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ORIGINAL PAPER

Loktanella soesokkakensis sp. nov., isolated from the junction between the North Pacific Ocean and a freshwater spring

Sooyeon Park · Jung-Sook Lee · Keun-Chul Lee · Jung-Hoon Yoon

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Abstract A Gram-negative, aerobic, non-flagellated and rod-shaped or ovoid bacterial strain, designated DSSK1-5^T, was isolated from the junction between the North Pacific Ocean and a freshwater spring at Jeju island, South Korea. Strain DSSK1-5^T was found to grow optimally at 30 °C, at pH 7.0–8.0 and in the presence of 2.0–3.0 % (w/v) NaCl. A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences revealed that strain DSSK1-5^T fell within the clade comprising *Loktanella* species, clustering consistently with the type strains of *Loktanella hongkongensis* and *Loktanella cinnabarina*, with which it exhibited 98.9 and 98.4 % sequence similarity values, respectively. Sequence similarities to the type strains of the other recognized *Loktanella* species were 94.0–96.2 %. Strain DSSK1-5^T was found to contain Q-10 as the predominant ubiquinone and $C_{18:1}$ $\omega 7c$ as the major fatty acid. The major polar lipids of strain DSSK1-5^T were identified as phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol, one unidentified glycolipid and one unidentified aminolipid. The DNA G+C content of strain DSSK1-5^T was determined to be 67.6 mol% and its mean DNA–DNA relatedness values with *L. hongkongensis* JCM 12479^T and *L. cinnabarina* JCM 18161^T were 19 and 23 %, respectively. The differential phenotypic properties, together with the phylogenetic and genetic distinctiveness, revealed that strain DSSK1-5^T is separated from other *Loktanella* species. On the basis of the data presented, strain DSSK1-5^T is proposed to represent a novel species of the genus *Loktanella*, for which the name *Loktanella soesokkakensis* sp. nov. is proposed. The type strain is DSSK1-5^T (= KCTC 32425^T = CECT 8367^T).

Keywords *Loktanella soesokkakensis* sp. nov. · Novel species · Ocean · Freshwater spring

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S. Park · J.-H. Yoon (✉)
Department of Food Science and Biotechnology, Sungkyunkwan University, Jangan-gu, Suwon, South Korea
e-mail: jhyoon69@skku.edu

J.-S. Lee · K.-C. Lee
Korea Research Institute of Bioscience and Biotechnology (KRIBB), PO Box 115, Yuseong, Daejeon, South Korea

Introduction

During a screening of novel bacteria from the junction place, located at Jeju island of South Korea, where the North Pacific Ocean and a freshwater spring meet, many novel bacterial taxa have been isolated and characterized taxonomically (Park et al. 2013). One of these isolates, designated DSSK1-5^T, is described in this study. Comparative 16S rRNA gene sequence analysis indicated that the novel strain has its closest phylogenetic affiliation to the genus *Loktanella*, a member of the class *Alphaproteobacteria*. The genus

Loktanella was created by Van Trappen et al. (2004) with the descriptions of three novel species, including *Loktanella salsilacus* as the type species of the genus. At the time of writing, the genus *Loktanella* comprises 13 species with validly published names (http://www.bacterio.cict.fr/l/loktanella.html; Euzéby 1997). Members of the genus *Loktanella* have been isolated from Antarctic lakes and marine environments (Van Trappen et al. 2004; Lau et al. 2004; Ivanova et al. 2005; Weon et al. 2006; Yoon et al. 2007, 2013; Hosoya and Yokota 2007; Moon et al. 2010; Lee 2012; Tsubouchi et al. 2013). The aim of the present work was to determine the exact taxonomic position of strain DSSK1-5^T by using a polyphasic characterisation including chemotaxonomic and other phenotypic analyses, a detailed phylogenetic investigation based on 16S rRNA gene sequences and DNA–DNA hybridization.

Materials and methods

Bacterial strains and culture conditions

Water was collected from the junction (33°15′7″N, 126°37′26″E) between the North Pacific Ocean and a freshwater spring, called Soesokkak, at Jeju island of South Korea, and used as a source for the isolation of bacterial strains. Strain DSSK1-5^T was isolated by the standard dilution plating technique at 25 °C on marine agar 2216 (MA; Becton–Dickinson) and cultivated routinely at 30 °C on MA. Strain DSSK1-5^T was maintained on MA at 4 °C for short-term preservation and as a glycerol suspension (20 %, w/v in distilled water) at −80 °C for long-term preservation. Strain DSSK1-5^T has been deposited in the Korean Collection for Type Cultures (KCTC; South Korea) and in the Spanish Type Culture Collection (CECT; Spain) under the accession numbers KCTC 32425^T and CECT 8367^T, respectively.

