Complete Biosynthetic Pathway of the C_{50} Carotenoid Bacterioruberin from Lycopene in the Extremely Halophilic Archaeon *Haloarcula japonica*

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Running Title: Biosynthetic Pathway of C_{50} Carotenoid in *Ha. japonica*

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ABSTRACT

***Haloarcula japonica*, an extremely halophilic archaeon that requires high concentrations of NaCl for growth, accumulates the C_{50} carotenoid bacterioruberin. By homology analysis, a gene cluster, including *c0507*, *c0506,* and *c0505*, was found and predicted to be involved in the synthesis of bacterioruberin. To elucidate the function of the encoded enzymes, we constructed *Ha. japonica* mutants of these genes and analyzed carotenoids produced by the mutants. Our research showed that *c0507*, *c0506*, and *c0505* encoded a carotenoid 3,4-desaturase (CrtD), a bifunctional lycopene elongase and**

1,2-hydratase (LycJ), and a C_{50} carotenoid 2″,3″-hydratase (CruF), respectively. The above three carotenoid biosynthetic enzymes catalyze the reactions that convert lycopene to bacterioruberin in *Ha. japonica*. This is the first identification of functional CrtD and CruF in archaea, and elucidation of the complete biosynthetic pathway of bacterioruberin from lycopene.

IMPORTANCE

Haloarcula japonica, an extremely halophilic archaeon, accumulates the C_{50} carotenoid bacterioruberin (BR). In this study, we have identified three BR biosynthetic enzymes and have elucidated their functions. Among them, two enzymes were found in an archaeon for the first time. Our results revealed the biosynthetic pathway responsible for production of BR in *Ha. japonica* and provides a basis for investigating carotenoid biosynthetic pathways in other extremely halophilic archaea. Elucidation of the carotenoid biosynthetic pathway in *Ha. japonica* may also prove useful for producing the C_{50} carotenoid BR efficiently by employing genetically modified haloarchaeal strains.

Carotenoids are natural pigments synthesized by bacteria, archaea, algae, fungi, and plants (1). They are involved in photosynthesis as accessory pigments (2), functioning as antioxidants (3,4), light protection pigments (5,6), and membrane stabilizers (7). Until now, more than 750 carotenoids have been identified and classified as C_{30}, C_{40}, and C_{50} carotenoids, depending on the number of carbons in their carotene backbones (1). Most of these compounds are based on the symmetric C_{40} backbone, phytoene, which is formed by condensation of two molecules of geranylgeranyl pyrophosphate (GGPP, C_{20}PP) (8). Only a small number of C_{30} carotenoids, which arise from the fusion of two molecules of farnesyl pyrophosphate (FPP, C_{15}PP), and even

fewer C_{50} carotenoids, which are derived from the C_{40} structure by the addition of two 5-carbon (C_5) isoprene units, have been discovered to date (1).

In nature, several restricted groups of bacteria show accumulation of C_{50} carotenoids, including species belonging to the bacterial genera *Corynebacterium*, *Dietzia*, *Micrococcus*, and extremely halophilic archaea, such as *Halobacterium salinarum* (9,10). To date, only three biosynthetic pathways of cyclic C_{50} carotenoids, the ε-cyclic C_{50} carotenoid decaprenoxanthin in *Corynebacterium glutamicum* (11-13), the β-cyclic C_{50} carotenoid 2,2′-bis-(4-hydroxy-3-methybut-2-enyl)-β,β- carotene in *Dietzia* sp. CQ4 (14), and the γ-cyclic C_{50} carotenoid sarcinaxanthin in *Micrococcus luteus* NCTC2665 (15), have been described, based on their structures. The acyclic C_{50} carotenoid bacterioruberin (BR) is known to be produced in some extremely halophilic archaea. BR of *Haloferax volcanii* has been isolated and identified by chromatographic (TLC and HPLC), spectroscopic (MS, NMR, and CD), and chemical (silylation and methylation) methods (16). *Haloferax mediterranei* can also accumulate BR and a two-stage culture method has been applied to increase production of C_{50} carotenoids in the laboratory (17). In addition to studies on the antioxidant properties of BR (18), the regulating mechanism of BR biosynthesis in *Hb. salinarum* has been investigated (19). *Hb. salinarum* is also known to produce the light-induced proton pump bacteriorhodopsin, comprising retinal and bacterioopsin (20). The carotenoids and retinal biosynthetic pathways of *Hb. salinarum* have been briefly described previously (10). This report proposed that retinal and BR are synthesized from a common precursor, lycopene, which is generated from phytoene as in C_{40} carotenoid biosynthetic pathways. Although the enzyme Lye, which catalyzes the committed step in BR biosynthesis, has been described in *Hb. salinarum*, the details of the BR biosynthetic pathway remain unclear.

Haloarcula japonica is a predominantly triangular, disc-shaped extremely halophilic archaeon that requires high concentrations of NaCl for growth (21). In our previous study, we

found that *Ha. japonica* can produce isopentenyldehydrorhodopin (IDR, C_{45}), bisanhydrobacterioruberin (BABR, C_{50}), and monoanhydrobacterioruberin (MABR, C_{50}) as intermediates, and that BR is produced as the final product (22). In addition, it has been suggested that *Ha. japonica* also produces the retinal proteins, cruxrhodopsin (23) and cruxhalorhodopsin (24). Thus, retinal could also be synthesized from carotenoids in *Ha. japonica*. Based on the results of our previous study and the proposed carotenoids biosynthetic pathways in *Hb. salinarum* (10), the carotenoids and retinal biosynthetic pathways in *Ha. japonica* are suggested to be as shown in Fig. 1. Phytoene synthase (CrtB) condenses two GGPP molecules to yield the colorless carotenoid, phytoene. Lycopene is generated from phytoene via a series of desaturation reactions that are catalyzed by phytoene desaturase (CrtI). Subsequently, the steps are divided into the retinal biosynthetic pathway and the BR biosynthetic pathway. In retinal synthesis, the cyclization of lycopene to β-carotene is catalyzed by lycopene β-cyclase (CrtY), and β-carotene is then cleaved to form retinal (C_{20}) by β-carotene cleavage dioxygenase (Brp). In the BR biosynthetic pathway, lycopene is used as a precursor and is converted to BR by introduction of two C_5 isoprene units, two double bonds, and four hydroxyl groups to the lycopene. All of the related enzymes and the complete pathway for biosynthesis of BR from lycopene remain unknown.

In this study, we identified and characterized three main BR biosynthetic enzymes in *Ha. japonica*, namely, C0506, C0507, and C0505. They function as a bifunctional lycopene elongase and 1,2-hydratase (LyeJ), a carotenoid 3,4-desaturase (CrtD), and a C_{50} carotenoid 2″,3″-hydratase (CruF), respectively, to generate BR from lycopene. These findings contribute to elucidation of the complete carotenoid biosynthetic pathway in extremely halophilic archaea.

