Identification of a novel carotenoid, 2'-isopentenylsaproxanthin, by *Jejuia pallidilutea* strain 11shimoA1 and its increased production under alkaline condition

N. Takatani・K. Nishida・T. Sawabe・T. Maoka・K. Miyashita・M. Hosokawa

N. Takatani・K. Nishida・T. Sawabe・K. Miyashita・M. Hosokawa*

Faculty of Fisheries Sciences, Hokkaido University

3-1-1 Minato, Hakodate, Hokkaido 041-8611, Japan.

*corresponding author

E-mail:hoso@fish.hokudai.ac.jp

TEL&Fax:+81-138-40-5530

T. Maoka

Research Institute for Production Development, Kyoto, Japan.

Abstract

Carotenoids are a class of naturally occurring pigment, carrying out important biological functions in photosynthesis and involved in environmental responses including nutrition in organisms. Saproxanthin and myxol, which have monocyclic carotenoids with a γ-carotene skeleton, have been reported to show a stronger antioxidant activity than those with β-carotene and zeaxanthin. In this research, a yellow-orange bacterium of strain 11shimoA1 (JCM19538), was isolated from a seaweed collected at Nabeta Bay (Shizuoka, Japan). The 16S rRNA gene sequence of strain 11shimoA1 revealed more than 99.99% similarity with those of *Jejuia pallidilutea* strains in the family *Flavobacteriaceae*. Strain 11shimoA1 synthesized two types of carotenoids. One of them was (3*R*, 3'*R*)-zeaxanthin with dicyclic structure and another was identified as (3*R*, 2'*S*)-2'-isopentenylsaproxanthin; a novel monocyclic carotenoid with pentenyl residue at C-2' position of saproxanthin, using FAB-MS, ^{1}H-NMR and CD analyses. Culturing strain 11shimoA1 in an alkaline medium at pH 9.2 resulted in a markedly increased in production of 2'-isopentenylsaproxanthin per dry cell weight, but a decreased in zeaxanthin production as compared to their respective production levels in medium with pH 7.0. These carotenoids are likely to play some roles in the adaptation of the bacterium to the environmental conditions.

Keywords: 2'-isopentenylsaproxanthin, *Jejuia pallidilutea*, monocyclic carotenoid, alkaline condition, zeaxanthin

Introduction

Carotenoids are a naturally occurring yellow, orange and red pigments synthesized by photosynthetic organisms, such as higher plants, algae, and photosynthetic bacteria also by some species of non-photosynthetic bacteria, yeasts and fungi. To date, more than 750 different carotenoids have been isolated and identified from natural sources (Jackson et al. 2008). Carotenoids can be classified into three groups based on their structure; dicyclic, monocyclic, and acyclic. β-Carotene and lycopene are the representative of carotenoids with dicyclic and acyclic structure respectively which are abundant in vegetables and fruits. Monocyclic carotenoids are commonly distributed in cyanobacteria (Takaichi et al. 2001). Myxoxanthophyll (myxol glucoside) is a well-known example of a monocyclic carotenoid with a single β-ionone ring and sugar moiety which is only unique to members of the phylum *Cyanobacteria* (Goodwin 1980; Takaichi et al. 2005; Takaichi and Mochimaru 2007). Aglycone and myxol has been found in marine bacteria, along with saproxanthin (Yokoyama et al. 1995; Shindo et al. 2007).

These structurally diverse carotenoids have a variety of biological functions in photosynthesis and photo-protection (Naguib 2000; Sommerburg et al. 1999), in stabilizing membrane fluidity (Yokoyama et al. 1995), and as precursors for hormones (Milborrow 2001). Some kinds of carotenoids have attracted greater attention because of their beneficial effects in the prevention of serious diseases such as cancer (Tanaka et al. 2012), cardiovascular disease (Pashkow et al. 2008), and metabolic syndrome (Miyashita et al. 2011). Recently, myxol and saproxanthin, which are rarely found carotenoids in nature, have been reported to show stronger antioxidant activities than those of β-carotene and zeaxanthin against lipid peroxidation in the rat brain homogenate model (Shindo et al. 2007). In addition, both of these monocyclic carotenoids showed a neuroprotective effect (Shindo et al.

2007). These results suggest that monocyclic carotenoids including saproxanthin and myxol have potential to be used in nutraceutical and pharmaceutical industries. However, only few organisms produce monocyclic carotenoids compared to dicyclic and acyclic carotenoids. Therefore, it is important to identify organisms that produce these monocyclic carotenoids and to investigate the biological functions of these compounds.

Since marine bacteria has shown large diversity in term of carotenoid biosynthesis (Britton et al. 2004; Misawa 2011), the present study attempted to isolate marine bacteria which produced a novel monocyclic carotenoids. In addition, culture conditions, such as the carbon source and pH are also important factors in carotenoid biosynthesis in bacteria. Therefore, the effects of different pH and different carbon sources on cell growth and production of carotenoid were determined in this study. A detailed understanding of the factors that promote carotenoid biosynthesis will allow the development of an optimized fermentation medium for carotenoid production.

In the present study, a yellow-orange pigmented bacterium; strain 11shimoA1 (JCM19538), belonging to the species *Jejuia pallidilutea* in the family *Flavobacteriaceae* (Lee et al. 2009) was isolated. Strain 11shimoA1 synthesized a novel monocyclic carotenoid; 2'-isopentenylsaproxanthin, as well as zeaxanthin. The strain 11shimoA1 produced more 2'-isopentenylsaproxanthin in the alkaline medium (pH 9.2) than in medium with a pH of 7.0.

Materials and methods

Screening of pigment-producing marine bacteria

Sample of seaweeds and seawater were collected at Nabeta Bay in Shimoda (Shizuoka Prefecture of Japan) in June, 2011. Artificial seawater-soaked seaweed and seawater were conventionally plated on Marine Agar 2216 (Difco). The agar plates were incubated for several days at 25°C. After incubation, the pigmented colonies were isolated. Among pigmented colonies, the strain 11shimoA1 with yellow-orange pigment was selected. The strain 11shimoA1 (JCM19538) was then deposited in the Japan Collection of Microorganisms, RIKEN BioResource Center (Tsukuba, Japan).