Loktanella hongkongensis JCM 12479^T and *Loktanella cinnabarina* JCM 18161^T, which were used as reference strains for phenotypic characterization, fatty acid analysis and DNA–DNA hybridization, were obtained from the Japan Collection of Microorganisms (JCM), Japan.

Cell biomass of strain DSSK1-5^T for DNA extraction and for the analyses of isoprenoid quinones and polar lipids was obtained from cultures grown for 2 days at 30 °C in marine broth 2216 (MB; Becton–Dickinson). For cellular fatty acid analysis, the cell mass of strain DSSK1-5^T, *L. hongkongensis* JCM 12479^T and *L. cinnabarina* JCM 18161^T was harvested from MA plates after cultivation for 3 days at 30 °C.

Morphological, cultural, physiological and biochemical characterization

Cell morphology was examined by light microscopy (BX51; Olympus) and transmission electron microscopy (JEM1010; JEOL). The latter technique was also used to assess the presence of flagella on cells from an exponentially growing MA culture. For this purpose, the cells were negatively stained with 1 % (w/v) phosphotungstic acid and the grids were examined after being air-dried. The Gram reaction was determined by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Growth under anaerobic conditions was determined after incubation for 10 days in an anaerobic jar (MGC) with AnaeroPack (MGC) on MA and on MA supplemented with nitrate; the jar was kept overnight at 4 °C to establish anoxic conditions before incubation at 30 °C. Growth at 4, 10, 20, 25, 30, 35, 37, 40 and 45 °C was measured on MA to determine the optimal temperature and temperature range for growth. The pH range for growth was determined in MB adjusted to pH 4.5–9.5 (using increments of 0.5 pH unit) by using sodium acetate/acetic acid and Na_2CO_3 buffers. The pH values were verified after autoclaving. Growth in the absence of NaCl and in the presence of 0.5, 1.0, 2.0 and 3.0 % (w/v) NaCl was investigated in trypticase soy broth prepared according to the formula of the Becton–Dickinson medium except that NaCl was excluded and that 0.45 % (w/v) $MgCl_2{\cdot}6H_2O$ was added. Growth in the presence of 2.0–18.0 % NaCl (at increments of 1.0 %) was investigated in MB. Catalase and oxidase activities were determined as described by Lányí (1987). Hydrolysis of casein, starch, hypoxanthine, L-tyrosine and xanthine was tested on MA using the substrate concentrations described by Barrow and Feltham (1993). Hydrolysis of aesculin and Tween-80 and nitrate reduction were investigated as described previously (Lányí 1987) with the modification that artificial seawater was used for the preparation of media. Hydrolysis of gelatin and urea was investigated by using Nutrient gelatin and Urea agar base media (Becton–Dickinson), respectively, with the modification that

artificial seawater was used for the preparation of media. The artificial seawater contained (l^{-1} distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g $MgCl_2 \cdot 6H_2O$, 5.94 g $MgSO_4 \cdot 7H_2O$ and 1.3 g $CaCl_2 \cdot 2H_2O$ (Bruns et al. 2001). Utilization of various substrates for growth was tested as described by Baumann and Baumann (1981), using supplementation with 1 % (v/v) vitamin solution (Staley 1968) and 2 % (v/v) Hutner's mineral salts (Cohen-Bazire et al. 1957). Susceptibility to antibiotics was tested on MA plates using antibiotic discs (Advantec) containing the following (μg per disc unless otherwise stated): ampicillin (10), carbenicillin (100), cephalothin (30), chloramphenicol (100), gentamicin (30), kanamycin (30), lincomycin (15), neomycin (30), novobiocin (5), oleandomycin (15), penicillin G (20 U), polymyxin B (100 U), streptomycin (50) and tetracycline (30). Enzyme activities were determined, after incubation for 8 h at 30 °C, by using the API ZYM system (bioMérieux).

Molecular studies

Chromosomal DNA was extracted and purified according to the method described by Yoon et al. (1996), with the modification that RNase T1 was used in combination with RNase A to minimize contamination of RNA. The 16S rRNA gene was amplified by PCR as described previously (Yoon et al. 1998) using two universal primers, 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1512R (5′-ACGGTTACCTTGTTACGACTT-3′). Sequencing of the amplified 16S rRNA gene was performed as described by Yoon et al. (2003). Alignment of sequences was carried out with CLUSTAL W software (Thompson et al. 1994). Gaps at the 5′ and 3′ ends of the alignment were omitted from further analysis. Phylogenetic analyses were performed as described by Yoon et al. (2012).

