Muriicola jejuensis gen. nov., sp. nov., isolated from seawater

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An aerobic, yellow–orange-pigmented, Gram-staining-negative bacterium, designated strain EM44^T, was isolated from seawater on the eastern coast of Jeju Island, Korea. Growth was observed at 15–35 °C (optimum 25–30 °C), pH 6.5–9.0 (optimum pH 7.0–8.5) and between 1 and 5 % NaCl (w/v) (optimum 2–4 %). Cells of strain EM44^T were non-motile, straight rods and showed catalase and oxidase activities. The G+C content of the genomic DNA was 47.9 mol% and the major respiratory quinone was MK-6. The major fatty acids were iso-C_{15:0} G, iso-C_{15:0}, iso-C_{17:0} 3-OH and iso-C_{16:0} 3-OH. Strain EM44^T contained phosphatidylethanolamine as a major polar phospholipid. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain EM44^T fell within the family *Flavobacteriaceae* in the phylum *Bacteroidetes* and was most closely related to members of the genera *Eudoraea*, *Zeaxanthinibacter* and *Robiginitalea* with 92–94.5 % gene sequence similarities. On the basis of chemotaxonomic data and molecular properties, it is clear that strain EM44^T represents a novel genus within the family *Flavobacteriaceae*, for which the name *Muriicola jejuensis* gen. nov., sp. nov. is proposed. The type strain of the type species is EM44^T (=KCTC 22299^T=DSM 21206^T).

The family *Flavobacteriaceae* is one of the major phylogenetic lineages within the phylum *Bacteroidetes* (Bernardet *et al.*, 2002) and its members have been isolated from marine habitats (Cho & Giovannoni, 2004; Bowman & Nichols, 2005; Asker *et al.*, 2007; Manh *et al.*, 2008; Alain *et al.*, 2008; Yang & Cho, 2008; Nedashkovskaya *et al.*, 2009). Cultured isolates of the family *Flavobacteriaceae* are known to be proficient in degrading various complex biomolecules; hence, they are considered to be important members of the bacterial community involved in metabolic

activities in aquatic environments (Kirchman, 2002; Kahng *et al.*, 2009). In the course of investigations of bacterial communities in seawater (Kahng *et al.*, 2009; Lee *et al.*, 2009), a novel Gram-staining-negative bacterium producing yellow–orange pigments was isolated and investigated using a polyphasic approach.

A surface seawater sample was collected from the eastern coast of Jeju Island (33° 26' N 126° 54' E), South Korea. The seawater temperature was 21 °C and the pH was 7.8. The seawater sample was spread on marine agar 2216 (MA; Difco) at 25 °C for 5 days. Strain EM44^T was isolated and routinely grown on MA at 30 °C for 3 days under aerobic conditions, except where indicated otherwise. The strain was stored at –80 °C in marine broth (MB, Difco) supplemented with 10 % (v/v) glycerol for preservation.

Amplification and sequencing of the 16S rRNA gene of strain EM44^T was performed as described by Kim *et al.* (2008). Briefly, a single colony of strain EM44^T grown on MA was resuspended in 100 µl 5 % (w/v) Chelex-100

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Abbreviations: ML, maximum-likelihood; NJ, neighbour-joining; MP, maximum-parsimony.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EM44^T is EU443206.

Additional phylogenetic trees, a transmission electron micrograph of a cell of strain EM44^T and the results of the polar lipid analysis are available as supplementary figures with the online version of this paper.

