Muriicola marianensis sp. nov., isolated from seawater

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A Gram-stain-negative, aerobic, orange-pigmented, rod-shaped and non-motile bacterium, designated strain A6B8^T, was isolated from seawater of the Mariana Trench. The isolate grew at 4–50 °C (optimum 30–35 °C), at pH 6.5–8.0 (optimum pH 7.5) and with 0.5–4.0 % (w/v) NaCl (optimum 1.0–2.0 %). Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain A6B8^T was related most closely to the genus *Muriicola* and shared highest sequence similarity of 97.7 % with *Muriicola jejuensis* EM44^T. Chemotaxonomic analysis showed menaquinone 6 (MK-6) was the predominant isoprenoid and iso-$C_{15:0}$, iso-$C_{15:1}$ G and iso-$C_{17:0}$ 3-OH were the major cellular fatty acids. The polar lipid profile of strain A6B8^T included phosphatidylethanolamine, three unidentified aminolipids and four unidentified lipids. The genomic DNA G+C content was 47.1 mol%. The DNA–DNA relatedness value (23.3 %) clearly demonstrated that strains A6B8^T and *M. jejuensis* EM44^T were representatives of two different species. Based on the phenotypic, phylogenetic and chemotaxonomic characterizations, A6B8^T (=CGMCC 1.12606^T=KCTC 32436^T) is considered to be the type strain of a novel species of the genus *Muriicola*, for which the name *Muriicola marianensis* sp. nov. is proposed.

The single-species genus *Muriicola* was first proposed by Kahng *et al.* (2010) with the description of *Muriicola jejuensis*. This genus was classified within the family *Flavobacteriaceae*, which was proposed by Reichenbach (1989) and the description later emended by Bernardet *et al.* (2002). Isolates of the family *Flavobacteriaceae* are short to moderately long rods, Gram-negative, non-spore-forming, non-motile or motile by gliding and aerobic. The majority of members of the family *Flavobacteriaceae* are pigmented by carotenoid or flexirubin pigments or both. Most species within the family *Flavobacteriaceae* have been isolated from various coastal marine habitats, such as tidal flat sediment (Jeong *et al.*, 2013; Kim *et al.*, 2008; Yoon *et al.*, 2006), algae (Nedashkovskaya *et al.*, 2006; Park *et al.*, 2009) and coastal surface seawater (Hameed *et al.*, 2014). In this study, a novel *Flavobacteriaceae* strain was isolated from the Mariana Trench.

A seawater sample of 1000 m depth was selected from water column samples of the Mariana Trench (12.3508° N 145.2070° E) and transported to the lab without temperature control and conserved at 4 °C until used. The selected seawater was diluted using the tenfold dilution method and was plated on marine agar 2216 (MA; Difco). After 7 days of aerobic incubation at 28 °C, strain A6B8^T was picked out and a subculture was purified by restreaking. The strain was preserved at −80 °C in marine broth 2216 (MB; Difco) supplemented with 20 % (v/v) glycerol.

The growth temperature of strain A6B8^T was tested at 4, 17, 22, 25, 28, 30, 35, 37, 40 and 50 °C. The pH range for growth was determined at pH 5.0–10.0 by adding 30 mM different buffering agents including: MES (pH 5.0–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5) and CAPSO (pH 9.0–10.0). Salt tolerance was tested using MB with various concentrations of NaCl (0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 %, w/v). Cells in exponential phase (about 36 h) were used to characterize the morphology by both optical microscopy (BX40; Olympus) and transmission electron microscopy (JEM-1230; JEOL). Anaerobic growth was tested in modified MB, which contained sodium thiosulfate (20 mM), sodium sulfate (20 mM), sodium nitrite (5 mM) or sodium nitrate (20 mM) as electron receptors, under N_2 at 37 °C for 20 days (Kahng *et al.* 2010).

