Dokdonia donghaensis gen. nov., sp. nov., isolated from sea water

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Two Gram-negative, non-motile, non-spore-forming, slightly halophilic bacterial strains, DSW-1^T and DSW-21, were isolated from sea water and subjected to a polyphasic taxonomic study. They grew optimally at 30 °C and in the presence of 2 % (w/v) NaCl. Strains DSW-1^T and DSW-21 were characterized chemotaxonomically as containing MK-6 as the predominant menaquinone and iso-C_{15:0}, iso-C_{17:0} 3-OH and iso-C_{15:1} as the major fatty acids. Their DNA G + C content was 38 mol%. Strains DSW-1^T and DSW-21 exhibited four nucleotide differences in their 16S rRNA gene sequences and possessed a mean DNA–DNA relatedness level of 78 %. Phylogenetic analyses based on 16S rRNA gene sequences revealed that strains DSW-1^T and DSW-21 formed a distinct lineage within the family *Flavobacteriaceae*. The 16S rRNA gene sequences of strains DSW-1^T and DSW-21 had similarity levels of less than 92.2 % to the sequences of other members of the family *Flavobacteriaceae*. There were some differences in phenotypic properties between the two strains and *Cellulophaga* species, the nearest phylogenetic neighbours. On the basis of phenotypic, phylogenetic and genetic data, strains DSW-1^T (=KCTC 12391^T=DSM 17200^T) and DSW-21 were classified in a novel genus and species, *Dokdonia donghaensis* gen. nov., sp. nov.

Members of the *Cytophaga*–*Flavobacterium*–*Bacteroides* (CFB) group constitute one of the bacterial groups that exist dominantly in marine environments (Bowman *et al.*, 1997; Glöckner *et al.*, 1999; Kirchman, 2002). The CFB group is well known to be proficient in degrading biopolymers, such as cellulose and chitin (Kirchman, 2002). During the last few years, the number of novel genera assigned to the CFB group, including the recently described genera *Winogradskyella* (Nedashkovskaya *et al.*, 2005a), *Pibocella* (Nedashkovskaya *et al.*, 2005b), *Aquimarina* and *Stanierella* (Nedashkovskaya *et al.*, 2005c), *Roseivirga* (Nedashkovskaya *et al.*, 2005d), *Bizionia* (Nedashkovskaya *et al.*, 2005e), *Gramella* (Nedashkovskaya *et al.*, 2005f), *Sejongia* (Yi *et al.*, 2005) and *Marinicola* (Yoon *et al.*, 2005), has increased continuously. In this study, we describe two Gram-negative, slightly halophilic bacterial strains, DSW-1^T and DSW-21, which were isolated from sea water around a Korean island, Dokdo. The result of 16S rRNA gene sequence comparison indicated that the two strains are

phylogenetically affiliated to the CFB group. Accordingly, the aim of the present work was to investigate the taxonomic status of strains DSW-1^T and DSW-21 by a polyphasic taxonomic characterization.

Sea water collected from Dokdo was used as source for the isolation of bacterial strains. Strains DSW-1^T and DSW-21 were isolated by the standard dilution plating technique on marine agar 2216 (MA; Difco) at 25 °C. Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy. The presence of flagella was determined by transmission electron microscopy using 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 (initial 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 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 % units] was investigated in MB or trypticase soy broth. Growth at various temperatures (4–40 °C) was measured on MA. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on MA

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains DSW-1^T and DSW-21 are DQ003276 and DQ003277, respectively.

A table showing the cellular fatty acid composition of *Dokdonia donghaensis* gen. nov., sp. nov. and *Cellulophaga* species is available as supplementary material in IJSEM Online.

and on MA supplemented with nitrate, both of which had been prepared anaerobically using nitrogen. 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) with a modification that artificial sea water was used instead of distilled water. The artificial sea water contained (per litre of distilled water) 23.6 g NaCl, 0.64 g KCl, 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). The production of H_2S was tested as described previously (Bruns *et al.*, 2001). 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 carotenoids. 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). Enzyme activity was determined by using the API ZYM system (bioMérieux). 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 20E system (bioMérieux).

