Nubsella zeaxanthinifaciens gen. nov., sp. nov., a zeaxanthin-producing bacterium of the family *Sphingobacteriaceae* isolated from freshwater

Dalal Asker,^{1,2} Teruhiko Beppu² and Kenji Ueda²

Correspondence

Dalal Asker

dasker10@gmail.com

¹Department of Food Science, University of Massachusetts, 100 Holdsworth Way, Amherst, MA 01003, USA

²Life Science Research Center, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa 252-8510, Japan

A yellow-pigmented, Gram-negative, strictly aerobic, rod-shaped bacterium (TDMA-5^T) was isolated from a freshwater sample collected at Misasa (Tottori, Japan). The DNA G + C content was 38.6 mol%. Major fatty acids were iso-C_{15:0}, iso-C_{17:0} 3-OH and summed feature 4 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c). MK-7 was the predominant respiratory quinone. Zeaxanthin was the major carotenoid pigment produced; flexirubin-type pigments were not produced. TDMA-5^T was sensitive to gamma-irradiation. The strain degraded gelatin, casein, starch, Tween 80 and DNA. Phylogenetic analysis based on the 16S rRNA gene sequence placed TDMA-5^T in a distinct lineage in the family *Sphingobacteriaceae*, sharing 89.4–93.4 % sequence similarity with members of the nearest genus *Pedobacter*. Strain TDMA-5^T could be distinguished from the other members of the family *Sphingobacteriaceae* by a number of chemotaxonomic and phenotypic characteristics. Based on its unique phenotypic, genotypic and phylogenetic features, strain TDMA-5^T represents a novel genus and species, for which the name *Nubsella zeaxanthinifaciens* gen. nov., sp. nov. is proposed. The type strain of *Nubsella zeaxanthinifaciens* is TDMA-5^T (=NBRC 102579^T =CCUG 54348^T).

The family *Sphingobacteriaceae* belongs to the phylum *Bacteroidetes* and was first proposed by Steyn *et al.* (1998) to accommodate the genera *Sphingobacterium* and *Pedobacter*. Two additional genera, *Olivibacter* and *Parapedobacter*, have been proposed recently (Ntougias *et al.*, 2007; Kim *et al.*, 2007). The genus *Sphingobacterium*, the type genus of the family, was originally described by Yabuuchi *et al.* (1983) and at present comprises *Sphingobacterium antarcticum*, *S. faecium*, *S. multivorum*, *S. spiritivorum*, *S. thalophilum*, *S. mizutaii* and *S. daejeonense* (Kim *et al.*, 2006; Shivaji *et al.*, 1992; Steyn *et al.*, 1998; Takeuchi & Yokota, 1992; Yabuuchi *et al.*, 1983). Steyn *et al.* (1998) proposed the genus *Pedobacter* initially to accommodate the four heparinolytic species *Pedobacter africanus*, *Pedobacter heparinus*, *Pedobacter piscium* and *Pedobacter saltans*; nine further species, *Pedobacter caeni*, *Pedobacter cryoconitis*, *Pedobacter himalayensis*, *Pedobacter sandarakinus*, *Pedobacter aquatilis*, *Pedobacter ginsengisoli*, *Pedobacter suwonensis*, *Pedobacter*

panaciterrae and *Pedobacter roseus*, have since been added (Gallego *et al.*, 2006; Hwang *et al.*, 2006; Kwon *et al.*, 2007; Margesin *et al.*, 2003; Shivaji *et al.*, 2005; Steyn *et al.*, 1998; Ten *et al.*, 2006; Vanparys *et al.*, 2005; Yoon *et al.*, 2006, 2007).

Zeaxanthin (3,3'-dihydroxy-β-carotene) is a natural, fat-soluble, yellowish carotenoid that is found in some plants, algae and photosynthetic bacteria (Nelis & De Leenheer, 1989), where it serves as an accessory light-gathering pigment as well as a protectant against the toxic effects of ultraviolet radiation and oxygen radicals. Zeaxanthin is currently utilized as an ingredient in feed for fish and poultry to enhance the pigmentation of animal flesh and egg yolk. Additionally, this pigment has remarkable potential for use in pharmaceuticals, since it prevents age-related macular degeneration and tumour formation. 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).