Isolation and identification of carotenoids

The strain11shimoA1 was incubated for 2-3 days in a 300 ml Marine Broth 2216 (Difco) in a 500 ml Sakaguchi flasks on a rotary shaker at 140 rpm, 25°C.

After cultivation, the cell pellet was collected by centrifugation at 12,000 rpm, and the total lipid was extracted from the pellet using the method previously described by Folch et al. (1957). The total lipid was then applied onto a preparative thin-layer chromatography (TLC) plate with silica gel 60 (1 mm; Merck, Germany) and developed with n-hexane:ethyl acetate (4:6, v/v). Yellow and orange fractions (R_f = 0.35 of yellow, 0.46 of dark orange) were scraped and pigments were eluted from silica gel with acetone. To purify carotenoids, each pigmented fraction obtained by TLC was subjected to high performance liquid chromatography (HPLC) with Develosil ODS column (20 x 250 mm, Nomura Chemical, Japan), eluting with methanol:acetonitrile (7:3, v/v) at a flow rate of 8.0 ml/min. Carotenoids were detected with UV-VIS detector (Hitach L-2400) at 450 nm.

Spectroscopic analysis

The proton nuclear magnetic resonance (^{1}H-NMR) spectra of the carotenoids in $CDCl_3$ were measured at 500 MHz using a UNITY *INOVA*-500 NMR system (Varian, USA) with TMS as an internal control. The high-resolution fast atom bombardment-mass spectrometry (FAB-MS) spectra of the carotenoids were measured by a JMS-HX/HX110A mass spectrometer (JEOL, Japan) with m-nitrobenzyl alcohol as a matrix. The peak assignments of the ^{1}H-NMR spectra were made on the basis of ^{1}H-^{1}H COSY (correlated spectroscopy) and NOESY spectra and by comparison with previously reported data. The circular dichroism (CD) spectra were as recorded in ether at room temperature using a J-500 C spectropolarimeter (Jasco, Japan).

Molecular phylogeny

Bacterial genomic DNA of strain 11shimoA1 was purified using Wizard genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer's instructions. The 16S rRNA gene was amplified from the DNA using 24F and 1509R primer set (Sawabe et al. 1998). PCR was performed using GoTaq Green Master mix (Promega) according to the protocol provided by the company. The single PCR product was purified using the Gel and PCR purification system (Promega) and the sequences were determined using the SolGent DNA sequence service (Leave a Nest Co., Ltd.). The sequences were aligned and analyzed using the ClustalX (Thompson et al. 1997) and MEGA version 5 programs (Tamura et al. 2011), respectively. In the phylogenetic analysis, the sequences of small-subunit rRNA gene retrieved from the GenBank/EMBL/DDBJ database were used. The domain used to construct the phylogenetic tree was from the region of the small-subunit rRNA gene at the position

144 from 124 to 1378 (*E. coli* str. K12, AP012306 numbering, Fig. S1.). The phylogenetic
145 analyses were performed using three different methods, neighbor-joining (NJ),
146 maximum-likelihood (ML) and maximum-parsimony (MP) implemented in MEGA version 5.
147 The robustness of each topology was checked by NJ and 500 bootstrap replications. The tree
148 was drawn by MEGA version 5.
149
150 Nucleotide sequence accession number
151
152 The 16S rRNA sequence of the strain 11shimoA1 (JCM19538) is available in
153 GenBank/EMBL/DDBJ under the accession number of AB848003.
154
155 Cultivated conditions in carotenoid synthesis and cell growth
156
157 In order to investigate cultivated conditions in carotenoid synthesis of strain 11shimoA1, 50
158 mM D-glucose or sucrose was added into Marine Broth 2216. The pH of the culture media
159 (Marine Broth 2216) was adjusted by the addition of HCl or NaOH into Marine Broth 2216.
160 After cultivation of strain 11shimoA1 in 50 ml Marine Broth 2216 in 100 ml Erlenmeyer flask
161 for 2-3 days at 140 rpm and at temperature of 25^{o}C, total lipid was extracted by the method
162 previously described by Folch et al. (1957). As for the quantification analysis of carotenoids,
163 the total lipid was subjected to HPLC equipped with Develosil ODS-UG-5 column (4.6×250
164 mm, Nomura chemical, Japan) and was analyzed with diode array delivery detector L-7455
165 (Hitachi, Japan). Mobile phase of methanol:acetonitrile (7:3, v/v) was isocratically eluted at a
166 flow rate of 1.0 ml/min. The temperature of the column was maintained at 30^{o}C using column
167 oven L-7300 (Hitachi, Japan). Carotenoid content was calculated based on the calibration

curves of integrated peak areas by HPLC analysis and weight of carotenoid standards. Carotenoid levels were expressed in specific cellular units (mg/g cell dry weight (CDW)) (Bhosale and Bernstein 2004) or content per one ml of culture medium (μg/ml culture medium). For cell dry weight estimation, the culture was centrifuged at 12,000 rpm and the cell pellet was then dried at 105^{o}C to weight.

Cell growth was monitored by measuring the optical density at 600 nm using a U-2800A spectrophotometer (Hitachi, Japan) during cultivation.

Statistical analysis

Data is expressed as means ± standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA with *Dunnett's* test. A *P* value of less than 0.01 was considered to be a significant difference.

Results

Identification of pigments produced by strain 11shimoA1

Strain 11shimoA1 which was isolated from a type of seaweed (unidentified) produced yellow-orange pigmented colonies on the agar plates. After culturing the bacterium in Marine Broth 2216, two major peaks were detected by HPLC analysis in their total lipid extract (Fig. 1).

