Donghaeana dokdonensis gen. nov., sp. nov., isolated from sea water

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A Gram-negative, non-motile, non-spore-forming and rod-shaped bacterial strain, DSW-6^T, was isolated from sea water and subjected to a polyphasic study. Strain DSW-6^T grew optimally at 25 °C, in the presence of 2 % (w/v) NaCl and at pH 7.0–8.0. It was characterized chemotaxonomically as having MK-6 as the predominant menaquinone and iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{17:0} 3-OH and C_{15:0} as the major fatty acids (> 10 % of total fatty acids). Major polar lipids were phosphatidylethanolamine, unidentified phospholipids and amino-group-containing lipids that are ninhydrin-positive. The DNA G + C content was 36.9 mol%. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain DSW-6^T forms an independent line of descent within the family *Flavobacteriaceae*. The 16S rRNA gene sequence of strain DSW-6^T exhibited similarity values of less than 94.7 % to the sequences of other members of the family *Flavobacteriaceae*. Strain DSW-6^T could be distinguished from other phylogenetically related genera by differences in several phenotypic properties. Therefore, in view of the combined phenotypic and phylogenetic data, it is proposed that strain DSW-6^T (= KCTC 12402^T = DSM 17205^T) represents a novel genus and species, *Donghaeana dokdonensis* gen. nov., sp. nov.

Dokdo is an island located in the East Sea, Korea. Restriction of access to the island for a long time may have resulted in interesting microbial diversity, which led us to investigate the microbial community of soils and sea water from the island. In the course of screening micro-organisms from sea water in Dokdo, a bacterial strain, DSW-6^T, belonging to the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group, was isolated and characterized. CFB is one of the major bacterial groups in marine environments (Bowman *et al.*, 1997; Glöckner *et al.*, 1999; Kirchman, 2002). Accordingly, the aim of the present work was to determine the exact taxonomic position of strain DSW-6^T by a polyphasic taxonomic characterization.

Sea water provided the source for isolation of bacterial strains. A standard dilution plating technique on marine agar 2216 (MA; Difco) and incubation at 20 °C were used to isolate strain DSW-6^T. To investigate its morphological and physiological characteristics, strain DSW-6^T was routinely cultivated on MA at 25 °C. Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy. Transmission electron microscopy was also used to determine whether flagella are present in

cells from exponentially growing cultures. Gliding motility was investigated as described by Bowman (2000). The Gram reaction was determined by using the bioMérieux Gram Stain kit according to the manufacturer's instructions. The pH range for growth was determined in marine broth 2216 (MB; Difco) that was adjusted to various pH values (pH 4.5–10.5 at intervals of 0.5 pH units). The pH was adjusted prior to sterilization to various levels by the addition of HCl or Na₂CO₃. Growth in the absence of NaCl was investigated in R2A agar (Difco) and trypticase soy broth prepared according to the formula of the Difco medium except that no NaCl was used. Growth at various NaCl concentrations [0.5 % (w/v) and 1.0–10.0 % (w/v) at intervals of 1.0 %] was investigated in MB and trypticase soy broth (Difco). Growth at various temperatures (4–35 °C) was measured on MA. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on MA and on MA supplemented with nitrate that had both been prepared anaerobically in a nitrogen atmosphere. Catalase and oxidase activities and hydrolysis of casein and starch were determined as described by Cowan & Steel (1965). Hydrolysis of Tweens 20, 40, 60 and 80 was determined as described by Cowan & Steel (1965) with a modification that artificial sea water was used instead of distilled water. Hydrolysis of aesculin, gelatin and urea and nitrate reduction were determined as described by Lanyi (1987) using artificial sea water. Artificial sea water contained (per litre of distilled water) 23.6 g NaCl, 0.64 g KCl,