EXPERIMENTAL PROCEDURES

Microbial strains and growth conditions. All strains and plasmids used in this study are

listed in Table 1. The wild type and mutant strains of *Haloarcula japonica* (JCM 7785^T) were grown at 37°C in the dark with a complex medium, as described previously (25). The medium was supplemented with 8 μg/ml pravastatin (a gift from Daiichi Sankyo, Tokyo, Japan) instead of mevinoline, when required. *Escherichia coli* was cultured in LB medium at 37°C (26). Ampicillin (50 μg/ml) was added when required.

Isolation of genomic DNA and total RNA from *Ha. japonica*. *Ha. japonica* genomic DNA was isolated as described previously (27). Total RNA of *Ha. japonica* was prepared using Sepasol RNA I (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. The resulting total RNA was further treated with DNase I (GE Healthcare, Bucks, UK) to remove trace amounts of contaminating genomic DNA.

RT-PCR and PCR. Oligonucleotide primers for RT-PCR and PCR were purchased from Operon Biotechnologies (Tokyo, Japan). The oligonucleotide primers used in this study are listed in Table 2. RT-PCR was performed under the following conditions. Total RNA (1.0 μg) was reverse-transcribed at 45°C for 60 min in 20 μl of the reaction buffer, which contained 20 pmol of each primer (c0507-A, c0506-A, or c0505-A), 0.3 mM of each deoxynucleotide triphosphate, 2.5 mM manganese (II) acetate, 20 U RNase inhibitor (Toyobo, Osaka, Japan), and 5 U *rTth* DNA Polymerase (Toyobo). The cDNA generated was then amplified by PCR. PCR was carried out using KOD-Plus- or KOD-Dash (Toyobo) DNA polymerase, according to the manufacturer's instructions. Primer set c0507-S/c0507-A, c0506-S/c0506-A, or c0505-S/c0505-A was used to confirm the transcription of *c0507*, *c0506,* or *c0505* in the wild type and mutant strains of *Ha. japonica*.

Construction of *c0507*, *c0506*, and *c0505* mutant strains. Mutants with deletions and frame shift mutations were constructed by homologous recombination (28). The plasmid pWL102-Δ*c0507*, containing a disrupted *c0507*, was constructed as follows. The targeted gene, *c0507*, was amplified from *Ha. japonica* using the primer set c0507-S1/c0507-A1. The

amplified PCR fragment was ligated into the *Sma*I site of pUC119, yielding plasmid pUC119-*c0507*. A 652-bp DNA fragment, ranging from base numbers 378 to 1029 of *c0507,* was excised from pUC119-*c0507* by *Csp*45I and *Eco*RV digestion, followed by blunting and self-ligation, to yield plasmid pUC119-Δ*c0507*. Plasmid pUC119-Δ*c0507* was digested with *Eco*RI and *Hin*dⅢ, releasing a disrupted *c0507*. Plasmid pWL102 (29) was also digested with *Eco*RI and *Hin*dⅢ to eliminate the *Hf. volcanii* ori, and was then ligated to the disrupted *c0507*, yielding plasmid pWL102-Δ*c0507*.

The plasmids pDrHj2-Δ*c0506,* containing a disrupted *c0506*, and pDrHj2-Δ*c0505,* containing a disrupted *c0505*, were constructed as follows: The targeted genes, *c0506* and *c0505,* were amplified from *Ha. japonica* using the primer sets c0506-S1/c0506-A1 and c0505-S1/c0505-A1, respectively. Amplified PCR fragments were ligated into the *Sma*I site of pUC119, yielding plasmid pUC119-*c0506* and pUC119-*c0505*, respectively. A 292-bp DNA fragment, ranging from base numbers 271 to 562 of *c0506,* and a 221-bp DNA fragment, ranging from base numbers 318 to 538 of *c0505,* were removed from the plasmids pUC119-*c0506* and pUC119-*c0505* by EX-PCR, using the primer sets c0506-S3/c0506-A3 and c0505-S3/c0505-A3, respectively. This was followed by self-ligation, yielding plasmids pUC119-Δ*c0506* and pUC119-Δ*c0505*, respectively. Plasmid pUC119-Δ*c0506* was digested with *Eco*RI. After blunting, it was further digested with *Bam*HI, and the fragment containing the disrupted *c0506* was ligated to pDrHj2 (only containing the ColE1 ori), which had been digested with *Sma*I and *Bam*HI, to yield plasmid pDrHj2-Δ*c0506*. Fragment containing disrupted *c0505* was amplified from pUC119-Δ*c0505* using the primer set c0505-S1/c0505-A1, and the fragment was ligated to pDrHj2, which had been digested with *Sma*I, to yield plasmid pDrHj2-Δ*c0505*.

Plasmids pWL102-Δ*c0507*, pDrHj2-Δ*c0506,* and pDrHj2-Δ*c0505* were passaged through *E. coli* JM110 to avoid the restriction barrier formation of extremely halophilic archaea (30).

Transformation of *Ha. japonica* was performed using the polyethylene glycol method (27). Transformants were plated onto agar plates containing pravastatin. Pravastatin-resistant colonies were cultured in liquid medium without pravastatin for 96 h and plated onto agar plates without pravastatin in order to isolate recombinants in which the targeted gene on the genome was replaced with the corresponding disrupted gene. Gene disruption was confirmed by PCR analysis.

Complementation of mutant strains. Three plasmids pJ*c0507*, pJ*c0506*, and pJ*c0505*, were constructed as follows: The targeted genes, *c0507*, *c0506*, and *c0505,* were amplified from *Ha. japonica* using the primer sets c0507-S4/c0507-A4, c0506-S4/c0506-A4, and c0505-S4/c0505-A4, respectively. Amplified PCR fragments were ligated into the *Sma*I site of pUC119, yielding plasmids pUC*c0507*, pUC*c0506*, and pUC*c0505*, respectively. Plasmids pUC*c0507* and pUC*c0506* were digested with *Nde*I and *Bam*HI. The sequences containing targeted genes were ligated to pJFZ33 (a recombinant plasmid in which the *Ha. japonica csg* promoter sequence, the *Nde*I restriction site, the *Ha. japonica ftsZ2* gene sequence, and the *Bam*HI and *Nco*I restriction sites were inserted to the *E. coli*-haloarchaea shuttle vector pWL102), which had also been digested with *Nde*I and *Bam*HI, to yield plasmids pJ*c0507* and pJ*c0506*, respectively. Plasmid pUC*c0505* was digested with *Nde*I and *Nco*I. The sequence containing *c0505* was ligated to pJFZ33, which had also been digested with *Nde*I and *Nco*I, to yield a plasmid pJ*c0505*.

Plasmids pJ*c0507*, pJ*c0506*, and pJ*c0505* were passaged through *E. coli* JM110 and then transformed into respective mutant strains as described above.

DNA sequence accession number. The DNA sequence data of *c0507*, *c0506*, and *c0505* were deposited in the DNA data Data Bank of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL), and the GenBank nucleotide sequence database. Their accession numbers are LC008542, LC008543, and LC008544, respectively.