191 Peak 1 showed the same retention time (6.0 min) as that of the (all-*trans*) zeaxanthin
192 authentic standard. Analyses using positive ion high-resolution fast atom bombardment mass
193 spectrometry (FAB-MS) and ^{1}H-NMR (data not shown) confirmed the identification as
194 zeaxanthin (Fig.2). The CD spectrum was comparable to that of (3*R*, 3'*R*)-zeaxanthin as
195 described in a previous report by Buchecker and Noack (1995).
196 Peak 2 was detected at 8.1 min of retention time from the HPLC analysis (Fig. 1). This
197 carotenoid showed a maximum absorption at 472 nm and 502 nm; indicating the presence of a
198 myxol (saproxanthin)-type chromophore (Britton et al. 1995). The molecular formula of this
199 compound was determined to be $C_{45}H_{64}O_2$ from positive ion high-resolution FAB-MS data
200 (m/z 636.4901 [M^+] $C_{45}H_{64}O_2$ calc. 636.4907). This suggested that an isopentenyl group was
201 attached to the carotenoid moiety. The ^{1}H-NMR spectrum revealed the presence of 36 methyl
202 protons (12 methyl groups), six methylene protons, one oxy methine, one aliphatic methine,
203 and 18 olefinic protons in the molecule. These signals were assigned by COSY and NOESY
204 spectra and by comparisons with zeaxanthin, saproxanthin, myxoxanthophyll. ^{1}H-NMR data
205 is summarized in Table 1. The ^{1}H-NMR data of the carotenoid moiety was similar to those of
206 saproxanthin, except for the H-2' position (Englert 1995). The remaining part was assigned to
207 an isopentenyl moiety as shown in Table 1. In the case of saproxanthin, the methylene proton
208 at H-2 appears at 2.31 ppm as doublet. For the carotenoid produced by strain 11shimoA1,
209 however, a methine proton at H-2' (2.09 ppm) was multiplet. This indicated that an
210 isopentenyl group was attached at C-2' position of saproxanthin. COSY spectrum clearly
211 indicated the connection between carotenoid moiety at H-2' and isopentenyl moiety at H-1".
212 Therefore, the structure of this compound was determined to be 2'-(3-methylbut-2-enyl)-3',
213 4'-didehydro-1', 2'-dihydro-β, ψ-carotene-3, 1'-diol, which is a novel carotenoid with
214 modified saproxanthin binding isopentenyl group at C-2' position. Peak 2 is named

2’-isopentenylsaproxanthin (Fig. 2). CD of this compound showed [(EPA) nm (Δε) 225 (0), 232 (+2.0), 245 (0), 255 (-4.5), 285 (0), 307 (-3.2), 320 (0), 357 (+4.5), 378 (0), 420 (-2.3), 435 (0)], resembled those to myxoxanthophyll with having (3*R*,2’*S*) chirality (Britton et al. 1995). Therefore the (3*R*,2’*S*) chirality was proposed for 2’-isopentenylsaproxanthin (Fig.2).

Phylogenetic analysis of the strain 11ShimoA1

The phylogenetic analysis showed that strain 11shimoA1, which produces the novel carotenoid 2’-isopentenylsaproxanthin, belongs to the genus *Jejuia* in the family *Flavobacteriaceae*. In more details, the strain 11shimoA1 formed a robust clade with *Jejuia pallidilutea* type strains with more than 99.99% sequence similarity Fig. S1.). The clade was distinctively separated from the other clades involved with *Hyunsoonleella* spp. and unidentified and/or environmental clones.

Effect of additional sugars on growth and carotenoid production

Strain 11shimoA1 produced 2’-isopentenylsaproxanthin and zeaxanthin at 0.5 mg/g cell dry weight (CDW) and 1.6 mg/g CDW, respectively, in Marine Broth 2216 after 73 h of incubation at 140 rpm, 25^{o}C. To examine the effects of additional sugars as carbon source on growth and carotenoid production, strain 11shimoA1 was cultured in the media supplemented with 50 mM D-glucose or 50 mM sucrose. As shown in Fig. 3 (a), the growth of strain 11shimoA1 did not change after the addition of D-glucose or sucrose. On the other hand, D-glucose supplementation showed a markedly decreased of zeaxanthin content to 0.6 mg/g CDW after 73 h of culture (Fig. 3 (b)), and a slightly decreased of 2’-isopentenylsaproxanthin

content to 0.4 mg/g CDW. Sucrose supplementation did not affect cell growth or carotenoid production (Fig. 3).

Effect of pH of media on growth and production of carotenoid

In order to examine the effect of pH of the cultured medium on growth and production of carotenoid, strain 11shimoA1 was cultivated in Marine Broth 2216 and the pH was adjusted to 7.0, 8.8, or 9.2. The growth of strain 11shimoA1 was slower at pH 8.8 and pH 9.2 than at pH 7.0 (Fig. 4 (a)). However, after 72 h of culture in the medium of pH 8.8, the cell density of 11shimoA1 reached the same level as that in the medium with pH 7.0. Cell growth was slightly slower in the medium with pH 9.2 than in the medium with pH 7.0 and pH 8.8.

It is worth to note that, in the production of carotenoid, 2'-isopentenylsaproxanthin content (mg/g CDW) is increased when in culture media with higher pH (Fig. 4 (b)). In medium with pH 9.2, 2'-isopentenylsaproxanthin content was 1.1 mg/g CDW, as compared to 0.4 mg/g CDW in medium with pH 7.0. Since the final cell density was lower in medium with pH 9.2 than that in pH 7.0, the content of 2'-isopentenylsaproxanthin per volume of culture medium were compared. The 2'-isopentenylsaproxanthin content per milliliter of culture medium was higher in medium with pH 9.2 (1.2 μg/ml) than in the ones with pH 7.0 (0.6 μg/ml) after 138 h of culture. These data show that 2'-isopentenylsaproxanthin biosynthesis was enhanced in the strain 11shimoA1 under alkaline conditions. In contrast, zeaxanthin production was significantly decreased to 1.0 mg/g CDW at pH 9.2 compared to 1.3 mg/g CDW at pH 7.0. Thus, the pH of the culture medium is an important factor which affects the production of carotenoid 11shimoA1.

263 **Discussion**

264

265 In this study, 35 yellow-red pigmented bacteria were successfully isolated from seawater and
266 seaweeds collected at Nabeta Bay (Shizuoka, Japan). Among those bacteria, strain 11shimoA1
267 was selected for further analysis, as its colony color was yellow-orange, which was distinct
268 from the colony colors of the other strains.