Recently, we have isolated several zeaxanthin-producing marine bacteria from seawater samples collected on the Pacific coastline of Japan, and two have been identified as

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Nubsella zeaxanthinifaciens* TDMA-5^T is AB264126.

Maximum-likelihood and parsimony phylogenetic trees based on 16S rRNA gene sequences, a scanning electron micrograph of cells of strain TDMA-5^T and a detailed fatty acid profile are available as supplementary material with the online version of this paper.

representing novel genera and species within the family *Flavobacteriaceae* (Asker *et al.*, 2007a, b). We have also isolated several carotenoid-producing bacteria from freshwater samples that were collected at Misasa (Tottori, Japan), a region known for its high natural radioactivity (Kametani & Matsumura, 1983). A comprehensive taxonomic characterization based on 16S rRNA gene sequences has revealed a unique diversity of carotenoid producers that exists in this radioactive region (unpublished results), and a red-pigmented bacterium (TDMA-17) has been identified as representing a novel species within the genus *Sphingomonas* that produces astaxanthin (Asker *et al.*, 2007c). In this article, we report the results of a polyphasic taxonomic study of a unique heterotrophic, aerobic, yellow-pigmented bacterium (strain TDMA-5^T) also isolated from Misasa, which produces zeaxanthin. On the basis of biochemical and chemical criteria and the results of 16S rRNA gene sequence analysis, we propose that this isolate represents a novel genus within the family *Sphingobacteriaceae*.

Strain TDMA-5^T was isolated from a water sample collected at Misasa on 1 September 2005 by the conventional dilution-plating method using Luria-Bertani (LB) solid medium [l⁻¹: 10 g polypeptone (Difco), 5 g yeast extract (Difco), 5 g NaCl and 15 g agar]. Unless otherwise specified, all characteristics described hereafter are those of cells of TDMA-5^T grown on LB for 3 days at 30 °C. The phylogenetic position of the isolate was studied by standard analysis based on the 16S rRNA gene sequence. Genomic DNA of TDMA-5^T was extracted using a bacterial genomic DNA purification kit (Edge BioSystems). A DNA fragment that contained nearly the complete 16S rRNA

gene of TDMA-5^T (1422 bp) was amplified using the bacterial universal primers B8F (5'-AGAGTTTGATCC-TGGCTCAG; positions 8–27 based on *Escherichia coli* numbering) and B1492R (5'-GGTTACCTTGTTACG-ACTT; positions 1510–1492). The 16S rRNA gene was sequenced directly by using the BigDye Terminator v3.1 cycle sequencing kit on an ABI 3100 automated DNA sequencer (Applied Biosystems).

A sequence similarity search in the GenBank/EMBL/DDBJ nucleotide sequence databases performed using the BLASTN program (<http://www.ncbi.nlm.nih.gov/BLAST/>) revealed that strain TDMA-5^T belonged to the family *Sphingobacteriaceae*.

A neighbour-joining phylogenetic tree was constructed using the CLUSTAL W (Thompson *et al.*, 1994) and NJPlot (Perrière & Gouy, 1996) programs. The tree topology was estimated by a bootstrap analysis (Felsenstein, 1993) with 1000 resamplings of the dataset. Strain TDMA-5^T formed a clade together with several uncharacterized bacterial strains with sequence similarities of 96–97 %. This clade did not cluster robustly with any recognized species or genus (Fig. 1). Strain TDMA-5^T was nearest to the members of the genus *Pedobacter* (except *Pedobacter saltans*), with 16S rRNA gene sequence similarity ranging from 90.3 to 93.4 %. TDMA-5^T exhibited low 16S rRNA gene sequence similarity to the type strain of *Pedobacter saltans* (89.4 %). Sequence similarities between TDMA-5^T and other relatives ranged from 87.6 to 89.8 %; they include *Sphingobacterium* species (87.6–88.9 %), *Olivibacter sitien-sis* AW-6^T (89.8 %) and *Parapedobacter koreensis* Jip14^T (89.3 %).

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequences of strain TDMA-5^T and representative members of related genera of the family *Sphingobacteriaceae*. The tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). Bar, 2 substitutions per 100 nucleotide positions. Bootstrap values, expressed as percentages of 1000 replicates, are given at branching points; values above 50 % are shown. The 16S rRNA gene sequence from *Bacteroides caccae* ATCC 43185^T was used as the outgroup. GenBank accession numbers are shown in parentheses.

