Description of *Olleya aquimaris* sp. nov., isolated from seawater, and emended description of the genus *Olleya* Mancuso Nichols *et al.* 2005

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A Gram-stain-negative, non-flagellated, motile (by gliding), yellow-pigmented, rod-shaped bacterial strain, designated L-4^T, was isolated from seawater of Baekdo harbour in the East Sea, Korea. Strain L-4^T grew optimally at 37 °C, at pH 6.5–7.0 and in the presence of 2% (w/v) NaCl. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain L-4^T clustered with *Olleya marilimosa* CAM030^T, a member of the family *Flavobacteriaceae*. Strain L-4^T exhibited 16S rRNA gene sequence similarity values of 97.2% to *O. marilimosa* CAM030^T and less than 95.8% to other members of the family *Flavobacteriaceae*. Strain L-4^T and *O. marilimosa* CIP 108537^T contained MK-6 as the predominant menaquinone. The fatty acid and polar lipid profiles of strain L-4^T were similar to those of *O. marilimosa* CIP 108537^T. The DNA G + C content of strain L-4^T was 35 mol% and DNA–DNA relatedness between strain L-4^T and *O. marilimosa* CIP 108537^T was 7%. Differential phenotypic properties, together with its phylogenetic and genetic distinctiveness, enable strain L-4^T to be distinguished from *O. marilimosa*. On the basis of these data, strain L-4^T is considered to represent a novel species of the genus *Olleya* for which the name *Olleya aquimaris* sp. nov. is proposed; the type strain is L-4^T (=KCTC 22661^T =CCUG 58074^T). An emended description of the genus *Olleya* is also provided.

The genus *Olleya* was created by Mancuso Nichols *et al.* (2005) with *Olleya marilimosa* as the sole recognized species. Members of the genus *Olleya* can be distinguished from closely related members of the family *Flavobacteriaceae* on the basis of low 16S rRNA gene sequence similarities and differences in DNA G + C content and fatty acid profile (Mancuso Nichols *et al.*, 2005). In this study, a novel bacterial strain, designated L-4^T, isolated from seawater of Baekdo harbour in the East Sea, Korea, is described. Comparative 16S rRNA gene sequence analysis indicated that, phylogenetically, strain L-4^T is most closely related to members of the genus *Olleya*. The aim of the present work was to determine the exact taxonomic position of strain L-4^T by using a polyphasic approach that included determination of phenotypic properties and a detailed phylogenetic investigation based on 16S rRNA gene sequences.

The standard dilution plating technique was used for isolation of bacterial strains from seawater samples. Strain L-4^T was isolated on marine agar 2216 (MA; Difco) at 25 °C and cultivated routinely on MA at 37 °C. *O. marilimosa* CIP 108537^T was used as a reference strain for

DNA–DNA hybridization, phenotypic characterization and analyses of fatty acids, polar lipids and isoprenoid quinones. Cell morphology was examined by using light microscopy (E600; Nikon) and transmission electron microscopy (CM-20; Philips). The presence of flagella was assessed by using transmission electron microscopy of cells from an exponentially growing culture. For this purpose, cells were negatively stained with 1% (w/v) phosphotungstic acid and grids were examined after being air-dried. Gliding motility was investigated as described by Bowman (2000). The Gram reaction was determined using the bioMérieux Gram stain kit according to the manufacturer's instructions. Growth at 4, 15, 20, 25, 30, 37, 40 and 45 °C was measured on MA. The pH range for growth was investigated in marine broth 2216 (MB; Difco) adjusted to pH 4.0–10.0, in increments of 0.5 pH units, by using sodium acetate/acetic acid and Na₂CO₃ buffers. 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 Difco medium except that NaCl was omitted and 0.45% (w/v) MgCl₂·6H₂O or 0.06% (w/v) KCl was added. Growth in the presence of 2.0–8.0% (w/v) NaCl (in increments of 1.0%) was investigated in MB. Growth under anaerobic conditions was investigated in a Forma anaerobic chamber

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain L-4^T is FJ886713.

on MA and on MA supplemented with 0.1% (w/v) potassium nitrate, both of which had been prepared anaerobically under a nitrogen atmosphere. Catalase and oxidase activities were determined as described by Cowan & Steel (1965). Hydrolysis of casein, hypoxanthine, starch, xanthine, tyrosine and Tweens 20, 40, 60 and 80 was examined on MA using substrate concentrations described previously (Cowan & Steel, 1965). Nitrate reduction and hydrolysis of aesculin, gelatin and urea were assessed as described by Lányi (1987) with the modification that artificial seawater [containing (per litre distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5.94 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.3 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; Bruns *et al.*, 2001] was used for preparation of media. The presence of flexirubin-type pigments was investigated as described by Reichenbach (1992). Cellulase and xylanase activities were assessed by staining with a 0.1% (w/v) Congo red aqueous solution after incubation on MA supplemented with 0.5% (w/v) CM-cellulose (Sigma) or xylan (Sigma), respectively. Utilization of various substrates as sole carbon and energy sources 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). Acid production from carbohydrates was assessed as described by Leifson (1963). Susceptibility to antibiotics was tested on MA plates using antibiotic discs containing the following (in μg except where indicated): 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 assessed and additional biochemical tests were performed by using the API ZYM and API 20E systems (bioMérieux) incubated at 37 °C according to the manufacturer's instructions. Morphological, cultural, physiological and biochemical properties of strain L-4^T are given in the species description and in Table 1.

