Altererythrobacter ishigakiensis sp. nov., an astaxanthin-producing bacterium isolated from a marine sediment

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A Gram-negative, non-motile, non-spore-forming, halophilic rod, designated JPCMB0017^T, was isolated from a marine sediment of the coastal area of Okinawa, Japan. The isolate formed orange–red colonies on marine agar. Bacteriochlorophyll α was absent and sphingoglycolipid 1 and other carotenoids, including astaxanthin, adonixanthin and zeaxanthin, were present. Ubiquinone-10 (Q-10) was the main respiratory quinone and C_{18:1} ω 7c was the major cellular fatty acid. The G + C content of DNA was 59.1 mol%. Phylogenetic analysis based on 16S rRNA gene sequencing revealed that the isolate was a member of the genus *Altererythrobacter* in the family *Erythrobacteraceae*. Strain JPCMB0017^T exhibited 96.8% 16S rRNA gene sequence similarity with *Altererythrobacter marinus* H32^T. Unlike other members of the genus *Altererythrobacter*, strain JPCMB0017^T reduced nitrate. On the basis of genotypic and phenotypic data, a novel species is proposed to accommodate this isolate, with the name *Altererythrobacter ishigakiensis* sp. nov. The type strain is JPCMB0017^T (=NITE-AP48^T=ATCC BAA-2084^T=NBRC 107699^T).

Astaxanthin (3,3'-dihydroxy- β , β -caroten-4,4'-dione) is a carotenoid pigment with several essential biological functions and wide applications in the nutraceutical, cosmetic, food and animal feed industries (Lorenz & Cysewski, 2000). Astaxanthin is produced by micro-organisms such as green microalgae (Bar *et al.*, 1995; Renstrom *et al.*, 1981), red yeasts (Andrewes *et al.*, 1976) and members of diverse bacterial genera such as *Paracoccus* (Tsubokura *et al.*, 1999), *Sphingomonas* (Asker *et al.*, 2007), *Brevundimonas* (Iizuka & Nishimura, 1969) and *Bacillus* (Pane *et al.*, 1996).

The genus *Altererythrobacter* was proposed by Kwon *et al.* (2007). At present, this genus includes eight species with validly published names: *Altererythrobacter luteolus* (Yoon *et al.*, 2005), *A. epoxidivorans* (Kwon *et al.*, 2007), *A. indicus* (Kumar *et al.*, 2008), *A. marinus* (Lai *et al.*, 2009), *A. marensis*

(Seo & Lee, 2010), *A. dongtanensis* (Fan *et al.*, 2011), *A. namhicola* and *A. aestuarii* (Park *et al.*, 2011). The genus is phylogenetically related to four other genera, namely *Erythrobacter*, *Porphyrobacter*, *Erythromicrobium* and *Croceicoccus*, which together constitute the family *Erythrobacteraceae*. Members of the genus *Altererythrobacter* exhibit some common characteristics, such as non-motility, lack of bacteriochlorophyll (Bchl) α and the requirement for sodium for cell growth (Kumar *et al.*, 2008). Cells form yellow-pigmented colonies, but the origin of the colour remains to be clarified.

During the screening of astaxanthin-producing bacteria from marine sediments, we isolated a halophilic bacterium, designated JPCMB0017^T, which produced orange–red pigments. Marine sediments were collected from a site on the coast of Ishigaki island, Okinawa, Japan. Samples were suspended in sterile seawater and, after sedimentation, the supernatant was diluted 10- to 1000-fold and spread on 10-fold diluted marine agar 2216 (MA; Difco) or YPG medium [0.1% bacto-peptone, 0.05% yeast extract and 0.1% glucose; artificial seawater (Marine Art SF-1, Tomita Pharmaceutical Co., Ltd.)]. After incubation for 2 weeks at

Abbreviations: Bchl, bacteriochlorophyll; SGL-1, sphingoglycolipid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NITE-AP48^T is AB363004.

A supplementary figure and two supplementary tables are available with the online version of this paper.

25 °C, orange–red colonies were obtained, one of which was strain JPCCMB0017^T and was subsequently cultured in marine broth 2216 (MB; Difco).