DNA–DNA hybridization was performed fluorometrically by the method of Ezaki et al. (1989) using photobiotin-labelled DNA probes for cross-hybridization in 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. The DNAs of strain DSSK1-5^T, *L. hongkongensis* JCM 12479^T and *L. cinnabarina* JCM 18161^T were used individually as labelled DNA probes for reciprocal hybridization.

Chemotaxonomic characterization

Isoprenoid quinones were extracted and analysed as described by Komagata and Suzuki (1987), using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. The isoprenoid quinones were eluted by a mixture of methanol/isopropanol (2:1, v/v) using a flow rate of 1 ml min^{-1} at room temperature and detected by UV absorbance at 275 nm. For cellular fatty acid analysis, the physiological age of cells was standardized by observing the growth development on the agar plates followed by harvesting them from the same quadrant on the agar plates according to the standard MIDI protocol (Sherlock Microbial Identification System, version 6.1). Fatty acids were saponified, methylated and extracted using the standard protocol of the MIDI. The fatty acids were analysed by GC (Hewlett Packard 6890) and identified by using TSBA6 database of the Microbial Identification System (Sasser 1990). Polar lipids were extracted according to the procedure described by Minnikin et al. (1984), and separated by two-dimensional TLC using chloroform/methanol/water (65:25:3.8, v/v/v) for the first dimension and chloroform/methanol/acetic acid/water (40:7.5:6:1.8, v/v/v/v) for the second dimension as described by Minnikin et al. (1977). Individual polar lipids were identified by spraying with molybdophosphoric acid, molybdenum blue, ninhydrin and α-naphthol reagents (Minnikin et al. 1984; Komagata and Suzuki 1987) and with Dragendorff's reagent (sigma).

The DNA G+C content was determined by the method of Tamaoka and Komagata (1984) with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC equipped with a YMC ODS-A (250 × 4.6 mm) column. The nucleotides were eluted by a mixture of 0.55 M $NH_4H_2PO_4$ (pH 4.0) and acetonitrile (40:1, v/v), using flow rate of 1 ml min^{-1} at room temperature and detected by UV absorbance at 270 nm.

Results and discussion

Morphological, cultural, physiological and biochemical characteristics

Strain DSSK1-5^T was found to be aerobic, Gram-negative, non-flagellated and non-spore-forming rods

or ovals (Supplementary Fig. 1). Strain DSSK1-5^T was observed to grow optimally at 30 °C, at pH 7.0–8.0 and in the presence of 2.0–3.0 % (w/v) NaCl. Strain DSSK1-5^T was able to grow at temperatures between 10 and 37 °C, whereas one of its closest phylogenetic neighbours, the type strain of *L. cinnabarina*, did not grow at 10 or 37 °C (Tsubouchi et al. 2013). It was determined to grow in the presence of up to 16.0 % (w/v) NaCl, whereas the type strains of *L. hongkongensis* and *L. cinnabarina* were able to grow in the presence of up to only 14.0 and 8.0 % (w/v) NaCl, respectively (Lau et al. 2004; Tsubouchi et al. 2013). Strain DSSK1-5^T was found to show catalase and oxidase activities but no urease activity. Strain DSSK1-5^T was found to be unable to reduce nitrate to nitrite. Strain DSSK1-5^T was found to be resistant to polymyxin B, where as the type strains of *L. hongkongensis* and *L. cinnabarina* were found to be susceptible to polymyxin B. Strain DSSK1-5^T was also found to be resistant to kanamycin, lincomycin and tetracycline, but susceptible to ampicillin, carbenicillin, cephalotin, chloramphenicol, gentamicin, neomycin, novobiocin, oleandomycin, penicillin G and streptomycin.

Morphological, physiological and biochemical characteristics of strain DSSK1-5^T are given in the species description and in Table 1.

