Muricauda olearia sp. nov., isolated from crude-oil-contaminated seawater, and emended description of the genus *Muricauda*

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A Gram-staining-negative, rod-shaped and non-motile bacterium, designated CL-SS4^T, was isolated from crude-oil-contaminated seawater from the west coast of Korea and was investigated by means of a polyphasic taxonomic approach. Strain CL-SS4^T grew optimally at 25–30 °C, at pH 6.8–7.7 and in the presence of 2–3 % sea salts. The major fatty acids were iso-C_{17:0} 3-OH, iso-C_{15:0} and iso-C_{15:1} G. The major isoprenoid quinone was MK-6. The DNA G + C content was 50.7 mol%. Analysis of the 16S rRNA gene sequence of strain CL-SS4^T revealed that it was a member of the genus *Muricauda*, sharing 95.7–96.9 % sequence similarity with the type strains of *Muricauda* species and being most closely related to *Muricauda aquimarina* SW-63^T. Phylogenetic analyses based on 16S rRNA gene sequences confirmed that strain CL-SS4^T formed a deep lineage related to the genus *Muricauda*. Strain CL-SS4^T was distinguishable from recognized *Muricauda* species on the basis of differential phenotypic characteristics and DNA G + C content. Based on these results, it is proposed that strain CL-SS4^T represents a novel species of the genus *Muricauda*, for which the name *Muricauda olearia* sp. nov. is proposed. An emended description of the genus *Muricauda* is also proposed. The type strain is CL-SS4^T (=KCCM 90075^T=JCM 15563^T).

The genus *Muricauda*, a member of the family *Flavobacteriaceae*, was established by Bruns *et al.* (2001) with *Muricauda ruestringensis* as the type species. Some cells of this non-motile bacterium, which was isolated from intertidal sediment, displayed long and relatively thick appendages. Three other *Muricauda* species with similar appendages were subsequently isolated from a salt lake (*Muricauda flavescens* and *Muricauda aquimarina*; Yoon *et al.*, 2005) and tidal flat sediment (*Muricauda lutimaris*; Yoon *et al.*, 2008). In this study, a marine bacterium isolated from crude-oil-contaminated seawater was subjected to a polyphasic taxonomic analysis and allocated to the genus *Muricauda*.

Crude-oil-contaminated seawater was collected from the west coast of Korea. An aliquot (50 µl) of seawater was spread on marine agar 2216 (MA; Difco) and incubated under aerobic conditions at 30 °C for a week. Strain

CL-SS4^T was isolated by streaking on fresh MA and subsequent incubation. The purification procedure was repeated four times. The strain was maintained on MA at 30 °C and preserved in marine broth 2216 (MB; Difco) supplemented with 30 % (v/v) glycerol at –80 °C.

For 16S rRNA gene amplification by PCR, DNA was extracted from a single colony by using a boiling method (Englen & Kelley, 2000). The crude extract served as the DNA template for amplification, which included *Taq* DNA polymerase (Promega) and primers 27F and 1492R (Lane, 1991). The PCR product was purified by using an AccuPrep PCR Purification kit (Bioneer) and direct sequence determination of the purified 16S rRNA gene was performed with an Applied Biosystems automated sequencer (ABI 3730XL) at Macrogen, Seoul. The almost-complete 16S rRNA gene sequence of strain CL-SS4^T (1417 bp) was obtained and compared with available 16S rRNA gene sequences in GenBank using BLASTN searches (Altschul *et al.*, 1990). The sequence of strain CL-SS4^T was manually aligned with those of related species in the family

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CL-SS4^T is EU888117.

Flavobacteriaceae, obtained from the GenBank and Ribosomal Database Project II (Cole *et al.*, 2007) databases, using known 16S rRNA secondary-structure information. Phylogenetic trees were obtained by using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. An evolutionary distance matrix for the neighbour-joining method was generated according to the model of Jukes & Cantor (1969). The robustness of tree topologies was assessed by bootstrap analyses based on 1000 replications for the neighbour-joining and maximum-parsimony methods and 100 replications for the maximum-likelihood method. Alignment analysis was carried out using the jPHYDIT program (Jeon *et al.*, 2005), and phylogenetic analyses were carried out using MEGA4 (Tamura *et al.*, 2007) and PAUP 4.0 (Swofford, 1998). Likelihood parameters were estimated by using the hierarchical ratio test in MODELTEST, version 3.04 (Posada & Crandall, 1998).

