Maribacter dokdonensis sp. nov., isolated from sea water off a Korean island, Dokdo

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Two Gram-negative, non-spore-forming, slightly halophilic gliding bacterial strains, DSW-8^T and DSW-9, were isolated from sea water off a Korean island, Dokdo, of the East Sea, Korea, and their taxonomic position was investigated by a polyphasic study. The two strains grew optimally at 30 °C and in the presence of 2–3 % (w/v) NaCl. Strains DSW-8^T and DSW-9 were characterized chemotaxonomically as containing MK-6 as the predominant menaquinone and iso-C_{17:0} 3-OH, iso-C_{15:0} and iso-C_{15:1} as the major fatty acids. Major polar lipids were phosphatidylethanolamine, two unidentified phospholipids, an unidentified glycolipid and an amino group-containing lipid that was ninhydrin-positive. Their DNA G + C contents were 36.1 and 35.9 mol%. Phylogenetic analyses based on 16S rRNA gene sequences showed that strains DSW-8^T and DSW-9 fell within the genus *Maribacter* of the family *Flavobacteriaceae*. Strains DSW-8^T and DSW-9 exhibited no difference in their 16S rRNA gene sequences and possessed a mean DNA–DNA relatedness level of 89 %. Strains DSW-8^T and DSW-9 exhibited 16S rRNA gene sequence similarity levels of 96.9–98.0 % to the type strains of the four recognized *Maribacter* species, but their low level of DNA–DNA relatedness with these species demonstrated that they constitute a distinct *Maribacter* species. On the basis of phenotypic and phylogenetic data and genetic distinctiveness, strains DSW-8^T (=KCTC 12393^T = DSM 17201^T) and DSW-9 were classified in the genus *Maribacter* as members of a novel species, for which the name *Maribacter dokdonensis* sp. nov. is proposed.

The genus *Maribacter* was first described by Nedashkovskaya *et al.* (2004), with four species, *Maribacter sedimenticola*, *Maribacter aquivivus*, *Maribacter orientalis* and *Maribacter ulvicola*. The genus *Maribacter* is characterized chemotaxonomically by having MK-6 as the predominant menaquinone, iso-C_{15:0}, iso-C_{17:0} 3-OH and iso-C_{15:1} as the major fatty acids and DNA G + C contents of 35.0–39.0 mol% (Nedashkovskaya *et al.*, 2004). Phylogenetic analysis based on 16S rRNA gene sequences showed that the genus falls within the family *Flavobacteriaceae* of the phylum *Cytophaga–Flavobacterium–Bacteroides* (Nedashkovskaya *et al.*, 2004). In this study, we report on the detailed taxonomic characterization of two *Maribacter*-like bacterial strains, DSW-8^T and DSW-9, which were isolated from sea water at Dokdo, an island of the East Sea, Korea.

Sea water collected in Dokdo, Korea, provided the source for isolation of bacterial strains. Strains DSW-8^T and DSW-9 were isolated by the standard dilution plating technique on marine agar 2216 (MA; Difco) at 30 °C. The type strains of

the four *Maribacter* species were used as reference strains for DNA–DNA hybridization; *M. sedimenticola* KCTC 12966^T, *M. aquivivus* KCTC 12968^T, *M. orientalis* KCTC 12967^T and *M. ulvicola* KCTC 12969^T were obtained from the Korean Collection for Type Cultures, Taejeon, Korea. 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). 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 lacking NaCl. Growth at various NaCl concentrations was investigated in MB and trypticase soy broth (Difco). 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 and on MA supplemented with nitrate, both of which had been prepared

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains DSW-8^T and DSW-9 are AY960749 and AY960750, respectively.