Cell biomass of strains DSW-1^T and DSW-21 for DNA extraction and for isoprenoid quinone and polar lipid analyses was obtained by cultivation for 2 days in MB at 30 °C. Chromosomal DNA was isolated and purified according to the method described previously (Yoon *et al.*, 1996), with the exception that ribonuclease T1 was used in combination with ribonuclease 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). The DNA G + C content was determined by the method of Tamaoka & Komagata (1984) with a modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC. DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replicates for each sample. The highest and lowest values obtained in each sample were excluded,

and the means of the remaining three values were quoted as DNA–DNA relatedness values. Isoprenoid quinones were extracted according to the method of Komagata & Suzuki (1987) and analysed using reversed-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 strains DSW-1^T and DSW-21 was harvested from agar plates after incubation for 3 days on MA at 30 °C. The fatty acid methyl esters were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990).

Morphological, cultural, physiological and biochemical characteristics of strains DSW-1^T and DSW-21 are given in the genus and species descriptions (see below) or are shown in Table 1, together with those of *Cellulophaga* species. Strains DSW-1^T and DSW-21 produced carotenoid pigments (absorption maxima at 453 and 477–478 nm) but no flexirubin-type pigments. The two strains produced no acid from the substrates used in this study. The predominant isoprenoid quinone found in strains DSW-1^T and DSW-21 was menaquinone-6 (MK-6) at a peak area ratio of approximately 96–97%. Strains DSW-1^T and DSW-21 had cellular fatty acid profiles that contained large amounts of branched, hydroxy and straight-chain fatty acids; the major components (>5% of total fatty acids) were iso- $\text{C}_{15:0}$ (26.0 and 25.6%), iso- $\text{C}_{17:0}$ 3-OH (15.2 and 16.4%), iso- $\text{C}_{15:1}$ (13.6 and 13.3%), summed feature 3 (i.e. $\text{C}_{16:1\omega7c}$ and/or iso- $\text{C}_{15:0}$ 2-OH) (7.5 and 7.9%), iso- $\text{C}_{16:0}$ 3-OH (7.2 and 9.2%) and anteiso- $\text{C}_{15:0}$ (4.5 and 5.5%) (supplementary table in IJSEM Online). Phosphatidylethanolamine was the only phospholipid identified in strains DSW-1^T and DSW-21, and other major polar lipids were two unidentified phospholipids, an unidentified glycolipid and an amino-group-containing lipid that was ninhydrin-positive.

Almost complete 16S rRNA gene sequences of strains DSW-1^T and DSW-21 determined in this study comprised 1479 nucleotides, representing approximately 96% of the *Escherichia coli* sequence. There were four nucleotide differences between the 16S rRNA gene sequences of the two strains. Phylogenetic trees based on 16S rRNA gene sequences showed that strains DSW-1^T and DSW-21 formed a distinct lineage of descent within the family *Flavobacteriaceae* (Fig. 1). In the phylogenetic tree based on the neighbour-joining algorithm, strains DSW-1^T and DSW-21 joined the phylogenetic clade comprising *Cellulophaga* species. The two strains exhibited 16S rRNA gene sequence similarity values of 90.4–92.2% with respect to the type strains of recognized *Cellulophaga* species and of less than 91.7% with respect to the other species used in the phylogenetic analysis (Fig. 1). The DNA G + C content of strains DSW-1^T and DSW-21 was 38.3 mol%. The

Table 1. Differential phenotypic characteristics of *Dokdonia donghaensis* gen. nov., sp. nov. and *Cellulophaga* species