Table 1 Differential phenotypic characteristics of strain DSSK1-5^T and the type strains of two phylogenetically related *Loktanella* species

Characteristic	1	2	3
Growth at			
10 °C	+	+[a]	–[a]
37 °C	+	+[a]	–[a]
Maximum NaCl concentration for growth	16	14[a]	8[a]
Hydrolysis of aesculin	+	–[a]	+[a]
Utilization of			
L-Arabinose	+	–	+
D-galactose	+	–	+
Maltose	+	+	–
D-xylose	+	–	+
Acetate	+	–	+
Enzyme activity (API ZYM)			
α-Galactosidase	–	–	+
Susceptibility to			
Kanamycin	–	–	+
Polymyxin B	–	+	+
DNA G+C content (mol%)	67.6	65.9[a]	69.3[a]

Strains: DSSK1-5^T; 2, *L. hongkongensis* JCM 12479^T; 3, *L. cinnabarina* JCM 18161^T. Data obtained from this study unless otherwise indicated. All strains are positive for catalase and oxidase activities[a]; utilization of D-cellobiose, D-fructose, D-glucose, D-mannose, sucrose, citrate, L-malate, pyruvate and succinate; activity of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase and α-glucosidase; and susceptibility to ampicillin, carbenicillin, cephalothin, chloramphenicol, gentamicin, neomycin, novobiocin, oleandomycin, penicillin G and streptomycin. All strains are negative for motility[a]; Gram-staining; nitrate reduction[a]; hydrolysis of[a] casein, Tween-80 and urea; utilization of D-trehalose, benzoate, formate, salicin and L-glutamate; activity of lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidas, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase; and susceptibility to lincomycin and tetracycline Symbol: +, positive reaction; –, negative reaction

[a] Data of two reference strains taken from Lau et al. (2004) and Tsubouchi et al. (2013)

Phylogenetic analysis and DNA–DNA relatedness

The almost complete 16S rRNA gene sequence of strain DSSK1-5^T determined in this study comprised 1385 nucleotides (GenBank/EMBL/DDBJ accession number KC987356). In a neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, strain DSSK1-5^T clustered with the type strains of *L. hongkongensis* with a bootstrap resampling value of 92.8 %, and this cluster joined the type strain of *L. cinnabarina* with a bootstrap resampling value of 99.8 % (Fig. 1). The relationships between strain DSSK1-5^T and the type strains of *L. hongkongensis* and *L. cinnabarina* were also maintained in the trees constructed using the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). Strain DSSK1-5^T exhibited 16S rRNA gene sequence similarity values of 98.9 and 98.4 % to the type strains of *L. hongkongensis* and *L. cinnabarina*, respectively, and of 94.0–96.2 % to the type strains of the other *Loktanella* species.

Strain DSSK1-5^T exhibited DNA–DNA relatedness values of 19 ± 8 and 23 ± 5 % to *L. hongkongensis* JCM 12479^T and *L. cinnabarina* JCM 18161^T, respectively.

Chemotaxonomic characteristics

The predominant isoprenoid quinone detected in strain DSSK1-5^T was ubiquinone-10 (Q-10) which

