Zeaxanthinibacter enoshimensis gen. nov., sp. nov., a novel zeaxanthin-producing marine bacterium of the family *Flavobacteriaceae*, isolated from seawater off Enoshima Island, Japan

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A novel marine, Gram-negative, yellow-pigmented, rod-shaped, strictly aerobic, gliding and oxidase- and catalase-positive bacterium (strain TD-ZE3^T) was isolated from a seawater sample collected off the Pacific coastline of Japan near Enoshima Island (Fujisawa, Kanagawa). Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain TD-ZE3^T belonged to a distinct lineage in the family *Flavobacteriaceae*, with 90.5 % sequence similarity with the nearest species *Robiginitalea biformata*. Strain TD-ZE3^T could be distinguished from other members of the family *Flavobacteriaceae* by a number of chemotaxonomic and phenotypic characteristics. The DNA G + C content was 46.5 mol%. The major fatty acids were iso-C_{15:0}, iso-C_{17:0} 3-OH and iso-C_{17:1} ω9c and menaquinone-6 was the only respiratory quinone. Zeaxanthin was the major carotenoid pigment produced; flexirubin-type pigments were not produced. Strain TD-ZE3^T degraded gelatin, starch and Tween 80. Based on its unique phenotypic, genotypic and phylogenetic features, strain TD-ZE3^T represents a novel taxon, for which the name *Zeaxanthinibacter enoshimensis* gen. nov., sp. nov. is proposed. The type strain is TD-ZE3^T (=NBRC 101990^T = CCUG 53613^T).

At the time of writing, the family *Flavobacteriaceae* (Bernardet *et al.*, 2002; Reichenbach, 1989) in the phylum *Bacteroidetes* comprises 44 genera. Many members of this family were isolated from various marine and/or Antarctic habitats (Bowman *et al.*, 1997, 1998; Bowman, 2000; Bowman & Nichols, 2002, 2005; Cho & Giovannoni, 2004; Nedashkovskaya *et al.*, 2004d; Nichols *et al.*, 2005; Pinhassi *et al.*, 2006; Suzuki *et al.*, 2001). These bacteria are known to be proficient in degrading various biopolymers such as cellulose, chitin and pectin (Kirchman, 2002); hence, they are considered to be important members of the bacterial community involved in metabolic activities in aquatic environments.

In order to study the industrial application of microbial carotenoid production, we screened bacterial strains that produce useful carotenoids, including zeaxanthin. Zeaxanthin (3,3'-dihydroxy-β-carotene) is an oxygenated, yellow carotenoid that is found naturally in corn, alfalfa and marigolds (Nelis & De Leenheer, 1989). This pigment is currently used as an ingredient in feed for fish and poultry,

to enhance the pigmentation of animal flesh and egg yolk. In addition, zeaxanthin has a remarkable potential for use in pharmaceuticals as it prevents age-related macular degeneration. Micro-organisms that produce zeaxanthin belong to various taxa; they include *Synechocystis* sp. PCC 6803 (Lagarde *et al.*, 2000), *Erwinia herbicola* (Hundle *et al.*, 1993), *Paracoccus zeaxanthinifaciens* (Berry *et al.*, 2003; McDermott *et al.*, 1973) and *Sphingobacterium multivorum* (Alcantara & Sanchez, 1999; Rosa-Putra *et al.*, 2001). Here, we report the isolation and characterization of a novel zeaxanthin-producing marine bacterium that belongs to the family *Flavobacteriaceae*.

We isolated a new strain, designated TD-ZE3^T, by plating a seawater sample collected off the Pacific coastline of Japan near Enoshima Island (Fujisawa, Kanagawa; 35.3° N 139.5° E) on 10 April 2005 onto marine agar 2216 (MA; Difco) and incubating at 30 °C for 48 h. Unless otherwise specified, all characteristics described hereafter are those of cells of strain TD-ZE3^T grown on MA for 48 h at 30 °C.