Cell biomass for DNA extraction and for the analyses of isoprenoid quinones and polar lipids was obtained from cultures grown for 3 days in MB at 37 °C. 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 by RNA. 16S rRNA gene amplification was performed using two universal primers as described previously (Yoon *et al.*, 1998) and the PCR products were purified by using a QIAquick PCR purification kit (Qiagen). 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 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

Table 1. Differential phenotypic characteristics of strain L-4^T and *O. marilimosa* CIP 108537^T

Strains: 1, *Olleya aquimaris* sp. nov. L-4^T; 2, *O. marilimosa* CIP 108537^T. All data are from this study except for growth at 37 °C, anaerobic growth and spore formation for *O. marilimosa*; these data are from Mancuso Nichols *et al.* (2005). +, Positive; w, weakly positive; –, negative. Both strains are positive for: gliding motility; hydrolysis of gelatin and Tweens 20, 40, 60 and 80; acid production from maltose and sucrose; susceptibility to carbenicillin, chloramphenicol, lincomycin, novobiocin and oleandomycin; and catalase, oxidase, alkaline phosphatase, esterase lipase (C8), leucine arylamidase, cystine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities. Both strains are negative for: anaerobic growth; Gram-staining; spore formation; production of flexirubin-type pigments; nitrate reduction; hydrolysis of agar, hypoxanthine and xanthine; production of acetoin, H_2S and indole; utilization of cellobiose, D-fructose, D-galactose, sucrose, trehalose, D-xylose, acetate, benzoate, formate, L-malate, pyruvate, succinate, salicin and L-glutamate; acid production from L-arabinose, cellobiose, D-fructose, D-galactose, myo-inositol, lactose, D-mannitol, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, D-sorbitol and D-xylose; susceptibility to ampicillin, gentamicin, kanamycin, neomycin, penicillin G, polymyxin B, streptomycin and tetracycline; and cellulase (CM-cellulose), xylanase, urease, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, lipase (C14), trypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, α -mannosidase and α -fucosidase activities.

Characteristic	1	2
Growth at 37 °C	+	–
Hydrolysis of:		
Starch	+	–
L-Tyrosine	–	+
Aesculin	+	+*
Casein	+	+*
Utilization of:		
L-Arabinose	+	–
D-Mannose	–	+
Citrate	–	w
D-Glucose	–	–†
Maltose	–	–†
Acid production from:		
D-Glucose	–	+
D-Mannose	–	w
Trehalose	+	–
Susceptibility to cephalothin	+	–
Activity of (API ZYM):		
Esterase (C4)	+	–
Valine arylamidase	–	+
α -Chymotrypsin	+	–
N-Acetyl- β -glucosaminidase	–	–†
Major polar lipids‡	1 AL, 2 L	1 AL, 1 L
DNA G + C content (mol%)	35	42§

*Reported as negative by Mancuso Nichols *et al.* (2005).

†Reported as positive by Mancuso Nichols *et al.* (2005).

‡AL, Unidentified aminolipid; L, unidentified lipid.

§Data from this study obtained by the HPLC method; value of 49 mol% (thermal denaturation method) reported for *O. marilimosa* CAM030^T by Mancuso Nichols *et al.* (2005).

spraying with the appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). For cellular fatty acid analysis, cell mass of strain L-4^T and *O. marilimosa* CIP 108537^T was harvested from MA plates after cultivation for 3 days at 30 °C. Fatty acids were extracted and fatty acid methyl esters were 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 the modification that DNA was hydrolysed using nuclease P1 (Sigma) and the resultant nucleotides were analysed by reversed-phase HPLC. DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using 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 are quoted as DNA–DNA relatedness values.

The almost-complete 16S rRNA gene sequence of strain L-4^T determined in this study comprised 1443 bp, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. In the phylogenetic tree constructed using the neighbour-joining algorithm, strain L-4^T joined *O. marilimosa* CAM030^T, a member of the family *Flavobacteriaceae*, with a bootstrap confidence value of 98.5 % (Fig. 1). The relationship between strain L-4^T and *O. marilimosa* CAM030^T was also found in phylogenetic trees constructed using the maximum-likelihood and maximum-parsimony algorithms. Strain L-4^T exhibited

97.2 % 16S rRNA gene sequence similarity to *O. marilimosa* CAM030^T, 94.4–95.8 % to the type strains of *Lacinutrix* species and less than 95.4 % to strains of other species used in the phylogenetic analysis.

