Pseudoruegeria aestuarii sp. nov., of the family *Rhodobacteraceae*, isolated from a tidal flat

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A Gram-stain-negative, non-motile, aerobic and rod-shaped bacterium, designated strain MME-001^T, was isolated from the tidal flat of Muui-do in the Republic of Korea. Phylogenetic trees based on the 16S rRNA gene sequence showed that strain MME-001^T belonged to the genus *Pseudoruegeria* in the family *Rhodobacteraceae* and that it shared the highest 16S rRNA gene sequence similarity with *Pseudoruegeria sabulilitoris* GJMS-35^T (98.0 % similarity of the 16S rRNA gene). Growth of strain MME-001^T occurred in the presence of 1.0–7.0 % (w/v) NaCl at 15–40 °C and pH 7.0–9.0, with optimal growth in the presence of 2.0–3.0 % (w/v) NaCl at 25–30 °C and pH 7.0. Ubiquinone-10 was the major respiratory quinone. Major polar lipids were phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and an unidentified lipid. The major cellular fatty acids were $C_{18:1}\omega7c$ and $C_{16:0}$. The genomic DNA G+C content was 62 mol%. DNA-DNA hybridization values between strain MME-001^T and *P. sabulilitoris* KCTC 42111^T, '*Pseudoruegeria limi*' KCTC 32460, *Pseudoruegeria lutimaris* KCTC 22690^T, *Pseudoruegeria aquimaris* KCTC 12737^T and *Pseudoruegeriahaliotis* KACC 17214^T was 36±5, 57±7, 34±4, 18±5 and 21±3 %, respectively. Based on the phenotypic and phylogenetic taxonomical properties, this strain MME-001^T represents a novel species of the genus *Pseudoruegeria,* for which the name *Pseudoruegeria aestuarii* sp. nov. is proposed. The type strain is MME-001^T (=KCCM 43133^T=JCM 30751^T).

The genus *Pseudoruegeria,* a member of the class *Alphaproteobacteria,* was first proposed by Yoon *et al.* (2007). At the time of writing, the genus comprised five species, *Pseudoruegeria aquimaris* (Yoon *et al.*, 2007), *P. lutimaris* (Jung

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Abbreviations: DDH, DNA-DNA hybridization; DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MME-001^T is KP410678.

Two supplementary figures and a supplementary table are available with the online Supplementary Material.

et al., 2010), *P. haliotis* (Hyun *et al.*, 2013), *P. sabulilitoris* (Park *et al.*, 2014), and '*P. limi*' (Lee *et al.*, 2014). The major features of the genus *Pseudoruegeria* are that members are Gram-stain-negative, aerobic and rod-shaped, and have ubiquinone-10 as the predominant respiratory quinone. The major fatty acid is $C_{18:1}$ $\omega 7c$ and the common major polar lipid components are identified phosphatidylglycerol (PG), an unidentified aminolipid and an unidentified lipid (Yoon *et al.*, 2007; Jung *et al.*, 2010). The west coast regions of Korea, have widely developed tidal flats, from which many bacterial strains have been isolated. One of these isolates, designated strain MME-001^T, belonging to the genus *Pseudoruegeria* is described in this study. We determined the taxonomic position of strain MME-001^T by a polyphasic characterization that included determination of chemotaxonomic and other phenotypic properties, detailed phylogenetic analysis based on 16S rRNA gene sequences, and DNA–DNA hybridization.