Phylogenetic analyses based on the 16S rRNA gene sequence showed that strain CL-SS4^T belonged to the genus *Muricauda* in the family *Flavobacteriaceae* (Fig. 1). Strain CL-SS4^T was most closely related to *M. aquimarina* SW-63^T (96.9% sequence similarity) and to other members of the genus *Muricauda* (95.7–96.5% sequence similarity). The tree topologies inferred from the three tree-making algorithms showed that strain CL-SS4^T formed a coherent lineage with the clade comprising recognized *Muricauda* species (Fig. 1). The relationship was supported by high bootstrap values (neighbour-joining, 100%; maximum-parsimony, 98%; and maximum-likelihood, 89%). The low similarity value of less than 97% (Rosselló-Mora & Amann, 2001) and the

distinct phylogenetic position of strain CL-SS4^T indicated that the isolate represents a novel species of the genus *Muricauda*.

Morphological and physiological tests were performed as follows. Unless otherwise specified, all biochemical characteristics of strain CL-SS4^T and *M. aquimarina* KCCM 41646^T were based on cultures grown on MA at 30 °C. Except for ranges of temperature, pH and salinity for growth, fatty acids composition and genomic DNA G + C content, the other tests were performed with *M. aquimarina* KCCM 41646^T as the reference strain. Gram-staining was performed as described by Smibert & Krieg (1994). Flagellar and gliding motilities were assessed by using the hanging-drop method (Skerman, 1967) with cells grown in MB for 2 days. Cellular morphology and the presence of flagella and appendages were observed using transmission electron microscopy (EX2; JEOL). The presence of endospores was assessed by malachite green staining (Smibert & Krieg, 1994). The presence of flexirubin-type pigments was assessed as described by Gosink *et al.* (1998). Anaerobic growth was checked on MA, MA supplemented with potassium nitrate (0.1%, w/v) or ZOF medium (Lemos *et al.*, 1985) supplemented with agar (1.5%) incubated in the GasPak anaerobic system (BBL) at 30 °C for 10 days. The temperature range for growth was examined on the basis of colony formation on MA incubated at temperatures ranging from 5 to 45 °C, with increments of 5 °C. The pH range for growth was determined by assessing changes in OD₆₀₀ over the incubation period (up to 7 days) in MB at 30 °C. Prior to autoclaving, the pH was adjusted to pH 4–10 with increments of 0.5 pH unit using 1 M NaOH and 1 M HCl. The pH was measured after autoclaving and cooling and

Fig. 1. Neighbour-joining phylogenetic tree derived from the 16S rRNA gene sequences of strain CL-SS4^T and related members in the family *Flavobacteriaceae*. The sequence of *Bacteroides fragilis* ATCC 25285^T (X83935) was used as an outgroup. Bootstrap values from 1000 resamplings above 50% are shown at branch points. Solid circles indicate that the corresponding nodes were also recovered in the maximum-parsimony and the maximum-likelihood trees. Bar, 0.02 nucleotide substitution per site.

the following pH values were used to measure the ability to grow: pH 4.1, 4.7, 5.2, 5.9, 6.4, 6.8, 7.2, 7.7, 8.1, 8.5, 8.9, 9.4 and 9.9. The tolerance of strain CL-SS4^T to sea salts (Sigma) or NaCl was determined by assessing changes in OD₆₀₀ at 30 °C in ZoBell broth (1⁻¹: 5 g Bacto peptone, 1 g yeast extract, 0.1 g ferric citrate) supplemented with 0–10 % (with increments of 1 %) and 12 and 15 % (w/v) of sea salts or NaCl.