Total carotenoid extraction from *Ha. japonica*. The wild type and mutant strains of *Ha. japonica* were pre-cultured at 37°C. Four milliliters of pre-inoculum was transferred to a 2-L Erlenmeyer flask containing 400 mL of liquid medium and cultured for 240 h at 37°C in the dark. Cells were harvested by centrifugation (4°C, 4,400 × *g*, 20 min). Carotenoids were extracted from *Ha. japonica* essentially as described previously (22) with the following modifications: Cells were suspended in acetone/methanol (7:2, v/v) which contained 0.1% (w/v) antioxidant 2,6-di-*t*-butyl-*p*-cresol to avoid the destruction of carotenoids. After cell disruption by sonication, samples were centrifuged (4°C, 840 × *g*, 2 min) to obtain the supernatant. The pellets were re-extracted until all visible pigments were retained in the liquid phase. Finally, the supernatants were collected and evaporated to dryness in a vacuum. The dried carotenoid extract was used for following HPLC analysis of total carotenoids and mass spectrometric analysis of the purified carotenoids.

HPLC analysis of total carotenoids. The dried carotenoid extract was dissolved in a small volume of chloroform/methanol (3:1), and analyzed using an HPLC system (SCL-10A, Shimadzu, Kyoto, Japan) equipped with a μBondapak C_{18} column (3.9 × 300 mm, Waters, Milford, MA, USA). The eluent was methanol/water (9:1) for the first 10 min, followed by 100% methanol, at a flow rate of 1.5 ml/min (31). Absorption spectra of the carotenoids were recorded with a photodiode-array detector (SPD-M20A, Shimadzu) attached to the HPLC apparatus.

Mass spectrometric analysis of purified carotenoid. The dried carotenoid extract was dissolved in 2 ml of acetone/hexane (1:1), and was then applied onto a column of DEAE-Toyopearl 650M (Tosoh, Tokyo, Japan) to separate the polar lipids from the carotenoids (32). The column was washed with acetone/hexane (1:1) and the colored fractions were collected. After evaporating to dryness, the collected fractions were dissolved in 1 ml chloroform/methanol (3:1) and spotted onto HPTLC plates (1.13748, HPTLC silica gel 60 with concentrating zone, Merck Millipore, Darmstadt, Germany). The HPTLC was developed with petroleum

ether/acetone (7:1) in the dark and each separated fraction of carotenoids was collected. The collected silica powder was suspended with 1 ml chloroform/methanol (3:1), and then filtered through a PTFE 0.2-μm filter (Lab Lab, Tokyo, Japan). Carotenoids in each fraction were further separated using the HPLC system described above, with the following modification. Elution was performed with 100% methanol at a flow rate of 1.5 ml/min. Carotenoids in peaks were collected, and their relative molecular masses were measured using an MStation JMS-700 mass spectrometry system (JEOL, Tokyo, Japan) in the fast atom bombardment (FAB) mode, with *m*-nitrobenzyl alcohol as a matrix.

RESULTS

Identification of candidate genes. Recently, the draft genome sequence of *Ha. japonica* has been determined (33). By homology analysis, some ORFs (*c0507*, *c0506,* and *c0505*) were predicted as candidate genes encoding carotenoid biosynthetic enzymes. C0506 had a 31% amino acid sequence identity to lycopene elongase (CrtEb) from *Corynebacterium glutamicum*, and had a 60% amino acid sequence identity to lycopene elongase homolog (Lye) from *Hb. salinarum* (10). C0507 had a 29% amino acid sequence identity to the CrtI from *Pantoea ananatis* (34). Furthermore, C0507 also had a 26% and a 24% amino acid sequence identity to CrtD from *Rhodobacter capsulatus* (35) and *Deinococcus radiodurans* R1 (36), respectively. C0505, the homolog of CruF, had a 30% and a 31% amino acid identity to those of *Synechococcus* sp. PCC 7002 (37) and *Deinococcus radiodurans* R1 (38), respectively. In addition, *c0507*, *c0506,* and *c0505* cluster together on the genome of *Ha. japonica,* in this order (Fig. 2A), and RT-PCR analysis revealed that these genes were co-transcribed. Since carotenoid synthesis-related genes in some bacteria are assembled in clusters or in neighborhoods (34,39), these genes were predicted to be involved in the carotenoid synthesis of *Ha. japonica*.

Analysis of *c0506* and *c0507*. To characterize *c0506*, a mutant of this gene was constructed by homologous recombination, and designated as strain Δ*c0506*. Δ*c0506* showed the same growth rate as wild type *Ha. japonica*. The transcription of *c0507* and *c0505* in Δ*c0506* were confirmed by RT-PCR [Fig. 2B(b)]. The disruption of *c0506* did not affect the expression of the upstream gene *c0507*, although the transcription level of the downstream gene *c0505* was reduced to less than half as compared to that in wild type *Ha. japonica*. The cell suspension of Δ*c0506* was yellow in color, while the cell suspension of wild type *Ha. japonica* has a red color (Fig. 3). Thus, the carotenoids produced were likely to be different. The carotenoids produced by Δ*c0506* was analyzed by HPLC and compared with those of the wild type *Ha. japonica*. Five peaks were detected in wild type *Ha. japonica* and had been identified as BR (Fig. 3A, peak 1), MABR (peak 2), BABR (peak 3), IDR (peak 4), and lycopene (peak 5), respectively, in our previous study (Fig. 3 and Table 3) (22). In contrast, only one carotenoid (Fig. 3B, peak 5) was detected in Δ*c0506*. This carotenoid exhibited a retention time of 19.4 min and had a λ_{max} of 443, 469, and 500 nm, which corresponded to an acyclic carotenoid having 11 conjugated double bonds (n = 11). This was similar to peak 5 of wild type *Ha. japonica* [lycopene, with a retention time of 19.4 min and a λ_{max} of 442, 468, and 499 nm (n = 11)]. Mass spectrometric analysis showed that peak 5 of Δ*c0506* had a mass *m/z* of 536. Based on the retention time, absorbance spectrum, and mass spectrometric results, peak 5 of Δ*c0506* was identified as lycopene.

In Δ*c0506*, no C_{45} and C_{50} carotenoids were detected, suggesting that the biosynthetic pathway for carotenoids was discontinued at lycopene. Therefore, we deduced that *c0506* of *Ha. japonica* encodes a functional enzyme that catalyzes the conversion of lycopene to a BR precursor.

There seem to be two types of desaturation reactions in the carotenoid biosynthetic pathway of *Ha. japonica*. In the first reaction, CrtI converts phytoene into lycopene by the introduction of four double bonds. In the second reaction, CrtD forms double bonds at C-3,4 and C-3′,4′ of the lycopene derivatives. According to homology analysis results, C0507 shows amino acid

sequence homology to both CrtI and CrtD. To investigate the function of C0507, a mutant of *c0507* was constructed by homologous recombination, designated as strain Δ*c0507*. Δ*c0507* showed the same growth rate as wild type *Ha. japonica*. The transcriptions of *c0506* and *c0505* in Δ*c0507* were confirmed by RT-PCR, although their transcription levels were reduced to less than half as compared to those in wild type *Ha. japonica* [Fig. 2B(c)]. The cell suspension of Δ*c0507* was orange in color (Fig. 3D).

