Loktanella atrilutea sp. nov., isolated from seawater in Japan

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A Gram-negative, rod-shaped bacterium, IG8^T, was isolated from seawater off the Sanriku coast, Japan. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain IG8^T represented a separate lineage within the genus *Loktanella*; the highest 16S rRNA gene sequence similarity values were found with the type strains of *Loktanella salsilacus* (98.6%) and *Loktanella fryxellensis* (98.4%). DNA–DNA hybridization values between strain IG8^T and the type strains of *L. salsilacus* (27.9–36.1%) and *L. fryxellensis* (11.3–31.0%) were clearly below 70%, the generally accepted limit for species delineation. The DNA G + C content of strain IG8^T was 66.3 mol%. On the basis of DNA–DNA hybridization, some biochemical characteristics and 16S rRNA gene sequence comparison, it is proposed that the isolate represents a novel species, *Loktanella atrilutea* sp. nov. The type strain is IG8^T (=IAM 15450^T=NCIMB 14280^T).

The genus *Loktanella* was first described by Van Trappen *et al.* (2004) and contained three species, *Loktanella salsilacus*, *Loktanella fryxellensis* and *Loktanella vestfoldensis*. Recently, four more species, *Loktanella hongkongensis* (Lau *et al.*, 2004), *Loktanella agnita*, *Loktanella rosea* (Ivanova *et al.*, 2005) and *Loktanella koreensis* (Weon *et al.*, 2006), have been reported.

In this study, the taxonomic position of strain IG8^T, isolated from seawater off Masaki on the Sanriku coast, Iwate Prefecture, Japan, was determined. The seawater sample (0.05 ml) was spread onto a plate containing modified gelatin agar [0.75 × artificial seawater (ASW; 1 × ASW consists of 3% NaCl, 0.07% KCl, 1.08% MgCl₂·6H₂O, 0.54% MgSO₄·7H₂O and 0.1% CaCl₂·2H₂O), 0.4% gelatin, 0.025% peptone, 0.025% yeast extract, 0.001% FeSO₄·7H₂O, 0.001% Na₂HPO₄ and 1% agar] and incubated at 25 °C for a week. Strain IG8^T was purified and maintained at 25 °C on marine agar 2216 (MA; Difco).

16S rRNA gene sequences were obtained by direct sequencing of PCR-amplified DNA as described by Hosoya *et al.* (2006). The most closely related sequences were found using the BLAST program from the GenBank database (Altschul *et al.*, 1990). Multiple alignments were performed by using the CLUSTAL_X program (version 1.83; Thompson *et al.*, 1997). Nucleotide substitution rates (K_{nucl} ; Kimura, 1980) were determined and a phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987). Alignment gaps and unidentified

base positions were not taken into consideration in the calculation. Bootstrap analysis was based on 1000 trials. The results of the phylogenetic analysis based on 16S rRNA gene sequences showed that strain IG8^T belonged to the genus *Loktanella* (Fig. 1). The highest 16S rRNA gene sequence similarity values were found with the type strains of *L. salsilacus* (98.6%) and *L. fryxellensis* (98.4%); similarity values with the type strains of *L. vestfoldensis*, *L. hongkongensis*, *L. agnita*, *L. rosea* and *L. koreensis* were 95.5, 94.6, 94.6, 94.5 and 93.9%, respectively. *L. salsilacus* LMG 21507^T and *L. fryxellensis* LMG 22007^T, which are the closest relatives of strain IG8^T, were obtained from the BCCM/LMG Bacteria Collection (Belgium). Genomic DNA was extracted by the method of Saito & Miura (1963). For analysis of genetic relatedness, DNA–DNA hybridization was carried out at 49 °C for 4 h and measured fluorometrically using the method of Ezaki *et al.* (1989). Strain IG8^T showed relatively low DNA–DNA relatedness values to *L. salsilacus* LMG 21507^T (27.9–36.1%) and *L. fryxellensis* LMG 22007^T (11.3–31.0%). These values are significantly lower than that accepted as the phylogenetic definition of a species (Wayne *et al.*, 1987). The DNA G + C content was determined by HPLC according to the method of Mesbah *et al.* (1989). The DNA G + C content of strain IG8^T was 66.3 mol%; the DNA G + C contents of the reference strains *L. salsilacus* and *L. fryxellensis* are given in Table 1.