The genomic DNA of strain TD-ZE3^T was extracted using a bacterial genomic DNA purification kit (Edge BioSystems) and the nearly complete 16S rRNA gene sequence (1431 bp) was amplified using the bacterial universal primers B8F (5'-AGAGTTTGATCCTGGCTCAG-3'; nt 8–27 based on

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TD-ZE3^T is AB264057.

Scanning electron micrographs of cells of strain TD-ZE3^T are available as supplementary material in IJSEM Online.

Escherichia coli numbering) and B1492R (5'-GGTTAC-CTTGTTACGACTT-3'; nt 1508–1484 based on *Escherichia coli* numbering). The 16S rRNA gene fragment was sequenced directly using a BigDye Terminator v3.1 Cycle sequencing kit and an ABI 3100 automated DNA sequencer (Applied Biosystems).

A sequence similarity search in the GenBank/EMBL/DBJ nucleotide sequence databases performed using the program BLASTN (<http://www.ncbi.nlm.nih.gov/blast/>) revealed that strain TD-ZE3^T belonged to the family *Flavobacteriaceae*. A neighbour-joining phylogenetic tree was constructed using the programs CLUSTAL W (Thompson *et al.*, 1994) and NJ plot (Perriere & Gouy, 1996). The tree topology was estimated by using a bootstrap analysis (Felsenstein, 1993) with 1000 resamplings of the dataset. Strain TD-ZE3^T formed a clade together with several

uncharacterized bacterial strains with sequence similarities of 95–98 %. This clade did not cluster robustly with any recognized species or genus. Strain TD-ZE3^T shared 90.5 % 16S rRNA gene sequence similarity with *Robiginitalea biformata* HTCC 2501^T without significant bootstrap support (Fig. 1). Sequence similarities between strain TD-ZE3^T and other relatives ranged from 89.2 to 91.3 %; they include *Cellulophaga pacifica* (91.3 %), *Maribacter* species (90.2–90.4 %), *Zobellia* species (89.2–89.8 %), *Sediminicola luteus* (89.8 %) and *Arenibacter* species (89.6–89.7 %). A tree based on the maximum-likelihood method showed essentially the same topology (data not shown). The results of the phylogenetic analysis suggest that strain TD-ZE3^T represents a novel genus within the family *Flavobacteriaceae*.

Gram staining was performed according to the method described by Smibert & Krieg (1994) and bacterial cells were

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of strain TD-ZE3^T and representative members of related genera in the family *Flavobacteriaceae*. The tree was constructed using the neighbour-joining method (Saitou & Nei, 1987). GenBank accession numbers are shown in parentheses. Numbers at nodes indicate bootstrap values greater than 50 %. The 16S rRNA gene sequences of *Flexibacter flexilis* ATCC 23079^T (GenBank accession no. M62794) and *Chlorobium limicola* UdG_6037 (AJ299414) were used as out-groups (not shown). Bar, 0.02 substitutions per nucleotide position.

observed under a Zeiss Axioskop 2 microscope (Carl Zeiss Microimaging). Scanning electron microscopy was performed according to the method described by Bruns *et al.* (2001), using a model VE-8800 (Keyence). Flagellar and gliding motilities were determined using the hanging drop method and gliding motility was examined further using phase-contrast microscopy of growth margins of strain TD-ZE3^T grown for 1–2 days at 30 °C on 0.1 × MA [containing 0.1 % marine broth (MB; Difco) and 1 % agar] (Bowman, 2000). Anaerobic growth was assessed on MA incubated in a GasPak anaerobic system (BBL). The following tests were performed as described in each reference cited: casein hydrolysis (Norris *et al.*, 1985), Tween 80 hydrolysis (Baumann & Baumann, 1988), catalase activity, spore formation and hydrolysis of DNA and starch (Smibert & Krieg, 1994), and hydrolysis of chitin, carboxymethylcellulose (high viscosity; Sigma) and agar (Cowan & Steel, 1993). Oxidase activity was studied by spreading the bacterial cells on a cytochrome oxidase strip (BioChemika) to observe a colour change. Other enzyme activities, growth on carbohydrates, acid production from carbohydrates, nitrate reduction and the production of H₂S, indole and acetoin