The predominant isoprenoid quinone of strain L-4^T and *O. marilimosa* CIP 108537^T was MK-6, which is in line with all other members of the family *Flavobacteriaceae* (Bernardet & Nakagawa, 2006). The cellular fatty acid profile of strain L-4^T is shown in Table 2, together with that of *O. marilimosa* CIP 108537^T, which was also analysed in this study. The fatty acid profiles of the two strains were similar, although differences were observed in the proportions of some fatty acids (Table 2). The polar lipid profiles of the two strains were also similar. The major polar lipids of both strains were an unidentified aminolipid and one (*O. marilimosa* CIP 108537^T) or two (strain L-4^T) unidentified lipids. The DNA G+C content of strain L-4^T was 35 mol%, a value that was distinctly lower than that reported for *O. marilimosa* (Mancuso Nichols *et al.*, 2005).

The mean DNA–DNA relatedness between strain L-4^T and *O. marilimosa* CIP 108537^T was 7 %. Strain L-4^T could also be differentiated from *O. marilimosa* by differences in several phenotypic characteristics, including growth at 37 °C, hydrolysis of starch and L-tyrosine, utilization of and acid production from several substrates, activity of various enzymes and susceptibility to cephalothin (Table 1). The phylogenetic and genetic distinctiveness and differential phenotypic properties of strain L-4^T are sufficient to support its classification within a novel species of the genus *Olleya*, for which the name *Olleya aquimaris* sp. nov. is proposed.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of *Olleya aquimaris* sp. nov. L-4^T, *O. marilimosa* CAM030^T and related taxa. Bootstrap values (expressed as percentages of 1000 replications) >50 % are shown at branching points. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Lutibacter litoralis* CL-TF09^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Table 2. Cellular fatty acid compositions (%) of strain L-4^T and *O. marilimosa* CIP 108537^T

Strains: 1, *O. aquimaris* L-4^T; 2, *O. marilimosa* CIP 108537^T. Data are from this study. Fatty acids that represent <1.0 % in both strains were omitted. tr, Trace (<1 %); –, not detected.

Fatty acid	1	2
Straight-chain		
C _{15:0}	5.2	9.1
Unsaturated		
C _{15:1} ω6c	–	2.4
Branched		
iso-C _{14:0}	1.9	tr
iso-C _{15:0}	17.2	16.9
iso-C _{15:1}	15.7	15.0
anteiso-C _{15:0}	4.7	2.9
anteiso-C _{15:1}	1.7	1.4
iso-C _{16:0}	3.7	tr
iso-C _{16:1}	1.3	–
iso-C _{16:1}	–	1.1
Hydroxy		
C _{15:0} 2-OH	1.1	1.1
C _{15:0} 3-OH	tr	2.2
iso-C _{15:0} 3-OH	5.6	12.1
C _{16:0} 3-OH	tr	1.2
iso-C _{16:0} 3-OH	13.7	6.2
C _{17:0} 2-OH	2.0	1.2
iso-C _{17:0} 3-OH	12.0	9.9
Summed feature 3*	8.3	9.9

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

Emended description of the genus *Olleya* Mancuso Nichols *et al.* 2005

The description of the genus *Olleya* is as given by Mancuso Nichols *et al.* (2005) with the following amendments. The predominant menaquinone is MK-6. Common major polar lipids are an unidentified aminolipid and an unidentified lipid. The DNA G+C content is 35–42 mol% (by HPLC).

Description of *Olleya aquimaris* sp. nov.

Olleya aquimaris (a.qui.ma'ris. L. n. *aqua* water; L. gen. n. *maris* of the sea; N.L. gen. n. *aquimaris* of seawater).

Cells are Gram-stain-negative, non-flagellated, non-endo-spore-forming and rod-shaped (0.5–0.6 µm in diameter and 0.6–3.5 µm long). Motile by gliding. Strictly aerobic. Flexirubin-type pigments are not produced. Colonies on MA are circular, raised to slightly convex, glistening, smooth, yellow and 0.8–2.2 mm in diameter after incubation for 3 days at 37 °C. Optimal growth at 37 °C; growth occurs at 4 and 40 °C, but not at 45 °C. Optimal pH for

growth is between 6.5 and 7.0; growth occurs at pH 6.0, but not at pH 5.5. Growth occurs in the presence of 0.5–7.0 % (w/v) NaCl with optimum growth at approximately 2.0 % (w/v) NaCl. Mg²⁺ ions are required for growth. Catalase- and oxidase-positive. Nitrate is not reduced to nitrite. Aesculin, casein, gelatin, starch and Tweens 20, 40, 60 and 80 are hydrolysed, but CM-cellulose, hypoxanthine, L-tyrosine, urea, xanthine and xylan are not. Acetoin, H₂S and indole are not produced. The predominant menaquinone is MK-6. The major polar lipids are an unidentified aminolipid and two unidentified lipids. The major cellular fatty acids (>10 % of the total fatty acids) are iso-C_{15:0}, iso-C_{15:1}, iso-C_{16:0} 3-OH and iso-C_{17:0} 3-OH. Other phenotypic characteristics are given in Table 1.

The type strain is L-4^T (=KCTC 22661^T =CCUG 58074^T), isolated from seawater of Baekdo harbour in the East Sea, Korea. The DNA G+C content of the type strain is 35 mol%.

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