The following phenotypic characteristics were determined. Cell morphology was observed by using transmission electron microscopy (TEM) after negative staining with phosphotungstic acid. Gram staining was performed using the Hucker staining method as described by Smibert & Krieg (1994). Spore staining was performed using the

Abbreviation: ASW, artificial seawater.

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain IG8^T is AB246747.

Fig. 1. Neighbour-joining phylogenetic tree of *Loktanella atrilutea* and other related genera of the class *Alphaproteobacteria* based on 16S rRNA gene sequence analysis. Bootstrap values greater than 50 % are given at branch points. Bar, 0.01 nucleotide substitutions per site.

Schaeffer & Fulton staining method as described by Barrow & Feltham (1993). Salt tolerance was tested on R2A agar (Difco) supplemented with 0–10 % NaCl. In addition, the growth of colonies on nutrient agar (NA; Difco) and trypticase soy agar (TSA; BBL) was tested. Growth at different temperatures (8–37 °C) and pH values (range pH 5.0–9.0), cytochrome oxidase and catalase activities, and degradation of alginate were determined by previously described methods (Hosoya *et al.*, 2006). Hydrolysis of DNA was determined on DNA agar (Nissui) supplemented with 0.5 × ASW. Degradation of casein was tested on NA supplemented with 0.5 × ASW by the method of Smibert & Krieg (1994). Degradation of starch was tested on SP5 medium containing 0.2 % soluble starch by the method of Smibert & Krieg (1994). Hydrolysis of Tweens (20, 40, 60 and 80) was tested on modified Tween 80 medium [containing 0.1 % peptone, 0.5 × ASW, 1 % (v/v) Tweens and 2 % agar] according to the method of Barrow & Feltham (1993). Degradation of L-tyrosine was tested on

NA containing 0.5 % L-tyrosine, supplemented with 0.5 × ASW using the method of Barrow & Feltham (1993). Acid production from carbon sources was assessed using Leifson modified O/F medium incubated for 10 days (Smibert & Krieg, 1994). Tests with the commercial API ZYM and API 20E systems (bioMérieux) were generally performed according to the manufacturer's instructions. The API ZYM tests were read after 4 h incubation at 37 °C and API 20E tests were read after 48 h at 25 °C. Cells for inoculation onto the API test strips were suspended in 0.5 × ASW. For analysis of cellular fatty acids, cells were grown for 48 h at 25 °C on MA and were analysed using the GC-based Microbial Identification system (MIDI).

Strain IG8^T was a Gram-negative, aerobic, rod-shaped, non-sporulating bacterium. Cells were approximately 1.8–2.0 µm long by 0.5–1.0 µm wide and motile by means of a subpolar flagellum (Fig. 2). The isolate formed beige-coloured colonies that turned dark orange in the stationary phase. Phenotypic characteristics are given in Table 1 and in the species description. Phenotypically, strain IG8^T, *L. salsilacus* LMG 21507^T and *L. fryxellensis* LMG 22007^T differed from each other in their β-galactosidase and

Table 1. Differential characteristics of strain IG8^T, *L. salsilacus* LMG 21507^T and *L. fryxellensis* LMG 22007^T

Strains: 1, *L. atrilutea* sp. nov. IG8^T; 2, *L. salsilacus* LMG 21507^T; 3, *L. fryxellensis* LMG 22007^T.

