

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

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Received: 9 October 2013 / Revised: 7 March 2014 / Accepted: 17 March 2014 / Published online: 11 April 2014  
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**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  $\gamma$ -carotene skeleton, have been reported to show a stronger antioxidant activity than those with  $\beta$ -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 (3R, 3'R)-zeaxanthin with dicyclic structure and another was identified as (3R, 2'S)-2'-isopentenylsaproxanthin, a novel monocyclic carotenoid with pentenyl residue at C-2' position of saproxanthin, using FAB-MS,  $^1\text{H}$  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 as well as 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.  $\beta$ -Carotene and lycopene are the representative of carotenoids with dicyclic and acyclic structures, 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  $\beta$ -ionone ring and sugar moiety which is only unique to the members of the phylum *Cyanobacteria* (Goodwin 1980; Takaichi et al. 2005; Takaichi and Mochimaru 2007). Aglycone and myxol have 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 photoprotection (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 in carotenoids in nature, have been reported to show stronger antioxidant

**Electronic supplementary material** The online version of this article (doi:10.1007/s00253-014-5702-y) contains supplementary material, which is available to authorized users.

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activities than those of  $\beta$ -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 sproxanthin 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 the organisms that produce these monocyclic carotenoids and to investigate the biological functions of these compounds.

Since marine bacteria have 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 was 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 strain 11shimoA1 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 and 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 (20×250 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 ( $^1\text{H}$  NMR) spectra of the carotenoids in  $\text{CDCl}_3$  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  $^1\text{H}$  NMR spectra were made on the basis of  $^1\text{H}$ – $^1\text{H}$  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-500C 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 ribosomal RNA (rRNA) gene was amplified from the DNA using 24 F 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 from 124 to 1,378 (*Escherichia coli* str. K12, AP012306 numbering, Fig. S1). The phylogenetic analyses

were performed using three different methods, neighbor-joining (NJ), maximum-likelihood (ML), and maximum-parsimony (MP) implemented in MEGA version 5. The robustness of each topology was checked by NJ and 500 bootstrap replications. The tree was drawn by MEGA version 5.

#### Nucleotide sequence accession number

The 16S rRNA sequence of the strain 11shimoA1 (JCM19538) is available in GenBank/EMBL/DDBJ under the accession number of AB848003.

#### Cultivated conditions in carotenoid synthesis and cell growth

In order to investigate the cultivated conditions in carotenoid synthesis of strain 11shimoA1, 50 mM D-glucose or sucrose was added into Marine Broth 2216. The pH of the culture media (Marine Broth 2216) was adjusted by the addition of HCl or NaOH into Marine Broth 2216. After the cultivation of strain 11shimoA1 in 50 ml Marine Broth 2216 in 100-ml Erlenmeyer flask for 2–3 days at 140 rpm and at temperature of 25 °C, total lipid was extracted by the method previously described by Folch et al. (1957). As for the quantification analysis of carotenoids, the total lipid was subjected to HPLC equipped with Develosil ODS-UG-5 column (4.6×250 mm, Nomura Chemical, Japan) and was analyzed with diode array delivery detector L-7455 (Hitachi, Japan). Mobile phase of methanol/acetonitrile (7:3, v/v) was isocratically eluted at a flow rate of 1.0 ml/min. The temperature of the column was maintained at 30 °C using column 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 1 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 °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.