Antibiotic resistance was tested on MA plates using antibiotic discs including the following compounds (μg per disc unless otherwise stated): ampicillin (10), bacitracin (0.04 IU), carbenicillin (100), chloramphenicol (30), erythromycin (15),

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain A6B8^T is KC839612.

Four supplementary figures are available with the online Supplementary Material.

gentamicin (30), kanamycin (30), neomycin (30), novobiocin (30), polymyxin B (300 IU), penicillin G (10 IU), streptomycin (10), tetracycline (30) and vancomycin (30).

Single carbon source assimilation tests were performed using GN2 MicroPlates (Biolog) according to the manufacturer's instructions and as described by Park *et al.* (2009), except that cells were suspended with the GN2 medium supplemented with 2 % (w/v) sea salts. Catalase and oxidase activities, and the ability to hydrolyse casein, cellulose, gelatin, starch, Tweens 20, 40, 60 and 80 and L-tyrosine were tested according to Zhu *et al.* (2011). The methyl red test, Voges–Proskauer test and H_2S production were tested as described by Wu *et al.* (2010). Other enzyme activities and biochemical characteristics were tested using API 20NE and API ZYM kits (bioMérieux) at 30 °C according to the manufacturer's instructions. Acid production was investigated using an API 50CH kit (bioMérieux) at 30 °C and the strips were observed after incubation for 24 and 48 h. Leifson modified O/F medium was used for inoculation, which contained (per litre): 20 g NaCl, 5 g $MgCl_2 . 6H_2O$, 2 g $MgSO_4 . 7H_2O$, 1 g KCl, 0.5 g $CaCl_2$, 0.001 g $FeSO_4$, 0.5 g $(NH_4)_2SO_4$, 1 g trypticase peptone (BD), 0.1 g yeast extract (BD), 0.5 g Tris and 0.01 g phenol red (pH 7.5) (Leifson, 1963).

For pigment analysis, the KOH test was performed as described by Bernardet *et al.* (2002). Pigment was extracted according to Asker *et al.* (2007) and identified using HPLC.

Cells incubated at 30 °C for 3 days were used for chemotaxonomic identification. Total lipids were extracted and separated by two-dimensional TLC with silica gel 60 F_{254} plates (Merck), and further analysed as described by Zhang *et al.* (2013). Fatty acid methyl esters were obtained and analysed according to the instructions of the Microbial Identification System (MIDI; Microbial ID) with the standard MIS Library Generation Software. Isoprenoid quinones were extracted as described by Komagata & Suzuki (1987) and analysed by LC-MS (Tindall, 1990).

A Quick Bacteria Genomic DNA Extraction kit (DongSheng Biotech) was used to extract the genomic DNA and the 16S

Table 1. Differential phenotypic characteristics between strain A6B8^T and *M. jejuensis* EM44^T

All data are from this study. Both strains were positive for catalase, oxidase, nitrate reduction, H_2S production and hydrolysis of aesculin. Both strains were negative for hydrolysis of CM-cellulose, gelatin, starch, Tweens 20, 40, 60 and 80 and tyrosine, and methyl red and Voges–Proskauer tests. +, Positive; −, negative.

Characteristic	A6B8^T	*M. jejuensis* EM44^T
Temperature range (optimum) (°C)	4–50 (30–35)	15–35 (25–30)
pH range (optimum)	6.5–8.0 (7.5)	6.0–9.0 (7.0–8.5)
NaCl range (optimum) (w/v)	0.5–4.0 (1.0–2.0)	0.5–5.5 (2.0–4.0)
Growth on (GN2):		
Dextrin	−	+
D-Galactose	−	+
α-Lactose	−	+
Lactulose	−	+
Melibiose	−	+
Raffinose	−	+
Turanose	+	−
Xylitol	−	+
D-Gluconic acid	+	−
Glucosaminic acid	+	−
γ-Hydroxybutyric acid	−	+
DL-Lactic acid	+	−
Propionic acid	−	+
Succinamic acid	+	−
L-Alanine	+	−
L-Leucine	−	+
L-Ornithine	+	−
Putrescine	−	+
Acid production from:		
D-Mannose	−	+
Inulin	+	−
Glycogen	−	+
Xylitol	−	+
Potassium 5-ketogluconate	−	+
DNA G+C content (mol%)	47.1	46.6

