions 85, 96 and 212) appear to be essential for proton translocation to the Schiff base of the retinal (Mogi *et al.*, 1988; Otto *et al.*, 1990). No such experiments have been done for HR and SR yet, because of difficulties in the expression and purification of the protein, and the important residues for chloride ion transport and sensory functions are unknown. In this context, amino acid comparisons between bacterial rhodopsins isolated from other species could help to identify functionally important residues. A HR-like pigment from *N. pharaonis* has been identified, characterized and purified, and the amino acid sequence was recently determined (Bivin & Stoekenius, 1986; Duschl *et al.*, 1990; Lanyi *et al.*, 1990). On the basis of conserved

* Author for correspondence. Tel. (81) 484 62 1111 ext. 6333; fax (81) 484 65 8048.

Abbreviations: BR, bacteriorhodopsin; HR, halorhodopsin; SR, sensory rhodopsin; PR, phoborhodopsin; *bop*, bacteriorhodopsin; *hop*, haloopsin; *sop*, sensory opsin.

and conservatively replaced residues between *H. halobium* HR and *N. pharaonis* HR, several basic residues in the helical regions have been suggested to play a role in chloride ion transport.

In search of new bacterial rhodopsins, we first isolated several new halobacterial strains from crude solar salts commercially produced in Mexico and Australia. In this report, we examine the presence of BR-, HR-, SR- and PR-like pigments in five newly isolated halobacteria using flash spectroscopic measurements and light-induced ion transport measurements. In addition, we examine the homology of DNA from these organisms with the bacteriorhodopsin (*bop*) and haloopsin (*hop*) genes by DNA hybridization analysis.

Methods

Collection of crude solar salts and halobacterial strains. Crude solar salts were kindly provided by Japan Tobacco Inc. These salts were sent directly from the cargo boat and their origins identified as Guerrero Negro in Mexico, and Dampier, Macleod, Port Hedland and Shark Bay in Australia. *H. halobium* strain Flx3 (BR⁻, HR⁻, SR⁺, PR⁺) was a generous gift from Dr John L. Spudich. Strain ON1-bW (BR⁻, HR⁻, SR⁻, PR⁺ carotenoid⁻) arose from two separate spontaneous mutations of strain Flx3.

Isolation and characterization of new halobacteria from crude solar salts. The salts (~100 mg) were first dissolved in a complex medium used for growth of *H. halobium* (Oesterhelt & Stoekenius, 1974). After shaking vigorously for 6–8 d at 40 °C, the cultures were diluted about 1000 times with the medium and the dilutions were plated on complex medium solidified with 1.5% agar (Difco). Discrete red-purple coloured colonies were picked and inoculated into the complex liquid media. After 6–8 d growth, culture solutions were diluted, plated on the agar medium and discrete colonies picked. The cells from well-isolated colonies on the third liquid-culturing and plating were observed microscopically and checked for purity. We obtained seven different isolates from the five different solar salts. The cell shape and the motility of the isolates were monitored with infrared illumination selected by an infrared transmitting filter (Hoya IR-76) from a 12 V 50 W tungsten-halogen lamp beam (Philips 7023) and an infrared sensitive video camera (Sony XC-37) mounted on the phase contrast microscope (Nikon Optiphot XF-Ph).

Growth of cells and preparation of cell envelope vesicles. Newly isolated strains *H. halobium* R1 and ON1-bW were grown in the complex synthetic medium for 6–8 d. Because cells in early exponential growth phase contain larger amounts of PR than do stationary phase cells, cultures were used for the detection of PR-like pigment after 2 d growth. Cells were collected by centrifugation (8000 g, 15 min) and resuspended in 4 M-NaCl containing DNAase. Cell envelope vesicles were prepared by the freeze-thaw method. Resuspended cells were frozen at –80 °C overnight and thawed at room temperature. The suspension was washed with 4 M-NaCl three times by centrifugation (20000 g, 30 min). The envelope vesicles were further fractionated on a Ficoll density step gradient (25, 10 and 5% w/w) and the fraction between 10% and 5% was used for measurements of light-induced ion pump activities. In this preparation, the right-side-out envelope vesicles were mainly obtained as described (Li *et al.*, 1986). Protein concentration was determined by the Lowry method with bacteriorhodopsin as a standard.

