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Luteimonas dalianensis sp. nov., an Obligate Marine Bacterium Isolated from Seawater[§]

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A marine bacterial strain, designated OB44-3^T, was isolated from a crude oil-contaminated seawater sample collected near Dalian Bay, China. Cells of strain OB44-3^T were Gram-negative, aerobic, rod-shaped, and oxidase- and catalase-positive. The major fatty acids were branched-chain saturated iso-$C_{15:0}$ (27.9%) and unsaturated iso-$C_{17:1}$ *ω9c* (14.8%). The DNA G+C content was 64.6 mol%. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain OB44-3^T was a member of the genus *Luteimonas* (95–96% 16S rRNA gene sequence similarity); its closest neighbors were the type strains of *Luteimonas terricola* (96% sequence similarity), *Luteimonas mephitis* (96%), and *Luteimonas lutimaris* (96%). On the basis of phenotypic, chemotaxonomic, and phylogenetic distinctiveness, strain OB44-3^T was considered to represent a novel species of the genus *Luteimonas*. The name *Luteimonas dalianensis* sp. nov. is proposed, with strain OB44-3^T (=CGMCC 1.12191^T =JCM 18136^T) as the type strain.

Keywords: *Luteimonas dalianensis* sp. nov., marine bacterium, phylogenetic analysis

Introduction

The genus *Luteimonas*, a member of the family *Xanthomonadaceae* of the *Gammaproteobacteria*, was first proposed by Finkmann *et al.* (2000) with a single species, *Luteimonas mephitis*, isolated from a biofilter. Currently, the genus *Luteimonas* comprises ten recognized species, *L. mephitis* (Finkmann *et al.*, 2000), *L. composti* (Young *et al.*, 2007), *L. aestuarii* (Roh *et al.*, 2008), *L. aquatica* (Chou *et al.*, 2008), *L. marina* (Baik *et al.*, 2008), *L. terricola* (Zhang *et al.*, 2010), *L. lutimaris* (Park *et al.*, 2011), *L. cucumeris* (Sun *et al.*, 2012), *L. vadosa* (Romanenko *et al.*, 2013), and *L. huabeiensis* (Wu *et al.*, 2013), which have been isolated from such diverse habitats as a biofilter, tidal flat, fresh water, food waste, cucumber leaf, seawater, stratum water, sediment, and soil. In this study, we report the characterization of a novel marine bacterium, strain OB44-3^T, of the genus *Luteimonas*, which was isolated from a crude oil-contaminated seawater sample. This strain is physiologically adapted to growth in seawater, and requires sodium chloride (NaCl ≥2%) for growth, a hallmark of obligate marine bacteria. Sodium requirements have been studied extensively in Gram-negative marine bacteria and are indicative of highly evolved marine adaptations, such as respiration-dependent sodium ion pumps and/or a sodium-ependent membrane transport mechanism (MacLeod, 1965; Oh *et al.*, 1991).

Materials and Methods

Bacterial strains

Strain OB44-3^T was isolated from a crude oil-contaminated seawater sample collected near Dalian Bay, China (38°52′N 121°41′E). The artificial sea water medium (ASM) used for enrichment contained (per L of distilled water) 1 g crude oil, 20 g NaCl, 7.0 g $MgSO_4{\cdot}7H_2O$, 1 g NH_4NO_3, 0.7 g KCl, 2.0 g KH_2PO_4, 3.0 g Na_2HPO_4, and 10 ml trace element solution, pH 7.2. The trace element solution contained (per L of distilled water) 2 mg $CaCl_2$, 50 mg $FeCl_3{\cdot}6H_2O$, 0.5 mg $CuSO_4$, 0.5 mg $MnCl_2{\cdot}4H_2O$, and 10 mg $ZnSO_4{\cdot}7H_2O$. Aliquots of seawater (5 ml) were added to Erlenmeyer flasks containing 100 ml of medium, and the flasks were incubated for 7 days at 30°C on a rotary shaker (200 rpm). Then, 5 ml aliquots were removed and placed in fresh medium. After three sub-cultured, inocula from the flask were streaked out, and an isolate (designated OB44-3^T) was picked and sub-cultured on 2216E plates (Difco, USA) until a pure culture was obtained. The novel strain was deposited into the CGMCC (China General Microbiological Culture Collection Center) as CGMCC 1.12191^T and the JCM (Japan Collection of Microorganisms) as JCM 18136^T. The reference strain used for the DNA-DNA hybridization tests was *Luteimonas terricola* DSM 22344^T, obtained from the KCTC (Korean Collection for Type Cultures).