rRNA gene was amplified using universal primers 27F/1492R. The PCR product was cloned into pMD-19T vector (TaKaRa) for sequencing and the resulting sequence (1490 bp) was uploaded to the EzTaxon server (Kim *et al.*, 2012) for pair-wise alignment. The MEGA5 package (Tamura *et al.*, 2011) was used to perform multiple sequence alignment and for reconstruction of phylogenetic trees. Evolutionary distances for the neighbour-joining tree (Park *et al.*, 2009; Saitou & Nei, 1987), maximum-parsimony tree (Fitch, 1971) and maximum-likelihood tree (Felsenstein, 1981) were all calculated following the algorithm of Kimura's two-parameter model (Kimura, 1980). The G+C content of the genomic DNA was obtained by reversed-phase HPLC as described by Mesbah & Whitman (1989). DNA–DNA hybridization was performed using a DU800 spectrophotometer (Beckman Coulter) according to the method of thermal denaturation and renaturation as described by Zhang *et al.* (2010).

Cells of strain A6B8^T were Gram-stain-negative, rod-shaped (Fig. S1, available in the online Supplementary Material), aerobic, orange-pigmented and non-motile. The novel isolate grew at 4–50 °C (optimum 30–35 °C), at pH 6.5–8.0 (optimum pH 7.5) and with 0.5–4.0 % (w/v) NaCl (optimum 1.0–2.0 %). No growth was observed under anaerobic conditions. No flexirubin-type pigments were detected using the KOH test, although zeaxanthin was detected as a major component. Detailed physiological and biochemical characteristics are given in Table 1 and in the species description.

The polar lipid profile of strain A6B8^T was identical to that of *M. jejuensis* EM44^T, which both included phosphatidylethanolamine (PE), three unidentified aminolipids (AL1–3) and four unidentified lipids (L1–4) (Fig. S2).

The major cellular fatty acids of strain A6B8^T were iso-$C_{15:0}$ (37.2 %), iso-$C_{17:0}$ 3-OH (15.4 %) and iso-$C_{15:1}$ G (13.7 %). The fatty acid profiles of strain A6B8^T and the reference strain are given in Table 2. Compared with *M. jejuensis* EM44^T, strain A6B8^T had a significantly higher level of iso-$C_{15:0}$ and lower level of iso-$C_{16:0}$ 3-OH. The predominant quinone detected in strain A6B8^T was menaquinone 6 (MK-6), in accordance with data for *M. jejuensis* EM44^T.

On the basis of 16S rRNA gene sequence alignment, strain A6B8^T was related most closely to three single-species genera, namely *Muriicola*, *Eudoraea* and *Zeaxanthinibacter*, with similarities of 97.7, 95.5 and 93.3 %, respectively. Phylogenetic analysis showed that strain A6B8^T belonged to genus *Muriicola* by clustering with *M. jejuensis* EM44^T at high bootstrap support in the neighbour-joining, maximum-likelihood and maximum-parsimony trees (Fig. 1, Figs S3 and 4). The DNA–DNA relatedness value between strain A6B8^T and *M. jejuensis* EM44^T was only 23.3 %, which clearly indicated that strain A6B8^T represents a novel species of the genus *Muriicola* (Wayne, 1988). The genomic DNA G+C content of strain A6B8^T was 47.1 mol% (HPLC).

Table 2. Fatty acid composition (%) of strain A6B8^T and *M. jejuensis* EM44^T

All data were from this study. Fatty acids that represented <1 % in both strains are not shown. ECL, Equivalent chain length; TR, trace (<1 %); –, not detected. Major components (≥10 %) are highlighted in bold.