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4.53 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5.94 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.3 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Bruns *et al.*, 2001). Hydrolysis of hypoxanthine, tyrosine and xanthine was investigated on MA with the substrate concentrations described by Cowan & Steel (1965), i.e. 0.4, 0.5 and 0.4 % (w/v), respectively. The production of H_2S was tested as described previously (Bruns *et al.*, 2001). The presence of flexirubin-type pigments was investigated as described by Reichenbach (1992). Freeze-dried cells were extracted with acetone/methanol (1 : 1, v/v) to investigate the presence of carotenoid pigments; after removal of cells by centrifugation, the absorption spectrum of the supernatant was examined on a Beckman Coulter DU800 spectrophotometer. Acid production from carbohydrates was determined as described by Leifson (1963). Growth on several substrates was tested in a basal medium containing 0.2 g NaNO_3 , 0.2 g NH_4Cl and 0.05 g yeast extract in 1000 ml artificial sea water (Bruns *et al.*, 2001) as described by Suzuki *et al.* (2001). Susceptibility to antibiotics was tested on MA plates using antibiotic discs containing the following concentrations: polymyxin B, 100 U; streptomycin, 50 µg; penicillin G, 20 U; chloramphenicol, 100 µg; ampicillin, 10 µg; cephalothin, 30 µg; gentamicin, 30 µg; novobiocin, 5 µg; tetracycline, 30 µg; kanamycin, 30 µg; lincomycin, 15 µg; oleandomycin, 15 µg; neomycin, 30 µg; carbenicillin, 100 µg. Other physiological and biochemical tests were performed using the API ZYM and API 20E systems (bioMérieux); cell suspension to inoculate the systems was prepared by using cells cultivated for 3 days at 25 °C on MA and artificial sea water (Bruns *et al.*, 2001).

Cell biomass of strain DSW-6^T for DNA extraction and for isoprenoid quinone and polar lipid analyses was obtained by cultivation for 2 days in MB at 25 °C. Chromosomal DNA was isolated and purified according to the method described previously (Yoon *et al.*, 1996), with the exception that RNase T1 was used in combination with RNase A to minimize contamination with RNA. The 16S rRNA gene was amplified by PCR using two universal primers as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). Isoprenoid quinones were extracted according to the method of Komagata & Suzuki (1987) and analysed using reverse-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. Polar lipids were extracted according to the procedures described by Minnikin *et al.* (1984) and identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). For fatty acid methyl ester analysis, cell mass of strain DSW-6^T was harvested from agar plates after incubation for 3 days at 25 °C on MA. The fatty acid methyl esters were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G+C content was determined by the method of Tamaoka & Komagata (1984) with a modification that DNA was

hydrolysed and the resulting nucleotides were analysed by reverse-phase HPLC.

Morphological, cultural, physiological and biochemical characteristics of strain DSW-6^T are given in the species description (see later) or are shown in Table 1, together with those of some phylogenetically related genera. The acetone/methanol extract of the freeze-dried cells showed absorption maxima at 413, 441 and 465 nm, demonstrating the presence of carotenoids. Strain DSW-6^T produced no flexirubin-type pigments. The almost complete 16S rRNA gene sequence of strain DSW-6^T determined in this study comprised 1481 nucleotides, representing approximately 96 % of the *Escherichia coli* 16S rRNA sequence. In the phylogenetic trees based on 16S rRNA gene sequences, strain DSW-6^T formed a cluster with unidentified *Flavobacteriaceae* bacterium SW256, *Nonlabens tegetincola* and '*Stenothermobacter spongiae*' with which it exhibited 16S rRNA gene sequence similarity values of 93.3–98.2 % (Fig. 1). This cluster joined the clade comprising *Psychroflexus* species by a bootstrap confidence level of 54.2 % (Fig. 1). Strain DSW-6^T exhibited 16S rRNA gene sequence similarity levels of less than 90.8 % with respect to other species used in the phylogenetic analysis.

The predominant isoprenoid quinone detected in strain DSW-6^T was menaquinone-6 (MK-6) at a peak area ratio of approximately 94 %. The cellular fatty acid profile of strain DSW-6^T comprised (>1 % of total fatty acids) branched fatty acids iso-C_{15:0} (19.2 %), anteiso-C_{15:0} (11.1 %), iso-C_{17:1ω9c} (3.5 %), iso-C_{16:0} (1.5 %) and iso-C_{16:1} (1.3 %); hydroxyl fatty acids iso-C_{17:0} 3-OH (10.5 %), iso-C_{15:0} 3-OH (6.7 %), iso-C_{16:0} 3-OH (3.5 %), C_{15:0} 3-OH (3.3 %), C_{17:0} 2-OH (3.0 %), C_{15:0} 2-OH (1.8 %) and C_{16:0} 2-OH (1.5 %); straight-chain fatty acids C_{15:0} (10.2 %), C_{18:0} (2.0 %) and C_{16:0} (1.5 %); unsaturated fatty acids C_{15:1ω6c} (3.7 %), C_{17:1ω6c} (2.9 %) and C_{18:1ω5c} (1.7 %); and summed feature 3 comprising C_{16:1ω7c} and/or iso-C_{15:0} 2-OH (7.1 %). The phospholipid identified in strain DSW-6^T was phosphatidylethanolamine, and other major polar lipids were three unidentified phospholipids and three amino-group-containing lipids that were ninhydrin-positive. The DNA G+C content of strain DSW-6^T was 36.9 mol%.