The HPLC elution profile of carotenoids produced by Δ*c0507* is shown in Fig. 3D. The absorption spectrum indicated that peak 1′, peak 2′, peak 3′, peak 4′, peak 5′, peak 6′, and peak7′ had 9, 10, 9, 11, 11, 7, and 9 conjugated double bonds, respectively. Among these, peak 4′ and peak 5′, the main carotenoids in Δ*c0507*, possessed the most abundant conjugated double bonds, suggesting that peak 4′ and peak 5′ are the final products, or their derivatives. The absorption spectra of peak 4′ and peak 5′ are shown in Fig. 4. Mass spectrometric analysis also showed that peak 4′ and peak 5′ had a mass *m/z* of 708 (4 mass units more than BABR) and 622 (2 mass units more than IDR), respectively (Table 3). Based on these data, peak 4′ and peak 5′ were identified as TH-BABR and dihydroisopentenyldehydrorhodopin (DH-IDR, C_{45}), respectively. Since phytoene did not accumulate in Δ*c0507*, the desaturation reactions that convert phytoene into lycopene were not catalyzed by C0507, or the desaturation reactions were catalyzed cooperatively by C0507 and other phytoene desaturases. That is because, in a *Bradyrhizobium* strain, two distinct *crt* gene clusters are involved in the synthesis of its carotenoids (spirilloxanthin and canthaxanthin). Each cluster contains the genes *crtE* (GGPP synthase), *crtB*, and *crtI* leading to the common precursor lycopene (39). In addition, the main products of Δ*c0507*, TH-BABR and DH-IDR, lacked the double bonds at C-3,4 and C-3′,4′. Therefore, we deduced that C0507 functions as a CrtD, involved in the desaturation reactions that form double bonds at C-3,4 of DH-IDR and C-3′,4′ of DH-BABR. This is the first time that a CrtD has been identified functionally in archaea. Peak 1′, peak 2′, peak 3′, peak 6′, and peak 7′ remain

unidentified. Because the number of conjugated double bonds in these compounds was less than 11, and the number of conjugated double bonds in lycopene was 11, these peaks might be derivatives of lycopene precursors.

The biosynthetic pathway for carotenoids in Δ*c0506* was discontinued at lycopene, and Δ*c0507* accumulated DH-IDR and TH-BABR. C0506 was suggested to catalyze the conversion of lycopene to DH-IDR. The conversion contains two reactions, viz., introduction of a C_5 isoprene unit at C-2 and hydration at C-1,2 of the terminal of lycopene (Fig. 1). Therefore, C0506 in *Ha. japonica* is likely to be a bifunctional lycopene elongase and 1,2-hydratase. The *lye* gene of *Hb. salinarum*, a homolog of *c0506*, was expressed in *E. coli*, and the gene product catalyzes the conversion of lycopene to TH-BABR (10). In this conversion, introduction of a C_5 isoprene unit at C-2 and hydration at C-1,2 of the terminal of lycopene were both catalyzed by Lye. This result supports our prediction that C0506 would be a bifunctional enzyme. So we redefined Lye as a bifunctional enzyme, and designated C0506 from *Ha. japonica* as LyeJ.

In order to confirm the function of C0506 and C0507, the complementation study *in vivo* was performed. Plasmid pJ*c0506*, containing the intact *c0506* gene, was introduced into the Δ*c0506* and the transformant was named Δ*c0506*[pJ*c0506*]. HPLC analysis revealed that the biosynthesis of final product BR was restored (Fig. 3C and Table 3). However, the main products were BABR and MABR. That is probably the transcription level of the downstream gene *c0505* is still low in the complemented strain. Plasmid pJ*c0507*, containing the intact *c0507* gene, was introduced into the Δ*c0507*. The transformant Δ*c0507*[pJ*c0507*] restored the production of BR (Fig. 3E and Table 3). Since the transcription levels of the downstream genes, *c0506* and *c0505,* are probably still low in the complemented strain, the intermediates (MABR, BABR, IDR, and lycopene) accumulated and the proportion of final product diminished in Δ*c0507*[pJ*c0507*].

Analysis of *c0505*. The hydroxyl groups at C1 and C1′ of lycopene were introduced by C0506. However, the enzyme that introduces hydroxyl groups to the C3″ and C3‴ of BABR had not

been identified. C0505, the homolog of CruF, was selected as a candidate enzyme for this reaction. Its mutant was constructed by homologous recombination, and designated as strain Δ*c0505*. Δ*c0505* also showed the same growth rate as wild type *Ha. japonica*. The transcriptions of *c0507* and *c0506* in Δ*c0505* were confirmed by RT-PCR, and the disruption of *c0505* did not affect the expression of the upstream genes in the same cluster [Fig. 2B(d)].

The cell suspension of Δ*c0505* was red in color. In HPLC, only one peak (Fig. 3F, peak 3) was detected in Δ*c0505*, and the peak exhibited a retention time of 13.7 min and a λ_{max} of 468, 491, and 523 nm (n = 13; Table 3). Mass spectrometric analysis showed that peak 3 of Δ*c0505*, had a mass *m/z* of 704, compatible with that of BABR. Based on these data, peak 3 of Δ*c0505* was identified as BABR. These data demonstrated that the carotenoid biosynthetic pathway of Δ*c0505* was discontinued at BABR and indicated that C0505 catalyzes the reaction that introduces hydroxyl groups to C3″ and C3‴ of BABR to generate BR (Fig. 1). Therefore, C0505 in *Ha. japonica* is a C_{50} carotenoid 2″,3″-hydratase.

The *in vivo* complementation study was also assessed using the intact *c0505* gene-introduced transformant Δ*c0505*[p*Jc0505*]. HPLC analysis revealed that the carotenoid composition of Δ*c0505*[p*Jc0505*] was restored and similar to that of wild type *Ha. japonica* (Fig. 3G and Table 3).

DISCUSSION

The C_{50} carotenoid BR is known to accumulate in several extremely halophilic archaea (15-19), but the responsible biosynthetic pathway remains unclear. In this study, we have identified three main genes encoding enzymes responsible for the biosynthesis of BR from lycopene for the first time.