Strain MME-001^T was isolated from the tidal flat of Muuido in Incheon, Republic of Korea. Tidal flat samples were collected (37° 24′ 16″ N 126° 24′ 50″ E) in September 2014 and serially diluted into natural seawater agarose medium. To manufacture this medium, 0.5 g yeast extract and 1.5 % (w/v) agarose were added to 1 l natural seawater. The solution was autoclaved and supplemented with 1 ml trace element solution SL-6 (DSM medium no. 27) and 1 ml vitamin solution (Wolin, 1963) which was filtered using a 0.2 µm syringe filter. The plates were then incubated at 30 °C for 2 weeks. At the end of the incubation period, colonies were streaked on the same fresh medium at least four times to obtain pure colonies. The pure colonies were then transferred onto marine agar 2216 (MA). The pH was adjusted to 7.2–7 .4 with 1 M HCl or NaOH. One of the isolates was designated MME-001^T and routinely cultivated on MA at 30 °C. For analysis of the polar lipids, fatty acids, respiratory quinone and genomic DNA G+C content, and DNA–DNA hybridization experiments, the reference strains, *Pseudoruegeria sabulilitoris* KCTC 42111^T, '*P. limi*' KCTC 32460, *P. lutimaris* KCTC 22690^T and *P. aquimaris* KCTC 12737^T, were purchased from the Korean Collection for Type Cultures (Jeongup, Korea), and *P. haliotis* KACC 17214^T was bought from the Korean Agriculture Collection (Jeonju, Korea).

Genomic DNA of isolate MME-001^T was extracted using a HiYield Genomic DNA Mini Kit (Real Biotech Corporation). The 16S rRNA gene was amplified using PCR (Weisburg *et al.*, 1991) with the universal bacterial primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Lane, 1991) and the products were sequenced by CosmogeneTech. The nearest 16S rRNA gene full sequences were obtained using the universal bacterial primers, 27F, 337F (5′-GACTCC TACGGGAGGCWGCAG-3′), 518R (5′-ATTACCGCGGC TGCTGG-3′), 785F (5′-GGATTAGATACCCTGGTA-3′), and 1492R (Weisburg *et al.*, 1991) and assembled as described previously by Roh *et al.* (2008) using the SeqMan software (DNAStar). The 16S rRNA gene sequence of strain MME-001^T was aligned with sequences of related species with validly published names using SILVA (http://www.arb-silva.de/aligner) (Pruesse *et al.*, 2012). Phylogenetic neighbors were identified using BLAST (http://www.ncbi.nlm.nih.gov/blast/) and the EzTaxon-e server (http://www.ezbiocloud.net/eztaxon/) (Kim *et al.*, 2012). The evolutionary distances were calculated using Kimura's two-parameter model (Kimura, 1983). Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981), and maximum-parsimony (Fitch, 1971) methods using MEGA6 (Tamura *et al.*, 2013), and the bootstrap values were calculated based on 1000 replicates. In this study, the 16S rRNA gene sequence of strain MME-001^T was determined to be 1376 bp in length, and was related to those of members of the genus *Pseudoruegeria*. The 16S rRNA gene sequence similarities between strain MME-001^T and *P. sabulilitoris* GJMS-35^T, '*P. limi*' D-17, *P. lutimaris* HD-43^T, *P. aquimaris* SW-255^T, and *P. haliotis* WM67^T were 98.0, 97.4, 97.3, 96.9 and 96.7 %, respectively, while all others showed less than 96.4 % similarity. Upon phylogenetic analysis, strain MME-001^T was found to belong to the genus *Pseudoruegeria* (Fig. 1).

The morphological, physiological and biochemical characteristics of strain MME-001^T were examined by routine cultivation on MA at 30 °C. Cell morphology and the presence of flagella were determined by phase contrast microscopy (Primo Star; Carl Zeiss) and transmission electron microscopy (JEM-1010; JEOL), respectively. The motility was determined using semi-solid MA (containing 0.5 % agarose) after incubation at 30 °C for 7 days (Tittsler & Sandholzer, 1936). The Gram reaction was investigated using a Gram-stain kit (Bioworld) according to the manufacturer's instructions. Catalase and oxidase activities were performed as described by Smibert & Krieg (1994). Growth under anaerobic conditions was determined by incubating samples on MA in a GasPak EZ anaerobe gas generating pouch system with indicator (BD) for 2 weeks at 30 °C. Colonies of strain MME-001^T were circular, smooth, convex, glistening, pale yellow and 1.0 mm after incubation on MA at 30 °C for 3 days. The isolate was Gram-stain-negative, aerobic and rod-shaped. The cells were 0.5–0.6 µm wide by 2.4–2.5 µm long (Fig. S1, available in the online Supplementary Material). The motility of cells was not observed in semi-solid MA and flagella were not discernible upon transmission electron microscopy.