solution (Bio-Rad) and boiled for 10 min to prepare crude genomic DNA lysates. PCR amplification of 16S rRNA genes from the crude lysates was conducted using the universal primer F1 (5'-AGAGTTTGATCMTGGCTCAG-3') and R13 (5'-TACGGYTACCTTGTTACGACTT-3') as described previously (Lu *et al.*, 2006). The resulting 16S rRNA gene sequence (1487 nucleotides) was compared with available 16S rRNA gene sequences from GenBank using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>) to determine an approximate phylogenetic affiliation and aligned with sequences of closely related members by using the CLUSTAL W software program (Thompson *et al.*, 1994). Phylogenetic trees were constructed by using three different algorithms, neighbour-joining (NJ), maximum-likelihood (ML) and maximum-parsimony (MP) available in the PHYLIP software, version 3.6 (Felsenstein, 2002). Resulting tree topologies were evaluated by bootstrap analysis with the PHYLIP package based on 1000 resampled datasets. Sequence similarity values between the novel isolate and other related bacteria were evaluated using the EzTaxon nucleotide similarity search program (<http://147.47.212.35:8080>; Chun *et al.*, 2007).

Comparative 16S rRNA gene sequence analysis showed that strain EM44^T was most closely related to *Eudoraea adriatica* AS06/20a^T, *Zeaxanthinibacter enoshimensis* TD-ZE3^T and *Robiginitalea biformata* HTCC2501^T with 94.5 %, 92.9 % and 92.1 % sequence similarities, respectively. Phylogenetic analysis using the NJ algorithm based on

16S rRNA gene sequences indicated that strain EM44^T formed a distinct phylogenetic line from the genus *Eudoraea* within the family *Flavobacteriaceae* with a low bootstrap value (43 %), meaning that the phylogenetic topology was not stable (Fig. 1). Moreover, the topologies of phylogenetic trees built using the ML and MP algorithms showed that the isolate formed a phyletic lineage with the genus *Zeaxanthinibacter* within the family *Flavobacteriaceae* (see Supplementary Fig. S1 in IJSEM Online). Taxonomic analysis using the Ribosomal Database Project (RDP) classifier tool (<http://rdp.cme.msu.edu/classifier>, Naive Bayesian rRNA Classifier Version 2.0, Wang *et al.*, 2007) indicated that the new isolate might be classified as a member of the genus *Robiginitalea*. These phylogenetic analyses suggested that there was no genus group showing a clear phylogenetic relationship with strain EM44^T in the family *Flavobacteriaceae*.

Physiological characteristics of strain EM44^T were examined by growing the isolate on MA at different temperatures (5–50 °C at 5 °C intervals). The pH range for growth was determined in MB adjusted to pH 5.0–10.0 at intervals of 0.5 pH unit. The pH was adjusted prior to sterilization by the addition of HCl or NaOH and measured again after sterilization. NaCl requirements and tolerance were determined between 0–10 % (w/v) NaCl (increments of 1 %) in nutrient broth (NB) supplemented with modified artificial seawater (I⁻¹; 5.94 g MgSO₄·7H₂O, 4.53 g MgCl₂·6H₂O, 0.64 g KCl and 1.3 g CaCl₂). Gram staining was performed using a bioMérieux Gram Stain kit

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strain EM44^T and related taxa. Bootstrap values are shown in percentages of 1000 replicates, when greater than 50 %. *Flexibacter flexilis* ATCC 23079^T (GenBank accession no. M62794) was used as an outgroup (not shown). Bar, 0.01 changes per nucleotide position.

according to the manufacturer's instructions. Cell morphology, flagella and gliding motility were studied using phase-contrast microscopy and transmission electron microscopy (JEM-1010; JEOL) as described previously (Bernardet *et al.*, 2002; Jeon *et al.*, 2004). Antibiotic susceptibility tests were performed in duplicate as described previously (Reva *et al.*, 1995) using filter paper discs (diameter, 8 mm, Whatman) containing the following antibiotics (μg per disc unless otherwise indicated): ampicillin (10), polymyxin B (100 U), streptomycin (50), penicillin G (10 U), chloramphenicol (100), gentamicin (30), tetracycline (30), kanamycin (30), lincomycin (15), oleandomycin (15), neomycin (30), carbenicillin (100) and novobiocin (50). Oxidase activity was tested by oxidation of 1% (w/v) tetramethyl-*p*-phenylenediamine (Merck) and catalase activity was evaluated by the production of oxygen bubbles in 3% (v/v) aqueous hydrogen peroxide solution.