Light-induced ion pump activities. Light-induced pH changes were measured with a glass pH electrode (Radiometer GK2321C) in the following apparatus. About 1.5 ml of a sample suspension was stirred in a cylindrical glass pH chamber (inner diam. 15 mm). A projector lamp (300 W) was used as a light source. The light was passed through a heat-absorbing water bath, two infrared cut filters and an orange bypass filter (>540 nm). Temperature was kept at 25 °C using a water bath. pH measurements were performed under flowing argon.

Flash spectroscopic measurements. The monitoring beam was provided by a 12 V 100 W tungsten-halogen lamp (Philips 7027) passed through a monochromator (Otsuka Electronics UM-001). After passing through the sample and an additional UM-001 monochromator, the beam was focussed onto a photomultiplier (Hamamatsu Photonics R374). The photomultiplier output was fed into a current-voltage converter and then digitized with an A/D converter (Datal ADC-HZ12B, USA) and transferred to a microcomputer (Sharp MZ-2200). The actinic flash was delivered at 90° to the monitoring beam with a xenon flash (Sunpak auto622) passed through a red filter (Irie R-62) for $\lambda > 620$ nm light, and a 487 ± 6 nm interference filter (Irie KL-48) plus a short-cut filter (Irie Y-46) for detection of PR-like pigment. All measurements were performed at room temperature.

DNA isolation and hybridization. Genomic DNA from five new strains and *H. halobium* R1 was isolated and purified according to Vogelsang *et al.* (1983). Chromosomal DNA was restricted with a number of endonucleases (Takara) and ~1 µg of each digest was fractionated on 1.0% (w/v) agarose slab gels (6.0 × 10.7 cm) and blotted onto nylon membrane (Oncor) using the VacuGene Vacuum Blotting system (Pharmacia). Hybridization and detection were done using the Non-Isotopic System kit (Oncor), which uses the properties of biotin/streptavidin interactions to visualize nucleic acid. Biotin-labelled DNA probes were prepared by incorporating biotin-modified nucleotides into DNA molecules using a standard nick-translation technique. Hybridization was done in 10 ml Hybrisol III solution containing the denatured biotin-labelled probe (final concn. 12.5 ng ml⁻¹) at 42 °C overnight (14–16 h). After the hybridization, the membrane was washed for 30 min in 0.16 × SSC and 0.1% SDS at room temperature. Following the instructions, the blots were stained at 37 °C in the dark for ~16 h. Biotinylated lambda DNA cut with *Hind*III was used as molecular mass markers (Oncor).

Partial *bop* (position –36 to 839; numbering as in Dunn *et al.*, 1981) and *hop* (positions 364 to 1022; numbering as in Blanck & Oesterhelt, 1987) genes were synthesized by PCR to serve as probes. The nucleic acid sequence of probe genes was confirmed by the dideoxy method using a DNA sequencer (Applied Biosystems Model 373A).

Results

Seven well-isolated colonies were obtained from crude solar salts, and their features are summarized in Table 1. Purple membrane was isolated by washing in water from strain damp alone. From strains mac and mex, a red-purple-coloured membrane was isolated instead of purple membrane. Five strains (damp, mac, mex, port and shark) and *H. halobium* R1 were further investigated for the presence of retinal proteins.

Cell envelope vesicles of these five strains and *H. halobium* R1 were prepared, and flash-induced absorbance changes were measured. Fig. 1 shows the absorbance changes at 570 nm and 410 nm upon excitation with

Fig. 1. Flash-induced absorbance changes in the cell envelope vesicles prepared from *H. halobium* R1 and five new halobacterial strains. The changes at 410 nm and 570 nm absorbances were monitored for 100 ms after a red actinic flash light (>610 nm). The samples containing 0.1 mg protein ml^{-1} (strains R1 and damp), 0.3 mg protein ml^{-1} (strains mac, mex and port) and 2.0 mg protein ml^{-1} (strain shark) total membrane protein were suspended in 4 M-NaCl and 25 mM-PIPES at pH 7.0. The traces were the averages of 20 (strains R1, damp, mac, mex and port) and 40 (strain shark) flashes.