Fatty acid	A6B8^T	*M. jejuensis* EM44^T
Saturated		
$C_{15:0}$	3.1	5.9
Branched		
iso-$C_{14:0}$	–	1.2
iso-$C_{15:0}$	**37.2**	**15.8**
iso-$C_{16:0}$	3.3	5.3
iso-$C_{15:1}$ G	**13.7**	**11.8**
anteiso-$C_{15:0}$	1.9	2.8
iso-$C_{15:1}\omega6c$	TR	1.0
iso-$C_{16:1}$ H	TR	2.3
iso-$C_{17:1}\omega9c$	3.2	1.9
Unsaturated		
$C_{17:1}\omega6c$	TR	1.5
Hydroxy		
$C_{15:0}$ 3-OH	–	1.0
$C_{17:0}$ 2-OH	1.2	1.2
iso-$C_{15:0}$ 3-OH	5.1	5.3
iso-$C_{16:0}$ 3-OH	3.1	**12.5**
iso-$C_{17:0}$ 3-OH	**15.4**	**17.0**
Summed features*		
3	2.0	6.2
4	1.1	–
Unknown 13.565	3.5	1.5

*Summed feature 3 comprised $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH; summed feature 4 comprised iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B.

According to the results described above, strain A6B8^T should be classified within the genus *Muriicola*. In addition to the low DNA–DNA relatedness value, some phenotypic differences clearly distinguish strain A6B8^T from *M. jejuensis* EM44^T. For instance, strain A6B8^T utilized L-alanine, D-gluconic acid, glucosaminic acid, DL-lactic acid, succinamic acid, L-proline and turanose, which were not assimilated by *M. jejuensis* EM44^T. In addition, the major fatty acids (≥10 %) of strain A6B8^T were iso-$C_{15:0}$ (37.2 %), iso-$C_{17:0}$ 3-OH (15.4 %) and iso-$C_{15:1}$ G (13.7 %), while those of *M. jejuensis* EM44^T were iso-$C_{17:0}$ 3-OH (17.0 %), iso-$C_{15:0}$ (15.8 %), iso-$C_{16:0}$ 3-OH (12.5 %) and iso-$C_{15:1}$ G (13.7 %) (Table 2). Detailed differences in physiological and biochemical characterization between the two strains are summarized in Table 1.

Based on the phenotypic, phylogenetic and chemotaxonomic characteristics described above, strain A6B8^T is considered to represent a novel species of the genus *Muriicola*, for which the name *Muriicola marianensis* sp. nov. is proposed.

