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Detection and expression of a gene encoding a new bacteriorhodopsin from an extreme halophile strain HT (JCM 9743) which does not possess bacteriorhodopsin activity

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Abstract Membrane vesicles prepared from an extreme halophile strain, HT (JCM 9743), showed no bacteriorhodopsin activity. However, a DNA fragment, amplified by polymerase chain reaction (PCR), appeared to encode the C to G helices of a bacteriorhodopsin(bR)-like protein. With the PCR product as a probe, the gene coding for a novel bacteriorhodopsin was cloned from the genomic DNA of the strain HT. The open reading frame of the gene was ligated with the promoter region of the *bop* gene of *Halobacterium salinarum* bR, and expressed in a bR-deficient host strain, L33, using the plasmid vector pXLNov-R. The purplish membrane fraction purified from cells of a transformant exhibited a cyclic photoreaction characteristic of bacteriorhodopsin.

Key words Bacteriorhodopsin · Extreme halophile *Halobacterium salinarum* · Vector pXLNov-r · Photocycle

Introduction

Vitamin A aldehyde (retinal) is the basis of the visual pigments (Wald 1968) and is required also for photoenergy conversion in extremely halophilic archaea (Stoeckenius 1985). The well-studied species *Halobacterium salinarum* has four kinds of retinal-containing chromoproteins in the cell membranes: bacteriorhodopsin (bR, Oesterhelt and Stoeckenius 1971), halorhodopsin (hR, Mukohata et al. 1980), sensoryrhodopsin (sR, Bogomolni and Spudich 1982), and phoborhodopsin (pR, Tomioka et al. 1986). Two of these retinal proteins, bacteriorhodopsin (bR) and halorhodopsin (hR), are light-driven ion pumps. Bacteriorhodopsin transports protons from the inside to the outside of the cell and generates the transmembrane difference in the electrochemical potential for protons (Oesterhelt and Stoeckenius 1973). A cycle of conformational changes of bR molecules triggered by photoexcitation transports the protons (Stoeckenius 1985). This cyclic conformational change is called a photocycle, which is detectable by flash spectroscopy as a series of intermediates with distinct absorption spectra (Lozier et al. 1975).

Several new members of the bR family (light-driven proton pump family) have been reported in the last decade (Mukohata et al. 1988; Sugiyama et al. 1989, 1994; Uegaki et al. 1991; Otomo et al. 1992a,b; Tateno et al. 1994; Kitajima et al. 1996) Comparison of these bacteriorhodopsins revealed that the bR family is classified into at least three groups (Otomo et al. 1992b). In this paper we examine the presence of the bR activities and the encoding genes for the apoproteins in the type strains of established genera and a few unclassified strains of extreme halophiles. Two strains showed no bR activities although they had genes for bR-like proteins. One of these genes was cloned and expressed in a host *H. salinarum* strain L33 lacking bR (Needleman et al. 1991).

Materials and methods

Bacterial strains and isolation of chromosomal DNA

An extreme halophile strain, HT (JCM 9743), and *Halobacterium salinarum* (CCM 2090) were cultivated in JCM medium 168 (the same as DSM medium 372), an ordinary complex medium containing 20% NaCl (Kamekura and Dyall-Smith 1995) or in 25% NaCl-SGC (Sehgal and Gibbons complex medium). The taxonomic position of the strain HT is described in the Discussion. Other extreme

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halophiles were also cultivated in appropriate culture media and chromosomal DNAs were prepared as described previously (Kamekura and Dyall-Smith 1995). *Escherichia coli* strains and *H. salinarum* L33, used as hosts of plasmid vectors, were described previously (Needleman et al. 1991).

Measurement of bR and hR activities

Membrane vesicles were prepared from cells of various extreme halophiles, and cyclic photoreaction activities characteristic of halobacterial rhodopsins were measured as described before (Otomo et al. 1992a, Tomioka and Sasabe 1995). The detection limit of our spectroscopic measurement is about 60 bR molecules per cell.

Amplification of bR genes

Primer 1 (5′GAC TGG (CT)TG TTC AC(GC) AC(AG) CC, corresponding to the amino acid sequence DWLFTTP) and primer 2 [3′AAG TAC CA(GC) (GT)A(AG) CTG (GC)A, corresponding to the amino acid sequence FMV(L or I)D(V or L)] were synthesized, and polymerase chain reaction (PCR) was performed with chromosomal DNAs from various extreme halophiles as templates. PCR products were blunted, inserted to the *Sma*I site of pUC119, and introduced into *E. coli* JM109. Transformant colonies were grown up, their plasmid DNA isolated using Qiagen tip-20 columns, and the plasmid inserts sequenced by the chain termination method as described previously (Kamekura et al. 1992).

