Flavobacterium algicola sp. nov., isolated from marine algae

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A rod-shaped Gram-staining-negative, non-motile, aerobic and fucoidan-digesting strain, designated TC2^T, was isolated from marine algae collected from the coast of the Sea of Okhotsk at Abashiri, Hokkaido, Japan. The bacterium formed yellow, translucent, circular and convex colonies. Comparative 16S rRNA gene sequence analysis indicated that the strain belonged to the genus *Flavobacterium*, with the highest sequence similarities of 97.1 to 97.3% to the type strains of *Flavobacterium frigidarium*, *Flavobacterium frigoris*, *Flavobacterium limicola* and *Flavobacterium psychrolimnae*. DNA–DNA relatedness values between strain TC2^T and the above-mentioned species were lower than 28%. The genomic DNA G+C content was 33.9 mol%. The major respiratory quinone was menaquinone-6 and the predominant fatty acids were iso-C_{15:1} G, iso-C_{15:0}, iso-C_{15:0} 3-OH and summed feature 3 (which comprises iso-C_{15:0} 2-OH and/or C_{16:1}ω7c). Strain TC2^T could be differentiated from related species by several phenotypic characteristics. Thus, on the basis of these results, strain TC2^T represents a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium algicola* sp. nov. is proposed. The type strain is TC2^T (=NBRC 102673^T =CIP 109574^T).

The genus *Flavobacterium* belongs to the family *Flavobacteriaceae*, phylum *Bacteroidetes*, and comprises more than 50 species that have been found in various habitats, such as soil, sediment, microbial mats, fresh- and salt-water, sea ice and diseased fish. Members of the genus are also abundant in cold freshwater and marine ecosystems where they play a role in the uptake and degradation of organic materials (Bernardet & Bowman, 2006). The genus *Flavobacterium* comprises many psychrophilic and psychrotolerant species isolated from Antarctica, such as *Flavobacterium hibernum* (McCammon *et al.*, 1998); *F. gillisiae*, *F. tegetincola* and *F. xanthum* (McCammon & Bowman, 2000); *F. frigidarium* (Humphry *et al.*, 2001); *F. gelidilacus*, *F. degerlachei*, *F. frigoris*, *F. micromati*, *F. fryxellicola* and *F. psychrolimnae* (Van Trappen *et al.*,

2003, 2004, 2005); *F. antarcticum* (Yi *et al.*, 2005); *F. segetis* and *F. weaverense* (Yi & Chun, 2006); and *F. frigidimaris* (Nogi *et al.*, 2005). In addition, several cold-adapted species of the genus *Flavobacterium* have been described from regions other than Antarctica, e.g. *Flavobacterium limicola* from freshwater sediments (Tamaki *et al.*, 2003), and *Flavobacterium xinjiangense* and *Flavobacterium omnivorum* from the China No. 1 glacier (Zhu *et al.*, 2003).

This study deals with the taxonomic characterization of a fucoidan-digesting micro-organism that represents a novel species of the genus *Flavobacterium*. Fucoidan is a polysaccharide mainly composed of sulfated fucose that originates from brown macroalgae. It has been reported to exert several kinds of biological activities, including antithrombotic activity (Boisson-Vidal *et al.*, 2000), antitumor activity (Maruyama *et al.*, 2003) and inhibitory activity on viral and bacterial infections (Hoshino *et al.*, 1998; Shibata *et al.*, 2003). However, the potential benefits of fucoidan to the food industry have been hampered by its high molecular mass. Therefore, fucoidan-digesting micro-organisms and their enzymes, which digest fucoidan into low molecular mass compounds, are currently needed by

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains TC2^T, NBRC 102676^T, NBRC 102678^T, NBRC 102679^T and NBRC 103156^T are AB455265, AB455257, AB455259, AB455260 and AB455261, respectively.

DNA–DNA hybridization values between strain TC2^T and its closest phylogenetic neighbours are available as supplementary material with the online version of this paper.

the food industry. Hence, fucoidan-digesting micro-organisms were screened from the surface mucus of marine algae collected from the coast of the Sea of Okhotsk at Abashiri, Hokkaido, Japan.

For screening of fucoidan-degrading micro-organisms, a mineral synthetic liquid medium (MI-F) containing 0.5 % (w/v) GA-fucoidan as the sole carbon source was used (Sakai *et al.*, 1998; Nakagawa *et al.*, 2002a). The initial pH of the medium was adjusted to 7.0. GA-fucoidan was prepared from the holdfast of cultivated *Laminaria japonica* by a previously reported method (Ozawa *et al.*, 2006). Cultivation was performed aerobically with rotary shaking at 4 °C for 5 days. The liquid culture was subsequently inoculated on a MI-F plate for single colony isolation, and yellowish colonies of strain TC2^T were isolated. Strain TC2^T was subjected to a polyphasic taxonomy study after its ability to grow on GA-fucoidan as the sole carbon source had been confirmed using the MI-F liquid medium.