269 Strain 11shimoA1 was identified to *J. pallidilutea* based on16S rRNA gene sequencing. *J.*
270 *pallidilutea* EM39^{T} was proposed to be a novel genus and species in the family
271 *Flavobacteriaceae* (Lee et al. 2009). The strain EM39^{T} showed an orange colour of water
272 insoluble pigmentation. However, the chemical structures of the components from the
273 pigment have yet to be identified. This study first elucidated that the strain 11shimoA1
274 produced two types of carotenoid which chemical structures were determined by FAB-MS,
275 ^{1}H-NMR and CD analyses. One of the carotenoids was (3*R*, 3'*R*)-zeaxanthin; a yellow
276 xanthophyll. Zeaxanthin has dicyclic structure and is synthesized by some species of bacteria
277 including the *Flavobacterium* species (McDermott et al. 1974). Zeaxanthin and lutein are
278 normally found in the human eye and prevent age-related macular degeneration and
279 light-induced photoreceptor death (Beatty et al. 2001; Thomson et al. 2002). The nutraceutical
280 applications of zeaxanthin have been identified and well researched in the scientific world.

281 On the other hand, the other carotenoid was identified as a novel monocyclic carotenoid;
282 2'-isopentenylsaproxanthin. It is of a unique carotenoid structure comprising saproxanthin
283 (3′,4′-didehydro-1′,2′-dihydro-β,ψ-carotene-3,1′-diol) with a pentenyl residue at C-2' position.
284 Saproxanthin is synthesized by the marine bacterium strain 04OKA-13-27 (Shindo et al.
285 2007) and *Saprospira grandis* (Aasen and Liaaen-Jensen 1966). It has been reported to show
286 a stronger antioxidative activity than those of β-carotene and zeaxanthin against lipid

peroxidation in the rat brain homogenate (Shindo et al. 2007). In addition, another monocyclic carotenoid, myxol (3′,4′-didehydro-1′,2′-dihydro -β,ψ-carotene-3,1′,2′-triol) (Yokoyama and Miki 1995; Teramoto et al. 2004); which has a similar structure to that of saproxanthin, showed a neuro-protective effect against L-glutamate toxicity in the neuronal hybridoma cell line, N18-RE-105 (Shindo et al. 2007). Since the structure of 2'-isopentenylsaproxanthin identified in this study is similar to that of saproxanthin and myxol, it is likely to have a strong antioxidant and a neuro-protective effect.

The production of 2'-isopentenylsaproxanthin was affected by sugar supplementation and pH of the medium; a slight decrease using D-glucose supplementation and an increase in alkaline conditions. Zeaxanthin content (per CDW) decreased markedly and 2'-isopentenylsaproxanthin content (per CDW) showed a slightly decrease after 72 h of cultivation in Marine Broth 2216 added with 50 mM D-glucose. Nevertheless, the growth of strain 11shimoA1 was not affected by D-glucose supplementation. Sucrose supplementation did not show any effects on the production of carotenoid and the cell growth of strain 11shimoA1. Comparing to the study of the other type of *Flavobacterium* sp. in which the rapid growth and pigment production are supported by supplementation of glucose, sucrose, and xylose (Dasek et al. 1973; Sheperd and Dasek 1974; Sheperd et al. 1974; Shoeher and Wis 1972), it is possible for us to determine the metabolic diversity in production of carotenoid among Flavobacteria, which has recently been well studied especially in the genome survey (Fernández-Gómez et al. 2013).

In this study, effect of pH on growth production of carotenoid of strain 11shimoA1 was also evaluated the, since it was previously reported that *J. pallidilutea* EM39^{T} grew at pH 6.9-9.0 (Lee et al. 2009). In alkaline medium at pH 9.2, 2'-isopentenylsaproxanthin content per CDW was more than twice of that produced in the medium with pH 7.0 after 138 h of cultivation,

311 while growth rate of strain 11shimoA1 was slower and the final cell concentration was also
312 slightly lower. Therefore, the amount of 2'-isopentenylsaproxanthin and zeaxanthin per
313 volume of culture medium was compared at pH 9.2 and pH 7.0. Total carotenoid content per
314 CDW significantly increased by the incubation at medium with pH 9.2. Especially, the
315 content of 2'-isopentenylsaproxanthin content (1.2 μg/ml medium) was doubled that in the
316 medium with pH 7.0 (0.6 μg/ml medium) after 138 h cultivation. These data show that
317 2'-isopentenylsaproxanthin synthesis in strain 11shimoA1 was promoted under alkaline
318 conditions. In contrast, zeaxanthin content (per CDW and per ml culture medium) was lower
319 in medium with pH 9.2 than in the medium with pH 7.0. Thus, cultivation in alkaline
320 conditions markedly altered the composition of carotenoid in strain 11shimoA1 (Fig. 4 (b)).
321 In several bacteria, a large number of specific stress proteins are induced in harmful
322 environmental conditions such as pH, oxygen and light (Storz G and Hengge-Aronis R 2000).
323 The gene expressions are controlled by alternative sigma factors such as extractoplasmic
324 functions (ECF) subfamily. It has been reported that σ^W is one of ECF subfamily and is
325 induced by alkali shock in bacteria (Wiegert T et al. 2001). In addition, another ECF sigma
326 factor, CarQ is also known to regulate carotenogenesis in *Myxococcus xanthus* (Gorham et al.
327 1996). Therefore, it is suggested that carotenoid synthesis in strain 11 shimoA1 is regulated
328 by a sigma factor response to alkaline condition at pH 9.2. Long-chain C-50
329 bacterioruberin-type carotenoids have been isolated from several extremophilic bacteria and
330 archaea, including species of *Halobacterium, Haloferax* (D'Souza et al. 1997), and
331 psychrotrophic bacteria *Micrococcus roseus* (Jagannadham et al. 1991) and *Arthrobacter*
332 *agilis* (Fong et al. 2001). In these organisms, bacterioruberin-type carotenoids have been
333 reported to play roles in adaptation and survival to extreme environments. Long-chain
334 carotenoids may also reinforce the membrane bilayer to reduce O_2 diffusion in the cytoplasm