Cloning and sequencing of the bR gene from strain HT (*bop*HT)

A probe was prepared by the PCR with chromosomal DNA from the strain HT. The PCR products were labelled with digoxygenin-dUTP using a non-radioactive DNA labelling and detection kit (Boehringer Mannheim, Mannheim, Germany), and used in Southern blot and colony hybridization experiments.

The chromosomal DNA of the strain HT was digested with *Nae*I and electrophoresed in a 1% agarose gel, and 1.2-kb fragments were electro-eluted, which were then blunted, ligated with *Sma*I-cut pUC119, and used to transform *E. coli* JM 109 to ampicillin resistance. Transformants harboring plasmids containing the gene encoding the HT bR were selected by colony hybridization, and the nucleotide sequence of the insert was determined.

The nucleotide sequence for the gene, which we designate *bop*HT in this paper, has been assigned DDBJ accession number AB003751.

Plasmid vectors

The nonintegrating plasmid vector pXLNov-R, developed by the group of Lanyi and Needleman (Brown et al. 1995), was kindly provided by R. Needleman of Wayne State University. Selection markers were tetracycline for *E. coli* (10 μg/ml) and novobiocin for *H. salinarum* L33 (1 μg/ml), and the cloning sites were *Bam*HI and *Hin*dIII. pT7Blue T-vector was obtained from Novagen (Madison, WI, USA).

Expression of *bop*HT

To express the *bop*HT gene, the promoter region of the *H. salinarum* bR gene (Dunn et al. 1981; called *bop*HH in this paper) was ligated with the open reading frame of the *bop*HT. A 363-bp stretch preceding the triplet ATG encoding the N-terminal methionine of *bop*HH was amplified by PCR with primer 3 (5′GGGTGGATCCGTGAAGTCCGC) and primer 4 (3′GAGCAATCCATTCGAACGTAC). These primers were designed from the nucleotide sequence of *bop*HH from −360 to −340 and from −18 to +3, respectively. The underlined parts were modified to produce new restriction sites of *Bam*HI and *Hin*dIII, respectively. The PCR products were cloned into pT7Blue T-vector, which was used to transform *E. coli*. From a transformant, the plasmids were extracted and digested with *Bam*HI and *Hin*dIII. The extracted fragments were ligated with pXLNov-R which had been digested with *Bam*HI and *Hin*dIII. A plasmid obtained, pXLbopHH, was amplified in *E. coli*, extracted, and cut with *Hin*dIII.

Downstream of the ATG coding for the start methionine of *bop*HT was amplified by PCR with primers 5 (5′GGGTCAAGCTTTATGTGTTAC) and 6 (3′AAGGCCATTCGAACACACGTC). These primers were designed from the base sequence of *bop*HT from −12 to +9 and from 820 to 840, respectively (see Fig.1). The underlined parts are the new *Hin*dIII sites. The products were also cloned as already described, and 830-bp *Hin*dIII fragments were inserted to the *Hin*dIII-cut pXLbopHH. Since there was a *Bam*HI site at nucleotide 590 of the *Hin*dIII fragment of the *bop*HT, the directions of the inserted *Hin*dIII fragments were determined by digesting plasmid DNAs with *Bam*HI. The junction point of the final plasmid pXLNovHT9 was ascertained by sequencing, amplified, and used for the transformation of *H. salinarum* L33 to novobiocin resistance. Transformation was performed as described by Needleman et al. (1991).

A culture of the transformant, 63-9-1, was inoculated into 20 ml of 25% NaCl-SGC with novobiocin, and then transferred to 4 l of fresh SGC without novobiocin in a 5-l batch fermentor. After cultivation for several days at 37°C with an aeration of 3.5 l per min and no agitation, cells were harvested and suspended in 4 M NaCl.