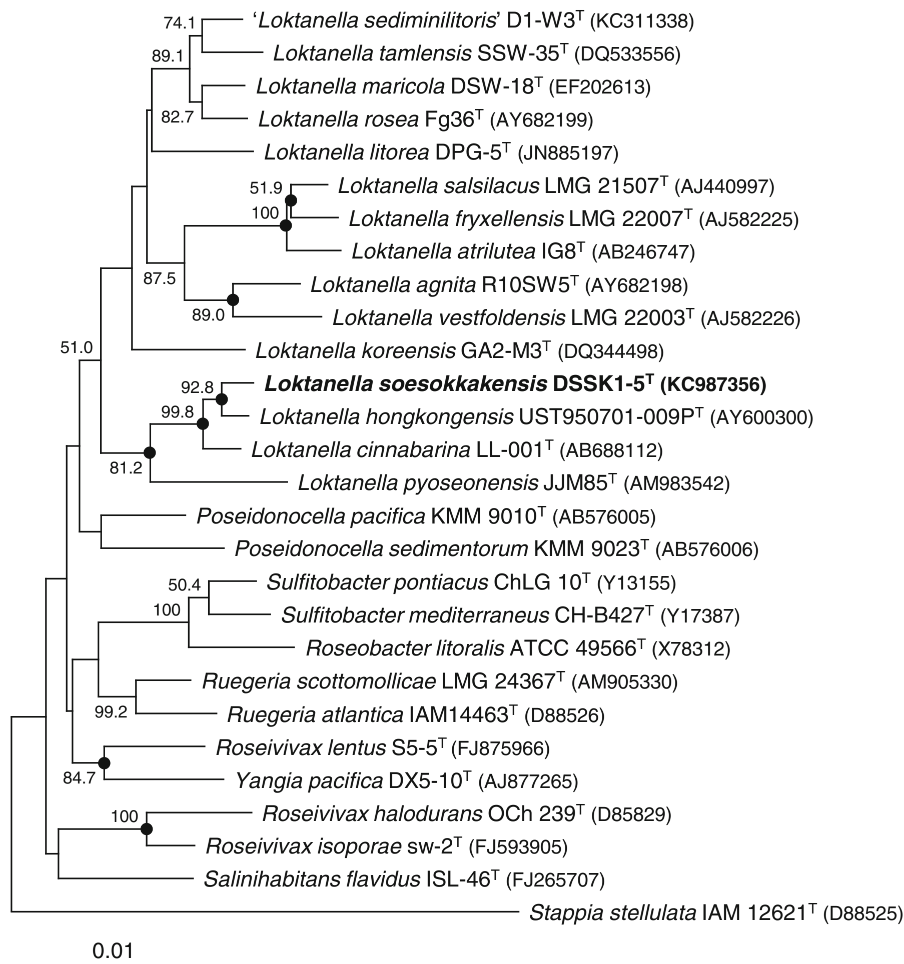

Fig. 1 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain DSSK1-5^{T}, the type strain of *Loktanella* species and representatives of some other members. Bootstrap values (expressed as percentages of 1,000 replications) of >50 % are shown at branching points. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum parsimony algorithms. *Stappia stellulata* IAM 12621^{T} (GenBank accession number, D88525) was used as an outgroup. Scale bar, 0.01 substitutions per nucleotide position

is compatible with that of the genus *Loktanella* (Moon et al. 2010; Lee 2012; Yoon et al. 2013; Tsubouchi et al. 2013). In Table 2, the fatty acid profile of strain DSSK1-5^{T} was compared with those of the type strains of two phylogenetically closely related *Loktanella* species grown and analysed under identical conditions and their fatty acid profiles were found to be very similar. The major fatty acid (>10 % of the total fatty acids) identified in strain DSSK1-5^{T} was $C_{18:1}$ $\omega7c$ (86.4 %). The fatty acid profile of strain DSSK1-5^{T} was also similar with those of the type strains of the other *Loktanella* species (Lee et al. 2012; Yoon et al. 2013). The major polar lipids detected in strain DSSK1-5^{T} were phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol, one unidentified glycolipid and one unidentified aminolipid; minor amounts of one unidentified glycophospholipid, one unidentified phospholipid and one unidentified lipid were also present (Fig. 2). The type strain of *L. salsilacus* was found to have major amounts of phosphatidylcholine and phosphatidylglycerol and a minor amount of phosphatidylethanolamine (Yoon et al. 2013). The type strains of the other *Loktanella* species, whose polar lipid data are known, have been described to have phosphatidylcholine and phosphatidylglycerol as the common major polar lipids

(Ivanova et al. 2005; Moon et al. 2010; Lee 2012; Yoon et al. 2013; Tsubouchi et al. 2013). The DNA G+C content of strain DSSK1-5^T was 67.6 mol%, a value in the range reported for members of the genus *Loktanella* (Table 1; Moon et al. 2010; Lee 2012).

Conclusion

The results obtained from the chemotaxonomic analyses and the phylogenetic analyses based on 16S rRNA gene sequences are sufficient to identify the taxonomic position of strain DSSK1-5^T as a member of the genus *Loktanella* (Moon et al. 2010; Lee 2012; Yoon et al. 2013; Tsubouchi et al. 2013). Strain DSSK1-5^T was differentiated from the phylogenetically closely related *Loktanella* species by differences in several phenotypic characteristics, including growth at 10 and 37 °C, aesculin hydrolysis, utilization of some substrates, susceptibility to kanamycin and polymyxin B and α-galactosidase activity (Table 1). These differences, in combination with phylogenetic and genetic distinctiveness of strain DSSK1-5^T, are sufficient to propose that the novel strain is separate from other *Loktanella* species (Wayne et al. 1987; Stackebrandt and Goebel 1994).