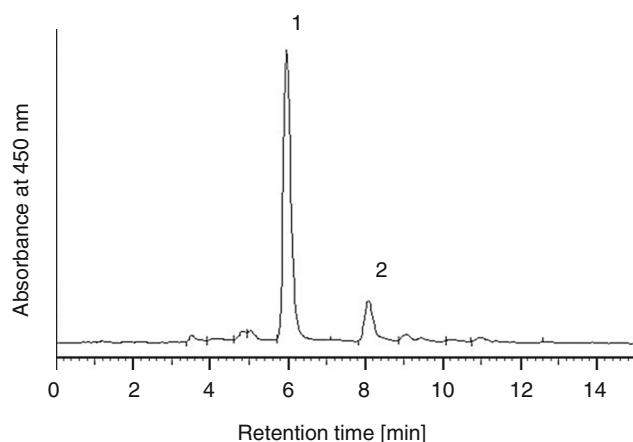
## Results

### Identification of pigments produced by strain 11shimoA1

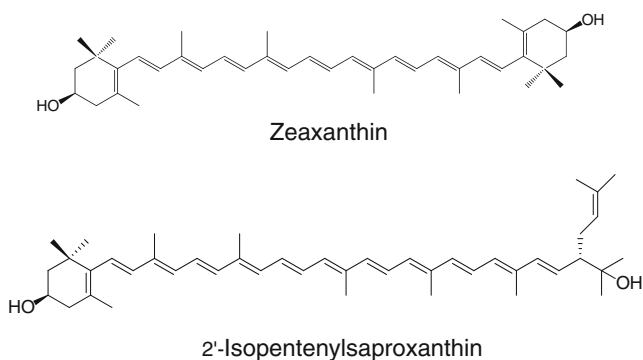
Strain 11shimoA1 which was isolated from a type of seaweed (unidentified) produced a yellow-orange pigmented colonies on the agar plates. After culturing the bacterium in Marine Broth 2216, two major peaks were detected by HPLC analysis in their total lipid extract (Fig. 1).

Peak 1 showed the same retention time (6.0 min) as that of the (all-*trans*) zeaxanthin authentic standard. Analyses using positive ion high-resolution fast atom bombardment mass spectrometry (FAB-MS) and <sup>1</sup>H NMR (data not shown) confirmed the identification as zeaxanthin (Fig. 2). The CD spectrum was comparable to that of (3*R*, 3'*R*)-zeaxanthin as described in a previous report by Buchecker and Noack (1995).

Peak 2 was detected at 8.1 min of retention time from the HPLC analysis (Fig. 1). This carotenoid showed a maximum absorption at 472 and 502 nm, indicating the presence of a myxol (saproxanthin)-type chromophore (Britton et al. 1995). The molecular formula of this compound was determined to be C<sub>45</sub>H<sub>64</sub>O<sub>2</sub> from positive ion high-resolution FAB-MS data (*m/z* 636.4901 [M<sup>+</sup>] C<sub>45</sub>H<sub>64</sub>O<sub>2</sub> calc. 636.4907). This suggested that an isopentenyl group was attached to the carotenoid moiety. The <sup>1</sup>H NMR spectrum revealed the presence of 36 methyl protons (12 methyl groups), 6 methylene protons, 1 oxy methine, 1 aliphatic methine, and 18 olefinic protons in the molecule. These signals were assigned by COSY and NOESY spectra and by comparisons with zeaxanthin, saproxanthin, and myxoxanthophyll. <sup>1</sup>H NMR data is summarized in Table 1. The <sup>1</sup>H NMR data of the carotenoid moiety were similar to those of saproxanthin, except for the H-2' position (Englert 1995). The remaining part was



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



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

assigned to an isopentenyl moiety as shown in Table 1. In the case of saproxanthin, the methylene proton at H-2 appears at 2.31 ppm as doublet. For the carotenoid produced by strain 11shimoA1, however, a methine proton at H-2' (2.09 ppm) was multiplet. This indicated that an isopentenyl group was attached at C-2' position of saproxanthin. COSY spectrum clearly indicated the connection between carotenoid moiety at H-2' and isopentenyl moiety at H-1''. Therefore, the structure of this compound was determined to be 2'-(3-methylbut-2-enyl)-3', 4'-didehydro-1', 2'-dihydro- $\beta$ ,  $\psi$ -carotene-3, 1'-diol, which is a novel carotenoid with modified saproxanthin binding isopentenyl group at C-2' position. Peak 2 is named 2'-isopentenylsaproxanthin (Fig. 2). CD of this compound showed [(EPA) nm ( $\Delta\epsilon$ ) 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.