To obtain the optimum growth temperature, strain MME-001^T was cultivated at 4, 10, 15, 20, 25, 30, 35, 40, 45 and 50 °C on MA for 2 weeks. The salinity range for growth of strain MME-001^T was determined with 0–10.0 % (w/v) NaCl using modified artificial seawater medium as described by Cha *et al.* (2013). The pH range for growth was determined in marine broth 2216 (MB) that was adjusted to pH 5.0–11.0 (in increments of 1.0 pH unit). Hydrolysis of starch and casein was determined according to the methods of Benson (2002). Hydrolysis of Tweens 20, 40, and 80 was determined according to the method

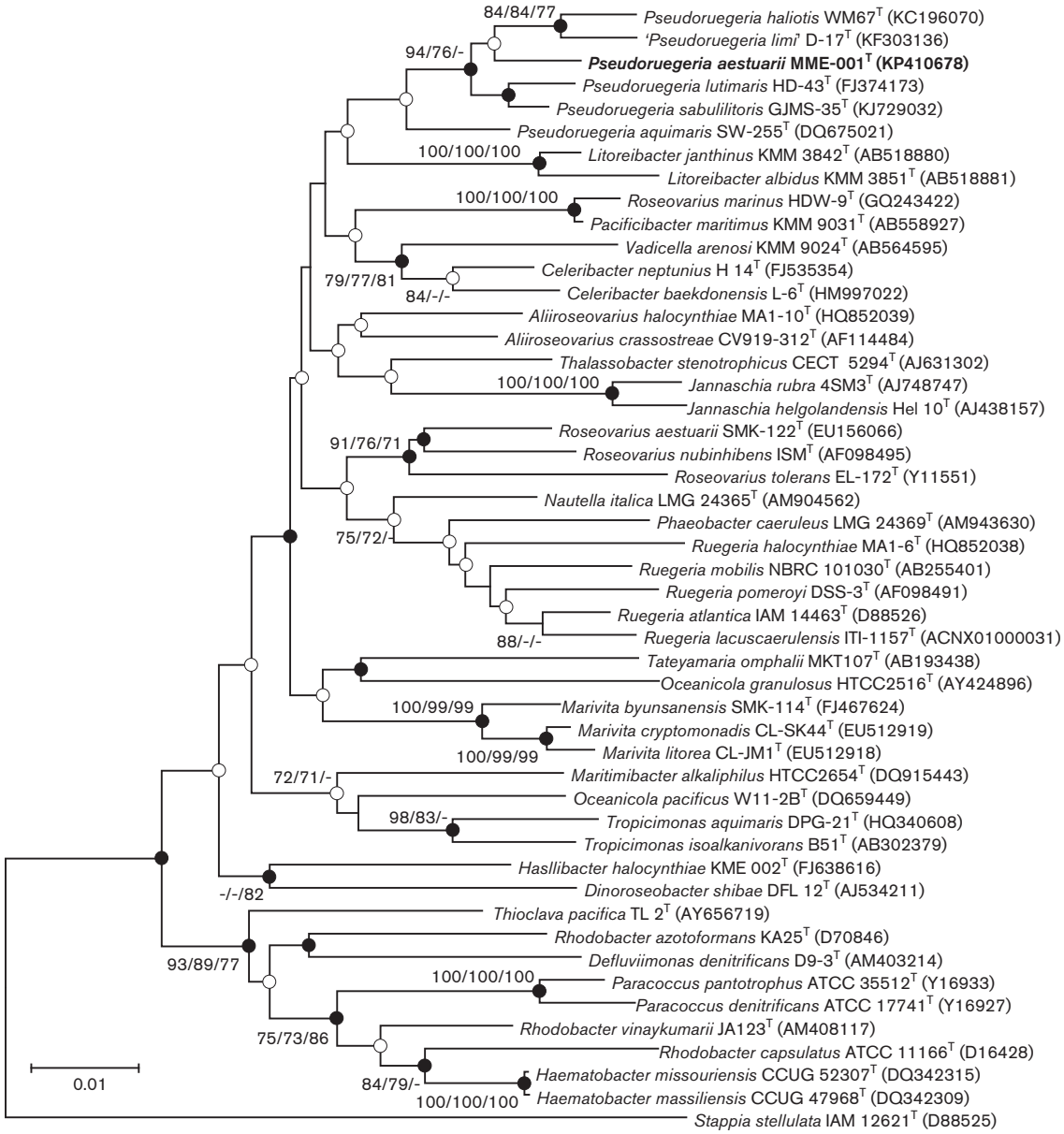

Fig. 1. Neighbour-joining (NJ) phylogenetic tree based on 16S rRNA gene sequences showing the position and relationship between strain MME-001^T and other species of the genus *Pseudoruegeria* and other related taxa. Numbers at nodes indicate bootstrap values (>70 %) calculated based on the NJ/maximum-likelihood (ML)/maximum-parsimony (MP) algorithms. Closed circles indicate that the corresponding nodes are also recovered by the ML and MP methods. Open circles indicate that the corresponding nodes are also recovered by the ML or MP methods. *Stappia stellulata* IAM 12621^T served as an outgroup. Bar, 0.01 substitutions per nucleotide position.

described by González *et al.* (1978). Hydrolysis of gelatin and L-tyrosine was evaluated as described by Smibert & Krieg (1994). Other enzyme activities were measured using the API 20NE and API ZYM systems (bioMérieux).