Species: 1, *Dokdonia donghaensis* gen. nov., sp. nov.; 2, *Cellulophaga baltica*, data from Johansen *et al.* (1999), Bowman (2000) and Nedashkovskaya *et al.* (2004); 3, *Cellulophaga fucicola*, data from Johansen *et al.* (1999), Bowman (2000) and Nedashkovskaya *et al.* (2004); 4, *Cellulophaga lytica*, data from Johansen *et al.* (1999), Bowman (2000) and Nedashkovskaya *et al.* (2004); 5, *Cellulophaga algicola*, data from Bowman (2000) and Nedashkovskaya *et al.* (2004); 6, *Cellulophaga pacifica*, data from Nedashkovskaya *et al.* (2004). +, Positive reaction; –, negative reaction; ND, not determined; v, variable reaction. Data in parentheses are for the type strain. All species are positive for catalase, NaCl requirement for growth and growth at 6 % NaCl. All species are negative for anaerobic growth and production of flexirubin-type pigments.

Characteristic	1	2	3	4	5	6
Oxidase	+	–*	–*	+	+	+
Gliding motility	–	+	+	+	+	+
Nitrate reduction	–	(–)*	+	–	+	+
Urease	–	(–)	+	–	–	ND
Growth at:						
8 % (w/v) NaCl	–	–	–	+†	+†	v (–)
4 °C	+	+	+	–†	+	+
37 °C	–	–	–	(+)	–	–
Hydrolysis of:						
Agar	–	+	+	+	+	+
Casein	+	+	–	v (–)	+†	–
Gelatin	–	+	–‡	v (+)	+	+
Starch	–	+	+	+	+	+
L-Tyrosine	–	+	+	+	+	ND
Tween 20	+	(+)	+	(+)	(–)	+
Tween 40	+	(–)	+	(+)	(+)	+
Tween 80	+	(+)	+	v (–)	+†	v (+)
Acid production from:						
L-Arabinose	–	(–)	+	v (–)	–	+
D-Cellobiose	–	(+)*	+	+	+†	+
D-Fructose	–	(+)	+	+	v (–)	ND
D-Galactose	–	(+)*	+	+	+†	+
D-Glucose	–	(+)*	+	+	+	+
Lactose	–	(–)	–	+	+	+
Maltose	–	(–)*	–	+	+	+
D-Mannose	–	(+)	–	+	+	ND
Melibiose	–	(–)	–	(–)	(+)	–
D-Raffinose	–	(–)	–	(–)	(–)	+
Sucrose	–	(+)	+	+†	+†	+
D-Xylose	–	(+)*	–	v (–†)	–	v (+)
D-Mannitol	–	(–)*	–	+†	–	–
L-Rhamnose	–	(–)	+	v (–)	–	–
Susceptibility to antibiotics						
Ampicillin (10 µg per disc)	–	(–)	–	(–)	(–)	v (–)
Carbenicillin	+	(–)	–	(–)	(–)	+
Streptomycin	–	(–)	–	(–)	(–)	v (+)
Tetracycline (30 µg per disc)	+	(–)	–	(–)	(+)	–
DNA G+C mol%§	38	(33–35)*	32–34	32–34	33–38	32–34

*Data taken from Johansen *et al.* (1999) and/or Bowman (2000); the opposite result was reported by Nedashkovskaya *et al.* (2004).

†Data taken from Bowman (2000); the opposite result for the type strain was reported by Nedashkovskaya *et al.* (2004).

‡Data taken from Johansen *et al.* (1999); the opposite result was reported by Bowman (2000) and Nedashkovskaya *et al.* (2004).

§Data (T_m and HPLC data) taken from Johansen *et al.* (1999), Bowman (2000) and Nedashkovskaya *et al.* (2004).

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of strains DSW-1^T and DSW-21 and representatives of some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the branch points. *Bacteroides fragilis* ATCC 25285^T was used as an outgroup. Dots indicate that the corresponding nodes were also recovered in the tree generated with the maximum-likelihood algorithm. Scale bar, 0.01 substitutions per nucleotide position.

mean DNA–DNA relatedness value of strains DSW-1^T and DSW-21 was 78 %, when their DNAs were used individually as labelled DNA probes for cross-hybridization, indicating that the two strains were members of the same genomic species (Wayne *et al.*, 1987). In view of the combined phenotypic, phylogenetic and genetic similarity, strains DSW-1^T and DSW-21 could be considered as members of the same species.