were examined using the commercial systems API 20E, API 20NE and API 50 CH (bioMérieux) according to the manufacturer's instructions and as described by Nichols *et al.* (2005). These systems were inoculated with bacterial suspensions in media containing 32 g synthetic sea salts l⁻¹ (Sigma). To determine the optimal growth temperature, strain TD-ZE3^T was cultivated on MA at 4, 10, 20, 30, 37, 40 and 45 °C. The requirement for NaCl was studied using a medium containing the following (l⁻¹): 5 g MgCl₂, 2 g MgSO₄, 0.5 g CaCl₂, 1 g KCl, 5 g Bacto peptone (Difco), 1 g Bacto yeast extract (Difco) and varying amounts of NaCl (1, 1.5, 3, 5, 7, 10 or 12 %) (pH adjusted to 7.5 using KOH). Growth at various pH values (pH 3.0–10.0) was evaluated in MB adjusted with HCl or NaOH. Phenotypic properties of strain TD-ZE3^T are given in the species description and those characteristics that differentiate strain TD-ZE3^T from related members of the family *Flavobacteriaceae* are listed in Table 1.

Cells of strain TD-ZE3^T were Gram-negative rods of 0.2–0.4 µm in width and 1.2–3.9 µm in length, motile by gliding and occasionally formed vesicle-like microstructures

Table 1. Characteristics that differentiate *Zeaxanthinibacter enoshimensis* gen. nov., sp. nov. TD-ZE3^T from related genera of the family *Flavobacteriaceae*

Taxa: 1, strain TD-ZE3^T; 2, *R. biformata* HTCC 2501^T (data from Cho & Giovannoni, 2004); 3, *Maribacter* (5 species) (Nedashkovskaya *et al.*, 2004d; Yoon *et al.*, 2005b); 4, *Pibocella ponti* KMM 6031^T (Nedashkovskaya *et al.*, 2005); 5, *Zobellia* (5 species) (Barbeyron *et al.*, 2001; Nedashkovskaya *et al.*, 2004b); 6, *Muricauda* (3 species) (Bruns *et al.*, 2001; Yoon *et al.*, 2005a); 7, *Arenibacter* (4 species) (Ivanova *et al.*, 2001; Nedashkovskaya *et al.*, 2003, 2004a, 2006); 8, *Cellulophaga* (4 species) (Bowman, 2000; Johansen *et al.*, 1999; Nedashkovskaya *et al.*, 2004c). +, Positive; –, negative; ND, not determined; v, variable.

Characteristic	1	2	3	4	5	6	7	8
Facultatively anaerobic	–	–	–	–	–	v	–	–
Oxidase/catalase	+ / +	+ / +	+ / +	+ / +	+ / +	+ / v	+ / +	+ / +
Pigmentation*	Y	O	Y	Y	Y/DO	Y	DO	Y
Gliding motility	+	–	+	+	+	v	v	+
Flexirubin pigments	–	–	–	–	+	–	–	–
Requirement of Na ⁺	+	+	+	+	+	+	v	+
Growth with 8 % NaCl	–	+	v	+	v	+	v	v
Growth at/in:								
7 % NaCl	+	+	v	+	v	+	v	v
4 °C	–	–	+	+	v	–	v	+
40 °C	+	+	–	–	v	+	v	v
42 °C	–	+	–	–	v	v	v	–
Acid from carbohydrates	+	–	v	+	+	+	+	+
Hydrolysis of:								
Agar	–	ND	v	–	+	–	–	+
Casein	–	–	–	+	v	–/ND	–	v
Gelatin	+	–	v	+	+	–	v	+
Starch	+	+	v	+	v	–	–	+
DNA	–	–	v	–	v	–/ND	v	v
Urea	–	–	–	–	–	–/ND	v	ND
Nitrate reduction	–	–	v	–	+	v	v	v
DNA G + C content (mol%)	46.5	55–56	35–39	35.5	36–43	41–45	37–40	32–34

*Abbreviations: DO, deep orange; O, orange; Y, yellow.