Morphology and physiological characteristics

To investigate the morphological and physiological characteristics, strain OB44-3^T was cultivated on 2216E medium at 28°C. Cell morphology and motility was examined using cells from the exponential growth phase with light microscopy (Olympus; x1000) and transmission electron microscopy (H-7650; Hitachi, Japan).

The Gram straining reaction of cells was carried out accor-

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ding to the classical procedure described by Doetsch (1981). Gliding motility was determined as described by Bowman (2000). The physiological properties of strain OB44-3^T were determined by the CGMCC using established procedures described by Gordon *et al.* (1974) and Yokota *et al.* (1993). Catalase activity, oxidase activity, enzyme activity, and acid production from different carbohydrates were determined by the CGMCC with Biolog GN2, API 20E, and API 20NE kits according to the manufacturer's instructions. The assimilation of single-carbon substrates was determined by the CGMCC with Biolog GN2 and API 20NE strips cultured at 28°C for 24 h. Growth at 4, 10, 15, 25, 30, 37, 42, and 45°C and at pH 4.0–10.0 (at intervals of 1.0 pH unit) was assessed after 5 days of incubation on 2216E agar. Growth on nutrient agar, trypticase soy agar (TSA; Difco), R2A agar (Difco), and LB agar (Difco) was also evaluated at 28°C. Salt tolerance was tested in LN medium (LB without NaCl) supplemented with 0–10% (w/v) NaCl after 10 days of incubation.

Phylogenetic analysis and DNA-DNA hybridization

Genomic DNA from strain OB44-3^T was extracted and purified according to standard procedures (Sambrook and Russell, 2001). The 16S rRNA gene cloned into pMD-18T vector (TaKaRa, Japan) was sequenced with an automated sequencer (Applied Biosystems model 3730). The 16S rRNA gene sequence of strain OB44-3^T was compared with known sequences found in the GenBank database using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was performed with MEGA5 program (Tamura *et al.*, 2011) after multiple alignments of the data via CLUSTAL_X program (Thompson *et al.*, 1997). A distance matrix method (distance options according to the Kimura two-parameter model), including clustering using the neighbor-joining method, and a discrete character-based maximum-parsimony method were used. In each case, bootstrap values were calculated based on 1,000 replications (Felsenstein, 1985). The taxonomic relationship between strain OB44-3^T and its phylogenetic relative was further examined using DNA-DNA hybridization. DNA-DNA hybridization values between OB44-3^T and *L. terricola* DSM 22344^T was performed fluorometrically, according to the method developed by Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was conducted in five replications for each sample. The highest and lowest values obtained for each sample were excluded, and the remaining three values were utilized in the calculation of hybridization values.

G+C content and analysis of cellar fatty acids

Cellular fatty acid analysis was carried out at the identification service from the CGMCC. The cell mass of strain OB44-3^T was harvested from tryptic soy agar (TSA) plates after incubation at 28°C for 2 days. The fatty acids were extracted, methylated and analyzed using the standard Sherlock MIDI (Microbial Identification) system (Sasser, 1990; Kämpfer and Kroppenstedt, 1996). The G+C content of the genomic DNA was determined by the CGMCC by thermal denaturation method (Mandel and Marmur, 1968), for which *Escherichia coli* K-12 (CGMCC 1.365) was used as a standard.

