Muricauda lutaonensis sp. nov., a moderate thermophile isolated from a coastal hot spring

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A yellow-pigmented, Gram-staining-negative, aerobic, non-motile, moderately thermophilic, rod-shaped bacterium, designated strain CC-HSB-11^T, was isolated from a coastal hot spring of Green Island (Lutao), located off Taituang, Taiwan. 16S rRNA gene sequence analysis demonstrated that it shared <94.4 % sequence similarity with *Muricauda* species. Menaquinone with six isoprene units (MK-6) was the major respiratory quinone and iso-C_{15:0}, iso-C_{15:1} G, iso-C_{15:0} 3-OH, iso-C_{16:0} 3-OH, iso-C_{17:0} 3-OH and summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1} ω7c/t) were the predominant fatty acids. The predominant polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. Six unidentified phospholipids and glycolipids also occurred as minor components. The DNA G + C content of strain CC-HSB-11^T was 46.4 ± 1 mol%. On the basis of 16S rRNA gene sequence similarities with other *Muricauda* species and differentiating fatty acid compositions and other phenotypic data, strain CC-HSB-11^T represents a novel species in the genus *Muricauda*, for which the name *Muricauda lutaonensis* sp. nov. is proposed. The type strain is CC-HSB-11^T (=BCRC 17850^T =KCTC 22339^T).

During investigations of the biodiversity of marine bacteria from water samples collected at a coastal hot spring (water temperature 55 °C) located on a volcanic island [Lutao (Green Island), 22° 40' N 121° 27' E] in the Pacific Ocean, off the east coast of Taiwan, strain CC-HSB-11^T was isolated on marine agar 2216 (MA; Difco) after incubation at 37 °C for 48 h. Subcultivation was done on MA at 37 °C for 2 days. The strain was preserved at −80 °C in marine broth 2216 (MB; Difco) with 20 % (v/v) glycerol or by lyophilization.

The 16S rRNA gene sequence of strain CC-HSB-11^T was determined and analysed as described previously (Young *et al.*, 2005). Analysis was performed by using the software package MEGA version 2.1 (Kumar *et al.*, 2001), after multiple data alignments with CLUSTAL X (Thompson *et al.*, 1997). A distance-matrix method (distance options according to Kimura's two-parameter model), including clustering by neighbour joining (Saitou & Nei, 1987) (Fig. 1), and a discrete character-based maximum-parsimony method (Kluge & Farris, 1969) were used. In each

case, bootstrap values were calculated based on 1000 replications. The 16S rRNA gene sequence of strain CC-HSB-11^T was a continuous stretch of 1438 bp. Sequence similarity calculations indicated that strain CC-HSB-11^T showed the greatest degree of similarity to *Muricauda aquimarina* SW-63^T (94.4 %), *M. lutimaris* SMK-108^T (94.3 %), *M. ruestringensis* B1^T (94.1 %) and *M. flavescens* SW-62^T (93.1 %). Lower sequence similarities (<92 %) were found with all other representatives of the family *Flavobacteriaceae* shown in Fig. 1. Strain CC-HSB-11^T formed a distinct branch supported by a high bootstrap percentage in the neighbour-joining (Fig. 1) and maximum-parsimony (data not shown) trees. DNA–DNA relatedness experiments were not carried out between strain CC-HSB-11^T and its closest phylogenetic neighbours in the genus *Muricauda*, as the 16S rRNA gene sequence similarities between strains were less than 95 % (Stackebrandt & Goebel, 1994).

Gram staining was performed as described by Gerhardt *et al.* (1994) and poly-β-hydroxybutyrate granules were sought by staining the cells with Sudan black. Cell morphology was observed by light microscopy (×1000, model A3000; Zeiss) and transmission electron microscopy (1200 EX; JEOL) using cells grown for 3 days at 37 °C on MA. The latter technique was also used to search for

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CC-HSB-11^T is EU564844.

The polar lipid profile of strain CC-HSB-11^T is available as supplementary material with the online version of this paper.

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain CC-HSB-11^T and representatives of the family Flavobacteriaceae. Bootstrap values based on 1000 replications are shown at branch nodes. The sequence of *Actibacter sediminis* JC2129^T was used as the outgroup. Bar, 0.01 substitutions per nucleotide position.

flagella or the kind of appendages reported for members of the genus *Muricauda* (Bruns *et al.*, 2001; Yoon *et al.*, 2005, 2008). Cells from exponentially growing cultures were negatively stained with 2 % (w/v) uranyl acetate and the grids were examined after being air dried. Gliding motility was investigated by using phase-contrast microscopy of a hanging-drop preparation from an MB culture (Bernardet *et al.*, 2002).