Purification of HT bR

The purplish membranes from transformant cells were partially purified according to the method described for the preparation of the purple membranes from *H. salinarum* R1 (Oesterhelt and Stockenius 1971), i.e., by dialysis against distilled water, repeated washing, and sucrose density gradient ultracentrifugation. HT bacteriorhodopsin was ex-

Table 1. Bacteriorhodopsin activities and the encoding genes

	bR activity[a]	Gene	Reference
Strains investigated previously			
Halobacterium halobium[b] strain S9	+	+	Dunn et al. 1981
sp.strain GRB	+		Soppa et al. 1989
sp.strain damp	+	+	Otomo et al. 1992a,b
sp.strain arg-3	+	+	Tateno et al. 1994
Halorubrum sp.strain aus-1	+	+	Sugiyama et al. 1989
sp.strain aus-2	+	+	Uegaki et al. 1991
sp.strains mac, mex, shark	+	+	Otomo et al. 1992a,b
strain SG1	+	+	Soppa et al. 1993
Haloarcula vallismortis	+	+	Kitajima et al. 1996
argentinensis	+	+	Tateno et al. 1994
mukohataei	+	+	Sugiyama et al. 1994
sp.strains port, mex2, shark2	+	+	Otomo et al. 1992a,b
Strains investigated in this study			
Halorubrum saccharovorum	–[c]	–	
lacusprofundi	–	–	
vacuolatum		–	
sodomense	+	+	
coriense	+	+	
distributum		+	
Haloarcula "sinaiiensis"		+	
hispanica		+	
Haloferax volcanii	–	–	
denitrificans		–	
gibbonsii	–	–	
mediterranei		–	
Halococcus morrhuae	–	–	
saccharolyticus		–	
salifodinae		–	
turkmenicus		–	
Natronococcus occultus	–		
Natronobacterium gregoryi		–	
Natronomonas pharaonis	–	–	
Natrialba asiatica strain 172P1		–	
strain B1T		–	
sp.strain SSL1		–	
Strain GSL11	–	+	
Strain HT	–	+	

[a] bR activity (–): below our detection limit of 60 bR molecules per cell.
[b] *H. halobium* is a synonym for *H. salinarum*. See Kamekura et al. (1997) for the taxonomy of extreme halophiles.
[c] K. Ihara, Personal communication.

tracted from the membrane with 30 mM octyl-thioglucoside (Saito and Tsuchiya 1984).

Results

Bacteriorhodopsin activities in halophilic archaeal genera

The presence of bR in extreme halophiles reported so far is summarized in the upper half of Table 1. Bacteriorhodopsins are found in representatives of three genera, *Halobacterium, Halorubrum*, and *Haloarcula*. In the present study we examined the presence of photoactive bRs in representatives of several other genera (lower half of Table 1). Extreme halophiles belonging to the genera *Haloferax, Halococcus, Natronobacterium, Natronomonas*, and *Natrialba* do not possess bR activities, though most isolates were pigmented bright red.

Alignment of the amino acid sequences of six bRs reported (Dunn et al. 1981; Otomo et al. 1992b; Mukohata 1994) had demonstrated that the helices C and G are highly conserved. This fact prompted us to search for any dormant genes, if present, that are not expressed in the host cells.

Detection of dormant *bop* genes

The primers 1 and 2 were designed from the conserved base sequences of the genes corresponding to the following amino acid sequences, DWLFTTP of the helix C, and FMV(L,I)D(V,L) of the helix G, respectively. Using these primers, PCR was performed to amplify the encoding genes. As expected, all strains which have been shown to exhibit bR activities gave clear DNA bands (Table 1). On

Fig. 1. Nucleotide sequence of the cloned *bop*HT and deduced amino acid sequence. The initiation and termination codons (ATG and TGA) are *underlined*. Amino acids cleaved off are shown in *small letters*. *Underlined* amino acid sequence was determined by protein sequencing

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                                GCCGGCGTA GACCCCCACC CAGAGGACGA GCGGCGACGA -361
CCACAGCGGA CCNCNGCCCG GNAACGTCGC TTGTACTGAC GACGGATAGG TGGCGACGAT -301
CGGAACGACG AGCGCGACAC GAGCAGCAGG CCGCTCTGGT AGACGAGTTT TTNTGGAATG -241
CGGCGCTTGA TCAGCGCGTA TCGCTCAACT CTNAGTCGTT CGTACGTCGA TTCGCCGAGG -181
AGTGCGTCCG CAATCGGTTC GTTCGGNTCA GTCGAGGTCA TCGGTTGTGG AGGGAGCGTT -121
GTGATCGGGC TATCCGACCG AACACAGAAC ANCCCAAAAT ACGNTGCGGT TTCATCTTCC  -61
ACGTCAAACT TCTGGACATA CACGGTACAA TAAGAGTTGT TTCCGTTAGG GTCAAGCAAT   -1