Table 1. Phenotypic characteristics of strain OB44-3^T and related *Luteimonas* species

Strains: 1, OB44-3^T; 2, *L. terricola* DSM 22344^T (data from Zhang *et al.*, 2010); 3, *L. aestuarii* KCTC 22048^T (Roh *et al.*, 2008); 4, *L. mephitis* 1953/27.1^T (Young *et al.*, 2007); 5, *L. composti* CC-YY255^T (Young *et al.*, 2007); 6, *L. aquatica* LMG 24212^T (Chou *et al.*, 2008); 7, *L. marina* KCTC 12327^T (Baik *et al.*, 2008); 8, *L. lutimaris* KACC 14929^T (Park *et al.*, 2011); 9, *L. vadosa* KMM 9005^T (Romanenko *et al.*, 2013); 10, *L. cucumeris* KCTC 23627^T (Sun *et al.*, 2012); 11, *L. huabeiensis* DSM 26429^T (Wu *et al.*, 2013). +, Positive; –, negative; W, weakly positive; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Isolation source	Seawater	Soil	Tidal flat	Biofilter	Food waste	Fresh water	Sea water	Tidal flat	Sediment	Cucumber	Stratum water
Colony color	Pale yellow	ND	Yellow	Yellow	Yellow	Yellow	Light yellow	Yellow to brown	Yellow	Yellow	Yellow
Growth at 37°C	+	–	+	–	+	+	+	+	+	+	+
Salinity range (%)	2–9	0–3	0–3	0–6	0–6	0–3	0–1	0–9	0–4	0–3	0–5
Assimilation of:											
Dextrin	–	–	+	–	–	–	+	–	–	+	+
Cellobiose	–	–	+	–	–	–	+	–	–	–	+
D-Mannose	–	–	–	ND	–	–	+	–	+	+	–
Maltose	–	–	+	–	+	–	+	+	+	+	–
D-Glucose	–	–	+	–	+	+	+	+	+	+	–
L-Arabinose	+	–	–	ND	+	–	+	–	+	–	–
Glycogen	+	–	+	ND	–	–	–	+	–	–	–
D-Mannitol	–	–	–	ND	–	–	+	+	+	+	–
D-Fructose	+	–	+	ND	–	–	–	+	–	+	+
D-Galactose	–	–	–	–	–	+	–	+	–	–	–
DNA G+C content (mol%)	64.6	72.0	64.7	67.0	68.1	70.3	67.6	69.6	ND	69.9	67.0

Results and Discussion

Morphological and physiological characteristics

Cells of strain OB44-3^T were Gram-negative, non-spore forming; non-motile, aerobic rods that were 0.32–0.47 μm wide and 1.02–1.24 μm long (Supplementary data Figs. 1–2). Strain OB44-3^T grew well on nutrient agar, TSA and LB agar, but not on R2A agar. Colonies of strain OB44-3^T on LB agar were circular, smooth with entire margins, translucent and pale yellow after 3 days of incubation at 28°C. Growth of strain OB44-3^T occurred in LN medium with 2–9% (w/v) NaCl (optimum 4%); no growth occurred in the absence of NaCl. Strain OB44-3^T grew at 15–37°C (optimum 28–30°C) and at pH 6.0–8.0 (optimum pH 7.0). In the API 20NE and Biolog GN2 kits, strain OB44-3^T assimilated glycogen, L-arabinose, D-fructose, D-allulose, methyl pyruvate, formic acid, beta-hydroxybutyric acid, alpha-oxobutyric acid, alpha-oxopentanoate, L-alanyl amine, D-alanine, L-alanine, L-alanylglycine, L-asparagine, L-glutamate, L-threonine, L-serine, inosine. Strain OB44-3^T differed from its closest strain *L. terricola* DSM 22344^T in its ability to utilize L-arabinose. The phenotypic characteristics of strain OB44-3^T are summarized in the species description and a comparison of selective characteristics with recognized members of the genus *Luteimonas* is given in Table 1.