Table 2. Whole-cell fatty acid profiles of *Zeaxanthinibacter enoshimensis* gen. nov., sp. nov. TD-ZE3^T and related genera in the family *Flavobacteriaceae*

Taxa: 1, strain TD-ZE3^T; 2, *R. biformata* HTCC 2501^T (data from Cho & Giovannoni, 2004); 3, *Maribacter* (5 species) (Nedashkovskaya *et al.*, 2004d; Yoon *et al.*, 2005b); 4, *Pibocella ponti* KMM 6031^T (Nedashkovskaya *et al.*, 2005); 5, *Zobellia* (5 species) (Barbeyron *et al.*, 2001; Nedashkovskaya *et al.*, 2004b); 6, *Muricauda* (3 species) (Bruns *et al.*, 2001; Yoon *et al.*, 2005a); 7, *Arenibacter* (4 species) (Ivanova *et al.*, 2001; Nedashkovskaya *et al.*, 2003, 2004a, 2006); 8, *Cellulophaga* (4 species) (Bowman, 2000; Johansen *et al.*, 1999; Nedashkovskaya *et al.*, 2004c). Data are reported as percentages of the total fatty acids. For genera that comprise several species, data are reported as a range of percentages of total fatty acids. tr, Trace amount (<1%); –, not detected. Fatty acids amounting to less than 1% in all taxa studied are not listed.

Fatty acid	1	2	3	4	5	6	7	8
Saturated acids:								
C _{14:0}	–	–	–	–	tr–1.0	–	–	–
C _{15:0}	7.7	5.5	3.5–14.5	4.2	7.5–14.4	5.1–13.2	4.2–16.0	9.3–14.3
C _{16:0}	tr	1.3	tr–1.4	1.0	tr–2.6	tr	tr–2.7	1.2–3.4
Branched saturated acids:								
iso-C _{14:0}	–	–	–	1.0	–	tr	–	–
iso-C _{15:0}	18.9	26.0	10.6–20.5	8.7	16.8–22.5	14.7–23.8	6.8–15.8	6.1–21.4
anteiso-C _{15:0}	2.3	3.7	tr–2.3	5.4	1.0–1.8	1.1–2.5	3.2–13.5	1.0–2.6
iso-C _{16:0}	1.1	–	tr–1.1	12.1	–	tr	–	tr–2.7
Monounsaturated acids:								
C _{14:1} ω5 <i>c</i>	–	–	–	tr–1.0	–	–	–	–
C _{15:1} ω6 <i>c</i>	tr	–	tr–4.8	1.5	1.1–3.2	tr	1.0–2.6	1.4–2.6
C _{16:1} ω7 <i>c</i>	–	–	–	–	–	–	–	9.0–19.2
C _{17:1} ω8 <i>c</i>	tr	–	–	–	–	tr	tr–2.4	–
C _{17:1} ω6 <i>c</i>	1.0	–	tr–1.7	4.7	1.0–1.2	tr–1.0	tr–3.0	–
C _{18:1} ω6 <i>c</i>	–	–	–	–	tr–1.1	–	–	–
Branched monounsaturated acids:								
iso-C _{15:1}	–	17.2	10.1–18.9	11.7	8.8–14.9	–	4.9–14.0	13.1–20.9
anteiso-C _{15:1}	–	–	–	2.1	0.0–1.8	–	tr–2.9	–
iso-C _{15:1} ω10 <i>c</i>	–	–	–	–	–	–	–	7.5–10.3
iso-C _{15:1} G	9.9	–	–	–	–	19.5–21.6	–	–
iso-C _{16:1}	–	–	–	6.2	–	tr	tr–1.7	–
iso-C _{16:1} ω6 <i>c</i>	–	–	–	–	–	–	–	1.1–2.2
iso-C _{17:1} ω9 <i>c</i>	13.3	–	2.3	5.2	2.4–5.1	1.3–1.5	2.2–5.3	–
anteiso-C _{17:1} ω9 <i>c</i>	–	–	–	2.3	–	–	–	–
iso-C _{17:1} ω7 <i>c</i>	–	–	–	–	–	–	–	5.1–6.7
anteiso-C _{17:1} ω7 <i>c</i>	–	–	–	–	–	–	–	tr–1.5
iso-C _{18:1}	–	–	0.0–2.4	–	–	–	–	–
Hydroxy acids:								
C _{15:0} 2-OH	–	–	–	1.8	–	tr	tr–1.0	–
C _{15:0} 3-OH	–	4.3	1.4–2.4	–	tr	1.0–1.8	tr–2.2	tr
C _{16:0} 3-OH	–	–	2.2–5.5	1.0	2.4–4.9	tr	1.0–3.8	tr–1.9
C _{17:0} 2-OH	tr	1.2	–	2.8	–	tr–1.4	1.0–5.1	–
C _{17:0} 3-OH	–	–	–	–	–	tr–1.3	–	–
iso-C _{15:0} 3-OH	2.7	–	2.9–5.4	tr	4.6–8.3	4.6–5.5	3.5–5.7	3.2–8.6
anteiso-C _{15:0} 3-OH	–	–	–	–	–	–	–	tr–1.8
iso-C _{16:0} 3-OH	2.4	–	1.0–2.5	5.9	–	1.7–4.6	1.6–7.2	5.0–7.3
iso-C _{17:0} 3-OH	16.5	25.8	11.6–29.2	5.6	15.1–25.9	17.3–20.9	6.9–21.9	2.8–20.8
Summed feature 3*	5.8	3.5	5.8–12.9	11.4	9.9–15.5	2.3–4.2	9.6–13.5	–
ECL 13.56†	11.0	–	–	–	1.4–2.4	4.8–6.5	–	–
Others	2.1	–	2.7–10.3	5.5	–	1.6–2.3	–	–