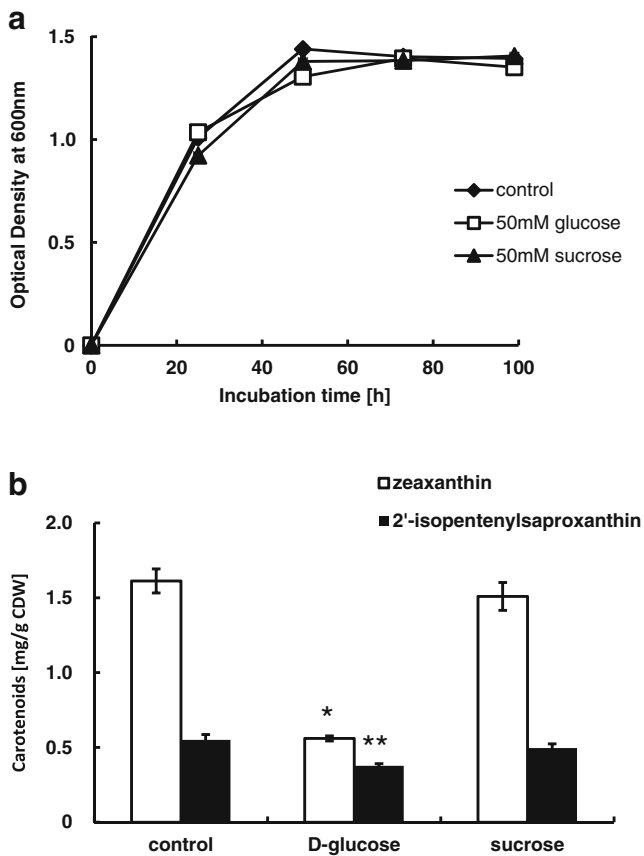
#### Effect of additional sugars on growth and carotenoid production

Strain 11shimoA1 produced 2'-isopentenylsaproxanthin and zeaxanthin at 0.5 and 1.6 mg/g CDW, respectively, in Marine Broth 2216 after 73 h of incubation at 140 rpm and at 25 °C. To examine the effects of additional sugars as carbon source on growth and carotenoid production, strain 11shimoA1 was cultured in the media

**Table 1**  $^1\text{H}$  NMR data of peak 2 produced by strain 11shimoA1

position	$\delta$ $^1\text{H}$	mult. J (Hz)
H-2 $\beta$	1.48	dd (12, 12)
H-2 $\alpha$	1.77	ddd (12, 5, 2)
H-3	4.00	m
H-4 $\beta$	2.04	dd (16, 10)
H-4 $\alpha$	2.39	ddd (16, 6, 2)
H-7	6.10	d (16)
H-8	6.15	d (16)
H-10	6.15	d (11)
H-11	6.62	dd (15, 11)
H-12	6.36	d (15)
H-14	6.25	d (11)
H-15	~6.64	m
H-16	1.08	s
H-17	1.08	s
H-18	1.74	s
H-19	1.97	s
H-20	1.97	s
H-2'	2.09	m
H-3'	5.50	dd (15, 9)
H-4'	6.17	d (15)
H-6'	6.13	d (11)
H-7'	6.60	dd (15, 11)
H-8'	6.37	d (15)
H-10'	6.25	d (11)
H-11'	6.64	dd (15, 11)
H-12'	6.38	d (15)
H-14'	6.28	d (12)
H-15'	~6.64	m
H-16'	1.23	s
H-17'	1.18	s
H-18'	1.92	s
H-19'	1.97	s
H-20'	1.98	s
H-1''	~2.00	m
H-1'''	~2.35	m
H-2''	5.07	t-like (7)
H-4''	1.61	s
H-5''	1.67	s

supplemented with 50 mM D-glucose or 50 mM sucrose. As shown in Fig. 3a, 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. 3b) 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).

