Loktanella maricola sp. nov., isolated from seawater of the East Sea in Korea

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A Gram-negative, non-motile and rod-shaped *Loktanella*-like bacterial strain, DSW-18^T, was isolated from seawater of the East Sea, Korea, and its exact taxonomic position was investigated by using a polyphasic approach. Strain DSW-18^T grew optimally at pH 7.0–8.0 and 25 °C in the presence of 2% (w/v) NaCl. It contained Q-10 as the predominant ubiquinone and C_{18:1}ω7c as the major fatty acid. The major polar lipids were phosphatidylcholine, phosphatidylglycerol and diphosphatidylglycerol. The DNA G + C content was 56.8 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain DSW-18^T fell within the cluster comprising *Loktanella* species. The levels of 16S rRNA gene sequence similarity between strain DSW-18^T and the type strains of recognized *Loktanella* species ranged from 94.4 to 98.4%. The DNA–DNA relatedness data and differential phenotypic properties, together with the phylogenetic distinctiveness, demonstrated that strain DSW-18^T was distinguishable from recognized *Loktanella* species. On the basis of the phenotypic, phylogenetic and genetic data, strain DSW-18^T represents a novel species of the genus *Loktanella*, for which the name *Loktanella maricola* sp. nov. is proposed. The type strain is DSW-18^T (=KCTC 12863^T=JCM 14564^T).

The genus *Loktanella* was created by Van Trappen *et al.* (2004) with the description of three novel species, *L. salsilacus*, *L. fryxellensis* and *L. vestfoldensis*. Subsequently, four other *Loktanella* species, *L. hongkongensis* (Lau *et al.*, 2004), *L. agnita* and *L. rosea* (Ivanova *et al.*, 2005) and *L. koreensis* (Weon *et al.*, 2006), have been described. Phylogenetic analyses based on 16S rRNA gene sequences have shown that the genus *Loktanella* belongs to the *Alphaproteobacteria* (Van Trappen *et al.*, 2004; Weon *et al.*, 2006). Here we report on the taxonomic characterization of a *Loktanella*-like bacterial strain, DSW-18^T, which was isolated from seawater off Dokdo in the East Sea, Korea.

Strain DSW-18^T was isolated by using the standard dilution plating technique at 25 °C on marine agar 2216 (MA; Difco). The type strains of two *Loktanella* species were used as reference strains for DNA–DNA hybridization; *L. rosea* LMG 22534^T was obtained from the Laboratorium voor Microbiologie Universiteit Gent (Ghent, Belgium) and *L. koreensis* KACC 11519^T was obtained from the Korean Agricultural Culture Collection (Suwon, Korea). The morphological, physiological and biochemical characteristics of strain DSW-18^T were investigated using routine cultivation on MA at 25 °C. Cell morphology was examined by using light microscopy (E600; Nikon) and

transmission electron microscopy. The presence of flagella was determined by using a Philips CM-20 transmission electron microscope with cells from exponentially growing cultures: for this purpose, the cells were negatively stained with 1% (w/v) phosphotungstic acid and the grids were examined after being air-dried. Growth under anaerobic conditions was determined after incubation in a Forma anaerobic chamber on MA and MA supplemented with nitrate, both of which had been prepared anaerobically using nitrogen. Growth in the absence of NaCl was investigated using trypticase soy broth prepared according to the formula of the Difco medium except that no NaCl was used. Growth at various NaCl concentrations was investigated using marine broth 2216 (MB; Difco) or trypticase soy broth (Difco). Growth at various temperatures (4–40 °C) was measured on MA. Growth on trypticase soy agar (TSA; Difco), nutrient agar (NA; Difco) and MacConkey agar (Difco) was tested at 25 °C. Catalase and oxidase activities and hydrolysis of casein, starch and Tweens 20, 40, 60 and 80 were determined as described by Cowan & Steel (1965). Hydrolysis of hypoxanthine, tyrosine and xanthine was tested on MA using the substrate concentrations described by Cowan & Steel (1965). Hydrolysis of aesculin, gelatin and urea and nitrate reduction were investigated as described by Lanyi (1987) with the modification that artificial seawater was used for preparation of the media. The artificial seawater contained (l⁻¹ distilled water): 23.6 g NaCl, 0.64 g KCl, 4.53 g

Abbreviation: BChl, bacteriochlorophyll.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DSW-18^T is EF202613.