It is evident from the phylogenetic tree (Fig. 1) that strain TDMA-5^T belongs to a distinct lineage in the family *Sphingobacteriaceae* that is most closely related to *Pedobacter* species excluding *Pedobacter saltans*, which clearly formed a monophyletic clade. *Pedobacter saltans* exhibited low 16S rRNA gene sequence similarity to other *Pedobacter* species (89.4–91.4%), and joined the cluster comprising *Sphingobacterium* species, *Olivibacter sitiensis* AW-6^T and *Parapedobacter koreensis* Jip14^T with a low bootstrap resampling value. These results agree with previous phylogenetic studies based on 16S rRNA gene sequences (Kim *et al.*, 2007; Kwon *et al.*, 2007; Ntougias *et al.*, 2007; Shivaji *et al.*, 2005; Ten *et al.*, 2006; Yoon *et al.*, 2006, 2007). Accordingly, *P. saltans* may have to be reclassified as a member of a new genus. Trees based on maximum-parsimony and maximum likelihood methods showed essentially the same topology (see Supplementary Fig. S1 in IJSEM Online). The results of phylogenetic analysis thus suggest that TDMA-5^T represents a novel genus within the family *Sphingobacteriaceae*.

The yellow pigments were extracted and analysed using HPLC-MS (LCMS-2010EV; Shimadzu) according to the method described previously (Asker *et al.*, 2007a, b). The menaquinone content was determined by an HPLC method (Collins, 1994). Fatty acid methyl esters of TDMA-5^T were extracted and analysed according to the standard protocol of the Sherlock Microbial Identification System (version 5.0; MIDI). The DNA G+C content was determined by an HPLC method (Mesbah & Whitman, 1989).

The yellow pigments of strain TDMA-5^T were identified as carotenoids, among which zeaxanthin was predominant [UV-Vis λ_{max} 451 and 476 nm; molecular mass ($M^+ + 1$), 569]. Menaquinone-7 (MK-7) was the major respiratory quinone. MK-7 is characteristic of the majority of species within the family *Sphingobacteriaceae*. The predominant cellular fatty acids were iso-C_{15:0} (29.4%), iso-C_{17:0} 3-OH (15.9%) and summed feature 4 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c) (20.1%). These fatty acids represented 65.4% of the total fatty acids of TDMA-5^T (Supplementary Table S1). The major fatty acids of all members of the family *Sphingobacteriaceae* are iso-C_{15:0}, iso-C_{17:0} 3-OH, iso-C_{15:0} 2-OH and C_{16:1}ω7c (Kim *et al.*, 2006, 2007; Margesin *et al.*, 2003; Ntougias *et al.*, 2007; Shivaji *et al.*, 2005; Steyn *et al.*, 1998; Vanparys *et al.*, 2005; Yoon *et al.*, 2006). However, TDMA-5^T differed from *Pedobacter* species, *Sphingobacterium* species, *Olivibacter sitiensis* AW-6^T and *Parapedobacter koreensis* Jip14^T by the presence of various minor fatty acid components including C_{17:0}, C_{15:0} 3-OH (except for *Parapedobacter koreensis* Jip14^T), C_{17:0} 3-OH, iso-C_{13:0}, iso-C_{14:0} and C_{17:1}ω6c (Table 1). Strain TDMA-5^T differed from members of all related genera by the absence of C_{16:1}ω5c.

The DNA G+C content of TDMA-5^T was 38.6 mol% (Table 1), in agreement with values of 36.9–44.2 mol% for the family *Sphingobacteriaceae* (Steyn *et al.*, 1998).