Phylogenetic analysis and DNA-DNA hybridization

An almost-complete 16S rRNA gene sequence of strain OB44-3^T consisting of 1,424 bp was obtained. Neighbor-joining tree (Fig. 1) showed that strain OB44-3^T was grouped with members of the genus *Luteimonas* and formed a distinct cluster with *L. terricola* DSM 22344^T (Zhang *et al.*, 2010). The relationship was supported by a high bootstrap value of 80% and the topologies of tree created by maximum-likelihood methods. Strain OB44-3^T showed the highest 16S rRNA gene sequence similarity, 96%, with *L. terricola* DSM 22344^T. Lower similarity values (95–96%) were found with the type strains of other *Luteimonas* species. To determine whether strain OB44-3^T represents a novel species of the genus *Luteimonas*, DNA-DNA hybridization values between OB44-3^T and *L. terricola* DSM 22344^T were estimated. The value obtained was 37%, which is low enough to confirm that strain OB44-3^T should be classified as representing a novel *Luteimonas* species.

G+C content and analysis of cellar fatty acids

The major cellular fatty acids of strain OB44-3^T were saturated iso-$C_{15:0}$ (27.9%), unsaturated iso-$C_{17:1}$ ω9*c* (14.8%) and anteiso-$C_{15:0}$ (12.4%). The fatty acid profile of strain OB44-3^T differs from those of other *Luteimonas* species by having