The almost complete 16S rRNA gene sequence, ranging from positions 28 to 1494 on the *Escherichia coli* numbering system (Brosius *et al.*, 1978), was determined according to previously published procedures (Nakagawa *et al.*, 2002b). The sequence obtained was aligned with those of representative members of the genus *Flavobacterium* using CLUSTAL_X (Thompson *et al.*, 1997) and then modified manually by referring to the 16S rRNA secondary structure of *E. coli* (Gutell *et al.*, 1994) and using the Se-Al v2.0 alignment editor (A. Rambaut; available at <http://tree.bio.ed.ac.uk/>). A phylogenetic tree was reconstructed using the neighbour-joining method (Saitou & Nei, 1987) and *K_{nuc}* values (Kimura, 1980). Alignment gaps and unidentified base positions were not taken into consideration in the calculations. The topology of the tree

was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. Analysis of the 16S rRNA gene sequences showed that strain TC2^T belonged to the genus *Flavobacterium* with the highest sequence similarities of 97.3, 97.1, 97.2 and 97.2 % to *F. frigidarium* NBRC 102676^T, *F. frigoris* NBRC 102678^T, *F. limicola* NBRC 103156^T and *F. psychrolimnae* NBRC 102679^T, respectively (Fig. 1). Trees obtained by the maximum-parsimony (Fitch, 1971, 1977) and maximum-likelihood (Felsenstein, 1981) methods showed almost the same topology. The 16S rRNA gene sequences of strains *F. frigidarium* NBRC 102676^T, *F. frigoris* NBRC 102678^T, *F. limicola* NBRC 103156^T and *F. psychrolimnae* NBRC 102679^T were determined again in this study in order to confirm published sequences and to ascertain the authenticity of the strains. Since only these four strains showed more than 97 % 16S rRNA gene sequence similarities with strain TC2^T, they were used as references for further taxonomic studies including phenotypic tests.

DNA–DNA hybridization between strain TC2^T and its four closest phylogenetic neighbours was performed using photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989). The hybridization temperature was 48.2 °C in 2×SSC buffer containing 25 % formamide. Approximately 70 % or greater DNA–DNA relatedness is considered evidence that two strains belong to the same species (Wayne *et al.*, 1987). The results indicated that strain TC2^T represented a distinct species as all values of DNA–DNA relatedness were below 28 % (see Supplementary Table S1 available in IJSEM Online).

The DNA G+C contents of strains TC2^T, *F. frigidarium* NBRC 102676^T, *F. frigoris* NBRC 102678^T, *F. limicola* NBRC 103156^T and *F. psychrolimnae* NBRC 102679^T were determined according to Mesbah *et al.* (1989). The values

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain TC2^T. *Flavobacterium aquatile* DSM 1132^T (AM230485) was used as the outgroup. Numbers at branches indicate confidence limits >50 % estimated by bootstrap analysis with 1000 resampling trials. Bar, 0.01 substitutions per nucleotide position.

obtained were 33.9, 33.9, 34.1, 33.9 and 34.0 mol%, respectively. The DNA G+C contents of the four reference type strains were determined again in this study using the same technique in order to compare the values to those previously obtained using different techniques (Humphry *et al.*, 2001; Tamaki *et al.*, 2003; Van Trappen *et al.*, 2004, 2005). The DNA G+C contents obtained in this study were almost identical to the published data.

To determine whole-cell fatty acid composition, strain TC2^T and the four reference strains were grown in tryptic soy broth (TSB; Difco) at 15 °C for 48 h. Fatty acid methyl esters were prepared and analysed according to the standard protocol of the Microbial Identification System (Microbial ID). The fatty acid compositions of strain TC2^T and its phylogenetic relatives are shown in Table 1. The major fatty acids of strain TC2^T were iso-C_{15:1} G (10.2 %), iso-C_{15:0} (18.5 %) and summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c, 24.1 %). These fatty acids occur in most members of the family *Flavobacteriaceae* (Bernardet *et al.*, 1996, 2002). The respective proportions of several fatty acids distinguished strain TC2^T from its closest relatives (Table 1).

Following extraction according to Nakagawa & Yamasato (1993) and determination using LC-MS analysis (8000 α ; Shimadzu), menaquinone-6 was found to be the major respiratory quinone in strain TC2^T, in line with all members of the family *Flavobacteriaceae*.