Gram staining was performed according to the method described by Smibert & Krieg (1994), and bacterial cells were observed under a Zeiss Axioskop 2 microscope. Scanning electron microscope observation was performed using a model VE-8800 (Keyence). Anaerobic growth was assessed on LB agar incubated in a GasPak anaerobic system (BBL). The following tests were performed as described in each cited reference: casein hydrolysis (Norris *et al.*, 1985) and catalase activity, spore formation and hydrolysis of Tween 80, DNA and starch (Smibert & Krieg, 1994). Oxidase activity was tested by using commercial cytochrome oxidase test strips (BioChemika). 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 50CH (bioMérieux) according to the manufacturer's instructions. To determine the optimal growth temperature, the strain was cultivated on LB agar at 4, 10, 20, 30, 37, 40 and 45 °C. To determine the survival rate after exposure to gamma radiation, cells of TDMA-5^T were grown on an LB plate and suspended in LB broth (5 ml). Aliquots of the cell suspension (1 ml) were irradiated at 2.35 kGy (13 Gy min⁻¹, ¹³⁷Cs source), diluted serially and plated onto LB agar. The plates were incubated aerobically at 30 °C. *E. coli* C600 was similarly treated. Percentage survival was determined by comparison with unirradiated cultures.

Cells of strain TDMA-5^T were Gram-negative rods (0.2–0.3 × 1.0–5.0 μm). A scanning electron micrograph is available as Supplementary Fig. S2. Spores were not observed. Strain TDMA-5^T formed deep yellow-pigmented, circular, convex, shiny, opaque and slimy colonies (2–3 mm in diameter) that are slightly adherent to agar, with entire margins after 3 days of incubation at 30 °C on LB agar plates. The temperature range for growth of TDMA-5^T was 18–40 °C (optimum, 30 °C). No growth occurred at 45 °C. The pH range for growth was pH 6.0–9.0. Strain TDMA-5^T was strictly aerobic and chemo-organotrophic. The strain exhibited positive reactions for oxidase and catalase. TDMA-5^T was sensitive to gamma-irradiation. Other phenotypic properties of strain TDMA-5^T are given in the species description and those characteristics that differentiate strain TDMA-5^T from related members of the family *Sphingobacteriaceae* are listed in Table 1.

Strain TDMA-5^T differed from the members of *Pedobacter* by having different minor fatty acids, being more mesophilic, being less psychrotolerant (no data were available for *Pedobacter caeni*, *Pedobacter africanus* or *Pedobacter saltans*) and being able to assimilate malate. TDMA-5^T differed from the members of *Sphingobacterium* by having different minor fatty acids, being less psychrotolerant, being able to assimilate malate, being unable to ferment glucose and being unable to hydrolyse urea. TDMA-5^T differed from *Olivibacter sitiensis* AW-6^T by having different minor fatty acids, having a narrower temperature growth range, being able to assimilate malate

Table 1. Differentiation of *Nubsella zeaxanthinifaciens* gen. nov., sp. nov. TDMA-5^T from related genera of the family *Sphingobacteriaceae*

Taxa: 1, strain TDMA-5^T; 2, *Pedobacter* (13 species) (data from Gallego *et al.*, 2006; Hwang *et al.*, 2006; Kwon *et al.*, 2007; Margesin *et al.*, 2003; Shivaji *et al.*, 2005; Steyn *et al.*, 1998; Ten *et al.*, 2006; Vanparys *et al.*, 2005; Yoon *et al.*, 2006, 2007); 3, *Sphingobacterium* (7 species) (Kim *et al.*, 2006; Shivaji *et al.*, 1992; Steyn *et al.*, 1998; Takeuchi & Yokota, 1992); 4, *Olivibacter sitiensis* AW-6^T (Ntougias *et al.*, 2007); 5, *Parapedobacter koreensis* Jip14^T (Kim *et al.*, 2007). In all studies, cells for fatty acid methyl ester analysis were grown in peptone-yeast extract media at 28–30 °C. +, Positive; –, negative; v, variable; tr, trace; ND, no data available.