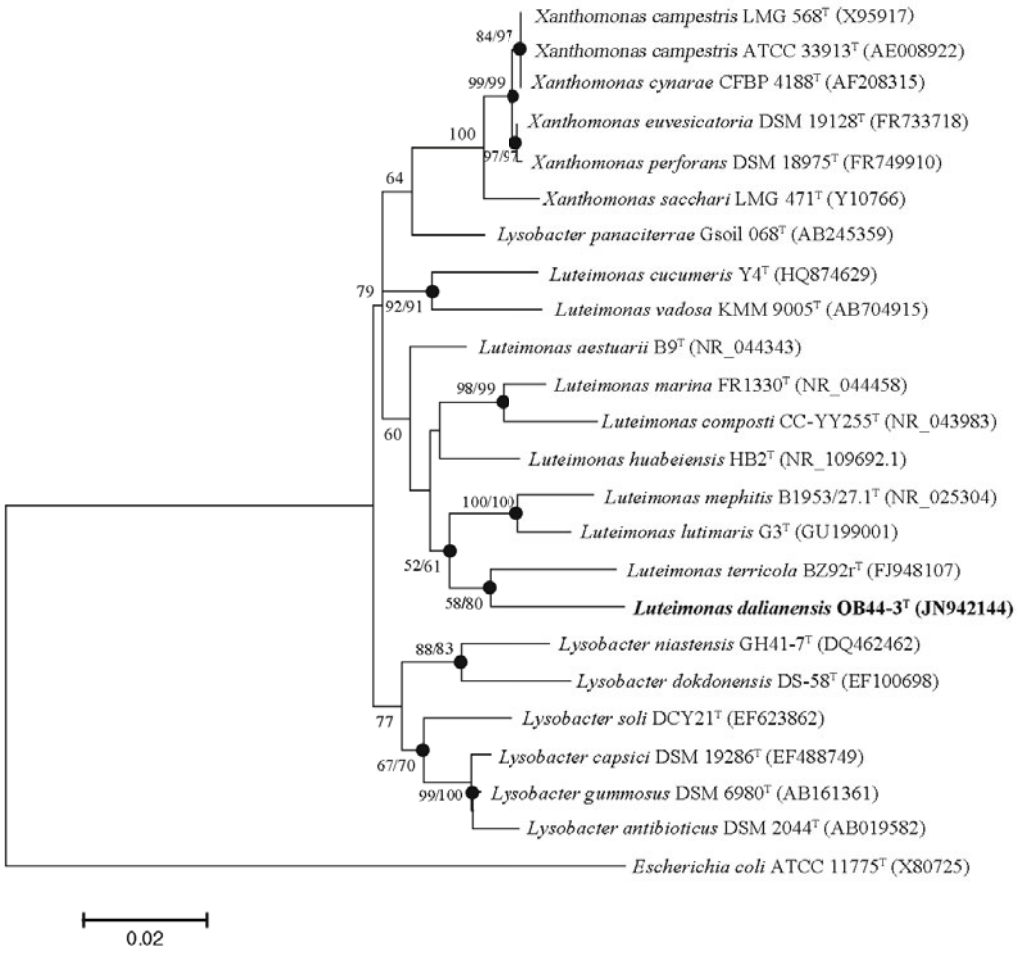

Fig. 1. Neighbor-joining phylogenetic tree based on nearly full length 16S rRNA gene sequences showing the positions of the strain *L. dalianensis* OB44-3^T and closely related species. Filled circles indicate generic branches that were also recovered using the maximum-parsimony algorithm. Bootstrap values over 50% (based on 1,000 replications) are shown at branching points. Bar, 0.02 substitutions per nucleotide position.

Table 2. Fatty acid compositions of strain OB44-3^T and related *Luteimonas* species

Strains: 1, OB44-3^T; 2, *L. terricola* DSM 22344^T (data from Zhang *et al.*, 2010); 3, *L. aestuarii* KCTC 22048^T (Roh *et al.*, 2008); 4, *L. mephitis* 1953/27.1^T (Young *et al.*, 2007); 5, *L. composti* CC-YY255^T (Young *et al.*, 2007); 6, *L. aquatica* LMG 24212^T (Chou *et al.*, 2008); 7, *L. marina* KCTC 12327^T (Baik *et al.*, 2008); 8, *L. lutimaris* KACC 14929^T (Park *et al.*, 2011); 9, *L. vadosa* KMM 9005^T (Romanenko *et al.*, 2013); 10, *L. cucumeris* KCTC 23627^T (Sun *et al.*, 2012); 11, *L. huabeiensis* DSM 26429^T (Wu *et al.*, 2013). –, Not determined.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11
Straight-chain saturated											
$C_{16:0}$	4.3	0.9	2.9	1.6	4.7	2.4	5.9	3.8	3.6	2.1	2.7
Branched saturated											
iso-$C_{11:0}$	5.9	18.4	14.5	5.2	6.5	4.0	6.4	11.1	5.1	6.0	12.4
anteiso-$C_{13:0}$	1.0	–	–	–	–	–	–	–	–	–	–
iso-$C_{14:0}$	1.0	0.3	0.5	1.5	1.9	0.3	0.2	1.2	0.8	1.7	4.9
iso-$C_{15:0}$	27.9	25.6	22.3	47.4	25.5	25.3	26.2	29.6	11.6	33.4	39.0
anteiso-$C_{15:0}$	12.4	0.7	4.9	3.0	2.7	6.2	4.5	1.9	1.4	4.8	0.8
iso-$C_{16:0}$	10.0	0.8	7.2	5.6	13.8	4.4	1.7	11.5	17.4	9.3	8.5
iso-$C_{17:0}$	8.3	3.9	11.5	5.5	5.5	21.5	14.5	7.1	11.2	8.9	2.8
Unsaturated											
$C_{16:1}$ *ω7c*/$C_{16:1}$ *ω6c*	2.0	–	–	–	–	–	–	–	–	–	–
iso-$C_{17:1}$ ω9c	14.8	24.9	19.8	18.7	25.5	22.3	26	17.0	–	20.8	–
Hydroxy											
iso-$C_{11:0}$ 3-OH	2.7	16.2	8.6	5.3	5.5	6.7	4.7	8.0	–	6.6	3.7
iso-$C_{13:0}$ 3-OH	3.0	–	–	–	–	–	–	–	–	–	3.0
iso-$C_{16:0}$ 3-OH	1.0	–	–	–	–	–	–	–	–	–	–

larger proportions of anteiso-$C_{15:0}$ (Table 2). The fatty acid profiles of strain OB44-3^T and the type strains of *Luteimonas* species are shown in Table 2. The DNA G+C content of strain OB44-3^T was 64.6 mol%.

