Polaribacter dokdonensis sp. nov., isolated from seawater

Jung-Hoon Yoon, So-Jung Kang and Tae-Kwang Oh

Correspondence
Jung-Hoon Yoon
jhyoon@kribb.re.kr

Korea Research Institute of Bioscience and Biotechnology (KRIBB), PO Box 115, Yusong, Taejeon, Korea

A Gram-negative, non-motile, non-spore-forming, slightly halophilic bacterial strain, DSW-5^T, was isolated from seawater off Dokdo, Korea, and subjected to a polyphasic taxonomic study. It grew optimally at 25–28 °C and in the presence of 2 % (w/v) NaCl. Strain DSW-5^T contained MK-6 as the predominant menaquinone and iso-C_{15:0}, iso-C_{15:1} and iso-C_{15:0} 3-OH as the major fatty acids. The major polar lipids detected were phosphatidylethanolamine, three unidentified phospholipids and an amino-group-containing lipid. The DNA G + C content was 30.0 mol%. Phylogenetic analyses based on 16S rRNA gene sequences revealed that strain DSW-5^T was most closely related to the genus *Polaribacter*. Similarity values between the 16S rRNA gene sequences of strain DSW-5^T and the type strains of recognized *Polaribacter* species were in the range 96.2–96.8 %. On the basis of its phenotypic properties and phylogenetic distinctiveness, strain DSW-5^T (=KCTC 12392^T=DSM 17204^T) was classified in the genus *Polaribacter* as the type strain of a novel species, for which the name *Polaribacter dokdonensis* sp. nov. is proposed.

The genus *Polaribacter* was first proposed by Gosink *et al.* (1998) with the description of three novel species and reclassification of *Flectobacillus glomeratus*. At the time of writing, the genus comprises four recognized species: *Polaribacter filamentus*, *P. franzmannii*, *P. glomeratus* and *P. irgensii* (Gosink *et al.*, 1998). Here we report on the detailed taxonomic characterization of a *Polaribacter*-like bacterial strain, DSW-5^T, which was isolated from seawater.

Seawater collected off Dokdo (37° 14' 12" N 131° 52' 07" E), Korea, was used as the source for isolation of bacterial strains. Strain DSW-5^T was isolated by the standard dilution plating technique on marine agar 2216 (MA; Difco) for 7 days at 25 °C. Cell morphology was examined by light microscopy and phase-contrast 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 prior to sterilization to various pH values (pH 4.5–10.5 at intervals of 0.5 pH units) 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 %] was investigated in MB or trypticase soy broth (Difco) at 25 °C. Growth at various temperatures (4–40 °C) was measured on MA. Growth under anaerobic conditions was determined after incubation at 25 °C in an anaerobic chamber on MA and on MA supplemented with nitrate, both of which had been prepared anaerobically using nitrogen. The ability to grow on various solid media was tested at 25 °C in nutrient agar (NA; Difco), NA plus 2.5 % (w/v) NaCl, trypticase soy agar (TSA; Difco), TSA plus 3 % (w/v) D-glucose, TSA plus 3 % (w/v) sucrose, TSA plus 2.5 % (w/v) NaCl, Levine EMB agar (Difco), MacConkey agar (Difco) and Mueller–Hinton agar (Difco). 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 seawater (ASW) 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 ASW was used instead of distilled water. ASW contained (per litre of distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O and 1.3 g CaCl₂·2H₂O (Bruns *et al.*, 2001). Hydrolysis of hypoxanthine, tyrosine and xanthine was investigated on MA with the substrate concentrations given by Cowan & Steel (1965). Hydrolysis of carboxymethylcellulose was tested as described by Bowman (2000) using MA as the basal medium. Hydrolysis of chitin (Sigma) was determined on solid and in liquid marine salts basal media (Baumann & Baumann, 1981) containing 0.5 % (w/v) chitin as the sole carbon source. The

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DSW-5^T is DQ004686.

presence of flexirubin-type pigments was investigated as described by Reichenbach (1992). H₂S production was tested according to the method of Bruns *et al.* (2001). 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 NaNO₃, 0.2 g NH₄Cl and 0.05 g yeast extract in 1000 ml ASW (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 discs containing the following antibiotics: 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) or carbenicillin (100 µg). Other physiological and biochemical tests were performed with the API 20E system (bioMérieux).

Cell biomass of strain DSW-5^T for DNA extraction and for isoprenoid quinone analysis 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 by 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 strain DSW-5^T was harvested from agar plates after incubation for 3 days on MA at 25 °C. 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.