The predominant menaquinone type (MK-6) of strain DSW-6^T was the same as that of all members of the family *Flavobacteriaceae* (Bowman *et al.*, 1998; Bernardet *et al.*, 2002). Phylogenetic analyses based on three different algorithms revealed that strain DSW-6^T forms a distinct phylogenetic lineage within the family *Flavobacteriaceae* (Fig. 1). The 16S rRNA gene sequence similarity values were too low (less than 94.7 %) to assign strain DSW-6^T to any recognized genus in the family. The fatty acid profiles, particularly proportions of the major fatty acids, also distinguished strain DSW-6^T from the phylogenetically related genera *Psychroflexus*, *Salegentibacter*, *Mesonina*, *Gillisia*, *Aquimarina*, *Stanierella* and *Gaetbulimicrobium*, although differences might partly be caused by different cultivation

Table 1. Differential phenotypic characteristics of *Donghaeana dokdonensis* gen. nov. and related genera

Genus: 1, *Donghaeana dokdonensis* gen. nov.; 2, *Psychroflexus*, data from Bowman *et al.* (1998) and Donachie *et al.* (2004); 3, *Salegentibacter*, data from Nedashkovskaya *et al.* (2004, 2005b); 4, *Mesonina*, data from Nedashkovskaya *et al.* (2003); 5, *Gillisia*, data from Van Trappen *et al.* (2004) and Nedashkovskaya *et al.* (2005c); 6, *Aquimarina*, data from Nedashkovskaya *et al.* (2005a); 7, *Stanierella*, data from Reichenbach (1989) and Nedashkovskaya *et al.* (2005a). All genera are positive for alkaline phosphatase (not determined for *Stanierella*), oxidase (variable for *Psychroflexus*) and hydrolysis of Tween 80 (variable for *Psychroflexus*). All genera are negative for acid production from D-cellobiose. +, Positive reaction; –, negative reaction; ND, not determined; V, variable reaction.

Characteristic	1	2	3	4	5	6	7
Catalase	+	+	+	+	+	+	–
Gliding motility	–	V	–	–	–	+	–
Cell size (µm)	0.4–0.6 × 1.0–30.0	0.2–1.5 × 2.0–50.0	0.5–0.7 × 2.5–5.3	0.4–0.5 × 1.6–2.3	0.5–0.7 × 3.0–4.0	0.3–0.5 × 5–7	0.3–0.4 × 1–40
Pigmentation	Orange	Orange	Yellow	Yellow	Yellow	Dark yellow to brown	Orange–red
Flexirubin-type pigments	–	–*	–	–	–	+	–
Growth at > 10 % NaCl	–	V	V	+	V	–	–
Growth at 37 °C	–	V	V	–	–	–	–
Nitrate reduction	–	V	V	–	–	–	–
H ₂ S production	–	–*	+	+	–	–	+
NaCl requirement for growth	+	V	V	+	V	+	+
β-Galactosidase activity	–	–*	+	ND	–†	ND	+
Hydrolysis of:							
Agar	–	–*	–	–	–	–	+
Casein	+	V	V	+	V	+	+‡
Gelatin	–	V	+	+	+	+	+
Starch	+	+	+	–	–	+	–
Urea	–	V	–	–	V	–	–
Tween 20	+	ND	+	+	V	+	+
Tween 40	+	ND	+	+	V	+	+
Acid production from:							
D-Glucose	–	V	V	–	V	–	–
Maltose	–	V	+	–	–	–	–
Utilization of:							
D-Glucose	–	ND	+	–	+	–	ND
D-Sucrose	–	ND	V	–	+	–	ND
Susceptibility to:							
Benzylpenicillin	+	ND	+	ND	–	–	–
Carbenicillin	+	ND	V	ND	+	+	–
Streptomycin	+	ND	V	ND	V	ND	+
Tetracycline	+	ND	+	ND	V	ND	ND
Major fatty acid(s)§ (> 10 %)	i-C _{15:0} , ai-C _{15:0} , i-C _{17:0} 3-OH, C _{15:0}	ai-C _{15:0} , ai-C _{15:1} ω10c, i-C _{16:0} 3-OH	i-C _{15:0} , i-C _{15:1} , C _{16:1} ω7c or i-C _{15:1}	i-C _{15:0} , i-C _{17:0} 3-OH	C _{17:0} 2-OH or i-C _{15:1}	i-C _{17:0} 3-OH, i-C _{15:0} , i-C _{15:1} , i-C _{17:1} ω9c	i-C _{17:0} 3-OH, i-C _{15:0} , i-C _{17:1} ω7c
DNA G+C content (mol%)	36.9	32–36	37–38	32.7–34.0	36–39	31.6–32.5	34

*Data for *Psychroflexus torquis* and *Psychroflexus gondwanensis*.