335 (Wisniewska and Subczynski 1998) and raise the hydrophobic barrier for polar molecules and
336 ions. Since 2'-isopentenylsaproxanthin content in strain 11shimoA1 was higher in alkaline
337 medium (pH 9.2) than in medium at pH 7.0, C-45 carbon chain 2'-isopentenylsaproxanthin,
338 but not C-40 chain zeaxanthin, might contribute to adaptation to this high-pH conditions.
339 Strain 11shimoA1 accumulated lycopene by the addition of nicotine, which inhibits β-end
340 cyclization and hydration or C-1, 2 double bond saturation (Takaichi et al. 1997; Britton
341 1990), in the medium (Fig. S2.). Further, chemical mutagenesis of the strain 11shimoA1 using
342 ethyl methanesulfonate produced a red-colony mutant, 11shimoA1R. In the 11ShimoA1R,
343 lycopene was detected as a major carotenoid (Fig. S3.). These results suggest that the strain
344 may have unique carotenoid synthetic pathways, which is converted to
345 2'-isopentenylsaproxanthin or zeaxanthin via lycopene. This also suggest that strain
346 11shimoA possesses a sensing system to regulate dicyclic or monocyclic carotenoids
347 production depending on the pH of the medium. In *Flavobacterium* p99-3 producing a
348 monocyclic carotenoid; myxol, which has similar structure with 2'-OH in the place of
349 isopentenyl residue of 2'-isopentenylsaproxanthin, it has been reported that CrtC and CrtD
350 which conduct desaturation and hydroxylation are conducted by CrtC and CrtD. We have just
351 analyzed the draft genome sequence of the strain 11shimoA1. On the basis of the genome
352 data, synthetic pathways of carotenoids and the gene regulations in strain 11shimoA1 could be
353 further elucidated.
354 In conclusion, the marine bacterium strain 11shimoA1 isolated from seaweed was
355 identified as a strain of *J. pallidilutea* in the family *Flavobacteriaceae* based on its 16S rRNA
356 gene sequences. Interestingly, the strain 11shimoA1 produced a novel monocyclic carotenoid,
357 2'-isopentenylsaproxanthin, as well as zeaxanthin. Culturing strain 11shimoA1 in medium

with a high pH enhanced 2'-isopentenylsaproxanthin synthesis. These results demonstrate that the bacterium may possess a unique physiological system in carotenoid production.

Acknowledgements This work was supported by Grants-in Aid for Scientific Research from MEXT (Ministry of Education, Culture, Sports, Science, and Technology of Japan) (No. 23380120 to M.H.) and Strategic Japanese-Brazilian Cooperative Program (JST-CNPq).

References

Aasen AJ, Liaaen-Jensen S (1966) The carotenoids of flexibacteria: II. A new xanthophyll from *Saprospira grandis*. Acta Chem Scand 20:811–81

Beatty S, Murray IJ, Henson DB, Carden D, Koh H, Boulton ME (2001) Macular Pigment and Risk for Age-Related Macular Degeneration in Subjects from a Northern European Population. Invest Ophthalmol Vis Sci 42:439-446

Bhosale P, Bernstein PS (2004) β-Carotene production by *Flavobacterium multivorum* in the presence of inorganic salts and urea. J Ind Microbiol Biotechnol 31:565–571

Britton G (1990) Carotenoid biosynthesis – an overview. In: Krinsky NI, Mathews-Roth MM, Taylor RF (eds) Carotenoids: chemistry and biology. Plenum Press, New York, pp 167–184

Britton G, Liaaen-Jensen S, Pfander H (2004) *Carotenoids Handbook*; Birkhauser Verlag: Basel, Switzerland.

Britton G, Liaaen-Jensen S, Pfander H (1995) Carotenoids volume 1a: isolation and analysis. Birkhäuser, Basel, Switzerland

Buchecker R, Noack K (1995) Circular dichroism. In: Britton G, Liaaen-Jensen S, Pfander H (eds) Carotenoids, vol. 1B. Birkhäuser, Basel, pp 63-116

Dasek J, Sheperd D, Traelnes RK (1973) Proced de production de zeaxanthin. Belgium Patent 790289

D'Souza SE, Altekar W, D'Souza SF (1997) Adaptive response of *Haloferax mediterranei* to low concentrations of NaCl (< 20 %) in the growth medium. Arch Microbiol 168:68-71

Englert G (1995) NMR spectroscopy. In: Britton G, Liaaen-Jensen S, Pfander H (eds) Carotenoids, vol. 1B. Birkhäuser, Basel, pp 147–160

406 Fernández-Gómez B, Richter M, Schüler M, Pinhassi J, Acinas SG, González JM and
407 Pedrós-Alió C (2013) Ecology of marine Bacteroidetes: a comparative genomics
408 approach. ISME J 7:1026-1037
409 Folch J, Lees M, Sloane-Stanley GH (1957) A simple method for the isolation and
410 purification of total lipids from animal tissues. J Biol Chem 226:497-509
411 Fong NJ, Burgess ML, Barrow KD, Glenn DR (2001) Carotenoid accumulation in the
412 psychrotrophic bacterium *Arthrobacter agilis* in response to thermal and salt stress.
413 Appl Microbiol Biotechnol 56:750-756
414 Goodwin TW (1980) The biochemistry of carotenoids, vol 1. Plants, 2nd edn. Chapman and
415 Hall, London
416 Gorham HC, McGowan SJ, Robson PR, Hodgson DA (1996) Light-induced carotenogenesis
417 in Myxococcus Xanthus: light-dependent membrane sequestration of ECF sigma factor
418 CarQ by anti-sigma factor CarR. Mol Microbiol. 19:171-186
419 Jackson H, Braun CL, Ernst H (2008) The chemistry of novel xanthophyll carotenoids. Am J
420 Cardiol 101:50D-57D
421 Jagannadham MV, Rao VJ, Shivaji S (1991) The major carotenoid pigment of a
422 psychrotrophic *Micrococcus roseus* strain: purification, structure, and interaction with
423 synthetic membranes. J Bacteriol 173:7911-7917
424 Lee DH, Kahng HY, Lee YS, Jung JS, Kim JM, Chung BS, Park SK, Jeon CO (2009) *Jejuia*
425 *pallidilutea* gen. nov., sp. nov., a new member of the family *Flavobacteriaceae* isolated
426 from seawater. Int J Syst Evol Microbiol 59:2148-2152
427 McDermott JCB, Brown DJ, Britton G, Goodwin TW (1974) Alternative Pathway of
428 Zeaxanthin Biosynthesis in a *Flavobacterium* species. Biochem J 144:231-243