ATGTGTTACG CTGCTCTAGC ACCACCCATG GCCGCAACAG TTGGCCCAGA ATTCATTTGG   60
m  c  y  a   a  l  a   p  p  m   A  A  T  V   G  P  E   F  I  W

TTATGGATCG GCACGATCGG CATGACCCTC GGAACCCTGG TATTCGTCGG TCGCGGACGT  120
L  W  I  G   T  I  G   M  T  L   G  T  L  V   F  V  G   R  G  R

GGCGTTCGTG ATCGGAAAAT GCAGGAGTTC TATATCATCA CGATCTTCAT CACAACCATC  180
G  V  R  D   R  K  M   Q  E  F   Y  I  I  T   I  F  I   T  T  I

GCCGCTGCCA TGTACTTCGC GATGGCAACC GGCTTCGGCG TCACCGAAGT CATGGTCGGC  240
A  A  A  M   Y  F  A   M  A  T   G  F  G  V   T  E  V   M  V  G

AACGAGGCGC TCACGATCTA CTGGGCGCCG TACGCCGACT GGCTGTTCAC GACGCCGCTG  300
N  E  A  L   T  I  Y   W  A  P   Y  A  D  W   L  F  T   T  P  L

CTGTTGCTCG ACCTCTCGCT GCTCGCCGGG GCGAACCGAA ACACGATCGC GACGCTGATC  360
L  L  L  D   L  S  L   L  A  G   A  N  R  N   T  I  A   T  L  I

GGCCTCGACG TTTTCATGAT CGGAACCGGC GCGATCGCAG CGCTCTCGTC CACCCCGGGT  420
G  L  D  V   F  M  I   G  T  G   A  I  A  A   L  S  S   T  P  G

ACCCGGTTCG CCTGGTGGGC GATCAGCACC GGTGCTCTGC TCGCCCTGCT GTACGTCCTC  480
T  R  F  A   W  W  A   I  S  T   G  A  L  L   A  L  L   Y  V  L

GTCGGGACGC TCTCCAAGAA CGCGCGCAAC CGGGCCCCCG AGGTCGCATC GCTGTTCGGG  540
V  G  T  L   S  K  N   A  R  N   R  A  P  E   V  A  S   L  F  G

AGACTCCGCA ACCTGGTTAT CGCGCTGTGG TTCCTCTACC CGGTGGTCTG GATCCTCGGC  600
R  L  R  N   L  V  I   A  L  W   F  L  Y  P   V  V  W   I  L  G

ACGGAAGGGA CGTTCGGCAT CCTTCCGCTG TACTGGGAAA CCGCGGCGTT CATGGTGCTC  660
T  E  G  T   F  G  I   L  P  L   Y  W  E  T   A  A  F   M  V  L

GACCTCTCGG CAAAGGTCGG ATTCGACGTG ATCCTGCTCC AGAGCCGCTC CGTCCTGGAG  720
D  L  S  A   K  V  G   F  D  V   I  L  L  Q   S  R  S   V  L  E

CGGGTCGCGA CGCCGACGGC TGCCCCGACC TGAGGCCGCT GCATCGACTC CCGACGGACG  780
R  V  A  T   P  T  A   A  P  T

GTGCCAGCGA CCGACGCGAT AGCAGAGACC CGGACGCTGT TCCGGTCTGC TCGTGTGCAG  840
CCGGC                                                              845
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the other hand, none of the species of the genera *Haloferax, Halococcus, Natronobacterium, Natronomonas*, or *Natrialba* gave any clear PCR products. Two species of the genus *Halorubrum* (*H. saccharovorum* and *H. lacusprofundi*) also did not give positive PCR products.

Interestingly, strain GSL11 and strain HT, which showed no bR activity, gave unambiguous PCR products with similar sizes to those from strains which exhibited bR activities. A membrane vesicle preparation of the strain HT did not show any halorhodopsin activity either.

The PCR-amplified DNA fragments of about 400 bp from the strains GSL11 and HT were sequenced and translated into 129 amino acids. There were only six amino acid-differences between the two sequences. All of the highly conserved regions of bR were present, suggesting that the two strains have genes encoding novel types of bRs.