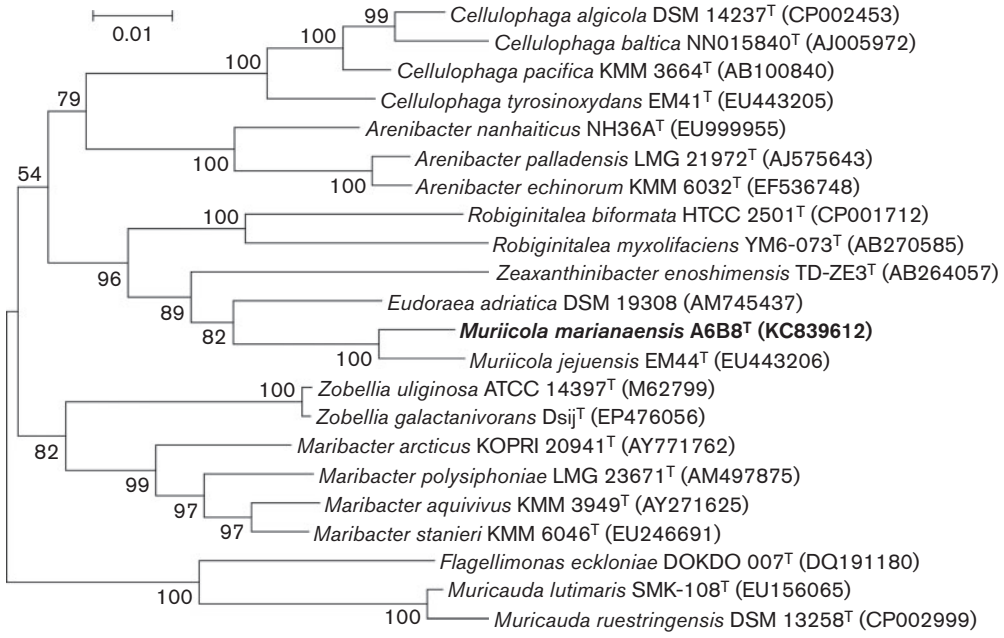

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationship between the novel isolate and other taxa in the family *Flavobacteriaceae*. Bootstrap values are based on 1000 replicates; only values >50 % are shown at branch points. Bar, 0.01 substitutions per nucleotide position.

Description of *Muriicola marianensis* sp. nov.

Muriicola marianensis sp. nov. (ma.ri.an.en′sis. N.L. masc. adj. *marianensis* referring to the Mariana Trench, from where the type strain was isolated).

Cells are 2.3–3.0 μm in length and 0.4–0.5 μm in width, Gram-stain-negative, rod-shaped, aerobic and non-motile. Colonies on MA are convex, orange-pigmented and circular with an entire margin after 3 days of incubation at 30 °C. No growth is observed under anaerobic conditions. Growth occurs at 4–50 °C (optimum 30–35 °C), at pH 6.5–8.0 (optimum pH 7.5) and with 0.5–4.0 % (w/v) NaCl (optimum 1.0–2.0 %). Sensitive to bacitracin, gentamicin, kanamycin, neomycin, polymyxin B and streptomycin. Positive for catalase and oxidase activities and H_2S production. No hydrolysis of casein, CM-cellulose, gelatin, starch, Tweens 20, 40, 60 and 80 or tyrosine. Negative in methyl red and Voges–Proskauer tests. In the GN2 MicroPlate, the following substrates are utilized for growth: α-cyclodextrin, D-fructose, gentiobiose, α-D-glucose, maltose, methyl β-D-glucoside, sucrose, trehalose, turanose, acetic acid, D-gluconic acid, D-glucosaminic acid, α-ketoglutaric acid, α-ketovaleric acid, DL-lactic acid, succinic acid, succinamic acid, L-alanine, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-ornithine and L-proline. Other compounds included in the GN2 MicroPlate are not utilized as sole carbon and energy sources. In the API 20NE system, nitrate is reduced to nitrite but not to nitrogen. Hydrolysis of aesculin and β-galactosidase are positive. Indole production, fermentation of D-glucose, arginine dihydrolase activity and hydrolysis of urea are negative. In the API ZYM system, tests for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase and *N*-acetyl-β-glucosaminidase are positive. Tests for lipase (C14), β-glucuronidase and α-fucosidase activities are negative. Weak activity is present for α-mannosidase. Acid is produced from D-glucose, D-fructose, D-mannose, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, D-lactose, melibiose, sucrose, trehalose, melezitose, raffinose, starch, gentiobiose, turanose and potassium 2-ketogluconate, but not from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xylose glycoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, inulin, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate or potassium 5-ketogluconate (API 50CH). The polar lipids include phosphatidylethanolamine (PE), three unidentified aminolipids (AL1–3) and four unidentified lipids (L1–4). The major fatty acids ($\geqslant$10 % of the total fatty acids) are iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and iso-$C_{15:1}$ G. The predominant respiratory quinone is MK-6.

The type strain, A6B8^T (=CGMCC 1.12606^T=KCTC 32436^T), was isolated from seawater of the Mariana

Trench at a depth of 1000 m. The genomic DNA G+C content of the type strain is 47.1 mol% (HPLC).

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