Growth of strain MME-001^T occurred at 15–40 °C in the presence of 1.0–7.0 % (w/v) NaCl at pH 7.0–9.0. The optimal growth ranges of this strain were at 25–30 °C in the presence of 2.0–3.0 % (w/v) NaCl at pH 7.0. Catalase and oxidase activities were positive. Strain MME-001^T hydrolyzed gelatin, but not starch, casein, Tweens 20, 40 and 80, or L-tyrosine. Additionally, this strain could not reduce nitrate to nitrite. The API 20NE test revealed that the strain was positive for β-glucosidase, protease and β-galactosidase. In API ZYM, the enzyme activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-

Table 1. Differential phenotypic characteristics of strain MME-001^T and related members of the genus *Pseudoruegeria*

Taxa: 1, MME-001^T (data from this study); 2, *P. sabulilitoris* KCTC 42111^T (Park *et al.*, 2014); 3, '*P. limi*' KCTC 32460 (Lee *et al.*, 2014); 4, *P. lutimaris* KCTC 22690^T (Jung *et al.*, 2010); 5, *P. aquimaris* KCTC 12737^T (Yoon *et al.*, 2007); 6, *P. haliotis* KACC 17214^T (Hyun *et al.*, 2013). All taxa are positive for catalase and oxidase, and enzyme activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), acid phosphatase, and naphthol-AS-BI-phosphohydrolase. All taxa are negative for hydrolysis of L-tyrosine, indole production and enzyme activities of lipase (C14), β-glucuronidase, arginine dihydrolase, cystine arylamidase, trypsin, α-chymotrypsin, α-mannosidase, and α-fucosidase. +, Positive; −, negative.