Cloning and expression of the *bop*HT gene

The cloned gene *bop*HT was 1244 bp in length, and analysis of the sequence suggested it encoded a protein with 250 amino acids (Fig. 1). The plasmid pXLNovHT9 was transformed with *H. salinarum* strain L33, and fifty colonies were

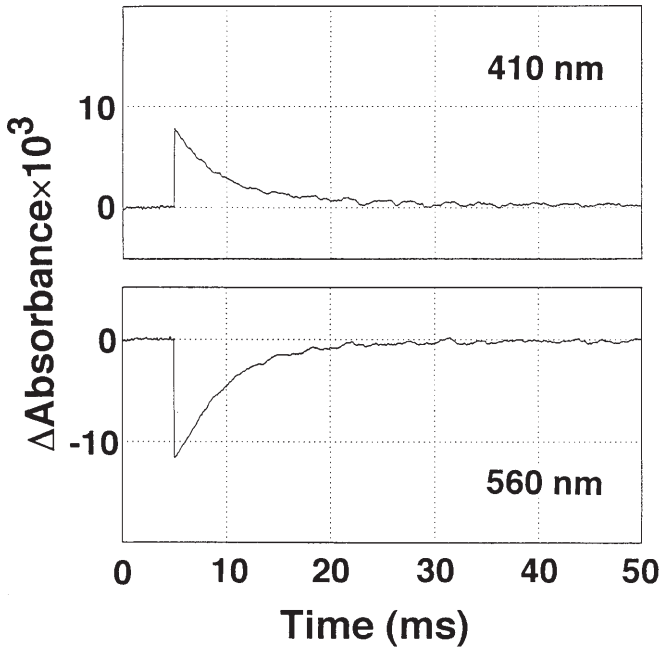

Fig. 2. Flash-induced absorbance changes in the purplish membrane fraction suspended in 4 M NaCl, 25 mM 1,4-piperazinediethanesulfonic acid (PIPES)/NaOH (pH7.2). An actinic red flash (620 ± 5 nm) was obtained through an interference filter (B-620, Asahi Spectra Co. Ltd, Tokyo, Japan). Absorbance changes at 560 nm and 410 nm were measured at 21°C

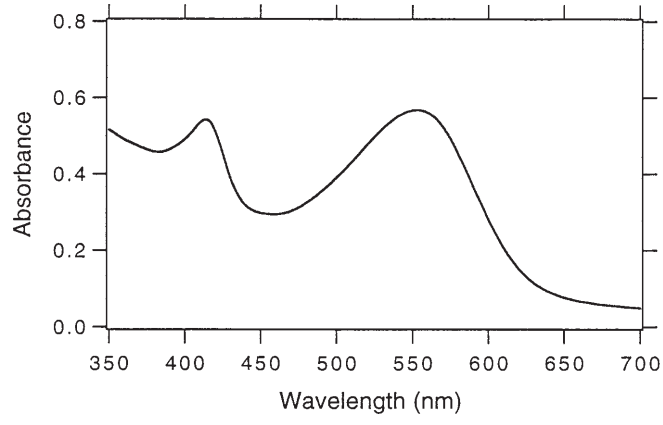

Fig. 3. Absorption spectrum of the purplish membrane fraction from transformant 63-9-1. The purplish membrane fraction was suspended in 4 M NaCl, 25 mM PIPES/NaOH (pH7.2)

inoculated into SGC medium with novobiocin and shaken at 37°C. Six colonies gave good growth in the medium, and two transformants, 60-9-1 and 63-9-1, turned slightly purple after prolonged incubation.

The photoactivity of bR in the purplish membrane fraction obtained from the transformant 63-9-1 was measured by flash spectroscopy. Absorbance changes induced by a red flash (620 ± 5 nm) were monitored in a 50-ms time window (Fig. 2). A transient decrease at 560 nm and an increase at 410 nm were observed. These absorbance changes are characteristic of the change of the original state of bR and that of the Mintermediate in the photocycle of bR, respectively (Lozier et al. 1975). Figure 3 shows the absorption spectrum of the purplish fraction. An absorption maximum at 552 nm is attributable to HT bR and another maximum at 413 nm indicates the presence of cytochromes. HT bR in the purplish membrane fraction is shifted to a shorter wavelength in comparison with *salinarum* bR.

A purified preparation of the purplish membrane was treated with sodium dodecyl sulfate (SDS) and electrophoresed in SDS-polyacrylamide gel. Proteins were extracted from the gel, and the amino acid sequence was determined using an ABI sequencer. The N-terminal amino acid sequence was AATVGPESIWLWIGTIGMTL, suggesting that the N-terminal 10 amino acids (written in small letters in Fig. 1) were cleaved off, as in the case of *salinarum* bR, in which 13 amino acids are cut off. Alignment of the amino acid sequence of the mature HTbR with those of other bRs showed that HTbR belongs to a novel, fourth group of bacteriorhodopsin-like proteins.