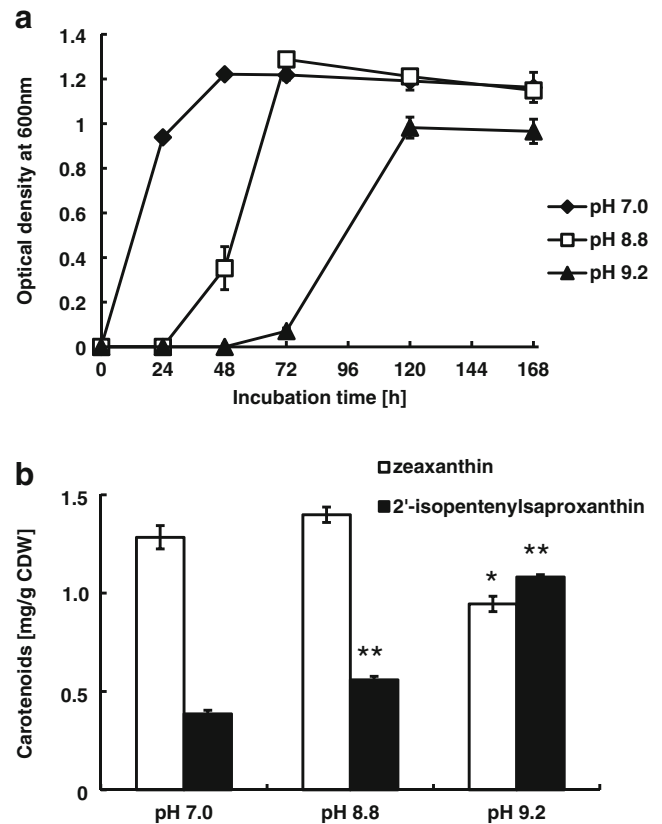


**Fig. 3** Effect of D-glucose or sucrose on cell growth (a) and carotenoid production (b). Growth was measured by monitoring optical density at 600 nm (a). Carotenoid production was measured after 73 h culture at 25 °C and 140 rpm. Carotenoid content is expressed as mg/g cell dry weight. Mean values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

#### 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 9.2 than at pH 7.0 (Fig. 4a). 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 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. 4b). 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 was compared. The 2'-isopentenylsaproxanthin content per milliliter of culture



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

medium was higher in medium with pH 9.2 (1.2  $\mu\text{g}/\text{ml}$ ) than in the ones with pH 7.0 (0.6  $\mu\text{g}/\text{ml}$ ) 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.

#### Discussion

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

Strain 11shimoA1 was identified to *J. pallidilutea* based on 16S rRNA gene sequencing. *J. pallidilutea* EM39<sup>T</sup> was



proposed to be a novel genus and species in the family *Flavobacteriaceae* (Lee et al. 2009). The strain EM39<sup>T</sup> showed an orange color of water insoluble pigmentation. However, the chemical structures of the components from the pigment have yet to be identified. This study first elucidated that the strain 11shimoA1 produced two types of carotenoid which chemical structures were determined by FAB-MS, <sup>1</sup>H NMR, and CD analyses. One of the carotenoids was (3*R*, 3'*R*)-zeaxanthin, a yellow xanthophyll. Zeaxanthin has dicyclic structure and is synthesized by some species of bacteria including the *Flavobacterium* species (McDermott et al. 1974). Zeaxanthin and lutein are normally found in the human eye and prevent age-related macular degeneration and light-induced photoreceptor death (Beatty et al. 2001; Thomson et al. 2002). The nutraceutical applications of zeaxanthin have been identified and well researched in the scientific world.

On the other hand, the other carotenoid was identified as a novel monocyclic carotenoid, 2'-isopentenylsaproxanthin. It is of a unique carotenoid structure comprising saproxanthin (3',4'-didehydro-1',2'-dihydro-β, ψ-carotene-3,1'-diol) with a pentenyl residue at C-2' position. Saproxanthin is synthesized by the marine bacterium strain 04OKA-13-27 (Shindo et al. 2007) and *Saprospira grandis* (Aasen and Liaaen-Jensen 1966). It has been reported to show 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'-triole) (Yokoyama and Miki 1995; Teramoto et al. 2004), which has a similar structure to that of saproxanthin, showed a neuroprotective 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 neuroprotective 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 the supplementation of glucose, sucrose, and xylose (Dasek et al. 1973; Sheperd and Dasek 1974; Sheperd et al. 1974; Shoehner and Wis 1972), it is possible for us to determine the metabolic diversity in the production of carotenoid among Flavobacteria, which has

recently been well studied especially in the genome survey (Fernández-Gómez et al. 2013).