The two strains contained the same predominant menaquinone type (MK-6) as members of the genus *Cellulophaga* and the other members of the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). They also shared the same major cellular fatty acids (iso-C_{15:0}, iso-C_{17:0} 3-OH and iso-C_{15:1}) with the *Cellulophaga* species (Bowman, 2000; Nedashkovskaya *et al.*, 2004; supplementary table in IJSEM Online), although differences in the relative proportions of some fatty acids (e.g. C_{15:0}, iso-C_{15:0} and C_{16:1}ω7c) were noticed. These differences might partly be caused by different cultivation conditions. Strains DSW-1^T and DSW-21 were differentiated from *Cellulophaga* species in some phenotypic properties, including gliding motility, acid production from carbohydrates and hydrolysis of some substrates (Table 1). The result of 16S rRNA gene sequence analyses and differential phenotypic properties were sufficient to categorize strains DSW-1^T and DSW-21 as members of a genus that is separate from the recognized genera of the family *Flavobacteriaceae*. Therefore, on the basis of the data presented, strains DSW-1^T and DSW-21 should be classified as members of a novel genus and species, for which the name *Dokdonia donghaensis* gen. nov., sp. nov. is proposed.

Description of *Dokdonia* gen. nov.

Dokdonia (Dok.do'ni.a. N.L. fem. n. *Dokdonia* named after Dokdo, an island located on the East Sea in Korea, from where the organisms were isolated).

Cells are Gram-negative, non-spore-forming rods or elongated rods. Strictly aerobic. Non-motile. Catalase- and oxidase-positive. The predominant menaquinone is MK-6. The type species is *Dokdonia donghaensis*.

Description of *Dokdonia donghaensis* sp. nov.

Dokdonia donghaensis (dong.ha.en'sis. N.L. fem. adj. *donghaensis* of Donghae, the Korean name of the East Sea of Korea, where Dokdo is located and from where the organism was isolated).

Exhibits the following properties in addition to those given in the genus description. Cells are 0.3–0.6 × 1.5–25.0 μm. Colonies on marine agar 2216 (MA) are circular, slightly convex, glistening, smooth, yellow in colour and 1.0–2.0 mm in diameter after incubation for 3 days at 30 °C. Growth occurs at 4 and 35 °C with an optimum temperature of 30 °C; growth does not occur at 36 °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 7 % (w/v) NaCl. Anaerobic growth does not occur on MA and on MA supplemented with nitrate. Aesculin and Tween 60 are hydrolysed, but hypoxanthine and xanthine are not. Indole and H₂S are not produced. Arginine dihydrolase, lysine

decarboxylase, ornithine decarboxylase and tryptophan deaminase are absent. In assays with the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase and valine arylamidase are present, but lipase (C14), cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase are absent. Susceptible to penicillin G, chloramphenicol, cephalothin, lincomycin and oleandomycin, but not to polymyxin B, gentamicin, novobiocin, kanamycin or neomycin. Growth occurs on peptone and tryptone as the sole carbon and nitrogen sources, but not on D-glucose, sucrose, 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 D-ribose, D-trehalose, D-sorbitol or *myo*-inositol. The major fatty acids (>10% of total fatty acids) are iso-C_{15:0}, iso-C_{17:0} 3-OH and iso-C_{15:1}. The major polar lipids are phosphatidylethanolamine, unidentified phospholipids, an unidentified glycolipid and an amino-group-containing lipid that is ninhydrin-positive. The DNA G+C content is 38 mol%. Other phenotypic properties are given in Table 1.

The type strain, DSW-1^T (= KCTC 12391^T = DSM 17200^T), was isolated from sea water. Another reference strain is DSW-21.

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