MgCl₂ · 6H₂O, 5.94 g MgSO₄ · 7H₂O and 1.3 g CaCl₂ · 2H₂O (Bruns *et al.*, 2001). H₂S production was tested as described by Bruns *et al.* (2001). Susceptibility to antibiotics was investigated on MA plates by using antibiotic discs containing the following amounts: 100 U polymyxin B, 50 µg streptomycin, 20 U penicillin G, 100 µg chloramphenicol, 10 µg ampicillin, 30 µg cephalothin, 30 µg gentamicin, 5 µg novobiocin, 30 µg tetracycline, 30 µg kanamycin, 15 µg lincomycin, 15 µg oleandomycin, 30 µg neomycin and 100 µg carbenicillin. Acid production from carbohydrates was determined as described by Leifson (1963). Utilization of various substrates for growth was tested as described by Baumann & Baumann (1981), using supplementation with 2% (v/v) Hutner's mineral salts solution (Cohen-Bazire *et al.*, 1957) and 1% (v/v) vitamin solution (Staley, 1968). Other physiological and biochemical tests were performed using the API 20E and API ZYM systems (bioMérieux). For *in vivo* pigment-absorption spectrum analysis, strain DSW-18^T was cultivated aerobically in the dark at 25 °C in MB. The culture was washed twice by centrifugation using MOPS buffer (MOPS/NaOH, 0.01 M; KCl, 0.1 M; MgCl₂, 0.001 M; pH 7.5) and disrupted by sonication with a Branson Sonifier 450. After removal of cell debris by centrifugation, the absorption spectrum of the supernatant was examined on a Beckman Coulter DU800 spectrophotometer.

Cell biomass for DNA extraction and for isoprenoid quinone and polar lipid analyses was obtained from cultures grown in MB at 25 °C. Chromosomal DNA was isolated and purified according to the method described by 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 analysed as described by Komagata & Suzuki (1987) using reversed-phase HPLC. For cellular fatty acid analysis, cell mass of strain DSW-18^T was harvested from MA plates after incubation for 3 days at 25 °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). Polar lipids were extracted according to the procedures described by Minnikin *et al.* (1984) and identified by using two-dimensional TLC followed by spraying with appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). The presence of phosphatidylcholine was identified by using Dragendorff's reagent (Sigma). The DNA G+C content was determined using the method of Tamaoka & Komagata (1984) with the modification that the DNA was hydrolysed using nuclease P1 (Sigma) and the resultant nucleotides were analysed by reversed-phase HPLC. DNA–DNA hybridization was performed fluorometrically by using the method of Ezaki *et al.* (1989) with photobiotin-labelled DNA probes and microdilution wells.

Hybridization was performed with five replications for each sample. The highest and lowest values obtained for 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 strain DSW-18^T are given in the species description (see below) or are shown in Table 1. Strain DSW-18^T produced bacteriochlorophyll (BChl) *a* aerobically in the dark. The sonicated cell extract showed absorption maxima at approximately 476 and 864 nm, indicating the presence of carotenoid and BChl *a*. The almost-complete 16S rRNA gene sequence of strain DSW-18^T determined in this study comprised 1419 nucleotides, representing approximately 96% of the *Escherichia coli* 16S rRNA gene sequence. In the phylogenetic tree constructed using the neighbour-joining algorithm, strain DSW-18^T fell within the clade comprising *Loktanella* species, joining the type strain of *L. rosea* at a bootstrap resampling value of 98.7% (Fig. 1). This tree topology was maintained in trees based on the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). Strain DSW-18^T exhibited 16S rRNA gene sequence similarity values of 98.4 and 96.9% to the type strains of *L. rosea* and *L. koreensis*, respectively, and of 94.4–95.2% to the type strains of the other *Loktanella* species. The fatty acid profile of strain DSW-18^T comprised (>0.5% of total fatty acids): unsaturated fatty acid C_{18:1ω7c} (75.9%); 11-methyl C_{18:1ω7c} (10.2%); straight-chain fatty acids C_{16:0} (3.9%), C_{18:0} (2.9%) and C_{17:0} (0.7%); hydroxy fatty acid C_{12:1} 3-OH (2.9%); and branched fatty acid iso-C_{17:0} 3-OH (1.3%). This fatty acid profile was similar to those of other recognized *Loktanella* species (Van Trappen *et al.*, 2004; Lau *et al.*, 2004; Ivanova *et al.*, 2005; Weon *et al.*, 2006). The predominant isoprenoid quinone detected in strain DSW-18^T was ubiquinone-10 (Q-10), at a peak area ratio of approximately 95%. The major polar lipids found in strain DSW-18^T were phosphatidylcholine, phosphatidylglycerol and diphosphatidylglycerol; a minor amount of phosphatidylethanolamine was also present. The DNA G+C content of strain DSW-18^T was 56.8 mol%, which was lower than those of other *Loktanella* species (Table 1). Strain DSW-18^T showed mean levels of DNA–DNA relatedness of 17 and 9%, respectively, to the type strains of two phylogenetically related species, *L. rosea* LMG 22534^T and *L. koreensis* KACC 11519^T, indicating that strain DSW-18^T represents a genomic species that is different from the two *Loktanella* species (Wayne *et al.*, 1987). The differential phenotypic properties, together with the phylogenetic distinctiveness and DNA–DNA relatedness data, were sufficient to categorize strain DSW-18^T as a member of a species that is distinct from recognized *Loktanella* species (Table 1) (Stackebrandt & Goebel, 1994; Wayne *et al.*, 1987). Therefore, on the basis of the data presented, strain DSW-18^T should be placed in the genus *Loktanella* as representing a novel species, for which the name *Loktanella maricola* sp. nov. is proposed.