Morphological, cultural, physiological and biochemical characteristics of strain DSW-5^T are given in the species description or are shown in Table 1, together with those of the four recognized *Polaribacter* species. The almost complete 16S rRNA gene sequence of strain DSW-5^T, comprising 1475 nt (approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence), was determined. Comparative 16S rRNA gene sequence analyses revealed that strain DSW-5^T was phylogenetically affiliated to the genus *Polaribacter* of the family *Flavobacteriaceae* (Fig. 1). In the neighbour-joining tree based on 16S rRNA gene sequences, strain

DSW-5^T joined the cluster comprising *Polaribacter* species with a bootstrap confidence level of 100 %, and the relationship between this clade and the cluster comprising *Tenacibaculum* species was supported by a bootstrap resampling value of 100 % (Fig. 1). Similar topologies were found in trees generated with the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). The 16S rRNA gene sequence of strain DSW-5^T exhibited similarity values of 96.2–96.8 % to those of the type strains of the four recognized *Polaribacter* species, 93.1–95.1 % to *Tenacibaculum* species and <90.2 % to other species used in the phylogenetic analysis (Fig. 1).

Strain DSW-5^T contained menaquinone-6 (MK-6), at a peak area ratio of approximately 96 %, as the predominant isoprenoid quinone. Strain DSW-5^T contained large amounts of branched, hydroxy, straight-chain and unsaturated fatty acids; the major components (>1 % of the total fatty acids) were iso-C_{15:0} (17.2 %), iso-C_{15:1} (15.4 %), iso-C_{15:0} 3-OH (14.3 %), iso-C_{17:0} 3-OH (8.4 %), C_{16:1}ω7c and/or iso-C_{15:0} 2-OH (6.6 %), C_{15:1}ω6c (5.1 %), iso-C_{16:0} 3-OH (5.0 %), iso-C_{13:0} (4.9 %), C_{15:0} 3-OH (3.7 %), C_{18:0} (3.2 %), C_{15:0} (2.3 %), C_{16:0} (1.7 %), iso-C_{16:1} (1.3 %), C_{17:1}ω6c (1.3 %), C_{16:0} 3-OH (1.3 %), iso-C_{14:0} (1.1 %) and C_{16:0} 2-OH (1.0 %). This fatty acid profile was similar to those of *Polaribacter* species analysed previously, although there were differences in the proportions of some fatty acids, perhaps because of differences in cultivation conditions, e.g. temperatures and cultivation media (Gosink *et al.*, 1998). The phospholipid identified in strain DSW-5^T was phosphatidylethanolamine, and other major polar lipids were three unidentified phospholipids and an amino-group-containing lipid that was ninhydrin-positive. The DNA G + C content of strain DSW-5^T was 30.0 mol%. These chemotaxonomic properties were in agreement with the result of monothetic phylogenetic classification of strain DSW-5^T as a member of the genus *Polaribacter* (Gosink *et al.*, 1998). Strain DSW-5^T was distinguishable from recognized *Polaribacter* species based on differences in some phenotypic characteristics (Table 1). The 16S rRNA gene sequence similarity data were sufficient to classify strain DSW-5^T in a species that is separate from all recognized *Polaribacter* species (Stackebrandt & Goebel, 1994). Therefore, on the basis of the data presented, strain DSW-5^T should be placed in the genus *Polaribacter* as the type strain of a novel species, for which the name *Polaribacter dokdonensis* sp. nov. is proposed.

Description of *Polaribacter dokdonensis* sp. nov.

Polaribacter dokdonensis (dok.do.nen'sis. N.L. masc. adj. *dokdonensis* of Dokdo, an island of Korea, from where the type strain was isolated).

Cells are Gram-negative, non-spore-forming, non-motile, aerobic rods (0.4–0.6 × 0.8–5.0 µm); coccoid cells are often observed. Colonies on MA are circular, smooth, glistening, convex, orange-coloured and 0.8–1.2 mm in diameter after incubation for 3 days at 25 °C. Optimal growth occurs at 25–28 °C; growth occurs at 4 and 33 °C, but not at 34 °C. Optimal pH for growth is 7.0–8.0; growth occurs at pH 5.5,

Table 1. Differential phenotypic characteristics of *Polaribacter* species

Species: 1, *P. dokdonensis* sp. nov.; 2, *P. filamentus*; 3, *P. franzmannii*; 4, *P. glomeratus*; 5, *P. irgensii*. Data for reference species were taken from Gosink *et al.* (1998). +, Positive; –, negative; w, weakly positive; ND, not determined. All species were positive for growth at 4 °C and on marine agar 2216. All species were negative for Gram-staining, urease, motility, production of flexirubin-type pigments, hydrolysis of chitin and cellulose, indole production, the Voges–Proskauer reaction, growth on NA, TSA plus 3 % (w/v) D-glucose, TSA plus 2.5 % (w/v) NaCl, EMB agar, MacConkey agar and Mueller–Hinton agar and utilization of D-ribose, DL-aspartate, L-leucine, L-proline, ethanol, methanol, acetate, benzoate, citrate, pyruvate and succinate.