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), 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 pigment was investigated as described by Reichenbach (1992). Acid

Table 1. Differential phenotypic characteristics of *Maribacter* species

Species: 1, *M. dokdonensis* sp. nov.; 2, *M. aquivivus*; 3, *M. orientalis*; 4, *M. sedimenticola*; 5, *M. ulvicola*. +, Positive reaction; –, negative reaction; w, weakly positive reaction. Data in parentheses are for the type strain. All species are Gram-negative, non-flagellated, rod-shaped, non-endospore-forming and strictly aerobic. All species are positive for catalase, oxidase, gliding motility, alkaline phosphatase, NaCl requirement, growth at 4–30 °C, growth in the presence of 1–5 % (w/v) NaCl, hydrolysis of Tween 40 and susceptibility to lincomycin and oleandomycin. All species are negative for hydrolysis of casein and urea, production of flexirubin pigments, production of indole and H_2S , Voges–Proskauer reaction, acid production from *myo*-inositol and D-sorbitol, utilization of citrate and susceptibility to benzylpenicillin, gentamicin, kanamycin, neomycin, polymyxin B and streptomycin.

Characteristic	1	2	3	4	5
Nitrate reduction	–	+	–	+	–
β -Galactosidase activity	–	+	+	–	+
Optimal temperature (°C) for growth	30	21–23	21–23	22–24	21–23
Growth at:					
6 % (w/v) NaCl	+	+	–	+	–
32 °C	+	–	+	+	+
Hydrolysis of:					
Agar	+	+	–	+	+
Gelatin	–	+	+	–	–
Starch	–	–	–	+	–
Tween 20	+	–	+	+	+
Tween 80	+	+	–	+	+
Acid production from:					
L-Arabinose	+	–	+	+	–
D-Cellobiose	+	–	+	–	+
D-Galactose	+	–	+	–	–
D-Glucose	+	–	+	–	+
Lactose	+	–	+	–	+
Maltose	+	–	+	–	+
Melibiose	(w) or +	–	+	–	–
L-Rhamnose	–	–	–	–	+
Sucrose	+	–	+	–	+
D-Xylose	+	–	+	–	–
D-Raffinose	(+) or w	–	–	–	–
D-Mannitol	+	–	–	–	–
Utilization of:					
L-Arabinose	–	–	+	–	–
D-Glucose	+	+	+	–	+
D-Mannose	+	+	+	–	+
Sucrose	+	+	+	–	+
Susceptibility to antibiotics					
Ampicillin (10 µg per disc)	–	–	–	–	+
Tetracyclin (30 µg per disc)	w	–	–	+	–
Carbenicillin (100 µg per disc)	–	+	+	+	+
DNA G + C mol%	35.9–36.1 (36.1)	35.0	39.0	37.5	35.9–36.7 (36.7)

production from carbohydrates was determined as described by Leifson (1963). Utilization of substrates as sole carbon and energy sources was tested as described by Baumann & Baumann (1981) using supplementation with 2% (v/v) Hutner's mineral base (Cohen-Bazire *et al.*, 1957) and 1% (v/v) vitamin solution (Staley, 1968). 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 with the API 20E system (bioMérieux).

Cell biomass of strains DSW-8^T and DSW-9 for DNA extraction and for isoprenoid quinone analysis was obtained from cultivation in MB at 30 °C. Cell mass of the type strains of the four *Maribacter* species was obtained by cultivation in MB at 22 °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). 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-8^T and DSW-9 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). 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 replications 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.

Morphological, cultural, physiological and biochemical characteristics of strains DSW-8^T and DSW-9 are given in the species description (see later) and in Table 1, together with those of the four *Maribacter* species. Almost complete 16S rRNA gene sequences of strains DSW-8^T and DSW-9, comprising 1486 nucleotides (approx. 96% of the *Escherichia coli* 16S rRNA sequence), were determined in this study. The 16S rRNA gene sequences of strains DSW-8^T and DSW-9 were identical. Comparative 16S rRNA gene sequence analyses revealed that strains DSW-8^T and DSW-9 were most closely affiliated to members of the genus *Maribacter* of the family *Flavobacteriaceae*. In the neighbour-joining tree based on 16S rRNA gene sequences, strains DSW-8^T and DSW-9 fell within the radiation of the cluster comprising the four recognized *Maribacter* species, joining *M. sedimenticola* at a bootstrap confidence level of 90.1% (Fig. 1). Similar tree topologies were found in the trees generated with the maximum-likelihood and maximum-parsimony algorithms (data not shown). The 16S rRNA gene sequences of strains DSW-8^T and DSW-9 exhibited similarity values of 96.9–98.0% to those of the type strains of the *Maribacter* species. The two strains exhibited 16S