Gram-staining, mobility and cell morphology were determined by phase-contrast microscopy and transmission electron microscopy (JEM 2000EX; JEOL). To evaluate the effect of 0–3.0 % (w/v) NaCl on growth, the bacterium was cultured in liquid BY medium (1.5 % bactopectone, 0.5 % yeast extract, 0.006 % KH₂PO₄, 0.005 % FeSO₄, 0.88 % MgCl₂, 0.18 % CaCl₂, 2.0 % NaCl; distilled water; pH 7.5) and NaCl was replaced with KCl in addition to the supplementation with NaCl. To evaluate the optimum pH for growth, the bacterium was cultured in liquid BY medium at pH 5.5–8.5. Colonies on MA were used for the analysis of cytochrome oxidase activity with a commercial paper disc (Oxidase test; bioMérieux) and catalase activity with 0.3 % hydrogen peroxide. Other biochemical tests, including that to determine nitrate reduction, were conducted using the API 20 NE system (bioMérieux), according to the manufacturer's instructions.

Genomic DNA was extracted by standard procedures (Sambrook *et al.*, 1989). The 16S rRNA gene was amplified by PCR and sequenced using universal primers: the forward primers corresponded to nucleotide positions 8–27 (5'-AGAGTTTGATCCTGGCTCAG-3') and 340–356 (5'-CCTACGGGAGGCAGCAG-3') of the *Escherichia coli*

16S rRNA gene and the reverse primers corresponded to positions 906–925 (5'-CCGTCAATTCCTTTAAGTTT-3') and 1492–1510 (5'-GGCTACCTTGTTACGACTT-3'). A partial sequence (1315 bp) was subjected to BLAST against sequences in the DDBJ database (Altschul *et al.*, 1990). The multiple sequence alignment was analysed using CLUSTAL W (Thompson *et al.*, 1994) and a phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) with MEGA version 4.0 (Kumar *et al.*, 2004).

For chemotaxonomic analyses, biomass was obtained from cultures grown in MB for 3 days at 30 °C. The G+C content of DNA was determined by the method of Katayama-Fujimura *et al.* (1984). Quinones were analysed by the methods described elsewhere (Nishijima *et al.*, 1997; Yokota *et al.*, 1992). Pigments were extracted from 50–100 mg dry cells using 5 ml dichloromethane/methanol (3:1, v/v). The extracts were dried using N₂ gas, resuspended in acetone and acetonitrile for TLC and HPLC, respectively, filtered through a 0.5 µm polytetrafluoroethylene membrane filter and subsequently loaded onto a HPLC column (Symmetry C18, 5 µm, 150 mm; Waters). The mobile phase was a mixture of acetonitrile/methanol/2-propanol (45:3:2, v/v). Pigments were monitored by measuring absorbance at approximately 480 nm. Carotenoid contents were quantified by comparing the peak area with those of the standard synthetic carotenoid

Table 1. Phenotypic characteristics of strain JPCCMB0017^T and the type strains of species of the genera *Altererythrobacter* and *Croceicoccus*

Strains: 1, JPCCMB0017^T (data from this study); 2, *A. marinus* H32^T (Lai *et al.*, 2009); 3, *A. marensis* MSW-14^T (Seo & Lee, 2010); 4, *A. luteolus* SW-109^T (Yoon *et al.*, 2005); 5, *A. indicus* MSSRF26^T (Kumar *et al.*, 2008); 6, *A. dongtanensis* JM27^T (Fan *et al.*, 2011); 7, *A. namhicola* KYW48^T (Park *et al.*, 2011); 8, *A. aestuarii* KYW147^T (Park *et al.*, 2011); 9, *A. epoxidivorans* JCS 350^T (Kwon *et al.*, 2007); 10, *Croceicoccus marinus* E4A9^T (Xu *et al.*, 2009). Data in parentheses were taken from this study. O, Orange; OR, orange–red; Y, yellow; +, positive; w, weakly positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10
Colony colour	OR	Y	Y	Y	Y	Y	O	Y	Y	Y
Oxidase	+	–	+	+	–	–	+	+	+	–
β-Galactosidase	+	w (–)	– (–)	ND (–)*	+	+	–	–	+	ND
Motility	–	–	+	–	–	–	–	–	–	+
DNA G+C content (mol%)	59.1	66.5	63.1	60.3	66.8	66.4	63.8	67.2	54.5	71.5
Nitrate reduction	+	– (–)	– (–)	– (–)	– (–)	–	–	–	– (–)	ND
Hydrolysis of:										
Aesculin	+	+	+	+	+	–	+	+	– (w)	+
Gelatin	–	– (–)	– (–)	– (–)	ND (–)†	–	–	–	– (–)	+
Utilization of:										
D-Glucose	–	– (–)	– (–)	+	+	+	–	–	– (–)	+
D-Mannose	–	– (–)	– (–)	– (–)	ND (–)	–	–	–	ND (–)	+
D-Mannitol	–	– (–)	– (–)	– (–)	– (–)	–	–	–	+	–
Maltose	–	+	– (–)	+	+	–	+	+	+	+
Malate	–	– (–)	+	– (–)	+	+	+	+	– (–)	+

*Also reported as negative by Fan *et al.* (2011).