Table 1. Summary of features of seven halobacterial strains newly isolated from crude solar salts

Salts from Guerrero Negro in Mexico, and Dampier, Macleod, Port Hedland and Shark Bay in Australia were collected. Strains were named after the place at which the salt was produced. The cell shape and the motility were monitored with the phase contrast microscope. The presence of purple membrane was tested by washing the total membrane fraction in water.

Name	Origin	Colour	Shape	Motility	Purple membrane
damp	Australia	Purple	Rods 1.0 ~ 6.0 μm	+	+
mac	Australia	Red	Rods 1.0 ~ 4.0 μm	+	—
mex	Mexico	Red	Rods 1.0 ~ 6.0 μm	+	—
port	Australia	Red	Rods 1.0 ~ 3.0 μm	+	—
shark	Australia	Orange	Rods 1.0 ~ 6.0 μm	$\pm$	—
mex 2	Mexico	Orange	Spheres 0.8 ~ 1.5 μm	+	—
shark 2	Australia	Orange	Rods 1.0 ~ 6.0 μm	+	—

a red flash light in the 100 ms time range. It is known that BR (absorption maximum ~ 570 nm) undergoes a photoreaction by light excitation, and then generates the long-lived M-intermediate (maximum at ~ 410 nm), whose recovery half-time is ~ 10 ms (Stoeckenius *et al.*, 1979). In all five strains, absorbance decreased at 570 nm and increased at 410 nm with a recovery half-time of 5–10 ms, indicating the presence of BR-like pigment in the cell envelope vesicles. This is further confirmed by transient absorbance changes at other wavelengths between 350 and 650 nm (Fig. 2). The flash-induced difference spectrum of all species shows the depletion maximum at ~ 580 nm and an absorbance increase at ~ 410 nm with the crossover point at ~ 460 nm. Therefore the absorbance changes, and the difference spectrum strongly indicates the presence of BR-like pigments. The recovery half-times at 410 nm in strains mac, mex, port and shark were faster than that found in *H. halobium* R1. Therefore the photocycle of the BR-like pigment in these strains is not identical to that in *H. halobium* R1. Assuming molar absorption coefficients similar to BR of *H. halobium*, we estimated that the

Fig. 2. Flash-induced absorbance difference spectra of *H. halobium* R1 and five new halobacterial strains. Each spectrum was obtained from an absorbance change 1 ms after the flash at various wavelengths (350 to 650 nm). The sample concentration is the same as described in Fig. 1.

BR-like pigments in the strains ranged from 0.3 to 104% of that in *H. halobium* R1 (Table 2).

Light-induced pH changes of the cell envelope vesicles were used to investigate light-induced ion pump activity (Fig. 3). In *H. halobium* R1, there was a rapid acidification of the external medium, followed by a slow alkalization. The fast acidification indicates the presence of BR molecules, which function as light-driven proton pumps from the inside to the outside of the vesicle. After denaturation of the other membrane proteins by the treatment with a weak acid at 40 °C (Kouyama *et al.*, 1987), the slow alkalization disappeared (data not shown). This slow alkalization is probably caused by the counterflux of proton due to the secondary ion transport system, such as a postulated Na⁺/H⁺ antiporter in *H. halobium* (Schobert & Lanyi, 1982). In the presence of the proton ionophore CCCP, which attenuates pH gradients, the pH change was smaller. An analogous light-induced pH change was found in the envelope vesicle from strain damp, implying similarities between it and *H. halobium* R1. In strains mac, mex and port, a rapid and extensive acidification in the external medium was observed without acid treatment of these vesicles, indicating that these strains contain BR-like pigment. In the presence of CCCP, a relatively large

Table 2. Relative amount of BR-like pigments in five halobacterial strains

The amount of the pigment was estimated from the light-induced absorbance changes at 410 nm and was expressed as percentage of the BR value for *H. halobium* R1.