The biosynthetic pathway for carotenoids in Δ*c0506* was discontinued at lycopene, and Δ*c0507* accumulated DH-IDR and TH-BABR; hence, C0506 was suggested to be a bifunctional

lycopene elongase and 1,2-hydratase, catalyzing the reaction that introduces a C_5 isoprene unit and a hydroxyl group to the terminal of lycopene. In strain Δ*c0507*, TH-BABR and DH-IDR were detected. Therefore, C0507 was proposed to be a carotenoid 3,4-desaturase and to be involved in the desaturation reactions that form double bonds at C-3,4 of DH-IDR and C-3′,4′ of DH-BABR. It seems that two types of possible biosynthetic pathways from lycopene to BABR exist in wild type *Ha. japonica*. The first type of pathway involves immediate conversion of DH-IDR, generated from lycopene by C0506, to IDR by C0507. Subsequently, C0506 further introduces a C_5 isoprene unit and a hydroxyl group to the other terminal of IDR to generate the C_{50} carotenoid DH-BABR, which is then desaturated by C0507 to form BABR. The second type involves C0506-mediated catalysis of reactions that introduce two C_5 isoprene units and two hydroxyl groups to each end of the C_{40} lycopene to generate the C_{50} TH-BABR, which is then converted to BABR by C0507. IDR was detected as an intermediate, but TH-BABR was not detected in the wild type *Ha. japonica*. Thus, it can be suggested that the former is the main pathway in wild type *Ha. japonica*. On the other hand, it has been proposed that the biosynthetic pathway of BABR from lycopene in *Hb. salinarum* involves the latter pathway (10); therefore, the carotenoid biosynthetic pathway in *Ha. japonica* is possibly different from that in *Hb. salinarum*.

Based on the proposed carotenoids and retinal biosynthetic pathways in *Ha. japonica* (Fig. 1), Δ*c0506* was expected to accumulate β-carotene or retinal beside lycopene. However, only lycopene was detected when Δ*c0506* was grown in the dark. Our previous study showed that the transcription of the cruxrhodopsin (a retinal protein) gene is regulated by high light intensity (23), and β-carotene was detected when *Ha. japonica* was grown in light (unpublished data). So it is possible that β-carotene could not be accumulated when Δ*c0506* is grown in the dark.

The carotenoid 1,2-hydratase catalyzes the synthesis of some carotenoids that contain hydroxyl groups, by hydration at the C-1,2 or C-1′,2′ double bond. Two types of carotenoid

1,2-hydratase (CrtC and CruF) have been discovered to date. The first type is the CrtC-type carotenoid 1,2-hydratase that has been primarily found in purple bacteria, such as *Rhodobacter capsulatus*, which produces spheroidene (35,40). The second type is the CruF-type carotenoid 1,2-hydratase that has been found in bacteria, such as *Synechococcus* sp. strain PCC 7002, which produces myxol-2′ fucoside (41), and two species of *Deinococcus* (38). C0505, the C_{50} carotenoid 2″,3″-hydratase from *Ha. japonica*, is a homolog of the CruF-type carotenoid 1,2-hydratase. This is the first time that a CruF has been identified functionally in archaea; even though C0505 catalyzes hydration at the C-2″,3″ and C-2‴,3‴ double bond of BABR instead of at the C-1,2 or C-1′,2′ double bond of lycopene. The carotenoid hydratases of the same CruF-type from different bacteria would have their own substrate specificities.

In genomic sequence analysis of *Ha. japonica*, *c0507* previously revealed its sequence homology to *crtI* of *P. ananatis* (34). However, our results demonstrated that C0507, the CrtD, is involved in desaturation reactions that form double bonds at C-3,4 of DH-IDR and C-3′,4′ of DH-BABR. Thus, the CrtI that converts phytoene to lycopene has not been identified. In addition, C0184, C1220, C1219, and C1158 from *Ha. japonica* also show amino acid sequence homology to GGPP synthase (CrtE), phytoene synthase (CrtB), lycopene β-cyclase (CrtY), and β-carotene cleavage dioxygenase (Brp), respectively. Additionally, further studies are necessary to investigate the remaining unidentified carotenoid biosynthetic enzymes in order to elucidate the complete carotenoid biosynthetic pathway in *Ha. japonica*.

It has been suggested that the antioxidant capacity of carotenoids is linked to the number of conjugated double bonds and hydroxyl groups (3,4,42). BR, which contains 13 conjugated double bonds and four hydroxyl groups, is regarded to be an effective free-radical scavenger. Our previous study has shown that the free-radical scavenging capacity of BR is much higher than that of β-carotene, which contains 11 conjugated double bonds (22). Moreover, BR is a

374 dipolar C_{50} carotenoid, and it was suggested to act as a "rivet" in the cell membrane, to increase
375 its rigidity and mechanical strength (7).
376 In summary, we have identified three carotenoid biosynthetic enzymes and have elucidated
377 their functions. Among them, CrtD and CruF were found in an archaeon for the first time. Our
378 results revealed the biosynthetic pathway responsible for production of BR from lycopene in *Ha.*
379 *japonica* and provides a basis for investigating carotenoid biosynthetic pathways in other
380 extremely halophilic archaea. Elucidation of the carotenoid biosynthetic pathway in *Ha. japonica*
381 may also prove useful for producing the C_{50} carotenoid BR efficiently by employing genetically
382 modified haloarchaeal strains.
383

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392
393

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516 in *Escherichia coli*. Nat. Biotechnol. **18:**843-846.
517

517 **TABLE 1** Bacterial strains and plasmids used in this study

518

519 * Ap^r, ampicillin resistance; Mev^r, pravastatin resistance.

520 **The pDrHj2 was constructed as follows. The sequence between *Sac*I and *Kpn*I in pUC119
521 was replaced with the mevinoline-resistance gene and the sequence between *Hin*dⅢ and *Xba*I
522 was replaced with the gene of a halophilic β-galactosidase (*bgaH*) under the control of *csg*
523 promoter. The *bgaH* gene was obtained from *Haloferax alicantei* and was in the opposite
524 direction to the mevinoline-resistance gene. The *csg* promoter is from *Haloarcula japonica*. *Ha.*
525 *japonica* has a large amount of a glycoprotein (CSG) on the cell surface, suggesting that the *csg*
526 promoter is powerful.

527

Strain and plasmid	Relevant properties*	Source or reference
Strain		
E. coli JM109	Host for cloning vectors	Laboratory stock
E. coli JM110	Host used for preparing plasmid that is free of Dam or Dcm methylation	Laboratory stock
Ha. japonica	Wild type (JCM 7785^T)	21
Δ*c0507*	*c0507* gene mutant of *Ha. japonica*	This work
Δ*c0506*	*c0506* gene mutant of *Ha. japonica*	This work
Δ*c0505*	*c0505* gene mutant of *Ha. japonica*	This work
Δ*c0507*[p*Jc0507*]	Δ*c0507* complement with p*Jc0507*	This work
Δ*c0506*[p*Jc0506*]	Δ*c0506* complement with p*Jc0506*	This work
Δ*c0505*[p*Jc0505*]	Δ*c0505* complement with p*Jc0505*	This work
Plasmid		
pUC119	*E. coli* cloning vector, Ap^r	Laboratory stock
pWL102	*E. coli*-haloarchaea shuttle vector, Ap^r, Mev^r	29
pDrHj2**	*E. coli*-haloarchaea shuttle vector, Ap^r, Mev^r	Laboratory stock
pWL102-Δ*c0507*	pWL102 derivative containing disrupted fragment of *c0507* gene	This work
pDrHj2-Δ*c0506*	pDrHj2 derivative containing disrupted fragment of *c0506* gene	This work

pDrHj2-Δ*c0505*	pDrHj2 derivative containing disrupted fragment of *c0505* gene	This work
pJFZ33	pWL102 derivative carrying *Ha. japonica csg* promoter and *ftsZ2* gene, Ap^r, Mev^r	27
p*Jc0507*	pJFZ33 derivative in which *ftsZ2* is replaced by the *c0507* gene	This work
p*Jc0506*	pJFZ33 derivative in which *ftsZ2* is replaced by the *c0506* gene	This work
p*Jc0505*	pJFZ33 derivative in which *ftsZ2* is replaced by the *c0505* gene	This work

528

529

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529 **TABLE 2** Sequences of the primers used in this study

530

531 **NdeI*, *BamHI*, and *NcoI* restriction sites introduced were underlined.