Table 1. Differential phenotypic characteristics of *Loktanella maricola* sp. nov. and *Loktanella* species

Taxa: 1, strain DSW-18^T (*L. maricola* sp. nov.); 2, *L. salsilacus*; 3, *L. fryxellensis*; 4, *L. vestfoldensis*; 5, *L. hongkongensis*; 6, *L. agnita*; 7, *L. rosea*; 8, *L. koreensis*. Data from Van Trappen *et al.* (2004), Lau *et al.* (2004), Ivanova *et al.* (2005), Weon *et al.* (2006) and this study. +, Positive reaction; –, negative reaction; w, weakly positive reaction; v, variable reaction; ND, not determined. All species are Gram-negative and non-motile, and positive for catalase. All species are negative for hydrolysis of agar (not determined for *L. hongkongensis*), casein and starch (not determined for *L. fryxellensis* and *L. hongkongensis*).

Characteristic	1	2	3	4	5	6	7	8
Colony pigmentation	Light orange	Beige	Pink–beige	Pink	Pink–white	Whitish	Pink	Beige
Diffusible pigment	–	–	–	–	Brown	–	–	–
Growth on:								
TSA	–	–	–	w	–	–	–	–
NA	–	–	–	w	–	–	–	–
Maximum growth temperature (°C)	34	30	25	37	44	35	35	30
Maximum NaCl concentration for growth (% w/v)	7	10	5	10	14	6	12	5
Oxidase	+	+	+	+	+	w	+	+
Nitrate reduction to nitrite	–	–	–	–	–	w	–	+
Hydrolysis of:								
Aesculin	–	+	+	+	ND	ND	ND	+
Gelatin	–	–	–	–	–	–	–	w
Tween 80	w	+	+	+	–	–	w	–
Urea	–	–	–	+	–	–	–	–
Enzyme activity (API ZYM):								
Trypsin	–	–	–	+	ND	–	–	–
α -Galactosidase	–	+	–	–	ND	+	–	–
β -Galactosidase	–	–	+	w	ND	ND	ND	w
β -Glucosidase	–	w	+	w	ND	ND	ND	w
Carbohydrate metabolism	+	–	–	–	v	–	–	–
DNA G + C content (mol%)	56.8	59–60	66	62–63	66	59	61	60

Description of *Loktanella maricola* sp. nov.

Loktanella maricola [ma.ri'co.la. L. n. *mare* sea; L. suff. *-cola* (from L. n. *incola*) a dweller, inhabitant; N.L. n. *maricola* inhabitant of the sea].

Cells are Gram-negative, non-motile and rod-shaped (0.3–0.6 × 0.8–3.0 µm). Colonies on MA are circular, slightly convex, glistening, light orange in colour and 1.0–2.0 mm in diameter after 7 days incubation at 25 °C. Growth does not occur on MacConkey agar. Growth occurs at 4 and

34 °C, but not at 35 °C. Optimal pH for growth is between 7.0 and 8.0; growth occurs at pH 5.5, but not at pH 5.0. Growth occurs in the presence of 7 % (w/v) NaCl, but not in the absence of NaCl or in the presence of more than 8 % (w/v) NaCl. Anaerobic growth does not occur on MA and on MA supplemented with nitrate. Hypoxanthine and Tweens 20, 40 and 60 are hydrolysed, but tyrosine and xanthine are not. H₂S and indole are not produced. BChl *a* is produced. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase are

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of *Loktanella maricola* sp. nov. strain DSW-18^T, other *Loktanella* species and some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) of >50 % are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Stappia stellulata* IAM 12621^T (GenBank accession no. D88525) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

absent. In assays with the API ZYM system, alkaline phosphatase, esterase (C4) and esterase lipase (C8) are present and leucine arylamidase and acid phosphatase are weakly present, but lipase (C14), valine arylamidase, cystine arylamidase, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase, β -glucuronidase, α -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase are absent. Utilizes the following substrates as carbon and energy sources: D-cellobiose, D-galactose, D-glucose, D-fructose, acetate, citrate, succinate, L-malate and pyruvate. L-Arabinose, maltose, D-mannose, sucrose, D-trehalose, D-xylose, benzoate, formate, L-glutamate and salicin are not utilized. Acid is not produced from L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, D-melezitose, melibiose, D-raffinose, L-rhamnose, D-ribose, sucrose, D-trehalose, D-xylose, *myo*-inositol, D-mannitol or D-sorbitol. Sensitive to ampicillin, carbenicillin, cephalothin, chloramphenicol, gentamicin, neomycin, novobiocin, oleandomycin, penicillin G, streptomycin and tetracycline, but not to kanamycin, lincomycin or polymyxin B. The predominant ubiquinone is Q-10. The major fatty acid is C_{18:1} ω 7c. Major polar lipids are phosphatidylcholine, phosphatidylglycerol and diphosphatidylglycerol. The DNA G+C content of the type strain is 56.8 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, DSW-18^T (=KCTC 12863^T=JCM 14564^T), was isolated from seawater off Dokdo in the East Sea, Korea.

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