Characteristic	1	2	3	4	5
Cell morphology	Straight or curved rods	Filamentous	Irregular rods	Curved rods and coils	Filamentous
Cell size:					
Length (µm)	0.8–5.0	1.6–32.0	4.0–16.0	1.6–3.2	0.8–48.0
Diameter (µm)	0.4–0.6	0.5–1.2	0.8–1.6	0.6–0.9	0.25–0.5
Coccoid cells	+	+	+	–	+
Gas vesicles	–	+	+	–	+
Colony colour	Orange	Salmon	Orange	Orange	Orange
Oxidase	+	–	+	+	w
Catalase	+	+	+	w	+
β-Galactosidase	–	–	+	–	–
Nitrate reduction	–	–	–	+	–
Growth on solid media:					
NA + 2.5 % (w/v) NaCl	–	+	–	–	–
TSA	–	–	–	+	–
TSA + 3 % (w/v) sucrose	–	–	–	+	–
Growth at:					
15 °C	+	+	–	+	–
25 °C	+	–	–	–	–
30 °C	+	–	–	–	–
Hydrolysis of:					
Aesculin	–	+	+	–	–
Gelatin	–	w	+	+	–
Starch	+	w	w	+	w
Utilization of:					
Casamino acids	–	+	+	+	+
Sucrose	–	–	–	+	–
L-Glutamate	–	+	–	+	–
DL-Malate	–	–	–	–	+
Glycerol	–	+	+	–	–
Absorption maxima (nm) of ethanol extracts	448–449, 472†	451, 475, 506	451, 506	451, 476, 505	450, 475, 506
DNA G + C content (mol%)	30.0	32 ± 1	32 ± 1	33.2	31

*Reported as negative by McGuire *et al.* (1987).

†An identical result for *P. dokdonensis* was obtained from acetone/methanol extracts.

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 or in the presence of >6 % (w/v) NaCl. Growth does not occur under anaerobic conditions on MA or on MA supplemented with nitrate. Tweens 20, 40, 60 and 80 are hydrolysed, but casein, hypoxanthine, xanthine and L-tyrosine are not. H₂S is not produced. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase are absent. In assays with the API ZYM system, alkaline phosphatase, esterase lipase (C8), leucine

arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase are present, esterase (C4) and cystine arylamidase are weakly present, and lipase (C14), α-chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase are absent. Peptone and tryptone are utilized as sole carbon and nitrogen sources; D-glucose, D-galactose, D-fructose, D-cellobiose and D-trehalose are not utilized as sole carbon sources. Acid is produced from D-fructose and D-raffinose, but not from

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of strain DSW-5^T, *Polaribacter* species and representatives of some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) >50% are shown at the branch points. *Cytophaga hutchinsonii* IAM 12607^T was used as an outgroup. Dots indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. Bar, 0.01 substitutions per nucleotide position.

L-arabinose, D-cellobiose, D-galactose, D-glucose, lactose, maltose, D-mannose, D-melezitose, melibiose, L-rhamnose, D-ribose, sucrose, D-trehalose, D-xylose, *myo*-inositol, D-mannitol or D-sorbitol. Susceptible to penicillin G, chloramphenicol, ampicillin, cephalothin, novobiocin, tetracycline, lincomycin, oleandomycin and carbenicillin, but not to polymyxin B, streptomycin, gentamicin, kanamycin or neomycin. The predominant menaquinone is MK-6. The major fatty acids (>10% of the total fatty acids) are iso-C_{15:0} (17.2%), iso-C_{15:1} (15.4%) and iso-C_{15:0} 3-OH (14.3%). The major polar lipids are phosphatidylethanolamine, three unidentified phospholipids and an amino-group-containing lipid. The DNA G+C content is 30.0 mol% (HPLC). Other phenotypic properties are given in Table 1.

The type strain, DSW-5^T (= KCTC 12392^T = DSM 17204^T), was isolated from seawater off Dokdo in the East Sea of Korea (also known as the Sea of Japan).

Acknowledgements

This work was supported by the 21C Frontier program of Microbial Genomics and Applications (grant MG05-0401-2-0) from the Ministry of Science and Technology (MOST) of the Republic of Korea. We are grateful to the Ulleung County Administration and the Cultural Heritage Administration of the Republic of Korea for aiding access to Dokdo, Korea.

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