429 Milborrow BV (2001) The pathway of biosynthesis of abscisic acid in vascular plants: a
430 review of the present state of knowledge of ABA biosynthesis. J Exp Bot 52:1145-1164
431 Misawa N (2011) Carotenoid β-ring hydroxylase and ketolase from marine
432 bacteria-promiscuous enzymes for synthesizing functional xanthophylls. Mar Drugs
433 9:757-771.
434 Miyashita K, Nishikawa S, Beppu F, Tsukui T, Abe M, Hosokawa M (2011) The allenic
435 carotenoid fucoxanthin, a novel marine nutraceutical from brown seaweeds. J Sci Food
436 Agric 91:1166-1174
437 Naguib YM (2000) Antioxidant activities of astaxanthin and related carotenoids. J Agric Food
438 Chem 48:1150–1154
439 Pashkow FJ, Watumull DG, Campbell CL (2008) Astaxanthin: a novel potential treatment for
440 oxidative stress and inflammation in cardiovascular disease. Am J Cardiol
441 101:58D-68D
442 Sawabe T, Sugimura I, Ohtsuka M, Nakano K, Tajima K, Ezura Y, Christen R (1998) *Vibrio*
443 *halioticoli* sp. nov., a nonmotile alginolytic marine bacterium isolated from the gut of
444 the abalone *Haliotis discus hannai*. Int J Syst Bacteriol 48:573-580
445 Sheperd D and Dasek J (1974) Proced de preparation de zeaxanthin. Belgium Patent 816876
446 Sheperd D, Dasek J, Carels M (1974) Proced de production de zeaxanthin. Belgium Patent
447 816767
448 Shoeher A, Wis O (1972) Proced pour la preparation de zeaxanthin. Belgium Patent 770744
449 Shindo K, Kikuta K, Suzuki A, Katsuta A, Kasai H, Yasumoto-Hirose M, Matsuo Y, Misawa
450 N, Takaichi S (2007) Rare carotenoids, (3R)-saproxanthin and (3R,2′S)-myxol, isolated
451 from novel marine bacteria (*Flavobacteriaceae*) and their antioxidative activities. Appl
452 Microbiol Biotechnol 74:1350-1357

453 Sommerburg OG, Siems WG, Hurst JS, Lewis JW, Kliger DS, van Kuijk FJ (1999) Lutein
454 and zeaxanthin are associated with photoreceptors in the human retina. Curr Eye Res
455 19:491-495
456 Storz G and Hengge-Aronis R (2000) Bacterial stress responses. Washington, DC: Amrican
457 Society for Microbiology.
458 Tanaka T, Shnimizu M, Moriwaki H (2012) Cancer chemoprevention by carotenoids.
459 Molecules 17:3202-3242
460 Takaichi S, Maoka T, Masamoto K (2001) Myxoxanthophyll in Synechocystis sp. PCC 6803
461 is myxol 2'-dimethylfucoside, (3R, 2'S)-myxol 2'-(2,4-di-O-methyl-a-L-fucoside), not
462 rhamnoside. Plant Cell Physiol 42:756-762
463 Takaichi S, Mochimaru M (2007) Carotenoids and carotenogenesis in cyanobacteria: unique
464 ketocarotenoids and carotenoid glycosides. Cell Mol Life Sci 64:2607-2619
465 Takaichi S, Mochimaru M, Maoka T, Katoh H (2005) Myxol and 4-ketomyxol 2'-fucosides,
466 not rhamnosides, from Anabaena sp. PCC 7120 and Nostoc punctiforme PCC 73102,
467 and proposal for the biosynthetic pathway of carotenoids. Plant Cell Physiol
468 46:497-504
469 Takaichi S, Wang ZY, Umetsu M, Nozawa T, Shimada K, Madigan MT (1997) New
470 carotenoids from the thermophilic green sulfur bacterium *Chlorobium tepidum*:
471 1',2'-dihydro-gamma-carotene, 1',2'-dihydrochlorobactene, and OH-chlorobactene
472 glucoside ester, and the carotenoid composition of different strains. Arch Microbiol
473 168:270-276
474 Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S (2011) MEGA5:
475 Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary
476 Distance, and Maximum Parsimony Methods. Mol Biol Evol 28:2731-2739

Teramoto M, Rählert N, Misawa N, Sandmann G (2004) 1-Hydroxy monocyclic carotenoid 3,4-dehydrogenase from a marine bacterium that produces myxol. FEBS Lett 570:184-188

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies formultiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876-4882

Thomson LR, Toyoda Y, Langner A, Delori FC, Garmett KM, Craft N, Nichols CR, Cheng KM, Dorey CK (2002) Elevated retinal zeaxanthin and prevention of light-induced photoreceptor cell death in quail. Invest Ophthalmol Vis Sci 43:3538-3549

Wiegert T, Homuth G, Versteeg S, Schumann W (2001) Alkaline shock induces the Bacillus subtillis sigma(W) regulon. Mol Microbiol 41: 59-71

Wisniewska A, Subczynski WK (1998) Effects of polar carotenoids on the shape of the hydrophobic barrier of phospholipid bilayers. Biochim Biophys Acta 1368:235-246

Yokoyama A, Miki W (1995) Isolation of myxol from a marine bacterium *Flvobacterium* sp. associated with a marine sponge. Fish Sci 61:684–686

Yokoyama A, Sandmann G, Hoshino T, Adachi K, Sakai M, Shizuri Y (1995) Thermozeaxanthins, New Carotenoid-glycoside-esters from Thermophilic Eubacterium *Thermus thermophilus*. Tetrahedron Lett 36:4901-4904

501 **Figure Legends**

502

503 **Fig. 1** HPLC chromatogram of pigments produced by strain 11shimoA1. Strain 11shimoA1
504 was cultivated in Marine Broth 2216 for 48 h at 25°C and 140 rpm. Pigments in total lipid
505 extract from the strain 11shimoA1 were analyzed by HPLC equipped with ODS column.
506 Mobile phase was methanol: acetonitrile (7:3, v/v) and flow rate was 1.0 ml/min. Pigments
507 were detected at 450 nm using UV detector.

508

509 **Fig. 2** Structure of carotenoids produced by strain 11shimoA1.