Characteristic	1	2	3	4	5	6
Optimal growth range of:						
Temperature (°C)	25–30	28–30	25	25–30	37	30
NaCl concentration (%, w/v)	2.0–3.0	2.0	2.0–3.0	2.0–3.0	2.0	1.0
pH	7.0	7.0–8.0	7.0–7.5	7.5–8.5	7.0–8.0	7.0–8.0
Hydrolysis of:*						
Casein	−	−	−	−	−	+
Starch	−	−	−	+	−	−
Tween 20	−	−	−	+	−	−
Tween 40	−	−	−	+	−	+
Tween 80	−	−	−	−	−	+
Gelatin	+	−	−	−	−	+
Reduction of nitrate to nitrite*	−	+	+	+	−	−
Glucose fermentation*	−	+	−	−	−	−
Urease activity*	−	−	−	−	+	+
Enzyme activity*						
Leucine arylamidase	+	+	+	+	+	−
Valine arylamidase	−	−	−	−	+	−
α-Galactosidase	−	−	+	−	+	−
β-Galactosidase	+	+	+	−	+	−
α-Glucosidase	+	+	+	−	+	+
β-Glucosidase	+	−	+	−	+	−
N-Acetyl-β-glucosaminidase	−	−	+	−	+	−
DNA G+C content (mol%)	62	64.1	63.6	73.5	67	66.5

*Data from this study.

glucosidase and β-glucosidase were positive. Other characteristics are provided in Table 1.

To observe utilization of carbohydrates, 1 % (w/v) acetate, L-arabinose, benzoate, cellobiose, citrate, formate, D-fructose, D-galactose, D-glucose, L-glutamate, glycerol, inositol, lactose, malate, maltose, D-mannitol, D-mannose, melibiose, L-ornithine, pyruvate, raffinose, L-rhamnose, D-ribose, D-sorbitol, succinate, sucrose, trehalose and D-xylose were added to modified MB from which the peptone was removed and yeast extract was reduced to 0.01 % (w/v) (González *et al.*, 1997). Acid production was measured as described by Leifson (1963) with the following carbohydrates (1 %, w/v): L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, D-mannose, melibiose, L-rhamnose, D-ribose, trehalose and D-xylose. The test results of carbohydrate utilization and acid production are given in the species description and Table S1.

Susceptibility to antibiotics was determined by spreading the bacterial suspension on MA plates and using discs containing the following antibiotics (μg ml^{-1} unless otherwise stated): ampicillin (10), carbenicillin (100), cephalothin (30), ciprofloxacin (10), erythromycin (25), gentamicin (30), kanamycin (30), lincomycin (15), neomycin (30), norfloxacin (20), novobiocin (10^1), penicillin G (20 UI), polymyxin B (100 UI), streptomycin (50) and tetracycline (30). Strain MME-001^T was sensitive to carbenicillin, erythromycin, gentamicin, kanamycin, neomycin, novobiocin, penicillin G, polymyxin B and streptomycin, but resistant to ampicillin, cephalothin, ciprofloxacin, lincomycin, norfloxacin and tetracycline.

Strain MME-001^T for analysis of quinone and polar lipids was cultivated on MA at 30 °C for 3 days. The isoprenoid quinones were extracted from freeze-dried cells using chloroform/methanol (2 : 1, v/v) (Collins & Jones, 1981) and identified using an HPLC system (YL9100; Younglin). The polar lipids were extracted according to the protocols described by Minnikin *et al.* (1984) and identified by two-dimensional TLC followed by spraying with the appropriate detection reagents (Minnikin *et al.*, 1984; Komagata &

Suzuki, 1987): sulfuric acid-ethanol (1 : 2, v/v) for total lipids, Zinzadze reagent (5 % of ethanolic molybdatophosphoric acid) for total phospholipids, ninhydrin for amino-containing lipids, Dragendorff reagent for phosphatidylcholine, and α-naphthol reagent for glycolipids. The cellular fatty acid composition was analysed as described by Miller (1982) using an Agilent 6890 gas chromatography system and a crosslinked methyl siloxane column (HP-1; A30 m×0.320 mm×0.25 μm). The profile analysis was determined using the Sherlock MIS Software version 6.2, based on the TSBA6 database (Sasser, 1990). The identities of fatty acid methyl esters detected by MIDI were further confirmed by GC-MS using an Agilent GC Series 6890 GC-MS. The genomic DNA G+C content was determined by the method described by Mesbah & Whitman (1989) using reversed-phase HPLC.