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of strains DSW-8^T and DSW-9, the *Maribacter* species and representatives of some other related taxa. Bootstrap values (expressed as percentages of 1000 replicates) greater than 50% are shown at the branching points. *Flavobacterium aquatile* ATCC 11947^T was used as an outgroup. Scale bar, 0.01 substitutions per nucleotide position.

rRNA gene sequence similarity values of 93.4–94.2 % to *Zobellia* species, 90.1–91.4 % to *Muricauda* species and less than 90.9 % to other species used in the phylogenetic analysis (Fig. 1).

The predominant isoprenoid quinone found in strains DSW-8^T and DSW-9 was menaquinone-6 (MK-6) at a peak area ratio of approximately 93–95 %. Strains DSW-8^T and DSW-9 had cellular fatty acid profiles that contained large amounts of straight-chain, branched and hydroxyl fatty acids; the major fatty acids were iso-C_{17:0} 3-OH, iso-C_{15:0} and iso-C_{15:1} (Table 2). These fatty acid profiles were similar to those of the *Maribacter* species, although there were differences in the proportions of some fatty acids that might have been caused by different cultivation conditions (Table 2). Phosphatidylethanolamine was the only phospholipid identified in strains DSW-8^T and DSW-9, and other major polar lipids were two unidentified phospholipids, an unidentified glycolipid and an amino group-containing lipid that was ninhydrin-positive. These chemotaxonomic properties support the attribution of strains DSW-8^T and DSW-9 to the genus *Maribacter* (Nedashkovskaya *et al.*, 2004).

The DNA G + C contents of strains DSW-8^T and DSW-9 were 36.1 and 35.9 mol%. The mean level of DNA–DNA relatedness between strains DSW-8^T and DSW-9 was 89 %, when their DNAs were used individually as labelled DNA probes for cross-hybridization, indicating that the two strains are members of the same genomic species, as suggested by the results of the phenotypic and phylogenetic analyses. The two strains differed from the other *Maribacter* species by several phenotypic characteristics (Table 1), and the level of their DNA–DNA relatedness with the type strains of these species was 11–21 % (Wayne *et al.*, 1987). Therefore, on the basis of the data presented, strains DSW-8^T and DSW-9 should be placed in the genus *Maribacter* as members of a novel species, for which the name *Maribacter dokdonensis* sp. nov. is proposed.

Description of *Maribacter dokdonensis* sp. nov.

Maribacter dokdonensis (dok.do.nen'sis. N.L. masc. adj. *dokdonensis* pertaining to Dokdo, the Korean island from where the strains were isolated).

Cells are rods, 0.3–0.4 × 0.8–4.0 µm. Colonies are circular or rhizoid, glistening, slightly convex, yellow in colour and 1.0–2.0 mm in diameter after incubation for 3 days on MA at 30 °C. Optimal growth temperature is 30 °C; growth occurs at 4 and 35 °C, but not at 36 °C. Optimal pH for growth is 7.0–8.0; growth occurs at pH 5.5, but not at 5.0. Optimal growth occurs in the presence of 2–3 % (w/v) NaCl; growth does not occur in the absence of NaCl and in the presence of greater than 10 % (w/v) NaCl. Aesculin, tyrosine and Tween 60 are hydrolysed, but hypoxanthine and xanthine are not. Anaerobic growth does not occur on MA and on MA supplemented with nitrate. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase

Table 2. Cellular fatty acid composition (%) of *Maribacter* species

Strains: 1, *M. dokdonensis* DSW-8^T; 2, *M. dokdonensis* DSW-9; 3, *M. aquivivus* KMM 3949^T; 4, *M. orientalis* KMM 3947^T; 5, *M. sedimenticola* KMM 3903^T; 6, *M. ulvicola* KMM 3951^T. –, Not detected. Fatty acids that represented <0.5 % in all strains were omitted.