The tests for oxidase and catalase activities and hydrolysis of casein, hypoxanthine, tyrosine and xanthine were performed according to Smibert & Krieg (1994). Nitrate reduction and hydrolysis of gelatin, Tweens 20, 40, 60 and 80 and urea were tested according to Hansen & Sørheim (1991). H₂S production was tested as described by Bruns *et al.* (2001). In addition, other enzyme activities were assayed using the API ZYM kit (bioMérieux) according to the manufacturer's instructions, except that the cell suspension was prepared using artificial seawater (1⁻¹: 24 g NaCl, 10.9 g MgCl₂·6H₂O, 4 g Na₂SO₄, 1.5 g CaCl₂·2H₂O, 0.7 g KCl, 0.2 g NaHCO₃, 0.1 g KBr, 0.027 g H₃BO₃, 0.03 g SrCl₂·6H₂O, 0.003 g NaF; Lyman & Fleming, 1940). Acid production from carbohydrates was tested as described by Lemos *et al.* (1985). Carbon utilization was tested using a basal broth medium supplemented with yeast extract (Bruns *et al.*, 2001) containing 0.2 % carbon source. A negative control without a carbon source was also employed and did not show any growth. Growth was monitored by OD₆₀₀ using a spectrophotometer (Ultraspec 2000; Pharmacia Biotech) for 15 days. Resistance to antibiotics was determined on MA incubated at 30 °C for 7 days by using the disc-diffusion plate method (Bauer *et al.*, 1966).

The phenotypic characteristics of strain CL-SS4^T are given in the species description and Table 1. The following phenotypic characteristics were unique to strain CL-SS4^T: inability to produce α -glucosidase and to utilize fructose and mannose as sole carbon source, and ability to utilize glutamate and pyruvate as sole carbon source and to produce acid from D-ribose (Table 1). Strain CL-SS4^T differed from its closest phylogenetic neighbour *M. aquimarina* by hydrolysis of gelatin, α -chymotrypsin, β -glucosidase and trypsin activities and utilization of carbon substrates (Table 1).

The fatty acid methyl esters in whole cells of strain CL-SS4^T grown on MA at 30 °C for 3 days were analysed by gas chromatography according to the instructions of the Microbial Identification System at KCCM in Seoul. DNA was extracted by using the method of Marmur (1961). The genomic DNA G+C content was analysed by HPLC (HP 100; Hewlett Packard) and analysis of deoxyribonucleosides was performed as described by Mesbah *et al.* (1989). Lambda phage DNA was used as a standard. Isoprenoid quinone composition was determined according to Minnikin *et al.* (1984) and analysed by HPLC as described by Collins (1985), using *M. aquimarina* KCCM 41646^T (Yoon *et al.*, 2005) as the reference strain.

The major fatty acids of strain CL-SS4^T were iso-C_{17:0} 3-OH (20.5 %), iso-C_{15:0} (18.7 %) and iso-C_{15:1} G (17.1 %), and its detailed fatty acid composition is shown in Table 2. The fatty acid profile of strain CL-SS4^T was similar to those of type strains of the four recognized *Muricauda* species grown under the same conditions though in slightly different proportions (Table 2). The major menaquinone of strain CL-SS4^T was menaquinone-6 (MK-6) in line with the other *Muricauda* species (Yoon *et al.*, 2005, 2008). These chemotaxonomic data supported the attribution of strain CL-SS4^T to the genus *Muricauda* (Bruns *et al.*, 2001; Yoon *et al.*, 2005, 2008). The DNA G+C content of strain CL-SS4^T was 50.7 mol%, a value significantly higher than the range reported for *Muricauda* species (Table 1).

Overall, the phenotypic data and phylogenetic inference obtained in this study indicate that strain CL-SS4^T should be assigned as representing a novel species of the genus *Muricauda*, for which the name *Muricauda olearia* sp. nov. is proposed. An emended description of the genus *Muricauda* is also proposed.

Emended description of the genus *Muricauda* Bruns *et al.* 2001, emend. Yoon *et al.* 2005

The description is as given by Bruns *et al.* (2001) and Yoon *et al.* (2005) with the following modifications. The DNA G+C content is 41–51 mol%. The major isoprenoid quinone is MK-6.

Description of *Muricauda olearia* sp. nov.

Muricauda olearia (o.le.a'ri.a. L. fem. adj. *olearia* pertaining to oil, referring to its source of isolation).