†Also reported as negative by Lai *et al.* (2009).

solutions. Fatty acids were analysed by the methods described in Yabuuchi *et al.* (1990, 1999). Dried cells (3 mg) were added to 2 ml HCl/methanol (1:19, w/v). The mixture was shaken vigorously and incubated at 90 °C for 45 min. Fatty acid methyl esters were extracted rapidly from cooled mixtures by washing with 1.5 ml n-hexane. The obtained samples were analysed by GC/MS (QP2010; Shimadzu) using a capillary column (CBP5-M25-025; Shimadzu) under the following conditions: ionizing voltage, 70 eV; ionizing current, 60 µA; interface temperature, 250 °C; injection-port temperature, 240 °C; and helium gas pressure, 100 kPa. The initial column temperature was 80 °C for 2 min and was ramped to 240 °C at a rate of 4 °C min⁻¹. Fatty acid methyl esters were identified by comparing the retention time of peaks and the mass spectra of samples with those of a standard fatty acid mixture (Funakoshi).

The presence of sphingoglycolipids (SGLs) in cells was analysed according to Kawahara *et al.* (1991). Dried cells were homogenized with chloroform/methanol (4:1, v/v) to remove phospholipids. For the extraction of SGLs, the residual cells were homogenized with chloroform/methanol (1:1, v/v) at room temperature and then with chloroform/methanol (1:3, v/v) at 80 °C for 60 min with stirring. The extracts were combined and applied to a silica-gel 60 column (70–230 mesh; Merck, Germany). SGLs were eluted using chloroform/methanol (2:1 and 1:3) and the elution profile was obtained by TLC using silica-gel 60 (aluminium plates; Merck). The SGL-containing fractions were pooled and hydrolysed with 0.1 M NaOH at 100 °C for 30 min. The hydrolysates were dialysed, lyophilized and applied to a second silica-gel 60 column to obtain purified SGLs. The chemical structure of

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (1315 bp) showing the relationship of strain JPCCMB0017^T with representatives of closely related taxa in the family *Erythrobacteraceae*. Bootstrap values (1000 replications; >50 %) are shown at branch nodes. Bar, 0.01 substitutions per nucleotide position.

SGLs was determined by electrospray-ionization tandem mass spectroscopy (ESI-MS/MS; Micro LC System Ultra-PlusII, Micro-Tech Scientific; LCQ-DECA XP mass spectrometer, Thermo Finnigan).

Colonies of strain JPCCMB0017^T on MA were orange-red, round, convex and smooth. Cells were non-motile rods, approximately 0.3–0.5 µm wide and 1.5–2.0 µm long. Ultrathin sections of cells revealed the typical arrangement of a Gram-negative cell envelope (see Supplementary Fig. S1, available in IJSEM Online). Table 1 compares the phenotypic characteristics of strain JPCCMB0017^T with the type strains of the eight recognized species of the genus *Altererythrobacter* and *Croceicoccus marinus*. Strain JPCCMB0017^T tested positive for oxidase and for the hydrolysis of aesculin, but gave a negative result for the hydrolysis of gelatin, and these characteristics were similar to those of the genus *Altererythrobacter*. On the other hand, strain JPCCMB0017^T could be readily differentiated from members of the

genus *Altererythrobacter* on the basis of several phenotypic properties, such as nitrate reduction, β-galactosidase activity and substrate utilization. Strain JPCCMB0017^T could be differentiated from *C. marinus* E4A9^T on the basis of several phenotypic characteristics. Non-motility, lack of BChl α and the specific sodium-ion requirement for growth indicated that strain JPCCMB0017^T could not be affiliated with the genera *Erythromicrobium* (Yurkov *et al.*, 1994) or *Porphyrobacter* (Hiraishi & Imhoff, 2005) or with *Erythrobacter longus* (Shiba & Simidu, 1982) or *Erythrobacter litoralis* (Yurkov *et al.*, 1994). The temperature range for growth of strain JPCCMB0017^T was 25–40 °C and the optimum conditions for growth were 35 °C, 1.5–2.0 % (w/v) NaCl and pH 7.5. Cells required NaCl for growth, which indicated that the isolate was halophilic. The isolate was strictly aerobic and chemo-organotrophic and positive for catalase, but was negative in tests for arginine dihydrolase and urease. Q-10 was determined to be the main respiratory quinone. SGL-1 was present and BChl α was absent. The DNA base