510

511 **Fig. 3** Effect of D-glucose or sucrose on cell growth (a) and carotenoid production (b).
512 Growth was measured by monitoring optical density at 600 nm (a). Carotenoid production
513 was measured after 73 h culture at 25°C, 140 rpm. Carotenoid content is expressed as mg/g
514 cell dry weight. Mean values are shown (n=3) (b). *$P < 0.01$ vs. zeaxanthin content in control.
515 **$P < 0.01$ vs. 2'-isopentenysaproxanthin content in control.

516

517 **Fig. 4** Effect of culture medium pH on growth (a) and carotenoid production (b) of strain
518 11shimoA1. Growth was measured by optical density at 600 nm (a). Carotenoid production
519 was measured after 138 h culture at 25°C, 140 rpm. Mean values are shown (n=3). Carotenoid
520 content is expressed as mg/g cell dry weight (b). *$P < 0.01$ vs. zeaxanthin content in cells
521 grown in pH 7.0 medium. **$P < 0.01$ vs. 2'-isopentenysaproxanthin content in cells grown in
522 pH 7.0 medium.

523

524

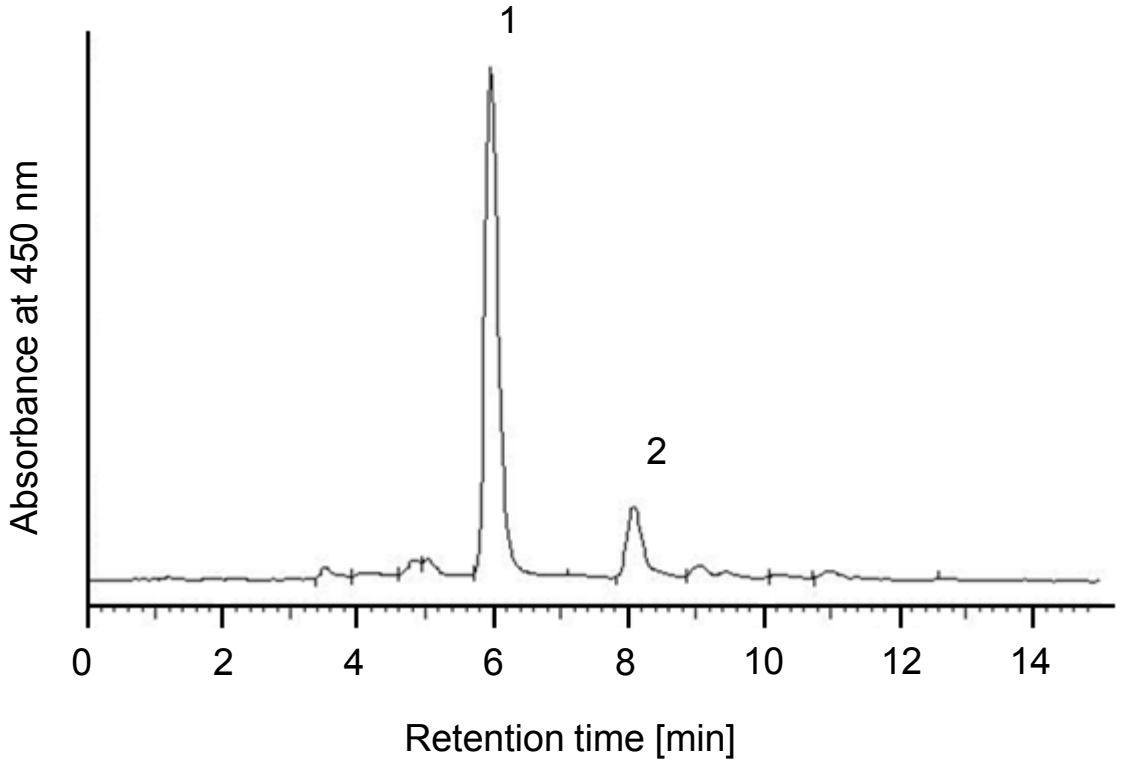
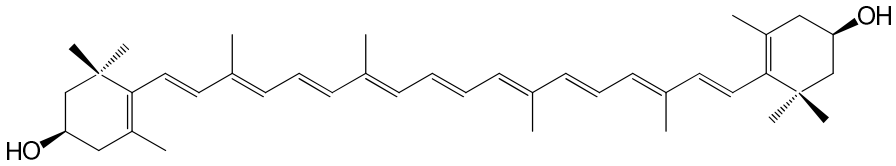
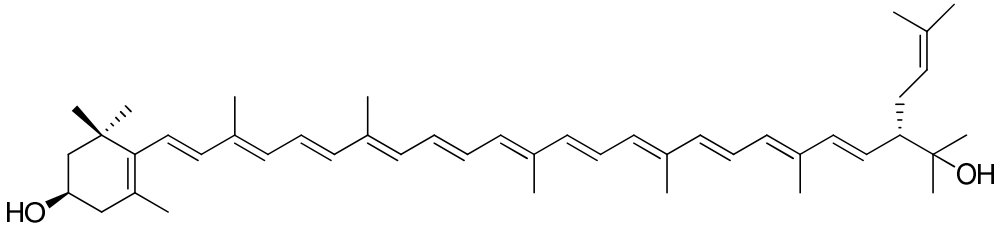


Fig. 1



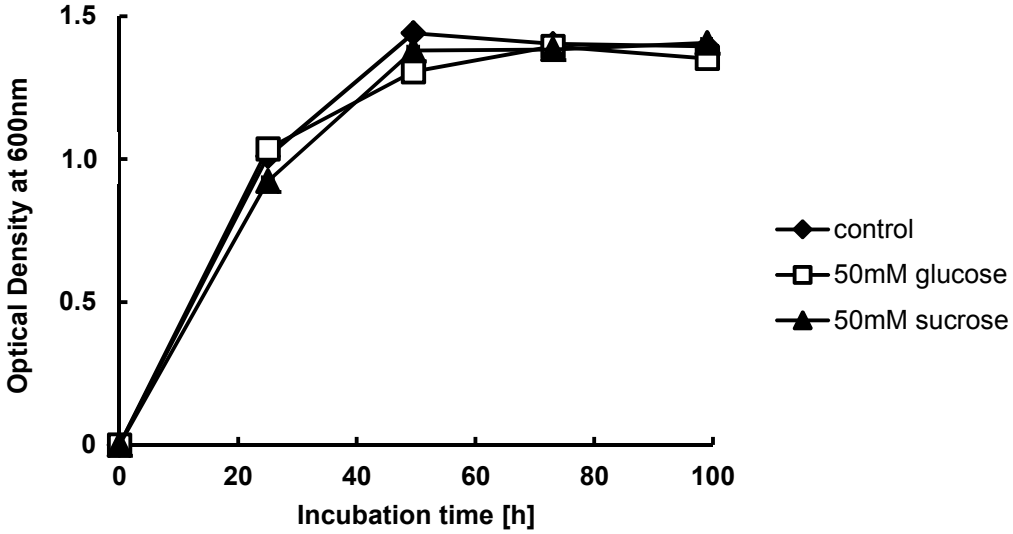
Zeaxanthin



2'-Isopentenylsaproxanthin

Fig. 2

(a)