The production of flexirubin type pigments was investigated using the KOH test as detailed in the minimal standards for the description of new taxa in the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). Carotenoid composition was characterized by using HPLC (model LC-20A, Shimadzu) equipped with a diode array detector (SPD-M20A, Shimadzu) and a reversed-phase column (250 $\times$ 4.6 mm, Kromasil, Akzo Nobel, 35 °C) and acetonitrile/methanol/tetrahydrofuran [5.8:3.5:0.7 (by vol.)] as a mobile phase (Asker *et al.*, 2007). Zeaxanthin was identified and confirmed from its retention time and absorption spectrum.

Hydrolysis of casein, Tween 80, Tween 20, aesculin, urea, tyrosine, starch and carboxymethylcellulose was investigated on MA after 7 days incubation at 30 °C according to previously described methods (Lányi, 1987; Smibert & Krieg, 1994). Nitrate reduction was performed according to the method of Lányi (1987). Additional enzymic activities and biochemical features were determined using API ZYM and API 20NE kits at 30 °C as recommended by the manufacturer (bioMérieux). The utilization or oxidation of carbon sources was determined using the GN MicroPlate system (Biolog) according to the manufacturer's instructions. Anaerobic growth was assessed on MA under anaerobic (with 4–10% CO₂) conditions using the GasPak Plus system (BBL) at 30 °C for 20 days.

Strain EM44^T grew well at 30 °C on MA. When tested on MA, growth of the strain was observed at temperatures between 15 and 35 °C, with optimum growth at 25–30 °C. Growth occurred between pH 6.5 and pH 9.0, with optimum pH 7.0–8.5. Bacterial cells were Gram-staining-negative straight rods that were 0.4–0.6 μm wide and 1.7–2.3 μm long (see Supplementary Fig. S2 in IJSEM Online). Strain EM44^T grew in NB supplemented with modified artificial seawater containing 1–6% (w/v) NaCl; optimal growth occurred in the range of 2–4% NaCl. The yellow–orange pigments of strain EM44^T were identified as carotenoids, among which zeaxanthin was predominant (UV-Vis λ_{max} of 451 and 476 nm) (Asker *et al.*, 2007).

Physiological and biochemical characteristics of strain EM44^T are shown in Table 1 and are detailed in the genus and species descriptions.

Isoprenoid quinones were analysed using a HPLC (model LC-20A, Shimadzu) equipped with a diode array detector (SPD-M20A, Shimadzu) and a reversed-phase column (250 $\times$ 4.6 mm, Kromasil, Akzo Nobel) as described previously (Komagata & Suzuki, 1987). Whole-cell fatty acids of strain EM44^T were analysed according to the instructions of the Microbial Identification System (MIDI; Microbial ID, Inc.) on cells grown on MA at 30 °C for 3 days. Analysis of isoprenoid quinones was carried out using the methods described by Komagata & Suzuki (1987). The DNA G + C content was determined using a HPLC fitted with a reversed-phase column (GROM-SIL 100 ODS-2FE, GROM) according to the method of Tamaoka & Komagata (1984). The major respiratory lipoquinone was menaquinone-6 (MK-6). The major cellular fatty acids were iso-C_{15:1} G (21.2%), iso-C_{15:0} (20.7%), iso-C_{17:0} 3-OH (12.5%), iso-C_{16:0} 3-OH (10.3%), summed feature 3 (comprising C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH, 5.8%), C_{15:0} (5.4%), iso-C_{15:0} 3-OH (4.9%), anteiso-C_{15:0} (4.4%), iso-C_{16:0} (3.9%), iso-C_{16:1}

Table 1. Differential characteristics of strain EM44^T and members of related genera of the family *Flavobacteriaceae*

Strains: 1, EM44^T (data from this study); 2, *Eudoraea adriatica* AS06/20a^T (Alain *et al.*, 2008); 3, *Zeaxanthinibacter enoshimensis* TD-ZE3^T (Asker *et al.*, 2007); 4, *Robiginitalea biformata* DSM 15991^T (Cho & Giovannoni, 2004). All strains are positive for the following characteristics: aerobic, catalase activity, oxidase activity and hydrolysis of aesculin (API 20NE). All strains are negative for the following characteristics: Gram staining, growth at 4 °C and 45 °C, flexirubin pigments, hydrolysis of carboxymethylcellulose and indole production (API 20NE). +, Positive; –, negative; (+), weakly positive; Y, yellow; O, orange; ND, not determined.