Discussion

Otomo et al. (1992b) have assigned a bacteriorhodopsin produced by the *Halobacterium* strain damp to the group of *salinarum* bR, and those from strains mac and mex to the archaerhodopsin group produced by strains aus-1 and 2. The other bR-like proteins from strains port, shark2, and mex2 formed another group of bacteriorhodopsins. Afterwards, these bR-like proteins were shown to form a group designated as cruxrhodopsins (Tateno et al. 1994). Assignments to genera in Table 1 of the seven strains used by Otomo et al. (1992b) are based on partial base sequences (740 bp) of 16S rRNA genes (Kamekura and Tomioka, unpublished data).

Species of the genera *Haloferax* and *Halococcus* did not give any PCR products with a combination of the primer 1 and 2, thus suggesting that the absence of the gene coding for the bR-like proteins might be a taxonomic marker for these genera. The genus *Halorubrum* seems to be divided into two subgroups, one with bR activities and another without the activity, even without detectable genes. There remains, however, a possibility that the designed primers 1 and 2 were not appropriate to amplify the unknown "genes."

One of the authors of this paper, Kamekura, obtained the strain HT used in this study, courtesy of Roger Latta, from the culture collection of the National Research Council of Canada (NRC) in 1984. This strain has been described as *H. trapanicum* NCIMB 767 in previous studies on the taxonomy of *Halobacteriaceae* and shown to be closely related to the strain GSL11 (L11) by 16S rRNA gene comparison and polar lipid profiles (Kamekura and Seno 1993, Kamekura and Dyall-Smith 1995). Recently, the type strain of *H. trapanicum*, NRC 34021, has been transferred to a new genus, *Halorubrum* (McGenity and Grant 1995; Oren

and Ventosa 1996). There has been some confusion, however, on the taxonomic position of *H. trapanicum*, as discussed by Kamekura and Dyall-Smith (1995). Thus, we designate the strain used in this work as strain HT until the confusion is resolved by comparative studies (Oren and Ventosa 1997).

Expression of the *bop*HT gene was first attempted by using the plasmid vector pMSD30 and *Haloferax volcanii* WFD11 as a host. This system, with novobiocin as a selection marker, has been successfully applied for the production of the extracellular serine proteinase halolysin R4 from the extreme halophile *Haloferax mediterranei* (Kamekura et al. 1996). The *bop*HT was subcloned into the *Sma*I site of pMDS30, and a transformed *H. volcanii* was cultivated in the presence of 5 μM retinal. The membrane vesicles prepared, however, did not show any bR activity. The combination of pXLNov-r and *H. salinarum* L33 has proven an excellent host–vector system for the expression of the *bop*HT.

The mechanism of the light-driven proton pumping by bacteriorhodopsin from *H. salinarum* and *Halobacterium* sp. GRB has been extensively studied by site-specific mutagenesis (Krebs and Khorana 1993; Soppa et al. 1989). Asp85 serves as the acceptor for the proton from the isomerized retinylidene Schiff base, and Asp96 participates in reprotonation of this group. Several other residues have been shown to affect the bR properties when substituted by other amino acids. These amino acid residues, as well as those forming the retinal pocket (Henderson et al. 1990) are conserved in the HT bR.

The detection limit of our spectroscopic measurement of bR is about 60 molecules per cell. Bogomolni and Spudich (1982) estimated their detection limit as 80 bR molecules per cell and determined the bR content of a strain of *H. salinarum*, S9-P, as 1.9×10^5 per cell. Thus, the absence of bR activity in the membrane vesicles of the strain HT means that the content of bR molecules, if present, is less than 0.03% of that of the strain S9-P. We thus consider that the reason for the absence of bR activity may be a defective promoter region.

H. salinarum loses its ability to produce purple membrane at a frequency of about 10^{-4}. Analyses of these mutants have revealed the existence of at least two additional genes, designated *brp* (bacterio-opsin related protein) and *bat* (bacterio-opsin activator), that are required for the *bop* expression. A series of different insertion element mutants located either in *bop*, *brp*, *bat*, or the intergenic region were shown to greatly reduce or abolish transcription of all three genes (Dennis 1993). Although there is no information on the *brp* or *bat* related genes in the strain HT, it is possible that some insertion element(s) reside(s) in these genes or region, but not in the *bop*HT itself. This was shown by the successful expression of the *bop*HT using the 360-bp intergenic region of *brp* and *bop*HH as a promoter.

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