Name	BR-like (%)
R1	100
damp	104
mac	19
mex	20
port	22
shark	0.3

alkalization of the external medium was observed in strains mac and mex but not port, suggesting the presence of a light-induced proton uptake. In strains mac and mex, therefore, there is a pigment functioning as an electrogenic light-induced ion pump which generates inside-negative membrane potential. In addition, this alkalization depended on chloride concentration of the medium (data not shown). These data strongly suggest the presence of a HR-like pigment which is known to function as a light-driven chloride pump from outside to

Fig. 3. Light-induced pH change of cell envelope vesicles suspension from various halobacterial strains. Proton transport was followed by pH change with (lower curve) and without (upper curve) CCCP (50 μ M). The vesicles were suspended in 3 M-KCl and 1 mM-HEPES buffer at pH 6.5. The sample contained 1 mg protein ml⁻¹ (in the case of shark, the protein concentration was 2 mg ml⁻¹). All measurements were done at 25 °C. Start and stop of the light illumination are indicated by arrows.

inside of the cell membrane. However, in view of the lack of alkalization in the presence of CCCP, strain port probably contains only BR-like pigment and not HR-like pigment. Slight acidification was detected in strain shark, indicating the presence of a small amount of BR-like pigment.

To determine whether these strains contain homologues of the *bop* and *hop* genes of *H. halobium* R1, total genomic DNA of the five strains was analysed by Southern blot hybridization. Although significant hybridization with the *bop* gene probe was observed for the DNA from strain damp (Fig. 4b; lanes 3 and 9), only faint signals of DNA fragments from other strains were detected (lanes 4–7 and 10–13) in the same blot. This result indicates that the gene encoding the BR-like pigment found in strain damp is homologous to that of *H. halobium* R1 and that the genes in other strains are not homologous. In the case of the *hop* gene, significant hybridization with DNA fragments of strain damp was also observed, again suggesting the presence of a homologous *hop* gene of *H. halobium* R1 (Fig. 4d; lanes 3 and 9). Faint hybridization bands were observed with DNA fragments from strains mac, mex and port (Fig. 4d; lanes 4–6 and 10–12), implying the presence of sequences with some homology to the *hop* gene of *H. halobium* R1. After a longer exposure, we could also observe a fainter signal from DNA fragments of strain shark (Fig. 4d; lanes 7 and 13).

After excitation of *H. halobium* R1 with the flash light (>620 nm), the absorption change at 570 nm does not recover completely for 100 ms, because of the presence of slow-cycling pigment (recovery half-time ~750 ms) in the cell envelope vesicles. This pigment is known as sensory rhodopsin, and has been suggested to mediate both the attractant and repellent photoresponse (Spudich & Bogomolni, 1984; Tomioka *et al.*, 1986b). Fig. 5 shows the flash-induced absorbance changes at 570 nm in cell envelope vesicles from various strains within the 3 s to

Table 3. Presence of BR-, HR-, SR- and PR-like pigment in five new halobacterial strains

The presence and relative amounts of the pigments was estimated from flash-induced absorbance change measurements and ion pump activity measurements, and are indicated by the number of plus signs. PR-like pigment of strain port was detected only in membrane from cells after 2 d of growth. The presence of HR- and PR-like pigments in strain damp was not confirmed.

Name	BR	HR	SR	PR
damp	+++	(+)	+	(+)
mac	++	++	+	ND
mex	++	++	+	ND
port	++	ND	ND	+
shark	+	ND	+	++

ND, Not determined.