Table 2. Cellular fatty acid compositions of strain JPCCMB0017^T and type strains of species of the genus *Altererythrobacter*

Strains: 1, JPCCMB0017^T (this study); 2, *A. marinus* H32^T (Lai *et al.*, 2009); 3, *A. marensis* MSW-14^T (Seo & Lee, 2010); 4, *A. luteolus* SW-109^T (Yoon *et al.*, 2005); 5, *A. indicus* MSSRF26^T (Kumar *et al.*, 2008); 6, *A. dongtanensis* JM27^T (Fan *et al.*, 2011); 7, *A. namhicola* KYW48^T (Park *et al.*, 2011); 8, *A. aestuarii* KYW147^T (Park *et al.*, 2011); 9, *A. epoxidivorans* JCS 350^T (Kwon *et al.*, 2007). Data in parentheses were taken from this study. Fatty acids representing <1.0 % of the total in all strains are not shown.

Fatty acid (%)	1	2	3	4	5	6	7	8	9
Saturated									
C _{12:0}	—	— (2.2)	— (—)	— (—)	— (—)	—	—	—	— (—)
C _{14:0}	3.0	0.2 (2.3)	— (4.4)	— (3.6)	— (1.4)	—	—	—	— (2.3)
C _{15:0}	2.0	— (4.1)	1.6 (1.1)	— (—)	— (—)	—	3.2	—	— (1.5)
C _{16:0}	8.1	6.7 (15.0)	13.0 (17.6)	9.0 (14.5)	6.8 (17.9)	7.1	5.8	14.6	3.1 (13.7)
C _{17:0}	1.1	— (1.4)	— (—)	— (—)	— (—)	—	—	—	— (—)
C _{18:0}	1.2	2.4 (12.0)	3.7 (2.0)	— (2.3)	— (2.5)	—	—	—	— (3.0)
Cyclic									
C _{17:0} cyclo	—	—	— (—)	— (—)	— (—)	—	—	—	6.9 (4.3)
C _{19:0} ω8c cyclo	—	0.3 (—)	— (—)	— (—)	— (—)	—	—	—	— (—)
Unsaturated									
C _{16:1} ω5c	5.0	2.3 (3.8)	1.9 (7.0)	3.3 (21.2)	— (3.1)	2.8	—	—	2.2 (20.3)
C _{17:1} ω6c	1.6	4.3 (1.1)	6.8 (—)	5.1 (—)	5.2 (—)	19.8	19.9	2.9	— (—)
C _{17:1} ω8c	1.7	— (—)	— (—)	— (—)	1.0 (—)	—	2.1	—	— (—)
C _{18:1} ω5c	—	3.5 (—)	2.2 (2.0)	3.4 (2.2)	2.0 (2.8)	—	—	—	— (—)
C _{18:1} ω7c	45.5	44.3 (24.4)	52.8 (29.4)	47.7 (33.4)	76.7 (54.2)	34.0	35.2	36.1	46.9 (35.1)
C _{18:1} ω9c	—	— (—)	— (—)	— (—)	— (—)	—	—	7.9	— (—)
11-Methyl C _{18:1} ω7c	—	25.4 (14.2)	— (8.8)	7.1 (6.8)	— (—)	2.2	—	11.0	— (—)
Hydroxy									
C _{12:0} 2-OH	1.2	— (—)	— (—)	— (—)	— (—)	—	—	—	— (—)
C _{14:0} 2-OH	13.3	3.0 (8.6)	1.3 (11.9)	3.4 (5.3)	7.2 (12.5)	5.6	7.5	—	— (3.5)
C _{15:0} 2-OH	6.9	0.3 (—)	— (—)	— (—)	— (—)	7.4	3.5	—	— (—)
C _{16:0} 2-OH	6.3	1.6 (5.0)	1.4 (7.7)	3.6 (4.8)	— (1.9)	12.5	—	9.0	— (—)
C _{16:1} 2-OH	—	0.1 (—)	— (—)	— (—)	— (—)	—	—	—	— (—)
C _{18:1} 2-OH	—	0.2 (—)	— (—)	— (—)	— (—)	1.6	—	—	— (—)
iso-C _{16:0} 3-OH	—	0.8 (—)	— (—)	1.0 (—)	— (—)	0.9	—	—	— (—)
Summed feature*	—	4.5 (—)	8.8 (2.6)	13.9 (1.9)	1.0 (—)	6.1	22.7	18.4	32.9 (3.2)

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. The summed feature consisted of one or more of C_{14:0} 3-OH, C_{16:1}ω7c and iso-C_{15:0} 2-OH.

composition was 59.1 mol%. More details of the physiological characteristics of strain JPCCMB0017^T are given in the species description and in Supplementary Table S1.