Characteristic	1	2	3
β-Galactosidase	–	+	–
α-Glucosidase	+	+	–
Acid production from:			
Arabinose	+	+	–
Cellobiose	+	+	–
Glycerol	–	+	–
Inositol	–	+	+
Rhamnose	+	–	+
Sorbitol	+	–	+
Trehalose	–	+	+
DNA G + C content (mol%)	66.3	62.2	67.4

Fig. 2. TEM of a negatively stained cell of strain IG8^T. Bar, 2 µm.

α -glucosidase activities and in their ability to produce acid from arabinose, cellobiose, glycerol, inositol, rhamnose, sorbitol and trehalose. The major cellular fatty acid of strain IG8^T, *L. salsilacus* LMG 21507^T and *L. fryxellensis* LMG 22007^T was 18:1 ω 7c; however, strain IG8^T also contained the fatty acid 11-methyl 18:1 ω 7c, which was not detected in *L. salsilacus* or *L. fryxellensis* (Table 2).

On the basis of DNA–DNA hybridization, phylogenetic analysis and phenotypic characteristics, strain IG8^T is proposed as a novel species of the genus *Loktanella*, *Loktanella atrilutea* sp. nov.

Description of *Loktanella atrilutea* sp. nov.

Loktanella atrilutea (at.ri.lu'te.a. L. adj. *ater* -tra -trum dark; L. adj. *luteus* -a -um orange; N.L. fem. adj. *atrilutea* dark orange).

Cells are Gram-negative, aerobic and rod-shaped, approximately 1.8–2.0 $\times$ 0.5–1.0 μ m. Motile by means of a subpolar flagellum. Colonies on MA plates are round and beige-coloured; older colonies are dark orange. Cytochrome oxidase- and catalase-positive. Optimal growth temperature is 25–30 °C; able to grow at 8 °C, but not at 37 °C. The pH range for growth is 6.0–8.0. Growth occurs at NaCl concentrations of 0–8% on R2A agar, but no growth is observed on NA or TSA. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, α -glucosidase and β -glucuronidase. Positive for degradation of Tweens (20, 40, 60 and 80). Acid is produced from arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, rhamnose, sorbitol, sucrose and xylose. Does not decompose alginate, citrate, DNA, starch, tyrosine or urea. Does not reduce nitrate. Does not produce acetoin, H₂S or

Table 2. Fatty acid profiles of strain IG8^T, *L. salsilacus* LMG 21507^T and *L. fryxellensis* LMG 22007^T

Strains: 1, *L. atrilutea* sp. nov. IG8^T; 2, *L. salsilacus* LMG 21507^T; 3, *L. fryxellensis* LMG 22007^T. Values are percentages (w/w) of total fatty acids. –, <1% total fatty acid or not detected.

Fatty acid	1	2	3
10:0 3-OH	1.5	1.3	1.3
16:0	9.5	3.2	4.0
18:1 ω 7c	74.4	85.5	86.3
18:0	2.2	1.5	2.9
11-Methyl 18:1 ω 7c	6.3	—	—
Summed feature 2*	3.8	—	1.5
Summed feature 3*	—	2.7	—
Summed feature 7*	1.0	2.1	3.2

*Summed features represent the following combinations of fatty acids: 2, 12:0 aldehyde, 16:1 iso I, 14:0 3-OH and/or unknown 10.928; 3, 16:1 ω 7c and/or 15:0 iso 2-OH; 7, 19:1 ω 6c, 19:0 cyclo ω 10c and/or unknown 18.846.

indole. Negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, lipase (C4), valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, β -galactosidase, β -glucosidase, *N*-acetyl- β -glucosamidase, α -mannosidase and α -fucosidase. Does not produce acid from dulcitol, glycerol, inositol, mannitol or trehalose. The major fatty acid is 18:1 ω 7c. The DNA G + C content of the type strain is 66.3 mol%.

The type strain, IG8^T (=IAM 15450^T=NCIMB 14280^T), was isolated from seawater off the Sanriku coast, Japan.

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