The predominant respiratory quinone of strain MME-001^T was ubiquinone-10 (at a peak area ratio of approximately 95 %), which is the same as other members of the genus *Pseudoruegeria* as described by Yoon *et al.* (2007). The composition of polar lipids was phosphatidylcholine (PC), phosphatidylethanolamine (PE), PG, diphosphatidylglycerol (DPG), and four unidentified lipids (Fig. S2). The polar lipid profile of strain MME-001^T was similar to the type strains of *P. sabulilitoris* KCTC 42111^T, showing PC, PE and PG as major polar lipids. The common major polar lipids of the genus *Pseudoruegeria* were PC, PE, PG, an unidentified aminolipid, and an identified lipid (Jung *et al.*, 2010); however, the unidentified aminolipid was not detected from strain MME-001^T in the present study whereas it was detected from the reference strains, *P. sabulilitoris* KCTC 42111^T, *P. aquimaris* KCTC 12737^T and *P. lutimaris* KCTC 22690^T. Although DPG has been a well-known common 'minor' lipid in species of the genus *Pseudoruegeria* (Yoon *et al.*, 2007; Jung *et al.*, 2010; Park *et al.*, 2014), it was only detected from strain MME-001^T but not from *P. sabulilitoris* KCTC 42111^T, *P. aquimaris* KCTC 12737^T or *P. lutimaris* KCTC 22690^T in this study.

The fatty acid profile of strain MME-001^T is shown in Table 2 with strains of other species of the genus *Pseudoruegeria* analysed in this study. The major cellular fatty acids (>5 %) of strain MME-001^T were $C_{18:1}\omega7c$ (85.4 %) and $C_{16:0}$ (6.6 %), which are similar to other reference species of the genus *Pseudoruegeria*. Furthermore, the major fatty acid found in members of the family *Rhodobacteraceae* was reported to be $C_{18:1}\omega7c$ and the detected fatty acid, summed feature 8, which includes $C_{18:1}\omega7c$ (Pujalte *et al.*, 2014). The DNA G+C content of strain MME-001^T was 62 mol%, which is lower than the values of other members of the genus *Pseudoruegeria* (65–74 mol%) (Yoon *et al.*, 2007; Jung *et al.*, 2010; Hyun *et al.*, 2013; Lee *et al.*, 2014; Park *et al.*, 2014).

DNA–DNA hybridization (DDH) was performed fluorometrically by the membrane filter method using a DIG High Prime DNA Labelling and Detection Starter Kit II (Roche Applied Science). The DNA of strain MME-001^T was labelled and the other reference strains were immobilized, with the experiment repeated three times. The DNA–DNA relatedness between strain MME-001^T and *P. sabulilitoris* KCTC 42111^T, '*P. limi*' KCTC 32460, *P. lutimaris* KCTC 22690^T, *P. aquimaris* KCTC 12737^T, and *P. haliotis* KACC 17214^T was 36±5, 57±7, 34±4, 18±5 and 21 ±3 %, respectively. Reciprocal experiments were also performed with the labelled DNA of *P. sabulilitoris* KCTC 42111^T, resulting in a DNA–DNA relatedness value of 33 ±8 % with strain MME-001^T. Current prokaryotic systematics define DDH values of <70 % as indicative of a distinct species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). Thus the DDH values indicated that strain MME-001^T was a distinct member of the genus *Pseudoruegeria*.

Although some characteristics, such as the relatively low G+C content, absence of unidentified amino lipids, and DDH values, distinguished strain MME-001^T from species of the genus *Pseudoruegeria*, phylogenetic, phenotypic, and chemotaxonomic properties of strain MME-001^T revealed that it belonged to the genus *Pseudoruegeria* within the family *Rhodobacteraceae*. Therefore, based on the data presented herein, strain MME-001^T is considered to represent a novel species of the genus *Pseudoruegeria*, for which the name *Pseudoruegeria aestuarii* sp. nov. is proposed.