*Summed feature 3 comprises iso-C_{15:0} 2-OH and/or C_{16:1}ω7*c*.

†Unknown fatty acids designated by their equivalent chain-length (ECL).

(see Supplementary Fig. S1 in IJSEM Online). After incubation at 30 °C for 48 h, colonies on MA were circular, 2–4 mm in diameter, yellow-pigmented, shiny and convex, with smooth surfaces and entire translucent margins.

As no flexirubin-type pigments were detected using the simple KOH test detailed in the minimal standards for describing new taxa of the family (Bernardet *et al.*, 2002), the yellow pigments were extracted by agitating bacterial cells in methanol on a rotary shaker (100 r.p.m.) at 50 °C in the dark until the cells were bleached, followed by centrifugation at 5000 g for 5 min. The absorbance of the resultant supernatant was determined between $\lambda = 260$ and 700 nm at room temperature using a Hitachi U2000 spectrophotometer. Carotenoid composition was characterized by using HPLC-MS (LCMS-2010EV; Shimadzu) using a Shim-Pack FC-ODS (150 $\times$ 4.6 mm, 5 μ m particle size; column temperature 35 °C) and acetonitrile/methanol/tetrahydrofuran (5.8:3.5:0.7, by vol.) as a mobile phase at a flow rate of 0.8 ml min⁻¹. Zeaxanthin was identified from its retention time, absorption spectrum and molecular mass. Commercial zeaxanthin (DHI; Water & Environment) was used as a standard. The menaquinone content was determined by using an HPLC method (Collins, 1994) with an extract of cells of *Cellulophaga lytica* as a reference for menaquinone-6 (MK-6; Johansen *et al.*, 1999). The fatty acid methyl esters of strain TD-ZE3^T were extracted and analysed according to the standard protocol of the Sherlock Microbial Identification System (version 5.0; MIDI).

The yellow pigments of strain TD-ZE3^T were identified as carotenoids, among which zeaxanthin was predominant [UV-Vis λ_{max} of 451 and 476 nm and molecular mass ($M + H$)⁺, 569]. MK-6 was found to be the only respiratory quinone. The predominant cellular fatty acids were iso-C_{15:0}, iso-C_{17:0} 3-OH, iso-C_{17:1} ω 9c, iso-C_{15:1} G, C_{15:0} and an unknown fatty acid with an equivalent chain-length (ECL) of 13.56. These fatty acids represented 77.3 % of the total fatty acids of strain TD-ZE3^T. The detailed fatty acid composition of strain TD-ZE3^T is shown in Table 2. It differed from those reported for *R. biformata* HTCC 2501^T by the absence of iso-C_{15:1} and the presence of iso-C_{15:1} G, iso-C_{17:1} ω 9c and ECL 13.56 (Table 2). Strain TD-ZE3^T differed from members of all related genera except *Muricauda* species by the absence of iso-C_{15:1}.