The pH range for growth was determined in MB that was adjusted prior to sterilization to pH 3–11 (in 0.5 pH unit intervals) using appropriate biological buffers (Chung *et al.*, 1995). Verification of the pH after autoclaving revealed only minor changes. Growth in MB at 10, 15, 20, 25, 30 and 35 °C and at 35–60 °C in 2 °C intervals was measured after 3 days of incubation. Anaerobic growth was assessed in MB and on MA supplemented with potassium nitrate (5 mM) incubated in an Oxoid AnaeroGen system (Miller *et al.*, 1995). Growth with 1–10 % (w/v) NaCl in 1 % increments was investigated in MB. The requirement of strain CC-HSB-11^T for natural seawater and artificial sea salts was evaluated on R2A agar (Difco) and in tryptic soy broth (Difco) with and without the addition of 60 % (v/v) seawater or 0, 1, 2, 3, 4, 5, 7 and 10 % (w/v) artificial sea salts (Sigma) (Lee, 2007). Growth with NaCl as the only salt was studied on R2A and trypticase soy agars supplemented with 0–9 % (w/v) NaCl. Growth under these different conditions was recorded by measuring the OD₅₉₅ of the broth cultures with time.

The presence of flexirubin-type pigments was investigated as described by Reichenbach (1992) and Bernardet *et al.* (2002). Catalase and oxidase activities and hydrolysis of casein, starch and Tweens 20, 40, 60 and 80 were assessed as described by Cowan & Steel (1965). Hydrolysis of aesculin, gelatin and urea and reduction of nitrate were investigated as described by Lányi (1987) with the modification that artificial seawater was used for the preparation of media. The artificial seawater contained (per litre distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O and 1.3 g CaCl₂·2H₂O (Bruns *et al.*, 2001). H₂S production was tested as described by Bruns *et al.* (2001). Acid production from carbohydrates was determined as described by Leifson (1963). Other biochemical characteristics were

investigated with the GN-II system (Biolog), API ZYM, API 20E, API 20NE and ATB PSE (bioMérieux) systems. Morphological features, especially the presence of appendages (Fig. 2) and other phenotypic characteristics of strain CC-HSB-11^T, are listed in the genus and species descriptions and in Table 1.

Respiratory quinones of strain CC-HSB-11^T were extracted and separated as described by Minnikin *et al.* (1984) and analysed by HPLC as described by Collins & Jones (1980). Menaquinone with six isoprene units (MK-6) was the predominant respiratory quinone (>91 %). The presence of MK-6 as the major respiratory quinone is in line with all members of the genus *Muricauda*.

Polar lipids were extracted and analysed by two-dimensional TLC according to Embley & Wait (1994). The predominant polar lipids of strain CC-HSB-11^T were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. Three unidentified phospholipids and three unidentified glycolipids were present in minor amounts (Supplementary Fig. S1, available in IJSEM online).

Fig. 2. Transmission electron micrograph showing appendages (arrow) found on a few cells of strain CC-HSB-11^T. Bar, 1 μm.

Table 1. Comparison of phenotypic characteristics of strain CC-HSB-11^T and type strains of recognized species in the genus *Muricauda*

Strains: 1, *Muricauda lutaonensis* sp. nov. CC-HSB-11^T; 2, *M. lutimaris* SMK-108^T; 3, *M. ruestringensis* DSM 13258^T; 4, *M. flavescens* SW-62^T; 5, *M. aquimarina* SW-63^T. Data for reference type strains were taken from Yoon *et al.* (2008) (*M. lutimaris* SMK-108^T) and Yoon *et al.* (2005) (remaining strains). All strains are Gram-staining-negative, non-spore-forming rods. All strains are positive for oxidase, alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, α -glucosidase and *N*-acetyl- β -glucosaminidase activities, hydrolysis of aesculin, tyrosine and Tween 20, utilization of D-fructose, cellobiose, sucrose and D-mannose and acid production from D-fructose, melibiose, D-mannose, cellobiose, lactose, sucrose, maltose, trehalose and raffinose. All strains are negative for growth without NaCl and at 4 °C, nitrate reduction, H₂S production, presence of β -glucuronidase and α -fucosidase activities, hydrolysis of starch and urea, utilization of citrate, lactate, formate, L-serine and mannitol and acid production from D-sorbitol, *myo*-inositol, D-ribose, D-mannitol and L-rhamnose. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5
Catalase activity	+	+	–*	+	+
Facultative anaerobe	–	+	+	–	–
Temperature for growth (°C)					
Maximum	55	39	40	44	44
Optimum	37–45	30	20–30	30–37	30–37
Hydrolysis of:					
Casein	+	+	–	–	–
Gelatin	–	+	–	–	–
Tweens 40, 60 and 80	–	–	+	+	+
Enzyme activity (API ZYM)					
Esterase (C4)	+	+	–	+	+
Lipase (C14)	+	–	–	–	–
Trypsin	+	–	–	w	+
α -Chymotrypsin	–	+	–	+	+
Acid phosphatase	+	+	+	–	+
Naphthol-AS-BI-phosphohydrolase	+	+	+	–	+
α -Galactosidase	+	–	–	–	–
β -Galactosidase	+	–	–	–	–
β -Glucosidase	+	+	+	–	+
α -Mannosidase	+	–	–	–	–
Utilization of (Biolog GN-II):					
Glucose	+	+	–	+	+
Lactose	+	–	+	+	+
Raffinose	+	–	+	+	+
Malate	–	–	+	–	–
Succinate	+	–	+	+	+
Alanine	–	–	+	–	–
Arginine	–	–	+	–	–
Acetate	+	–	–	–	–
Glutamate	+	–	–	–	–
Acid production from:					
D-Glucose	–	+	+	+	+
D-Xylose	–	–	+	–	–
L-Arabinose	–	+	+	–	–
Melezitose	+	+	+	+	–
D-Galactose	–	+	+	–	–
DNA G + C content (mol%)	46.4	41.1	41	45.2–45.4†	44.1–44.2†