Fig. 4. Southern blot hybridization analysis with the *bop* and *hop* genes in the genomic DNA from *H. halobium* strains R1 (lanes 2 and 8), damp (lanes 3 and 9), mac (lanes 4 and 10), mex (lanes 5 and 11), port (lanes 6 and 12) and shark (lanes 7 and 13). Lane 1 is biotinylated lambda DNA digested with *Hind*III. (a) Restriction patterns of the genomic DNA. Lanes 2 to 7: *Bam*HI cleavage; Lanes 8 to 13: *Pst*I cleavage. (b) Southern blot hybridization with a biotin-labelled probe prepared from the ~800 kbp *bop* gene fragment. The faint hybridizing bands in lanes 4 to 7 and 10 to 13 are marked by (◄). (c) Restriction patterns of the genomic DNA. Lanes 2 to 7: *Sac*I cleavage; lanes 8 to 13: *Sma*I cleavage. (d) Southern blot hybridization with a biotin-labelled probe prepared from the ~600 kbp *hop* gene fragment. The faint hybridizing bands in lanes 4 to 6 and 10 to 12 are marked by (◄). The faint hybridizing band after a longer exposure is marked by (●) in lanes 7 and 13. The *bop* and *hop* gene fragments were prepared using PCR technique as described in Methods.

100 s time range. A slow-cycling pigment with a recovery half-time of ~750 ms was observed in strain damp, suggesting the presence of a SR-like pigment similar to that of *H. halobium* R1. In strains mac, mex and shark, similar slow-cycling pigments were observed; however, the recovery half-time was more than 15 times slower than that found in SR of *H. halobium* R1. The flash-

induced absorbance difference spectra shown in Fig. 6 are similar to that found in SR from *H. halobium* R1 (Bogomolni & Spudich, 1982), suggesting the presence of SR-like pigment in strains damp, mac, mex and shark. A slow-cycling SR-like pigment was not observed in strain port.

PR is found in the cell membrane of the BR-, HR- and

Fig. 4 (cont.).

SR-negative mutant ON1-bW, which is derived from *H. halobium* R1 (Tomioka *et al.*, 1986a). This pigment functions as the receptor for negative phototaxis (Takahashi *et al.*, 1985). The absorption maximum of PR is ~490 nm and its photocycle is 2 to 3 times faster than that found in SR. Using an actinic flash of blue light (effective bandpass, 470–500 nm), only a PR pigment in the cell envelope vesicles should be excited. The flash-induced absorbance difference spectra and the transient absorbance changes at 450 nm are shown in Fig. 7. We detected a PR-like pigment only from the cell envelope vesicle of strains port and shark. The flash-induced difference spectrum and the kinetics of the absorbance change at 450 nm of strains port and shark are almost identical to those of ON1-bW. These results strongly

imply the presence of a PR-like pigment in these strains. We could not clearly detect the absorption changes between 350 and 650 nm in strains damp, mac and mex, because of the contributions from other absorption changes (BR, HR and carotenoid). A mutant strain without BR, HR and carotenoid is necessary to confirm the presence of PR-like pigment in the cell membrane from these strains.

Treatment with hydroxylamine is known to remove retinal from BR, HR, SR and PR (Oesterhelt *et al.*, 1974; Lanyi & Schobert, 1983; Tomioka *et al.*, 1986a). The cell envelope vesicles of all strains were exposed to 0.4 M-hydroxylamine at pH 7.0 in the light. After incubation for 24 h, there was a decrease in the flash-induced absorbance changes of BR-, SR- and PR-like pigments.

Fig. 5. Presence of SR-like pigment in the cell envelope vesicles from various strains determined by flash-induced absorbance difference changes. The change at 570 nm was monitored for 3 s (strains R1, damp and port), 100 s (strains mac and mex) and 50 s (strain shark) after a red actinic flash light (> 610 nm). The concentration of the total membrane protein was 1.0 mg ml^{-1} (strains R1, damp, mac, mex and port) and 2.0 mg ml^{-1} (strain shark) with 4 M-NaCl and 25 mM-PIPES at pH 7.0.

Addition of all-trans-retinal to these membranes after removal of hydroxylamine resulted in the reconstitution of photochemically active pigments, showing that all pigments required retinal (data not shown).

Discussion

Flash-induced absorbance changes and light-induced ion pump activities revealed that five newly isolated halobacterial strains have some retinal proteins. The presence of retinal pigments in the five strains is summarized in Table 3.