Gram-staining-negative, strictly aerobic, non-motile and non-spore-forming rods approximately 0.3–0.4 μ m wide and 1.6–4.4 μ m long. Appendages similar to those reported in other *Muricauda* species occur on some cells. After 3 days on MA at 30 °C, colonies are yellow, circular and convex, and approximately 1 mm in diameter. Grows at 15–40 °C (optimum, 25–30 °C) and at pH 5.2–9.4 (optimum, pH 6.8–7.7). Growth occurs at 1–6 % (w/v) sea-salt concentration (optimum, 2–3 %), but no growth occurs in media containing only NaCl. Flexirubin-type pigments are not produced. Positive for oxidase and catalase. Gelatin, tyrosine and Tweens 20, 40, 60 and 80 are hydrolysed, but casein, hypoxanthine, urea and xanthine are not. Negative for H₂S production and nitrate reductase. According to the API ZYM test, *N*-acetyl- β -glucosaminidase, acid and alkaline phosphatases, cystine arylamidase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase activities are present, but α -chymotrypsin, α -fucosidase, α - and β -galactosidases, α - and β -glucosidases, β -glucuronidase, lipase (C14), α -mannosidase and trypsin activities are absent. L-Arginine, cellobiose, glucose, glutamate, lactose, pyruvate, raffinose and sucrose are utilized as sole carbon sources, but acetate, alanine, benzoate, citrate, ethanol,

Table 1. Differential characteristics of strain CL-SS4^T and other *Muricauda* species

Strains: 1, *Muricauda olearia* sp. nov. CL-SS4^T (data from this study); 2, *M. aquimarina* SW-63^T (Yoon *et al.*, 2005, 2008 and this study); 3, *M. lutimaris* SMK-108^T (Yoon *et al.*, 2008); 4, *M. ruestringensis* B1^T (Bruns *et al.*, 2001; Yoon *et al.*, 2005); 5, *M. flavescens* SW-62^T (Yoon *et al.*, 2005). All strains are positive for the following characteristics: presence of oxidase, *N*-acetyl- β -glucosaminidase, alkaline phosphatase, cystine arylamidase, esterase lipase (C8), leucine arylamidase and valine arylamidase activities; hydrolysis of Tween 20 and tyrosine; utilization of cellobiose and sucrose; and acid production from cellobiose, D-fructose, D-glucose, lactose, D-mannose, raffinose, sucrose and trehalose. All strains are negative for the following characteristics: growth without NaCl and at 4 °C; nitrate reduction and H₂S production; presence of α -fucosidase, α - and β -galactosidases, β -glucuronidase, lipase (C14) and α -mannosidase activities; hydrolysis of hypoxanthine, urea and xanthine; utilization of acetate, benzoate, citrate, ethanol, formate, mannitol, methanol and L-serine; and acid production from *myo*-inositol and D-mannitol. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3	4	5
Facultative anaerobic growth	–	–	+	+	–
Optimal growth temperature (°C)	25–30	30–37*	30	20–30	30–37
Catalase activity	+	+	+	–†	+
Hydrolysis of:					
Casein	–	–	+	–	–
Gelatin	+	–	+	–	–
Tweens 40, 60 and 80	+	+	–	+	+
Enzyme activity (API ZYM)					
Acid phosphatase	+	+	+	+	–
α -Chymotrypsin	–	+	+	–	+
Esterase (C4)	+	+	+	–	+
α -Glucosidase	–	+	+	+	+
β -Glucosidase	–	+	+	+	–
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	–
Trypsin	–	+	–	–	w
Utilization of:					
Alanine	–	–	–	+	–
L-Arginine	w	–	–	+	–
Fructose	–	+	+	+	+
Glucose	+	+	+	–	+
Glutamate	+	–	–	–	–
Lactose	+	+	–	+	+
Mannose	–	+	+	+	+
Pyruvate	+	–	–	–	–
Raffinose	+	+	–	+	+
Succinate	–	+	–	+	+
Acid production from:					
L-Arabinose	–	–	+	+	–
D-Galactose	–	–	+	+	–
D-Ribose	+	–	–	–	–
D-Xylose	–	–	–	+	–
DNA G + C content (mol%)	50.7	44.1*	41.1	41	45.2

*Data from Yoon *et al.* (2005). For the other tests, congruent results were obtained from this study with Yoon *et al.* (2005, 2008).