The DNA G + C content of strain TD-ZE3^T, determined using HPLC (Mesbah & Whitman, 1989), was 46.5 $\pm$ 0.3 mol% (mean $\pm$ SD, three replicates). This value was 10 mol% lower than that of *R. biformata* HTCC 2501^T and higher than those of related members of the genera *Maribacter*, *Pibocella* and *Cellulophaga* (Table 1).

Based on the results of the 16S rRNA gene-based phylogenetic analysis and on the above-mentioned phenotypic features, we conclude that strain TD-ZE3^T represents a novel genus within the family *Flavobacteriaceae*, for which we propose the name *Zeaxanthinibacter enoshimensis* gen. nov., sp. nov.

Description of *Zeaxanthinibacter* gen. nov.

Zeaxanthinibacter (Ze.a.xan'thi.ni.bac'ter. N.L. neut. n. *zeaxanthinum* zeaxanthin; N.L. masc. n. *bacter* from Gr. n. *bakterion* rod; N.L. masc. n. *Zeaxanthinibacter* zeaxanthin-producing rod-like bacterium).

Gram-negative, non-spore-forming rods and motile by gliding. Strictly aerobic. Catalase- and oxidase-positive. MK-6 is the only respiratory quinone. Carotenoid pigments are produced, but not flexirubin-type pigments. The G + C content of the type strain of the type species is 46.5 mol%. The type species is *Zeaxanthinibacter enoshimensis*.

Description of *Zeaxanthinibacter enoshimensis* sp. nov.

Zeaxanthinibacter enoshimensis (en.o.shi.men'sis. N.L. masc. adj. *enoshimensis* pertaining to Enoshima Island in Japan, where the type strain was isolated).

Displays the following characteristics in addition to those given in the genus description. Cells are 0.2–0.4 μ m in width and 1.2–3.9 μ m in length; some cells show the presence of vesicle-like microstructures. On MA, colonies (2.0–4.0 mm in diameter) are convex, translucent, shiny and smooth and contain non-diffusible yellow carotenoid pigments with zeaxanthin as the major component. Growth occurs at 16.0–40.0 °C (optimum, 28.0–30.0 °C) and at pH 5.5–11.0 (optimum, 7.0–8.0). Optimal growth occurs in the presence of 4 % (w/v) NaCl; growth is inhibited in the absence of NaCl and in the presence of >8 % (w/v) NaCl. Aesculin, Tween 80, gelatin and starch are hydrolysed, but agar, DNA, casein, cellulose, chitin and urea are not. Indole and H₂S are not produced. Nitrate is not reduced. β -Galactosidase activity is positive. Tryptophan deaminase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase and urease activities are negative. Acid is produced from glucose, mannose, methyl α -D-mannoside, methyl α -D-glucoside, aesculin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, raffinose, arabinose and D-turanose. Glucose, maltose, mannose, mannitol, *N*-acetylglucosamine and gluconate are assimilated, but arabinose, capric acid, adipic acid, malate, trisodium citrate and phenylacetic acid are not. Predominant cellular fatty acids (representing 77.3 % of the total fatty acids) are iso-C_{15:0}, iso-C_{17:0} 3-OH, iso-C_{17:1} ω 9c, iso-C_{15:1} G, C_{15:0} and an unknown fatty acid with an ECL of 13.56. The DNA G + C content of the type strain is 46.5 mol%.

The type strain, TD-ZE3^T (=NBRC 101990^T = CCUG 53613^T), was isolated from a shallow seawater sample collected off the Pacific coastline of Japan near Enoshima Island.

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