Table 2 Cellular fatty acid compositions (%) of strain DSSK1-5^T and the type strains of two phylogenetically related *Loktanella* species

Fatty acid	1	2	3
Straight-chain			
$C_{16:0}$	5.1	6.1	10.7
$C_{17:0}$	0.6	–	–
$C_{18:0}$	2.4	1.7	3.1
Unsaturated			
$C_{18:1}$ *ω7c*	86.4	87.3	80.0
Hydroxy			
$C_{10:0}$ 3-OH	1.6	1.9	1.2
$C_{12:0}$ 3-OH	–	–	2.4
11-Methyl-$C_{18:1}$ *ω7c*	1.5	0.9	–
Summed feature 3[a]	1.2	1.1	2.6

Strains: 1, DSSK1-5^T; 2, *L. hongkongensis* JCM 12479^T; 3, *L. cinnabarina* JCM 18161^T. All data obtained from this study. Fatty acids that represented <0·5 % in all strains were omitted."—" not detected

[a] Summed feature 3 contained $C_{16:1}$ *ω7c* and/or $C_{16:1}$ *ω6c*

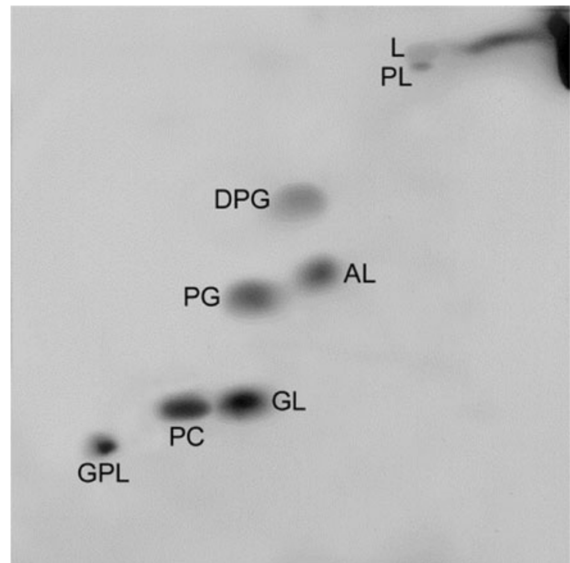

Fig. 2 Thin layer chromatogram of the polar lipids of *L. soesokkakensis* DSSK1-5^T. Spots were revealed by spraying the plates with 10 % ethanolic molybdophosphoric acid. *PC* phosphatidylcholine; *PG* phosphatidylglycero; *DPG* diphosphatidylglycero; *AL* unidentified aminolipi; *GL* unidentified glycolipi; *GPL* unidentified glycophospholipi; *PL* unidentified phospholipi; *L* unidentified lipid

Therefore, on the basis of the phenotypic, chemotaxonomic, phylogenetic and genetic data, strain DSSK1-5^T is considered to represent a novel species of the genus *Loktanella*, for which the name *Loktanella soesokkakensis* sp. nov. is proposed.

Description of *Loktanella soesokkakensis* sp. nov.

Loktanella soesokkakensis (so.e.so.kkak.en'sis. N.L. fem. adj. *soesokkakensis* pertaining to Soesokkak, from where the type strain was isolated).

Cells are Gram-negative, non-spore-forming, non-flagellated and rod-shaped or ovoid, approximately 0.3–1.0 µm in diameter and 0.8–6.0 µm in length. Colonies on MA are circular to slightly irregular, raised, smooth, glistening, light orange in colour and 2.0–3.0 mm in diameter after incubation for 3 days at 30 °C. Optimal growth occurs at 30 °C; growth occurs at 10 and 40 °C, but not at 4 and 45 °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 1.0–16.0 % (w/v) NaCl with an optimum of approximately 2.0–3.0 % (w/v) NaCl. Anaerobic growth does not occur on MA and on MA

supplemented with nitrate. Catalase- and oxidase-positive. Nitrate is not reduced to nitrite. Aesculin, hypoxanthine and L-tyrosine are hydrolysed but casein, gelatin, starch, Tween-80, urea and xanthine are not. L-Arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, maltose, D-mannose, sucrose, D-xylose, acetate, citrate, L-malate, pyruvate and succinate are utilized but D-trehalose, benzoate, formate, salicin and L-glutamate are not. In assays with the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase and α-glucosidase activities are present but lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent. The predominant ubiquinone is Q-10. The major fatty acid (>10 % of the total fatty acids) is $C_{18:1}$ $\omega 7c$. The major polar lipids are phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol, one unidentified glycolipid and one unidentified aminolipid. The DNA G+C content of the type strain is 67.6 mol%.

The type strain, DSSK1-5^T (= KCTC 32425^T = CECT 8367^T), was isolated from the junction between the ocean and a freshwater spring at Jeju island of South Korea. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DSSK1-5^T is KC987356.

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