Taxonomic conclusion

It is clear from the 16S rRNA gene sequence comparison and DNA-DNA hybridization data that strain OB44-3^T represents a novel species of the genus *Luteimonas* (Wayne *et al.*, 1987). In addition, strain OB44-3^T differs from the type strains of the other species of the genus *Luteimonas* mainly by its growth characteristics: this strain requires sodium for growth, and no growth occurs in the absence of NaCl, which has not been described for other *Luteimonas* species. Based on the phenotypic, phylogenetic, and genomic evidence, strain OB44-3^T was identified as a representative of a novel species of *Luteimonas*, for which the name *L. dalianensis* sp. nov. is proposed.

Description of *Luteimonas dalianensis* sp. nov.

Luteimonas dalianensis (da.li.an.en'sis. N.L. masc. adj. *dalianensis* pertaining to Dalian, a city in China, from where the type strain was isolated).

Cells are Gram-negative, aerobic, non-spore forming, non-motile, and rod-shaped, approximately 0.32–0.47 μm wide and 1.01–1.24 μm long. Colonies grown on LB agar are convex, small circular, smooth, translucent, and pale yellow after 3 days of incubation at 28°C. Growth occurs at 15–37°C (optimum 28–30°C); no growth occurs below 4°C or above 45°C. The pH range for growth is 6.0–8.0 (optimum pH 7.0). No growth occurs in the absence of NaCl, and no growth occurs in 9.0% (w/v) NaCl. Positive for oxidase and catalase-reaction. Positive for citric acid utilization, but negative for hydrolysis of aesculin and gelatin. Nitrate reduction, acetoin production (Voges-Proskauer reaction), indole production and activities of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease and tryptophan deaminase are negative (API 20E). Negative for assimilation of D-glucose, D-mannose, D-mannitol, maltose, N-acetyl-galactosamine, N-acetylglucosamine, alpha-cyclodextrin, dextrin, Tween 40, Tween 80, ribitol, D-arabitol, D-cellobiose, i-erythritol, L-fructose, D-galactose, dextrinose, inositol, alpha-D-lactose, lactulose, D-melibiose, methyl glucoside, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, succinic acid methyl, caproic acid, cis aconic acid, citric acid, D-galactose acid lactone, D-galacturonic acid, D-gluconic acid, beta-methyl glucoside, D-glucuronic acid, alpha-hydroxybutyric acid, gamma-hydroxybutyric acid, beta-mandelic acid, itaconic acid, alpha-ketoglutaric acid, D,L-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharinic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, aspartic acid, glycyl aspartic acid, glycyl glutamic acid, L-histidine, hydroxy proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, D,L-carnitine, gamma-amino butyric acid, urocanic acid, uridine, thymidine, phenyl ethylamine, putrescine, 2-amino ethanol, 2,3-butanediol, glycerol, glycerol phosphate, D-glucose-1-phosphate, D-glucose-6-phosphate. Utilizes glycogen, L-arabinose, D-fructose, D-allulose, methyl pyruvate, formic acid, beta-hydroxybutyric acid, alpha-oxobutyric acid, alpha-oxopentanoate L-alanyl amine, D-alanine, L-alanine, L-alanylglycine, L-asparagine, L-glutamate, L-threonine, L-serine, inosine (API 20 NE and Biolog GN2).

The major cellular fatty acids of strain OB44-3^T were saturated iso-$C_{15:0}$, unsaturated iso-$C_{17:1}$ *ω9c*, anteiso-$C_{15:0}$, and iso-$C_{16:0}$. Minor amounts of iso-$C_{17:0}$, iso-$C_{11:0}$, iso-$C_{16:0}$, iso-$C_{13:0}$ 3-OH, iso-$C_{11:0}$ 3-OH, $C_{16:1}$ *ω7c*/$C_{16:1}$ *ω6c*, $C_{16:0}$ 3-OH, anteiso-$C_{13:0}$, iso-$C_{14:0}$. The DNA G+C content of the

type strain is 64.6 mol%. The type strain OB44-3^T (=CGMCC 1.12191^T =JCM 18136^T) was isolated from a crude oil-contaminated seawater sample in Dalian, China.

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