*Reported as negative by Bruns *et al.* (2001) but positive by Yoon *et al.* (2005).

†Range of values for two strains, including the type strain.

Fatty acid methyl esters were obtained from cells cultivated on MA for 3 days at 30 °C by saponification, methylation and extraction as described by Kämpfer & Kroppenstedt (1996) and separated by gas chromatography (model

5898A; Hewlett Packard). Peaks were automatically integrated and fatty acid names and percentages were determined using the Microbial Identification standard software package (Sasser, 1990). The detailed fatty acid

profile of strain CC-HSB-11^T is compared with those of the type strains of *Muricauda* species in Table 2.

For determination of the G+C content, DNA was prepared and degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture was then separated by HPLC. The DNA G+C content of strain CC-HSB-11^T was 46.4 mol%, a value

Table 2. Comparison of cellular fatty acid compositions of strain CC-HSB-11^T and type strains of recognized species in the genus *Muricauda*

Strains: 1, *M. lutaonensis* sp. nov. CC-HSB-11^T; 2, *M. lutimaris* SMK-108^T; 3, *M. ruestringensis* DSM 13258^T; 4, *M. flavescens* SW-62^T; 5, *M. aquimarina* SW-63^T. Data for reference type strains were taken from Yoon *et al.* (2008) (*M. lutimaris* SMK-108^T) and Yoon *et al.* (2005) (remaining strains). All strains were cultivated for 3 days on MA at 30 °C. Fatty acids representing less than 0.5% in all strains were omitted. ECL, Equivalent chain-length; tr, traces (<0.5%); –, not detected/not reported.

Fatty acid (%)	1	2	3	4	5
Straight-chain					
C _{15:0}	–	7.6	13.2	12.4	5.9
C _{16:0}	1.5	tr	tr	0.6	tr
Branched					
iso-C _{13:0}	2.0	–	–	–	–
iso-C _{14:0}	2.5	–	–	–	0.6
iso-C _{15:0}	23.9	14.5	14.7	16.4	23.7
iso-C _{15:1} G	18.2	21.3	20.5	19.9	21.6
anteiso-C _{15:0}	3.0	1.4	1.1	2.1	2.0
iso-C _{16:0}	1.4	tr	–	0.6	0.5
iso-C _{16:1} G	1.1	–	–	–	–
iso-C _{17:1} ω9c	–	1.8	1.4	1.3	1.5
Unsaturated					
C _{15:1} ω6c	–	0.6	0.9	0.9	0.9
C _{17:1} ω6c	–	0.9	1.0	0.5	0.5
C _{17:1} ω8c	–	0.5	0.7	tr	–
C _{18:1} ω9c	1.0	–	–	–	–
Hydroxy					
C _{15:0} 2-OH	0.7	tr	0.5	0.6	tr
C _{15:0} 3-OH	–	2.6	1.8	1.0	1.4
iso-C _{14:0} 3-OH	0.5	–	–	–	–
iso-C _{15:0} 3-OH	6.0	7.8	4.6	4.7	5.2
C _{16:0} 3-OH	0.6	0.5	tr	0.8	0.6
iso-C _{16:0} 3-OH	7.6	4.0	1.7	2.9	4.0
C _{17:0} 2-OH	1.5	1.4	0.7	1.3	0.7
C _{17:0} 3-OH	tr	0.8	1.3	0.7	tr
iso-C _{17:0} 3-OH	18.9	24.6	20.9	19.9	17.3
Summed feature 3*	7.1	3.3	4.2	4.1	2.3
Unknown					
ECL 11.543	–	tr	0.6	–	0.8
ECL 13.565	–	1.7	6.5	5.4	4.8
ECL 16.582	–	1.9	1.7	1.6	1.3

*Summed features represent two or three fatty acids that could not be separated by the Microbial Identification System. Summed feature 3 contained iso-C_{15:0} 2-OH and/or C_{16:1} ω7clt.

slightly above the range reported for *Muricauda* species (41–45.4 mol%).