In this study, the effect of pH on growth production of carotenoid of strain 11shimoA1 was also evaluated, since it was previously reported that *J. pallidilutea* EM39<sup>T</sup> 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, while growth rate of strain 11shimoA1 was slower and the final cell concentration was also slightly lower. Therefore, the amount of 2'-isopentenylsaproxanthin and zeaxanthin per volume of culture medium was compared at pH 9.2 and 7.0. Total carotenoid content per CDW significantly increased by the incubation at medium with pH 9.2. Especially, the content of 2'-isopentenylsaproxanthin content (1.2 μg/ml medium) was doubled that in the medium with pH 7.0 (0.6 μg/ml medium) after 138 h cultivation. These data show that 2'-isopentenylsaproxanthin synthesis in strain 11shimoA1 was promoted under alkaline conditions. In contrast, zeaxanthin content (per CDW and per ml culture medium) was lower in medium with pH 9.2 than in the medium with pH 7.0. Thus, cultivation in alkaline conditions markedly altered the composition of carotenoid in strain 11shimoA1 (Fig. 4b).

In several bacteria, a large number of specific stress proteins are induced in harmful environmental conditions such as pH, oxygen, and light (Storz G and Hengge-Aronis R 2000). The gene expressions are controlled by alternative sigma factors such as extracytoplasmic functions (ECF) subfamily. It has been reported that σ<sup>W</sup> is one of the ECF subfamily and is induced by alkali shock in bacteria (Wiegert T et al. 2001). In addition, another ECF sigma factor, CarQ is also known to regulate carotenogenesis in *Myxococcus xanthus* (Gorham et al. 1996). Therefore, it is suggested that carotenoid synthesis in strain 11 shimoA1 is regulated by a sigma factor response to alkaline condition at pH 9.2. Long-chain C-50 bacterioruberin-type carotenoids have been isolated from several extremophilic bacteria and archaea, including species of *Halobacterium*, *Haloferax* (D'Souza et al. 1997), and psychrotrophic bacteria, *Micrococcus roseus* (Jagannadham et al. 1991) and *Arthrobacter agilis* (Fong et al. 2001). In these organisms, bacterioruberin-type carotenoids have been reported to play roles in adaptation and survival to extreme environments. Long-chain carotenoids may also reinforce the membrane bilayer to reduce O<sub>2</sub> diffusion in the cytoplasm (Wisniewska and Subczynski 1998) and raise the hydrophobic barrier for polar molecules and ions. Since 2'-isopentenylsaproxanthin content in strain 11shimoA1 was higher in alkaline medium (pH 9.2) than in medium at pH 7.0, C-45 carbon chain 2'-isopentenylsaproxanthin, but not C-40 chain zeaxanthin, might contribute to the adaptation to this high-pH conditions.

Strain 11shimoA1 accumulated lycopene by the addition of nicotine, which inhibits  $\beta$ -end cyclization and hydration or C-1, 2 double bond saturation (Takaichi et al. 1997; Britton 1990), in the medium (Fig. S2). Further, chemical mutagenesis of the strain 11shimoA1 using ethyl methanesulfonate produced a red-colony mutant, 11shimoA1R. In the 11ShimoA1R, lycopene was detected as a major carotenoid (Fig. S3). These results suggest that the strain may have unique carotenoid synthetic pathways, which is converted to 2'-isopentenylsaproxanthin or zeaxanthin via lycopene. This also suggest that strain 11shimoA possesses a sensing system to regulate dicyclic or monocyclic carotenoids production depending on the pH of the medium. In *Flavobacterium* p99-3 producing a monocyclic carotenoid, myxol, which has similar structure with 2'-OH in the place of isopentenyl residue of 2'-isopentenylsaproxanthin, it has been reported that CrtC and CrtD which conduct desaturation and hydroxylation are conducted by CrtC and CrtD. We have just analyzed the draft genome sequence of the strain 11shimoA1. On the basis of the genome data, synthetic pathways of carotenoids and the gene regulations in strain 11shimoA1 could be further elucidated.

In conclusion, the marine bacterium strain 11shimoA1 isolated from seaweed was identified as a strain of *J. pallidilutea* in the family *Flavobacteriaceae* based on its 16S rRNA gene sequences. Interestingly, the strain 11shimoA1 produced a novel monocyclic carotenoid, 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).

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