Characteristic	1	2	3	4
Pigmentation	Y/O	–	Y	O
Growth with/at:				
8% NaCl	–	–	–	+
7% NaCl	–	–	+	+
1% NaCl	+	–	+	+
10 °C	–	–	–	+
37 °C	+	–	+	+
42 °C	–	–	–	+
Nitrate reduction	+	–	–	–
Gliding motility	–	–	+	–
Hydrolysis of:				
Agar	–	–	–	ND
Starch	–	–	+	+
Gelatin	–	–	+	–
β -Galactosidase	+	+	+	–
Urease	(+)	–	–	–
DNA G + C content (mol%)	47.9	38.9	46.5	56.4

G (1.9 %), C_{16:0} (1.6 %), iso-C_{17:1}ω9c (1.5 %), iso-C_{14:0} (1.2 %), unknown 13.56 (1.2 %), anteiso-C_{15:0} A (1.1 %), C_{17:0} 2-OH (1.1 %) and C_{16:0} 3-OH (0.9 %). The complete fatty acid composition of strain EM44^T and its closest phylogenetic neighbours is given in Table 2. The detailed fatty acid composition of strain EM44^T was different from those of related type strains. Polar phospholipids were dominated by a large amount of phosphatidylethanolamine and several amino group-containing lipids were also present (see Supplementary Fig. S3 in IJSEM Online). The polar lipid profile of strain EM44^T was similar to that of *E. adriatica* AS06/20a^T, the type species and only recognized representative of the genus *Eudoraea* (Alain *et al.*, 2008). The DNA G+C content of strain EM44^T was 47.9 mol%, which was quite different from that of *E. adriatica* AS06/20a^T (Alain *et al.*, 2008). Therefore, data

obtained using the polyphasic approach used in this study support the description of strain EM44^T as a new genus and novel species in the family *Flavobacteriaceae*, for which the name *Muriicola jejuensis* gen. nov., sp. nov. is proposed.

Description of *Muriicola* gen. nov.

Muriicola [Mu.ri.i'co.la. L. n. *muria* (or *muries*) salt liquor; L. suff. *-cola* (from L. n. *incola*) dweller, inhabitant; N.L. masc. n. *Muriicola* dweller in salt water].

Cells are non-motile by gliding, non-spore-forming and strictly aerobic rods without flagella. Nitrate is reduced to nitrite. Gram-staining-negative, catalase- and oxidase-positive. Major isoprenoid quinone is MK-6. Carotenoid pigments are produced with zeaxanthin as the major component, but flexirubin-type pigments are not produced. Predominant polar phospholipid is phosphatidylethanolamine. The G+C content of the type strain of the type species is 47.9 mol% (HPLC). The type species is *Muriicola jejuensis*.

Description of *Muriicola jejuensis* sp. nov.

Muriicola jejuensis (je.ju.en'sis. N.L. masc. adj. *jejuensis* of Jeju island, Republic of Korea, from where the type strain was isolated).