Description of *Pseudoruegeria aestuarii* sp. nov.

Pseudoruegeria aestuarii (aes.tu.a'ri.i. L. gen. n. aestuarii of a tidal flat).

Cells are Gram-stain-negative, non-motile, aerobic, rod-shaped and 0.5−0.6 μm in width by 2.4−2.5 μm in length. Colonies on MA are circular, convex, smooth, glistening, pale yellow and 1.0 mm in diameter after incubation for 3 days at 30 °C. Growth occurs in presence of 1.0–7.0 % (w/v) NaCl (optimum 2.0−3.0 %) at 15 and 40 °C (optimum 25−30 °C) and pH 7.0–9.0 (optimum pH 7.0). Na^+ is required for growth, nitrate is not reduced to nitrite and anaerobic growth does not occur on MA. Gelatin is hydrolysed, while starch, casein, Tweens 20, 40 and 80, and L-tyrosine are not. In the API 20NE kit, β-glucosidase, protease and β-galactosidase are positive, while indole production, glucose fermentation, and activities of arginine dihydrolase and urease are negative. In the API ZYM test, enzyme activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase and β-glucosidase are positive, but lipase (C14), valine arylamidase, cysteine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are negative. Utilizes acetate, L-arabinose,cellobiose, citrate, formate, D-fructose, D-galactose, D-glucose, L-glutamate, glycerol, inositol, lactose, malate, maltose, D-mannitol, D-mannose, melibiose, pyruvate, L-rhamnose, D-ribose, D-sorbitol, succinate, sucrose, trehalose and D-xylose as carbon and energy sources, but benzoate, L-ornithine and raffinose are not utilized. Acid is produced from L-arabinose, cellobiose,

Table 2. Cellular fatty acid contents (%) of strain MME-001^T and strains of related species of the genus *Pseudoruegeria*

Strains: 1, MME-001^T; 2, *P. sabulilitoris* KCTC 42111^T; 3, '*P. limi*' KCTC 32460; 4, *P. lutimaris* KCTC 22690^T; 5, *P. aquimaris* KCTC 12737^T; 6. *P. haliotis* KACC 17214^T. All data are from this study. TR, Trace <1.0 %; –, not detected.

Fatty acid (%)	1	2	3	4	5	6
Straight-chain						
$C_{16:0}$	6.6	7.1	6.5	6.2	TR	7.7
$C_{17:0}$	TR	TR	4.0	–	TR	1.0
$C_{18:0}$	2.6	2.0	1.8	3.2	3.2	2.0
Unsaturated						
$C_{18:1}\omega7c$	85.4	86.4	78.7	82.7	80.7	81.4
Hydroxy						
$C_{10:0}$ 3-OH	–	–	–	–	3.8	–
$C_{12:0}$ 3-OH	1.5	1.8	2.7	1.1	–	TR
11-Methyl $C_{18:1}\omega7c$	1.5	TR	TR	1.4	3.7	–
Cyclo $C_{19:0}\omega8c$	–	–	–	–	3.5	2.4
Summed feature*						
1	–	–	1.1	TR	–	TR
7	TR	TR	1.1	TR	TR	TR

*Summed features are groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 1 comprised iso-$C_{15:1}$ H and/or $C_{13:0}$ 3-OH; summed feature 7 comprised $C_{19:1}\omega7c$ and/or $C_{19:1}\omega6c$.

D-fructose, D-galactose, D-glucose, D-mannose, melibiose, L-rhamnose, D-ribose, trehalose and D-xylose. The predominant respiratory quinone is Q-10. The polar lipids are PC, PE, PG, DPG and four unidentified lipids. The major fatty acids are $C_{18:1}\omega7c$ and $C_{16:0}$.

The type strain, MME-001^T (=KCCM 43133^T=JCM 30751^T) was isolated from the tidal flat sediment of Muuido in Incheon, Republic of Korea. The genomic DNA G+C content of the type strain is 62 mol% (HPLC).

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