16S rRNA gene sequence analysis showed that the closest phylogenetic neighbours of strain CC-HSB-11^T were members of the genus *Muricauda* in the family *Flavobacteriaceae*, although the level of sequence similarity was rather low. Moreover, strain CC-HSB-11^T could be differentiated from all members of the genus *Muricauda* by a combination of growth, morphological and physiological characteristics as well as by several differences in the fatty acid profiles.

On the basis of these results, strain CC-HSB-11^T represents a novel species in the genus *Muricauda*, for which the name *Muricauda lutaonensis* sp. nov. is proposed.

Description of *Muricauda lutaonensis* sp. nov.

Muricauda lutaonensis (lu.tao.nen'sis. N.L. fem. adj. *lutaonensis* pertaining to Lutao, the geographical origin of the type strain).

Cells are Gram-staining-negative, strictly aerobic, non-spore-forming rods, displaying appendages and vesicles. Cells are 0.3–0.5 µm wide and 8.0–15.0 µm long. Devoid of flagellar and gliding motility. Growth is visible after 24 h of incubation on MA at 37 °C. Colonies on MA are shiny, translucent, golden yellow and circular with regular, non-spreading edges. Growth does not occur on tryptic soy, nutrient or yeast extract agars. Growth occurs at 25–55 °C (optimum 37–45 °C), but not at 20 or 60 °C. Growth occurs at pH 6–9 (optimum pH 8) and in the presence of 2–6% NaCl (optimum 3–5%) in MB. Growth does not occur in tryptic soy broth or on R2A agar without supplementation with sea salts or seawater. Growth does not occur on R2A and tryptic soy agars supplemented with NaCl only. Natural seawater or artificial sea salts (1–3%) are required. No anaerobic growth on MA supplemented with potassium nitrate. Nitrate is not reduced to nitrite. Flexirubin-type pigments are not produced and poly-β-hydroxybutyrate granules are not accumulated. Oxidase- and catalase-positive. Acid is not produced from D-glucose, D-xylose, L-arabinose, D-galactose, D-sorbitol, *myo*-inositol, D-ribose, D-mannitol or L-rhamnose. The following carbon sources are utilized in the Biolog GN II system: dextrin, cellobiose, D-fructose, gentiobiose, α-D-glucose, α-lactose, lactulose, maltose, D-mannose, melibiose, raffinose, sucrose, trehalose, turanose, pyruvic acid methyl ester, acetic acid, α-ketobutyric acid, succinic acid, L-glutamic acid, L-proline, L-threonine, inosine and α-D-glucose 1-phosphate. All other carbon sources in the GN II system are not utilized. Positive reactions (API 20E and 20NE) for the production of acetoin, hydrolysis of aesculin, β-glucosidase and β-galactosidase activities and assimilation of mannose. Sensitive to (1⁻¹) ampicillin/sulbactam (8/4–16/8 mg), ticarcillin (16 mg), ticarcillin/clavulonic acid (16/2 mg), piperacillin (16 mg), piperacillin/tazobactam (64/4 mg), ceftazidime (8–16 mg), cefepime (8–16 mg), imipenem (4–8 mg), meropenem (4–8 mg) and ciprofloxacin (1–2 mg); resistant to (1⁻¹) amikacin (16–32 mg), genta-

micin (4–8 mg), tobramycin (4–8 mg), cotrimoxazole (2–38 mg) and colistin (2 mg) (ATB PSE 5). Additional phenotypic features are given in Table 1. The fatty acid profile is characterized by large amounts of branched chain and hydroxy fatty acids (Table 2). The predominant polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, three unidentified phospholipids and three unidentified glycolipids. The major respiratory lipoquinone is MK-6. The DNA G + C content of the type strain is 46.4 ± 1 mol%.

The type strain is CC-HSB-11^T (=BCRC 17850^T =KCTC 22339^T), isolated from water of a coastal hot spring located on Luta, a small volcanic island in the Pacific Ocean off the eastern coast of Taiwan.

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