Characteristic	1	2	3	4	5
Growth at:					
5 °C	–	+*	v	+	–
40 °C	+	–	v	+	+
42 °C	–	–	v	+	+
Glucose fermentation	–	v	+	ND	+
Nitrate reduction	–	–	v	ND	–
Arginine dihydrolase	–	v*	ND	ND	–
Lipase	+	v	v	–†	–
Hydrolysis of:					
DNA	+	v	v	ND	ND
Gelatin	+	v	v	ND	–
Starch	+	v	v	–	ND
Aesculin	+	v	v	ND	+
Urea	–	–	v	ND	–
Assimilation of:					
Glucose	+	v	+	+	+
Arabinose	–	v	v	ND	–
Mannitol	–	v	v	–	+
Maltose	+	v	+	–	+
Malate	+	–	–	–	–
N-Acetyl-D-glucosamine	+	+	ND	ND	+
Minor fatty acids (%):					
C _{17:0}	+ (0.1)	–	–	–	–
C _{15:0} 3-OH	+ (1.3)	–	–	–	+ (1.7)
C _{17:0} 3-OH	+ (0.6)	–	–	–	–
iso-C _{13:0}	+ (0.6)	–	–	–	–
iso-C _{14:0}	+ (0.3)	–	–	–	–
C _{16:1ω5c}	–	v (tr–3.0)	v (tr–1.5)	+ (1.3)	–
C _{17:1ω6c}	+ (0.8)	–	–	–	–
DNA G + C content (mol%)	38.6	36.9–44.2	37.7–44.2	45.6	45.6

*No data available for *Pedobacter caeni*, *Pedobacter africanus* or *Pedobacter saltans*.

†Tween 20 is not hydrolysed.

and maltose, being able to hydrolyse starch and having a lower DNA G + C content. Finally, TDMA-5^T differed from *Parapedobacter koreensis* Jip14^T by having different minor fatty acids, being able to assimilate malate and not able to assimilate mannitol, being able to hydrolyse gelatin, being unable to ferment glucose and having a lower DNA G + C content.

Based on the result of 16S rRNA gene-based phylogenetic analysis and on the above-mentioned phenotypic features, we conclude that TDMA-5^T represents a novel genus and species within the family *Sphingobacteriaceae*, for which we propose the name *Nubsella zeaxanthinifaciens* gen. nov., sp. nov.

Description of *Nubsella* gen. nov.

Nubsella (Nub.sel'la. N.L. fem. dim. n. *Nubsella* arbitrary name derived from the acronym NUBS for Nihon University College of Bioresource Sciences, where the type species was first isolated).

Gram-negative, non-spore-forming rods. Strictly aerobic. Catalase- and oxidase-positive. MK-7 is the only respiratory quinone. Carotenoid pigments are produced. As determined by 16S rRNA gene sequence analysis, the genus *Nubsella* is a member of the family *Sphingobacteriaceae*. The G + C content of the type strain of the type species is 38.6 mol%. The type species of the genus is *Nubsella zeaxanthinifaciens*.

Description of *Nubsella zeaxanthinifaciens* sp. nov.

Nubsella zeaxanthinifaciens (ze.a.xan.thi.ni.fa'ci.ens. N.L. neut. n. zeaxanthinum zeaxanthin; L. part. pres. faciens making/producing; N.L. part. adj. zeaxanthinifaciens zeaxanthin-producing).

Displays the following characteristics in addition to those given in the genus description. Cells are motile, $0.2\text{--}0.3 \times 1.0\text{--}5.0\ \mu\text{m}$. Forms deep-yellow, circular, convex, shiny, opaque and slimy colonies that are slightly adherent to agar, with entire margins and a diameter of 2–3 mm on LB agar plates after 3 days incubation at 30 °C. Contains non-diffusible yellow carotenoid pigments, with zeaxanthin as the major component. Growth occurs between 18.0 and 40.0 °C (optimum, 30.0 °C) and between pH 6.0 and 9.0 (optimum, pH 6.0–7.0). Anaerobic growth does not occur. Tween 80, starch, gelatin, casein, aesculin and DNA are hydrolysed, while agar is not. Indole and H₂S are not produced. Nitrate is not reduced. Glucose, mannose, *N*-acetylglucosamine, maltose and malate are assimilated, but arabinose, mannitol, gluconate, capric acid, adipic acid, trisodium citrate and phenylacetic acid are not. In API 20E tests, β -galactosidase and the Voges–Proskauer test are positive; tests for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, hydrogen sulphide and indole production and urease are negative. Acid is not produced from D-glucose, melibiose, amygdalin, L-arabinose, D-mannitol, inositol, D-sorbitol, L-rhamnose or sucrose. Major fatty acids are iso-C_{15:0}, iso-C_{17:0} 3-OH and summed feature 4 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c).

The type strain is TDMA-5^T (=NBRC 102579^T =CCUG 54348^T), isolated from a freshwater sample collected at Misasa (Tottori, Japan).

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