Photocycling rate and flash-induced difference spectra of both BR-like and SR-like pigments in cell envelope vesicles strongly suggest that bacteriorhodopsin and sensory rhodopsin from strain damp are very similar to

those in *H. halobium* R1. Hybridization analysis also indicated that the DNA from strain damp contained sequences homologous to both the *bop* and *hop* genes of *H. halobium* R1 (Fig. 4). Strain damp DNA was also homologous to the sensory opsin (*sop*) gene of *H. halobium* R1 (data not shown). Therefore, strain damp could contain the same retinal proteins as *H. halobium* R1.

Although strains mac and mex were independently isolated from Australia and Mexico, flash-induced absorbance changes and light-induced ion pump activities indicate that mac and mex strains contain similar retinal proteins. By washing the envelope vesicles from these two strains in water, we isolated a red-purple-coloured membrane in contrast with purple membrane from *H. halobium* R1. SDS-PAGE of this red-purple-coloured patch showed a single band with a molecular mass of $\sim 26 \text{ kDa}$ (data not shown), which is similar to that of BR. This membrane patch was also air-dried and observed by electron microscopy. Some cracks, formed at an angle of 120° from each other, were observed (our unpublished data), implying that the BR-like pigment from strains mac and mex aggregates to form a two-dimensional crystalline lattice of the cell membrane, much like the purple membrane.

The light-induced acidification of the external medium of the cell envelope vesicles clearly indicates that strains mac and mex contain a large amount of BR-like pigment. This acidification was also detected in 4 M-NaCl , suggesting that strains mac and mex have no secondary ion transport systems, such as an Na^+/H^+ antiporter, postulated to exist in *H. halobium* (Schobert & Lanyi, 1982). The light-induced alkalization in the presence of CCCP showed the presence of a HR-like pigment. Although the above features of BR- and HR-like pigment are similar to those of *H. halobium* R1, the hybridization analysis for the total genomic DNA with *bop* and *hop* gene probes clearly indicated that the genes encoding the BR- and HR-like pigments were not homologous to those of *H. halobium* R1. In addition, the photocycle of the SR-like pigment differed from that of SR from *H. halobium* R1. Therefore, strains mac and mex and their retinal proteins are not identical to *H. halobium* R1.

Although strain port contained a relatively large amount of BR-like pigment, neither purple membrane nor the red-purple-coloured membrane patch was isolated by washing in water. In contrast with purple membrane, this BR-like pigment probably does not make a specialized patch in the cell membrane. Therefore, this BR-like pigment seems to be different from that of strains mac and mex.

Although the flash-induced absorbance difference spectrum of the SR-like pigment is almost identical to

Fig. 6. Flash-induced absorbance difference spectra of the SR-like pigment. Each spectrum was obtained from the absorbance change 300 ms (strains R1, damp and port) or 10 s (strains mac, mex and shark) after the flash at various wavelengths (350 to 700 nm). Sample concentration was as described in Fig. 5.

Fig. 7. Presence of PR-like pigment in the cell envelope vesicles from strains ON1-bw, port and shark determined by flash-induced absorbance changes. Flash-induced absorbance difference spectra and the change in absorbance at 450 nm for 1 s after an actinic flash light (effective bandpass, 470 to 500 nm) are shown. Spectra were obtained from the absorbance change 100 ms after the flash at various wavelengths (350 to 650 nm). In strain port, cells in early exponential growth phase (~2 d) were used. The concentration of the total membrane protein was 2.0 mg ml⁻¹ with 4 M-NaCl and 25 mM-PIPES at pH 7.0.

that of *H. halobium* R1, its photocycle in strains mac, mex and shark is more than 15 times slower than that of *H. halobium* R1. This photocycle results in an increase in its intermediate form, which presumably mediates the repellent photoresponse by near-UV light. Therefore, these strains may be more sensitive to UV light under physiological conditions. It was suggested that the charge displacement by the amino acid replacement (Asp-96 in BR is replaced by Tyr-87 in SR) of the proton donor to the Schiff base caused the slow-cycling of SR (Blanck *et al.*, 1989). If this assumption is correct, this SR-like pigment would have a more positively charged residue instead of Tyr in this position.