532

Primer*	Sequence	Usage
c0507-S	5′-GGTTGGCCTCCAGCTCATTG-3′	To confirm the transcription of target genes by RT-PCR
c0507-A	5′-CTCGTGGTCCGGCAGGAGTTC-3′	
c0506-S	5′-TCGCCCTGTTTCTGTACTTCAC-3′	
c0506-A	5′-GATATCTGGAATCGCCGAGAAGG-3′	
c0505-S	5′-CACCGAGAACCGGTTTACCATC-3′	
c0505-A	5′-ACTGCGGCATCTCGTAGATCC-3′	
c0507-S1	5′-ATGTGGCCACGAATGCCATAC-3′	To amplify the target genes by PCR for the construction of mutants
c0507-A1	5′-GGTCTGTCGGGAGTTCTGGC-3′	
c0506-S1	5′-GGACATCGCCTGAGTAATGCC-3′	
c0506-A1	5′-GGTCTTTGCCGCTACCCATAC-3′	
c0506-S3	5′-TCTGGGCGATGGGGATGCAC-3′	
c0506-A3	5′-TTTCGGATTGTGCTCGTCGATATCGG-3′	
c0505-S1	5′-GCTGTATGGGTAGCGGCAAAG-3′	
c0505-A1	5′-GAGCTGGCTGATTCCAAACGG-3′	
c0505-S3	5′-TGGATCCTGGAGCCGTAGCTATC-3′	
c0505-A3	5′-AGCTCGATGCCGTAGGAGTACAG-3′	
c0507-S4	5′-GAATTCCATATGAGTGACTTGTCCGGTG-3′	To amplify the intact target genes by PCR for the complementation study
c0507-A4	5′-GGGGATCCTCATTAGTGGTGGTGGTGGTGGGCGATGTCCTCGATGAGC-3′	
c0506-S4	5′-GAATTCCATATGCCAGAACTCCCGACAG-3′	
c0506-A4	5′-GGGGATCCTCATTAGTGGTGGTGGTGGTGGTGCCCATACAGCATCACCCAC-3′	
c0505-S4	5′-GAATTCCATATGGGTAGCGGCAAAGACC-3′	
c0505-A4	5′-GGCCATGGTCATTAGTGGTGGTGGTGGTGTGCCGCCAGACGGCCCGACCG-3′	

533

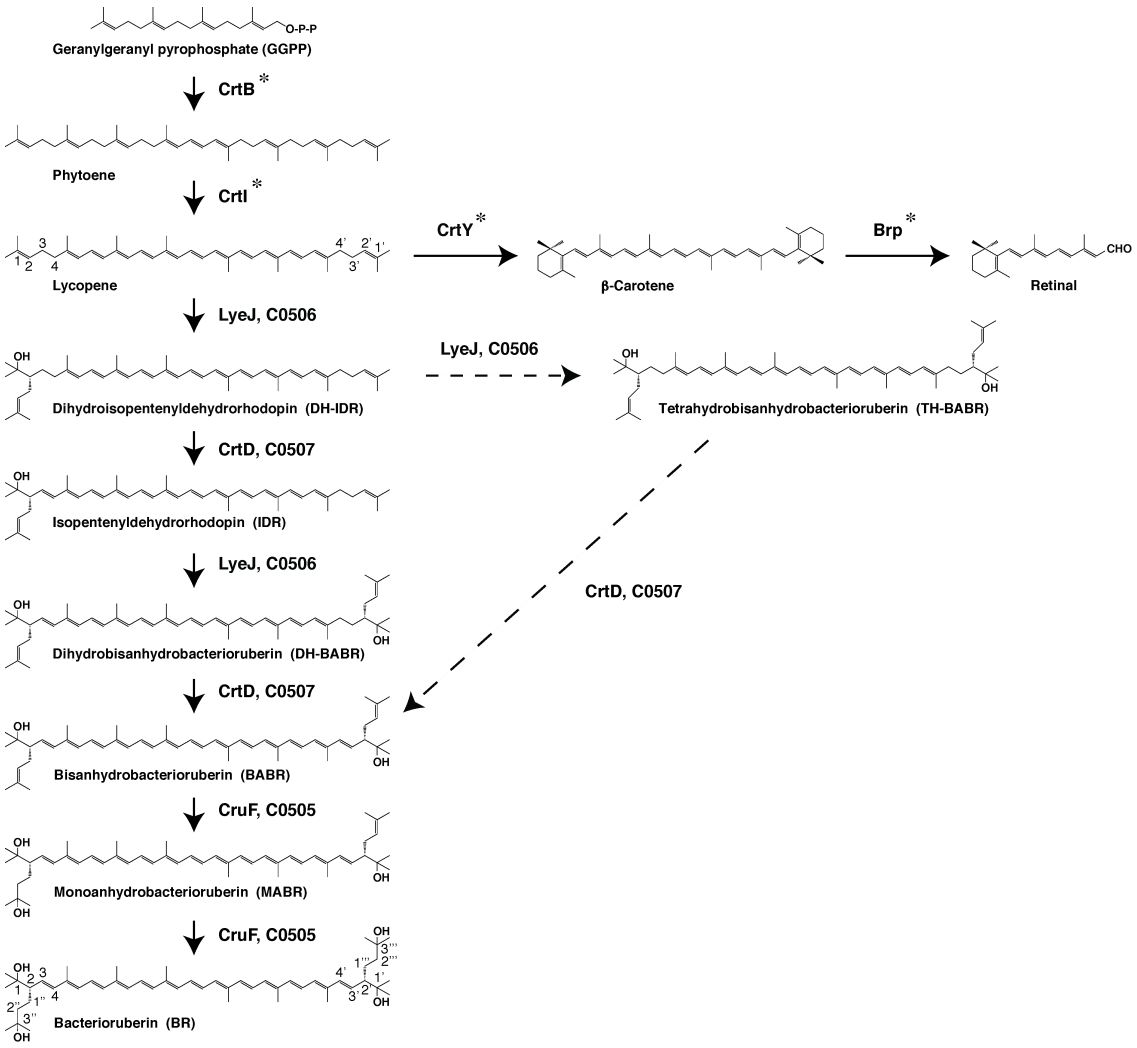
533 **TABLE 3** Characteristics of carotenoids extracted from wild type, mutants and their genetically
534 modified *Ha. japonica* strains
535
536 *BR, bacterioruberin; MABR, monoanhydrobacterioruberin; BABR, bisanhydrobacterioruberin;
537 IDR: isopentenyldehydrorhodopin; TH-BABR tetrahydrobisanhydrobacterioruberin; DH-IDR,
538 dihydroisopentenyldehydrorhodopin. Carotenoids were analyzed using HPLC equipped with a
539 μBondapak C_{18} column (3.9 × 300 mm, Waters) and eluted with methanol/water (9:1, v/v) for
540 the first 10 min, and then 100% methanol (1.5 ml/min). Absorption spectra of the carotenoids
541 were recorded with a photodiode-array detector attached to the HPLC apparatus (Fig. 3). The
542 relative molecular masses of the purified carotenoids were measured using an MStation
543 JMS-700 mass spectrometry system (JEOL) in the FAB mode with *m*-nitrobenzyl alcohol as a
544 matrix. N.D., not determined.
545
546