†Data for *Gillisia limnaea*.

‡Data taken from Nedashkovskaya *et al.* (2005a); a different result was obtained by Reichenbach (1989).

§i, iso; ai, anteiso. Data for the genus *Salegentibacter* are those of *Salegentibacter holothuriorum* and *Salegentibacter mishustinae*.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of strain DSW-6^T and representatives of some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the branching points. *Bacteroides fragilis* ATCC 25285^T was used as an out-group. Dots indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. Scale bar, 0.02 substitutions per nucleotide position.

conditions (Table 1). Strain DSW-6^T was also differentiated from phylogenetically related genera by differences in several phenotypic properties as shown in Table 1. Low 16S rRNA gene sequence similarity values between strain DSW-6^T and all other members of the family *Flavobacteriaceae*, together with differential phenotypic properties, suggest that strain DSW-6^T constitutes a new genus and species in the family *Flavobacteriaceae*, for which the name *Donghaeana dokdonensis* gen. nov., sp. nov. is proposed.

Description of *Donghaeana* gen. nov.

Donghaeana (Dong.hae.a'na. *Donghae*, the Korean name of the East Sea in Korea; L. fem. suff. -ana, suffix used with the sense of belonging to; N.L. fem. adj. used as a substantive *Donghaeana* pertaining to the East Sea of Korea, where Dokdo is located).

Cells are Gram-negative, non-spore-forming, rods or elongated rods (0.4–0.6 × 1.0–30.0 µm). Strictly aerobic. The predominant menaquinone is MK-6. The major polar lipids are phosphatidylethanolamine, unidentified phospholipids and amino-group-containing lipids that are ninhydrin-positive. The type species is *Donghaeana dokdonensis*.

Description of *Donghaeana dokdonensis* sp. nov.

Donghaeana dokdonensis (dok.do.nen'sis. N.L. fem. adj. *dokdonensis* of Dokdo, a Korean island, where the type strain was isolated).

Exhibits the following properties in addition to those given in the genus description. Cells are non-motile. Colonies on MA are circular, convex, glistening, smooth, orange-coloured and 0.8–1.4 mm in diameter after incubation for 3 days at 25 °C. Growth occurs from 4 to 32 °C with an

optimum temperature of 25 °C. Optimal pH for growth is 7.0–8.0; growth is observed at pH 5.5, but not at pH 5.0. Optimal growth occurs in the presence of 2% (w/v) NaCl; growth does not occur in the absence of NaCl and in the presence of greater than 8% (w/v) NaCl. Anaerobic growth does not occur on MA and on MA supplemented with nitrate. Aesculin and Tween 60 are hydrolysed, but hypoxanthine, xanthine and L-tyrosine are not. Indole is not produced. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase are absent. In assays with the API ZYM system, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase are present, but lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. Susceptible to chloramphenicol, cephalothin, novobiocin and lincomycin, but not to polymyxin B, ampicillin, gentamicin, kanamycin or neomycin. Growth occurs on peptone and tryptone as the sole carbon and nitrogen sources, but not on D-ribose, D-galactose, D-fructose, D-cellobiose, D-trehalose, Casamino acids, DL-aspartate, L-glutamate, L-leucine or L-proline. Acid is not produced from L-arabinose, D-fructose, D-galactose, lactose, D-mannose, melibiose, D-melezitose, D-raffinose, L-rhamnose, D-ribose, sucrose, D-trehalose, D-xylose, D-mannitol, D-sorbitol or myo-inositol. The major fatty acids (>10% of total fatty acids) are iso-C_{15:0} (19.2%), anteiso-C_{15:0} (11.1%), iso-C_{17:0} 3-OH (10.5%) and C_{15:0} (10.2%). The DNA G + C content is 36.9 mol%. Other phenotypic properties are given in Table 1.

The type strain, DSW-6^T (= KCTC 12402^T = DSM 17205^T), was isolated from sea water.

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