Morphological features of cells cultivated on tryptic soy agar (TSA; Difco) at 20 °C after 24 h were observed by using light microscopy. Colony morphology on TSA at 20 °C after 24 h was determined. Gliding motility was investigated by using phase-contrast microscopy of a hanging-drop preparation from a TSB culture (Bernardet *et al.*, 2002). Anaerobic growth was examined by using the Anaeropack (Mitsubishi Gas Chemical). Ability to grow on marine 2216 (Difco), nutrient (Oxoid) and TS agars was examined at 15 °C after 6 days. Growth at 5–40 °C (in increments of 5 °C) and at pH 3–11 (in increments of 0.5 pH unit at pH 5–8, and of 1 pH unit outside of this range) was evaluated on TSA and in TSB, respectively. The pH of the medium was adjusted using NaOH and HCl solutions and the media obtained were sterilized by filtration. The NaCl range for growth was determined in TSB supplemented with several concentrations of NaCl. TSB was prepared according to the composition of the Difco medium, except that NaCl was not added. The NaCl-free medium was then supplemented with 0, 2, 2.5, 3, 3.5, 4 and 5 % NaCl (w/v). The bathochromic shift test with 20 % (w/v) KOH (Fautz & Reichenbach, 1980) was used to detect flexirubin-type pigments. Congo red adsorption was tested by using the methods of Bernardet *et al.* (2002) and Surgalla & Beesley (1969). Only *F. frigidarium* NBRC 102676^T absorbed the dye when the method of Bernardet *et al.* (2002) was used, whereas Congo red absorption was observed after 14 days at 15 °C in strain TC2^T and all four reference strains when the method of Surgalla & Beesley

Table 1. Fatty acid composition (%) of strain TC2^T and related species of the genus *Flavobacterium*

Strains: 1, *F. algicola* sp. nov. TC2^T; 2, *F. frigidarium* NBRC 102676^T; 3, *F. frigoris* NBRC 102678^T; 4, *F. psychrolimnae* NBRC 102679^T; 5, *F. limicola* NBRC 103156^T. tr, Trace (less than 1 % of total); –, not detected. All data from this study. Fatty acids amounting to less than 1 % in all strains listed are not shown.

Fatty acid	1	2	3	4	5
C _{14:0}	1.1	tr	tr	tr	tr
iso-C _{15:1} G	10.2	5.4	5.5	2.5	3.1
anteiso-C _{15:0} A	tr	tr	1.2	tr	tr
iso-C _{15:0}	18.5	29.3	9.7	13.1	10.4
anteiso-C _{15:0}	6.7	3.4	18.9	8.2	12.5
C _{15:1} ω6c	1.1	1.5	3.8	3.4	3.4
C _{15:0}	1.0	3.0	2.5	4.3	8.7
iso-C _{16:1} H	–	1.1	1.3	2.8	3.6
iso-C _{16:0}	tr	1.0	1.8	1.6	2.1
C _{16:1} ω5c	1.1	tr	tr	tr	tr
C _{16:0}	7.3	2.0	1.7	1.5	2.2
iso-C _{15:0} 3-OH	9.9	11.1	5.9	5.4	5.7
iso-C _{17:1} ω9c	2.3	8.7	8.8	12.1	7.0
anteiso-C _{17:1} ω9c	tr	tr	tr	3.4	3.2
iso-C _{17:0}	tr	tr	tr	1.2	tr
C _{17:1} ω8c	tr	tr	tr	2.3	2.5
C _{17:1} ω6c	tr	2.0	3.0	5.1	4.8
iso-C _{16:0} 3-OH	–	1.0	3.6	2.4	3.7
C _{16:0} 3-OH	5.4	tr	1.2	tr	1.1
C _{18:1} ω5c	tr	tr	tr	1.0	tr
iso-C _{17:0} 3-OH	5.2	11.3	8.1	8.4	8.1
C _{17:0} 2-OH	tr	tr	1.7	1.1	1.9
C _{17:0} 3-OH	–	tr	tr	tr	1.0
Unknown*	1.0	2.1	1.2	1.9	1.2
Summed feature† 3	24.1	8.5	12.7	6.6	5.4
Summed feature 4	tr	1.9	1.0	6.6	1.7

*The equivalent chain-length (ECL) of the unknown fatty acid was 16.582.

†Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3, iso-C_{15:0} 2-OH and/or C_{16:1}ω7c; summed feature 4, anteiso-C_{17:1} B and/or iso-C_{17:1} I.

(1969) was used. Production of catalase, oxidase and H₂S, reduction of nitrate, degradation of aesculin, agar, alginate, casein, CM-cellulose, chitin, DNA, gelatin, pectin, starch and L-tyrosine, and production of brown diffusible pigment on L-tyrosine agar were examined according to the methods of Lewin & Lounsbery (1969), Smibert & Krieg (1981) and Barrow & Feltham (1993) by using one-fifth strength TSB as the basal medium. Tests in the commercial API ZYM, API 20NE and API 20E systems (bioMérieux) were performed by TechnoSuruga Laboratory (Shizuoka, Japan) according to the instructions of the manufacturer. The phenotypic characteristics of strain TC2^T are given in the species description and in Table 2.