Displays the following characteristics in addition to those in the genus description. Cells are 0.4–0.6 µm wide and 1.7–2.3 µm long. Colonies on MA are yellow–orange, low convex, circular with entire margins. Growth occurs at 15–35 °C (optimum 25–30 °C), at pH 6.5–9.0 (optimum pH 7.0–8.5) and in the presence of 1–5 % (w/v) NaCl (optimum 2–4 %). Anaerobic growth is not observed after 20 days at 30 °C on MA. Nitrate is reduced to nitrite, but nitrogen gas is not produced. Aesculin is hydrolysed, but Tween 20, Tween 80, gelatin, starch, carboxymethylcellulose, tyrosine, casein and urea are not hydrolysed. Alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, trypsin, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase and N-acetyl-β-glucosaminidase activities are present, but lipase (C14), β-glucuronidase and α-fucosidase activities are absent. Weak enzymic activities are observed for esterase (C4), α-chymotrypsin, naphthol-AS-BI-phosphohydrolase and α-mannosidase (API ZYM). Positive for Biolog GN2 MicroPlate system substrates α-cyclodextrin, dextrin, D-fructose, D-galactose, gentiobiose, α-D-glucose, α-D-lactose, lactulose, maltose, melibiose, methyl β-D-glucoside, raffinose, sucrose, D-trehalose, xylitol, acetic acid, γ-hydroxybutyric acid, α-ketoglutaric acid, α-ketovaleric acid, propionic acid, succinic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-leucine, L-proline, L-threonine and putrescine. Other organic substrates included in the Biolog GN2 microplates are not oxidized. Strain EM44^T is resistant to polymyxin B, streptomycin, ampicillin, gentamicin, tetracycline, kanamycin, neomycin and oleandomycin but sensitive to

Table 2. Cellular fatty acid content (%) of strain EM44^T and members of related genera of the family *Flavobacteriaceae*

Strains: 1, EM44^T (data from this study); 2, *Eudoraea adriatica* AS06/20a^T (Alain *et al.*, 2008); 3, *Zeaxanthinibacter enoshimensis* TD-ZE3^T (Asker *et al.*, 2007); 4, *Robiginitalea biformata* (n=2, Cho & Giovannoni, 2004). Data are expressed as percentages of total fatty acids. Fatty acids amounting to <1.0 % in all strains are not shown. tr, Trace amount (<1 %); –, not detected; ECL, equivalent chain-length.

Fatty acid	1	2	3	4
Straight chain				
C _{15:0}	5.4	–	7.7	5.5
C _{16:0}	1.6	tr	tr	1.3
Branched				
iso-C _{14:0}	1.2	–	–	tr
iso-C _{15:0}	20.7	29.4	18.9	26.0
iso-C _{16:0}	3.9	tr	1.1	tr
iso-C _{15:1}	–	–	–	17.2
iso-C _{15:1} G	21.2	16.0	9.9	–
anteiso-C _{15:0}	4.4	tr	2.3	3.7
anteiso-C _{15:1} A	1.1	–	–	–
iso-C _{16:1} G	1.9	–	–	–
iso-C _{17:1} ω9c	1.5	9.0	13.3	tr
Unsaturated				
C _{15:1} ω6c	–	1.1	tr	tr
C _{17:1} ω6c	–	tr	1.0	tr
Hydroxy				
C _{15:0} 3-OH	4.9	1.3	–	tr
C _{17:0} 2-OH	1.1	–	tr	1.2
iso-C _{15:0} 3-OH	–	7.3	2.7	4.3
iso-C _{16:0} 3-OH	10.3	1.4	2.4	tr
iso-C _{17:0} 3-OH	12.5	14.1	16.5	25.8
Summed feature 3*	5.8	11.6	5.8	3.5
Unknown ECL 13.56	1.2	2.3	11.0	–
Unknown ECL 16.58	–	1.2	–	–

*Summed features represent groups of two or three fatty acids which 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.

penicillin G, carbenicillin, lincomycin, chloramphenicol and novobiocin. The major cellular fatty acids are iso-C_{15:1} G, iso-C_{15:0}, iso-C_{17:0} 3-OH and iso-C_{16:0} 3-OH. Polar phospholipids are dominated by a large amount of phosphatidylethanolamine and several amino group-containing lipids are also present. The major isoprenoid quinone is menaquinone-6 (MK-6).

The type strain, EM44^T (=KCTC 22299^T=DSM 21206^T), was isolated from seawater on the eastern coast of Jeju Island, Korea. The DNA G + C content of the type strain is 47.9 mol% (HPLC).

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