At present, only one BR-like pigment (archaerhodopsin) in Australian *Halobacterium* (*Halobacterium* sp. aus-1) has been isolated, characterized and sequenced (Mukohata *et al.*, 1988; Sugiyama *et al.*, 1989). This pigment exists in the red-purple-coloured membrane patch (called claret membrane) similar to the BR-like pigment in strains mac and mex. A HR-like pigment, however, has not been detected in *Halobacterium* sp. aus-1. Therefore, this strain could be different from strains mac and mex. A number of *H. halobium* strains containing purple membrane have been isolated (Pfeifer, 1986). Their genetic features have been characterized and some differences between these strains have been revealed. However, the sequence of all these BRs have not yet been determined. Only BR in *H. halobium* strain GRB, which is genetically stable, has been sequenced and found to be identical to that of *H. halobium* (Soppa *et al.*, 1989). Halobacterial strains containing purple membranes could be widespread under extremely halophilic conditions, and strain damp, isolated from Australia, is one such.

Crude solar salts were kindly provided by Japan Tobacco Inc. We thank Mrs Yasuko Urabe for her assistance with DNA hybridization analysis. We would like to express our gratitude to Dr Naoki Kamo for his helpful discussion. This work was supported in part by a grant for 'Special Researchers' Basic Science Program' given by the Science and Technology Agency of the Japanese Government.

References

- BIVIN, D. B. & STOECKENIUS, W. (1986). Photoactive retinal pigments in haloalkaliphilic bacteria. *Journal of General Microbiology* **132**, 2167–2177.
- BLANCK, A. & OESTERHELT, D. (1987). The halo-opsin gene. II. Sequence, primary structure of halorhodopsin and comparison with bacteriorhodopsin. *EMBO Journal* **6**, 265–273.
- BLANCK, A., OESTERHELT, D., FERRANDO, E., SCHEGG, E. S. & LOTTSPEICH, F. (1989). Primary structure of sensory rhodopsin I, a prokaryotic photoreceptor. *EMBO Journal* **8**, 3963–3971.
- BOGOMOLNI, R. A. & SPUDICH, J. L. (1982). Identification of a third rhodopsin-like pigment in phototactic *Halobacterium halobium*. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 6250–6254.
- DUNN, R., MCCOY, J., SIMESK, J. M., MAJUMDAR, A., CHANG, S. H., RAJBHANDARY, U. L. & KHORANA, H. G. (1981). The bacteriorhodopsin gene. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 6744–6748.
- DUSCHL, A., LANYI, J. K. & ZIMANYI, L. (1990). Properties and photochemistry of a halorhodopsin from the Haloalkaliphile, *Natronobacterium pharaonis*. *Journal of Biological Chemistry* **265**, 1261–1267.
- HENDERSON, R., BALDWIN, J. M., CESKA, T. A., ZEMLIN, F., BECKMANN, E. & DOWNING, K. H. (1990). Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. *Journal of Molecular Biology*, **213**, 899–929.
- KOUYAMA, T., KOUYAMA, A. N. & ILEGAMI, A. (1987). Bacteriorhodopsin is a powerful light-driven proton pump. *Biophysical Journal* **51**, 839–841.
- KHORANA, H. G. (1988). Bacteriorhodopsin, a membrane protein that uses light to translocate protons. *Journal of Biological Chemistry* **263**, 7439–7442.
- LANYI, J. K. & SCHOBERT, B. (1983). Effects of chloride and pH on the chromophore and photocycling of halorhodopsin. *Biochemistry* **22**, 2763–2769.
- LANYI, J. K., DUSCHL, A., HATFIELD, G. W., MAY, K. & OESTERHELT, D. (1990). The primary structure of a halorhodopsin from *Natronobacterium pharaonis*. *Journal of Biological Chemistry* **265**, 1253–1260.
- LI, Q., GOVINDJEE, R. & EBREY, T. G. (1986). A proton release site on the C-terminal side of bacteriorhodopsin. *Photochemistry and Photobiology* **44**, 515–518.
- MATSUNO-YAGI, A. & MUKOHATA, Y. (1977). Two possible roles of bacteriorhodopsin; a comparative study of strains of *Halobacterium halobium* differing in pigmentation. *Biochemical and Biophysical Research Communications* **78**, 237–243.
- MOGI, T., STERN, L. J., MARTI, T., CHAO, B. H. & KHORANA, H. G. (1988). Aspartic acid substitutions affect proton translocation by bacteriorhodopsin. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 4148–4152.
- MUKOHATA, Y., SUGIYAMA, Y., IHARA, K. & YOSHIDA, M. (1988). An Australian halobacterium contains a novel proton pump retinal protein: archaerhodopsin. *Biochemical and Biophysical Research Communications* **151**, 1339–1345.
- OESTERHELT, D. & STOECKENIUS, W. (1971). Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. *Nature New Biology* **233**, 149–152.
- OESTERHELT, D. & STOECKENIUS, W. (1974). Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane. *Methods in Enzymology* **31**, 667–678.
- OESTERHELT, D., SCHUHMANN, L. & GRUBER, H. (1974). Light-dependent reaction of bacteriorhodopsin with hydroxylamine in cell suspensions of *Halobacterium halobium*: demonstration of an apomembrane. *FEBS Letters* **44**, 257–261.
- OTTO, H., MARTI, T., HOLZ, M., MOGI, T., STERN, L. J., ENGEL, F., KHORANA, H. G. & HEYN, M. P. (1990). Substitution of amino acids Asp-85, Asp-96, and Arg-82 in bacteriorhodopsin affects the proton release phase of the pump and pK of the Schiff base. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 1018–1022.
- PFEIFER, F. (1986). Insertion elements and genome organization of *Halobacterium halobium*. *Systematic and Applied Microbiology* **7**, 36–40.
- SCHOBERT, B. & LANYI, J. K. (1982). Halorhodopsin is a light-driven chloride pump. *Journal of Biological Chemistry* **257**, 10306–10313.
- SOPPA, J., OTOMO, J., STRAUB, J., TITTOR, J., MEESSEN, S. & OESTERHELT, D. (1989). Bacteriorhodopsin mutants of *Halobacterium* sp. GRB. II. Characterization of mutants. *Journal of Biological Chemistry* **264**, 13049–13056.
- SPUDICH, J. L. & BOGOMOLNI, R. A. (1984). The mechanism of colour discrimination by a bacterial sensory rhodopsin. *Nature, London* **312**, 509–513.
- STOECKENIUS, W., LOZIER, R. H. & BOGOMOLNI, R. A. (1979). Bacteriorhodopsin and the purple membrane of Halobacteria. *Biochimica et Biophysica Acta* **505**, 215–278.