Table 2. Phenotypic characteristics that differentiate strain TC2^T from related species of the genus *Flavobacterium*

Species: 1, *F. algicola* sp. nov. TC2^T; 2, *F. frigidarium* NBRC 102676^T; 3, *F. frigoris* NBRC 102678^T; 4, *F. psychrolimnae* NBRC 102679^T; 5, *F. limicola* NBRC 103156^T. All data from this study. +, Positive; –, negative; v, variable. All strains are positive for growth on TSA and for degradation of aesculin and are negative for gliding motility, production of H₂S and flexirubin pigments, degradation of CM-cellulose, pectin, chitin and DNA, and nitrate reduction. Data from the original descriptions (Humphry *et al.*, 2001; Tamaki *et al.*, 2003; Van Trappen *et al.*, 2004, 2005) are shown in parentheses.

Characteristic	1	2	3	4	5
Growth on nutrient agar	+	+	+(–)	+	+
Tolerance to 3 % NaCl	+	+	+	–	–
Growth at 25 °C	+	–	+	+	+
Congo red adsorption	+	+	+(–)	+(–)	+
Acid from carbohydrates	+	–	–	–	–
Degradation of:					
Gelatin	–	–(+)	–	–	–(+)
Casein	–	+	+	+	+
Starch	+	–	+	+	+
Agar	–	–	–	–	–(+)
Alginate	+	+(–)	–	–	–
Tyrosine	–	–	–(+)	–	–(+)
Brown pigment on tyrosine agar	–	–(+)	–	–	–(+)
ONPG hydrolysis	+	–	–	+	–
DNA G + C content (mol%)	3.9	33.9	34.1	34	33.9

The phylogenetic analysis and DNA–DNA hybridization clearly demonstrated that strain TC2^T represents a new species of the genus *Flavobacterium* that can be differentiated from closely related species by the phenotypic characteristics listed in Table 2. The name *Flavobacterium algicola* sp. nov. is proposed for this species.

Description of *Flavobacterium algicola* sp. nov.

Flavobacterium algicola (al.gi'co.la. L. fem. n. *alga* seaweed, alga; L. suff. *-cola* inhabitant, dweller; N.L. masc. n. *algicola* alga-dweller)

Cells are Gram-staining-negative, non-spore-forming, aerobic rods, 0.7–0.8 µm in diameter and 1.5–2.0 µm in length. Gliding motility is not observed. Colonies on TSA are yellow, translucent, circular and convex with entire margins. Growth occurs on marine 2216, nutrient and trypticase soy agars. The temperature range for growth is 5–25 °C (optimum, 20 °C). The pH range for growth is 5.5–8 (optimum, pH 7). The salinity range for growth is 0–3 % NaCl (optimum, 0 %). Flexirubin-type pigments are not produced. Congo red is absorbed. Brown diffusible pigment is not produced on L-tyrosine agar. Catalase and oxidase activities are present. Nitrate is not reduced. Hydrogen sulfide is not produced. Aesculin, alginate and

starch are degraded, but agar, casein, CM-cellulose, chitin, DNA, gelatin, pectin and tyrosine are not. *N*-acetyl-D-glucosamine, glucose, maltose, D-mannitol and D-mannose are utilized, but adipate, L-arabinose, caprate, citrate, gluconate, malate and phenylacetate are not utilized (API 20NE). Acids are produced from D-mannitol and sucrose, but not from L-arabinose, D-amygdalin, glucose, inositol, melibiose, L-rhamnose or D-sorbitol (API 20E). *N*-acetyl-D-glucosaminidase, acid phosphatase, alkaline phosphatase, leucine arylamidase and naphthol-AS-BI-phosphohydrolase activities are present, but α-chymotrypsin, esterase (C4), esterase-lipase (C8), α-fucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, lipase (C14), α-mannosidase, trypsin, cystine arylamidase and valine arylamidase activities are absent (API ZYM). The major respiratory quinone is menaquinone-6. The major fatty acids (more than 5 % of total) are iso-C_{15:1} G, iso-C_{15:0}, anteiso-C_{15:0}, C_{16:0}, iso-C_{15:0} 3-OH, C_{16:0} 3-OH, iso-C_{17:0} 3-OH and summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1ω7c}). The DNA G + C content of the type strain is 33.9 mol%.

The type strain is TC2^T (=NBRC 102673^T =CIP 109574^T) isolated from marine algae from the coast of the Sea of Okhotsk at Abashiri, Hokkaido, Japan.

Acknowledgements

We are grateful to Dr Takashi Ito, Ms Megumi Ando and Ms Tomomi Chida, Department of Food Science and Technology, Tokyo University of Agriculture, for their skilful assistance. This work was supported in part by the Research for Promoting Technological Seed from the Japan Science and Technology Agency (JST).

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