1.5
1.0
0.5
0
Optical Density at 600nm
0
20
40
60
80
100
Incubation time [h]
control
50mM glucose
50mM sucrose

(b)

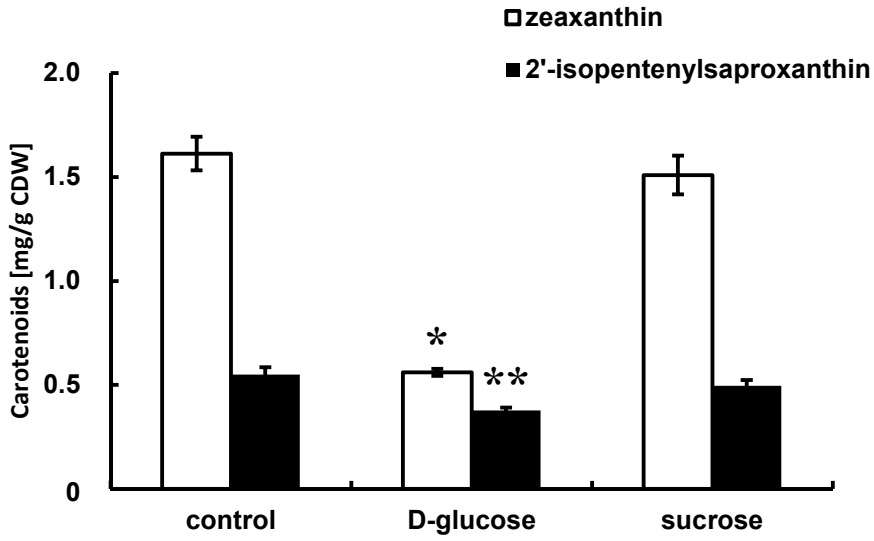
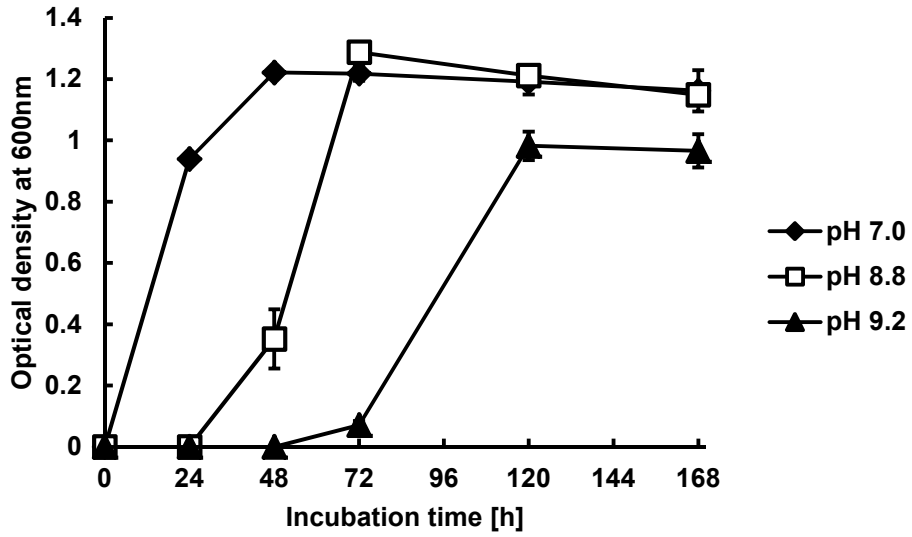

zeaxanthin
2'-isopentenylsaproxanthin
2.0
1.5
1.0
0.5
0
Carotenoids [mg/g CDW]
*
**
control
D-glucose
sucrose

Fig. 3

(a)


Optical density at 600nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
24
48
72
96
120
144
168
Incubation time [h]
pH 7.0
pH 8.8
pH 9.2

(b)

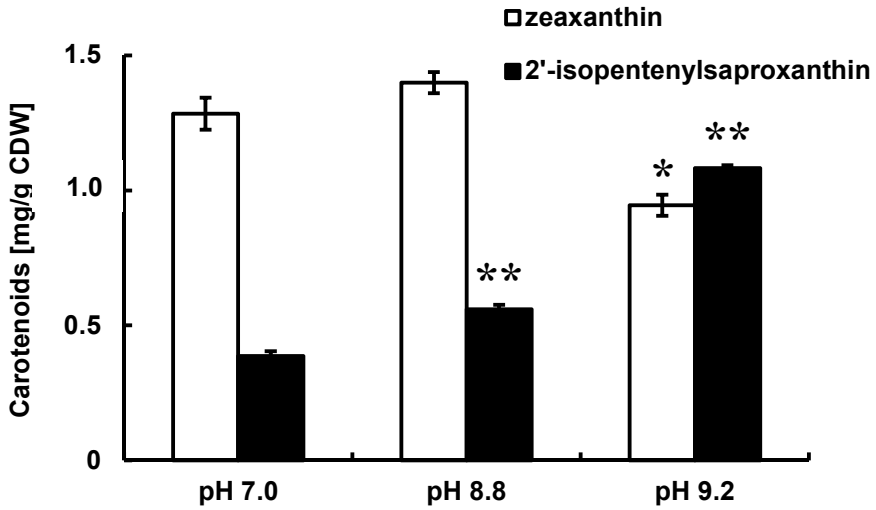

zeaxanthin
2'-isopentenylsaproxanthin
Carotenoids [mg/g CDW]
1.5
1.0
0.5
0
*
**
**
pH 7.0
pH 8.8
pH 9.2

Fig. 4