- SUGIYAMA, Y., MAEDA, M., FUTAI, M. & MUKOHATA, Y. (1989). Isolation of a gene that encodes a new retinal protein, achae-rhodopsin, from *Halobacterium* sp. aus-1. *Journal of Biological Chemistry* **264**, 20859–20862.
- TAKAHASHI, T., TOMIOKA, H., KAMO, N. & KOBATAKE, Y. (1985). A photosystem other than PS370 also mediates the negative phototaxis of *Halobacterium halobium*. *FEMS Microbiology Letters* **28**, 161–164.
- TOMIOKA, H., TAKAHASHI, T., KAMO, N. & KOBATAKE, Y. (1986*a*). Flash-spectrophotometric identification of a fourth rhodopsin-like pigment in *Halobacterium halobium*. *Biochemical and Biophysical Research Communications* **139**, 389–395.
- TOMIOKA, H., TAKAHASHI, T., KAMO, N. & KOBATAKE, Y. (1986*b*). Action spectrum of the photoattractant response of *Halobacterium halobium* in early logarithmic growth phase and role of sensory rhodopsin. *Biochimica et Biophysica Acta* **884**, 578–584.
- VOGELSANG, H., OERTEL, W. & OESTERHELT, D. (1983). The isolation of the bacterioopsin gene. *Methods in Enzymology* **97**, 226–241.
- WOESE, C. R., KANDLER, O. & WHEELIS, M. A. (1990). Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria, and Eucarya. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 4576–4579.