Strain	Peak no.	Retention time (min)	λ_{max} (nm) in HPLC eluent	Conjugated double bonds (*n*)	Mol. mass (*m/z*)	Calculated molecular mass	Carotenoid*
Ha. japonica	1	9.6	469, 492, 525	13	740	740	BR
	2	11.8	469, 491, 522	13	722	722	MABR
	3	13.8	467, 490, 521	13	704	704	BABR
	4	16.0	453, 479, 509	12	620	620	IDR
	5	19.4	442, 468, 499	11	536	536	Lycopene
Δ*c0506*	5	19.4	443, 469, 500	11	536	536	Lycopene
Δ*c0507*	4′	14.8	441, 466, 498	11	708	708	TH-BABR
	5′	16.7	440, 465, 495	11	622	622	DH-IDR
Δ*c0505*	3	13.7	468, 491, 523	13	704	704	BABR
Δ*c0506* [p*Jc0506*]	1	9.6	469, 491, 523	13	N.D.	740	BR
	2	11.8	468, 491, 523	13	N.D.	722	MABR

	3	13.8	466, 490, 522	13	N.D.	704	BABR
Δ*c0507* [pJ*c0507*]	1	9.6	468, 492, 524	13	N.D.	740	BR
	2	11.8	469, 491, 522	13	N.D.	722	MABR
	3	13.8	466, 490, 521	13	N.D.	704	BABR
	4	16.0	454, 480, 510	12	N.D.	620	IDR
	5	19.4	443, 468, 499	11	N.D.	536	Lycopene
Δ*c0505* [pJ*c0505*]	1	9.6	468, 492, 525	13	N.D.	740	BR
	2	11.8	467, 492, 522	13	N.D.	722	MABR
	3	13.8	466, 490, 520	13	N.D.	704	BABR
	4	16.0	454, 479, 509	12	N.D.	620	IDR

547

548



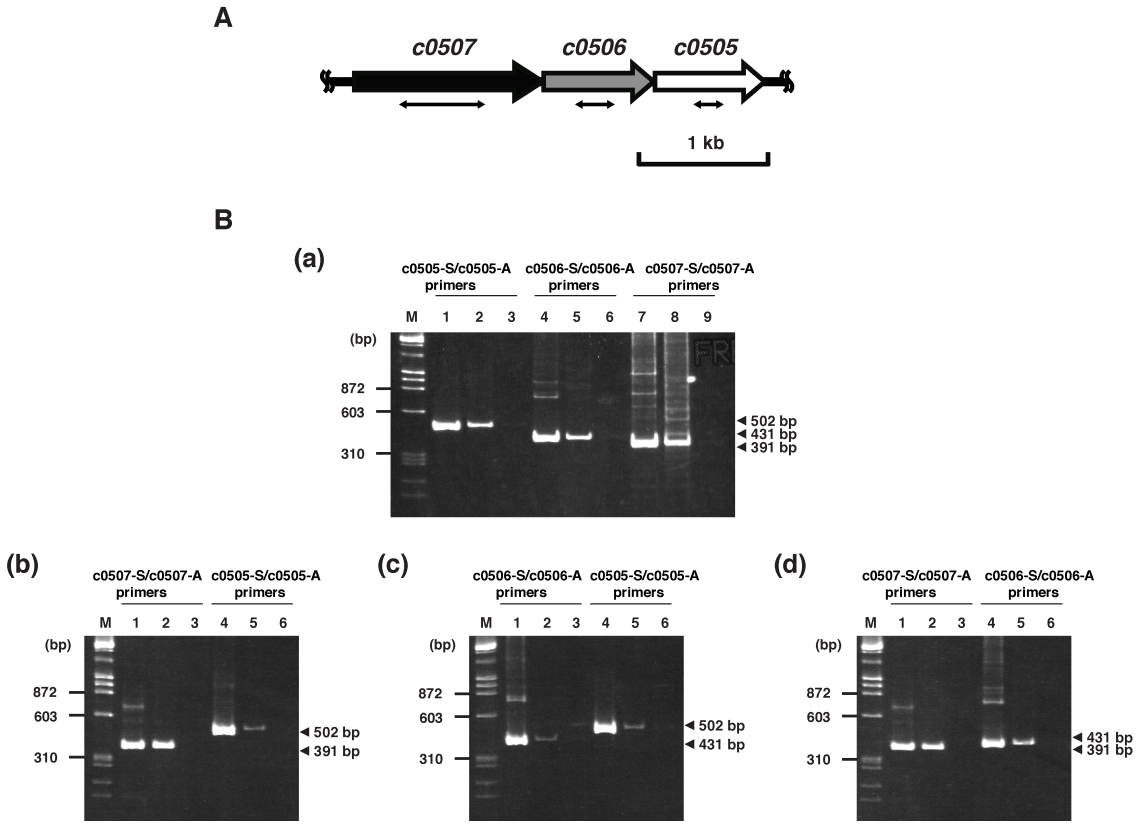
548

549

550 **FIG 1** Proposed main steps in the carotenoids and retinal biosynthetic pathways of *Haloarcula*
551 *japonica*. Solid arrows signify the main carotenoid biosynthetic steps. The dashed arrows
552 indicate other possible carotenoid biosynthetic steps. Asterisks indicate the unidentified
553 carotenoid biosynthetic enzymes.