Fatty acid	1	2	3	4	5	6
Straight-chain fatty acid						
C _{14:0}	0.9	0.9	1.0	0.5	0.5	0.9
C _{15:0}	3.8	3.5	14.5	12.3	6.3	8.1
C _{16:0}	1.4	1.3	0.5	1.2	1.0	1.0
Branched fatty acid						
iso-C _{13:0}	0.6	0.6	–	–	–	–
iso-C _{15:0}	19.7	20.3	12.3	10.6	20.5	13.6
iso-C _{15:1}	16.1	15.7	13.6	10.1	16.9	18.9
anteiso-C _{15:0}	0.3	0.4	1.3	2.3	1.2	1.9
iso-C _{16:0}	–	–	0.7	0.3	1.1	0.3
iso-C _{17:1ω5c}	–	–	1.4	–	–	1.2
iso-C _{17:1ω9c}	2.0	2.2	2.2	4.0	2.3	2.2
iso-C _{18:1}	–	–	–	2.4	–	–
Unsaturated fatty acid						
C _{13:1}	–	–	1.1	0.3	0.7	1.1
C _{14:1ω5}	–	–	1.0	–	–	0.9
C _{15:1ω6c}	0.6	0.6	4.8	2.5	1.7	1.6
C _{17:1ω6c}	0.2	0.2	1.7	1.3	0.5	0.5
Hydroxy fatty acid						
C _{15:0} 3-OH	1.4	1.4	2.3	1.5	2.4	1.5
iso-C _{15:0} 3-OH	5.0	5.0	3.2	2.9	5.4	4.1
C _{16:0} 3-OH	5.4	5.5	2.9	3.0	2.2	3.7
iso-C _{16:0} 3-OH	1.1	1.0	2.5	2.1	1.7	1.7
iso-C _{17:0} 3-OH	28.6	29.2	11.6	18.8	20.4	14.5
Summed feature*						
3	8.6	8.4	12.9	11.4	5.8	12.2
Unknown fatty acid(s)	2.7†	3.0†	10.2	5.9	7.9	10.3

*Summed feature represent groups of two or three fatty acids which could not be separated by GLC with the MIDI system. Summed feature 3 contained C_{16:1ω7c} and/or iso-C_{15:0} 2-OH.

†Sum of ECL (equivalent chain-length) 13.565 and 16.582.

and tryptophan deaminase are absent. In assays with the API ZYM system, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucosidase and *N*-acetyl-β-glucosaminidase are present, but lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, α-mannosidase and α-fucosidase are absent. D-Fructose, D-galactose, D-cellobiose, D-trehalose, D-xylose, maltose and salicin are utilized, but acetate, succinate, benzoate, L-malate, pyruvate, formate and L-glutamate are not utilized. Acid is produced from D-mannose, D-melezitose and D-trehalose, but acid is not produced from D-ribose. Acid production from D-fructose is variable (positive for the type strain). Susceptible

to novobiocin (5 µg), but not to cephalothin (30 µg). The predominant menaquinone is MK-6. The major fatty acids are iso-C_{17:0} 3-OH, iso-C_{15:0} and iso-C_{15:1}. The major polar lipids are phosphatidylethanolamine, two unidentified phospholipids, an unidentified glycolipid and an amino group-containing lipid that is ninhydrin-positive. The DNA G + C content is 35.9–36.1 mol% (HPLC) (36.1 mol% for type strain). Other characteristics are shown in Table 1.

The type strain, DSW-8^T (= KCTC 12393^T = DSM 17201^T), was isolated from sea water off Dokdo, an island located at the edge of the East Sea, Korea. Another reference strain is DSW-9.

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