Preliminary 16S rRNA gene sequence comparison with BLAST indicated that strain JPCCMB0017^T was related to the family *Erythrobacteraceae* and a phylogenetic dendrogram was constructed with members of the genera in this family. Strain JPCCMB0017^T was clearly placed within a cluster comprising the genera *Altererythrobacter* and *Croceicoccus* (Fig. 1). Strain JPCCMB0017^T was most closely related to the uncharacterized alphaproteobacterium strain JL991 (98.7% 16S rRNA gene sequence similarity) and *Altererythrobacter* sp. KYW2 (98.6%). The most closely related type strains of recognized species were *A. marinus* H32^T (96.8% 16S rRNA gene sequence similarity), *A. marensis* MSW-14^T (96.5%), *A. luteolus* SW-109^T (96.0%) and *A. indicus* MSSRF26^T (95.8%).

The major fatty acid in strain JPCCMB0017^T was octadecenoic acid (C_{18:1}ω7c, 45.5%). In addition, minor amounts of 2-hydroxy-dodecanoic acid (C_{12:0} 2-OH), 2-hydroxy-myristic acid (C_{14:0} 2-OH), 2-hydroxy-pentadecanoic acid (C_{15:0} 2-OH) and 2-hydroxy-hexadecanoic acid (C_{16:0} 2-OH) were found (Table 2). The fatty acid profile of the isolate was markedly similar to that of *A. indicus* (Kumar *et al.*, 2008), although there were differences in the proportions of some fatty acids.

Strain JPCCMB0017^T produced the carotenoids astaxanthin, adonixanthin, zeaxanthin, canthaxanthin and echinenone (see Supplementary Table S2). The above-mentioned pigments were identified in both MB and BY medium. In contrast, only zeaxanthin was detected in other members of the genus *Altererythrobacter*.

The phylogenetic and chemotaxonomic analysis revealed that strain JPCCMB0017^T is a member of the genus *Altererythrobacter*. However, the fatty acid composition and physiological characteristics discriminated the new isolate from members of the other species of the genus *Altererythrobacter*. Thus, we propose that strain JPCCMB0017^T represents a novel species, with the name *Altererythrobacter ishigakiensis* sp. nov.

Description of *Altererythrobacter ishigakiensis* sp. nov.

Altererythrobacter ishigakiensis (i.shi.ga.ki.en'sis. N.L. masc. adj. *ishigakiensis* of or belonging to Ishigaki island, Okinawa, Japan, where the type strain was isolated).

Cells are non-motile rods, Gram-negative and strictly aerobic (0.3–0.5 µm wide and 1.5–2.0 µm long). Colonies are orange-red, round, convex and smooth. The carotenoids astaxanthin, adonixanthin, echinenone, canthaxanthin and echinenone are present; astaxanthin, adonixanthin and zeaxanthin are the chief pigments. Optimum growth is observed at 35 °C, pH 7.5 and 1.5–2.0% (w/v) NaCl. Growth does not occur in the absence of NaCl. Ubiquinone-10 is the main quinone. Bchl *a* is not produced. Reduces nitrate, produces catalase, oxidase and β-galactosidase and hydrolyses

aesculin, but does not produce arginine dihydrolase, urease or indole or hydrolyse gelatin. Glucose acidification is not observed. Does not utilize D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, maltose, potassium gluconate, n-capric acid, adipic acid, malate, sodium citrate or phenylacetate. The major fatty acids are C_{18:1}ω7c and C_{14:0} 2-OH; the minor fatty acids are C_{14:0}, C_{15:0}, C_{16:0}, C_{17:0}, C_{18:0}, C_{16:1}ω5c, C_{17:1}ω6c, C_{17:1}ω8c, C_{12:0} 2-OH, C_{15:0} 2-OH and C_{16:0} 2-OH. SGL-1 is also produced.

The type strain, JPCCMB0017^T (=NITE-AP48^T=ATCC BAA-2084^T=NBRC 107699^T), was isolated from a marine sediment obtained from the coastal area of Okinawa, Japan. The DNA G+C content of the type strain is 59.1 mol%.

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