†Negative in Bruns *et al.* (2001) but positive in Yoon *et al.* (2005).

formate, fructose, mannitol, mannose, methanol, L-serine and succinate are not utilized. Acid is produced from cellobiose, D-fructose, D-glucose, lactose, D-mannose, raffinose, D-ribose, sucrose and trehalose, but not from L-arabinose, D-galactose, *myo*-inositol, D-mannitol or D-xylose. Sensitive to (μ g per disc) ampicillin (6), cephalixin (20), gentamicin (6), kanamycin (20), mitomycin C (0.6), penicillin G (6), polymyxin B (25), streptomycin (6) and

vancomycin (20), but resistant to chloramphenicol (20), erythromycin (10), nalidixic acid (20) and tetracycline (20). Major cellular fatty acids are iso-C_{17:0} 3-OH, iso-C_{15:0} and iso-C_{15:1} G. The DNA G + C content of the type strain is 50.7 mol%.

The type strain, CL-SS4^T (=KCCM 90075^T=JCM 15563^T), was isolated from crude-oil-contaminated seawater from the west coast of Korea.

Table 2. Cellular fatty acid composition (%) of strain CL-SS4^T and recognized *Muricauda* species

Strains: 1, *Muricauda olearia* sp. nov. CL-SS4^T (data from this study); 2, *M. aquimarina* SW-63^T (Yoon *et al.*, 2005); 3, *M. lutimaris* SMK-108^T (Yoon *et al.*, 2008); 4, *M. ruestringensis* DSM 13258^T (Yoon *et al.*, 2005); 5, *M. flavescens* SW-62^T (Yoon *et al.*, 2005). Fatty acids representing less than 0.5% in all strains were omitted. All strains were cultivated on MA at 30 °C for 3 days. ECL, Equivalent chain-length; tr, traces (<0.5%); –, not detected/not reported.

Fatty acid	1	2	3	4	5
Straight-chain					
C _{15:0}	10.7	5.9	7.6	13.2	12.4
C _{16:0}	0.5	tr	tr	tr	0.6
Branched					
iso-C _{14:0}	–	0.6	–	–	–
iso-C _{15:0}	18.7	23.7	14.5	14.7	16.4
anteiso-C _{15:0}	1.4	2.0	1.4	1.1	2.1
iso-C _{15:1} G	17.1	21.6	21.3	20.5	19.9
iso-C _{16:0}	tr	0.5	tr	–	0.6
iso-C _{17:1} ω9c	3.6	1.5	1.8	1.4	1.3
Unsaturated					
C _{15:1} ω6c	1.4	0.9	0.6	0.9	0.9
C _{17:1} ω6c	1.6	0.5	0.9	1.0	0.5
C _{17:1} ω8c	0.8	–	0.5	0.7	tr
Hydroxy					
C _{15:0} 2-OH	0.7	tr	tr	0.5	0.6
C _{15:0} 3-OH	1.9	1.4	2.6	1.8	1.0
iso-C _{15:0} 3-OH	4.4	5.2	7.8	4.6	4.7
C _{16:0} 3-OH	0.9	0.6	0.5	tr	0.8
iso-C _{16:0} 3-OH	1.4	4.0	4.0	1.7	2.9
C _{17:0} 2-OH	0.6	0.7	1.4	0.7	1.3
C _{17:0} 3-OH	1.2	tr	0.8	1.3	0.7
iso-C _{17:0} 3-OH	20.5	17.3	24.6	20.9	19.9
Summed feature 3*	7.1	2.3	3.3	4.2	4.1
Unknown					
ECL 11.543	–	0.8	tr	0.6	–
ECL 13.565	1.4	4.8	1.7	6.5	5.4
ECL 16.582	1.5	1.3	1.9	1.7	1.6

*Summed feature 3 contains C_{16:1} ω7c and/or iso-C_{15:0} 2-OH.

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