554

554

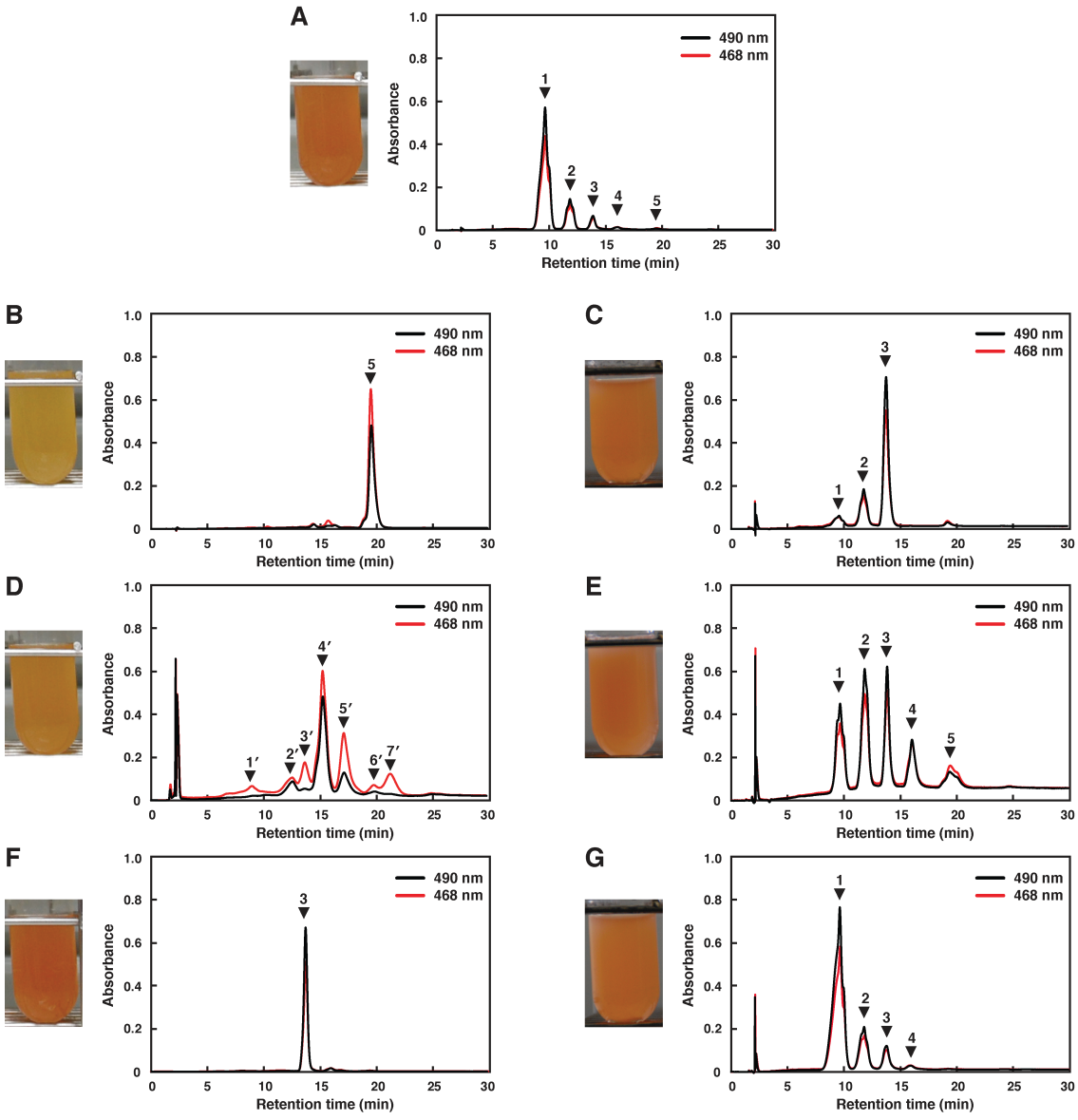


555

556 **FIG 2** The *c0507*/*c0506*/*c0505* gene cluster and agarose gel electrophoresis of the RT-PCR
557 products. A, the *c0507*/*c0506*/*c0505* gene cluster. The two direction arrows show the deleted
558 parts in the corresponding mutants: a 652-bp fragment, ranging from base numbers 378 to 1029
559 of *c0507,* was removed in Δ*c0507*; a 292-bp fragment, ranging from base numbers 271 to 562 of
560 *c0506*, was removed in Δ*c0506*; a 221-bp fragment, ranging from base numbers 318 to 538 of
561 *c0505*, was removed in Δ*c0505*. B, agarose gel electrophoresis of the RT-PCR product. (a) the
562 genomic DNA and total RNA were isolated from wild type *Ha. japonica*; (b), the genomic DNA
563 and total RNA were isolated from Δ*c0506*; (c), the genomic DNA and total RNA were isolated
564 from Δ*c0507*; (d), the genomic DNA and total RNA were isolated from Δ*c0505*. Lanes 1, 4, and

7, the positive control reaction product using genomic DNA as the template; Lanes 2, 5, and 8, the RT-PCR product; Lanes 3, 6, and 9, the negative control reaction product with total RNA not subjected to reverse transcription.

568



569

570 **FIG 3** Cell suspension color of wild type, mutants and their genetically modified *Ha. japonica*

571 strains, and HPLC elution profiles of carotenoids extracted from them. A, Wild type. B, Δ*c0506*.

572 C, Δ*c0506*[pJ*c0506*]. D, Δ*c0507*. E, Δ*c0507*[pJ*c0507*]. F, Δ*c0505*. G, Δ*c0505*[pJ*c0505*]. Peak 1,

573 bacterioruberin (BR); peak 2, monoanhydrobacterioruberin (MABR); peak 3,
574 bisanhydrobacterioruberin (BABR); peak 4, isopentenyldehydrorhodopin (IDR); peak 5,
575 lycopene; peak 4′, tetrahydrobisanhydrobacterioruberin (TH-BABR); peak 5′,
576 dihydroisopentenyldehydrorhodopin (DH-IDR). Peaks 1′, 2′, 3′, 6′, and 7′ might be the
577 derivatives of lycopene precursors. Eluent was methanol/water (9:1, v/v) for the first 10 min,
578 and then 100% methanol (1.5 ml/min).
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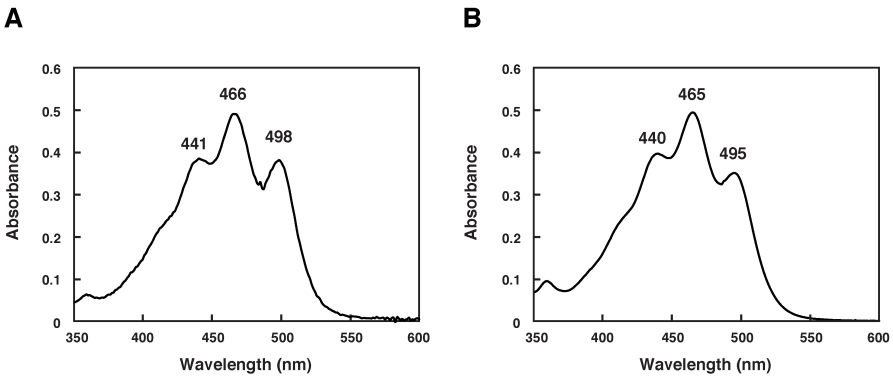

580

581 **FIG 4** Absorption spectra of the carotenoids extracted from the Δ*c0507*. A, peak 4′
582 [tetrahydrobisanhydrobacterioruberin (TH-BABR); λ_{max} = 441, 466, and 498 nm]; B, peak 5′
583 [dihydroisopentenyldehydrorhodopin (DH-IDR). λ_{max} = 440, 465, and 495 nm]. Spectra were
584 measured using a photodiode-array detector attached to the HPLC apparatus, with a mobile
585 phase of 100% methanol.