Pseudoruegeria lutimaris sp. nov., isolated from a tidal flat sediment, and emended description of the genus *Pseudoruegeria*

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A Gram-negative-staining, non-motile and rod-shaped bacterial strain, HD-43^T, was isolated from a tidal flat sediment collected from Hwang-do, an island of Korea. Strain HD-43^T grew optimally at pH 7.0–8.0, at 30 °C and in the presence of 2 % (w/v) NaCl. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain HD-43^T clustered with *Pseudoruegeria aquimaris* SW-255^T. It exhibited 96.6 % 16S rRNA gene sequence similarity and 79.4 % *gyrB* sequence similarity with *P. aquimaris* SW-255^T. Strain HD-43^T contained Q-10 as the predominant ubiquinone and $C_{18:1}\omega7c$ as the major fatty acid. The major polar lipids were phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminolipid, an unidentified glycolipid and an unidentified lipid. The DNA G+C content was 73.5 mol%. The mean DNA–DNA relatedness between strain HD-43^T and *P. aquimaris* SW-255^T was 5 %. Differential phenotypic properties demonstrated that strain HD-43^T is clearly distinguishable from *P. aquimaris*. On the basis of phenotypic, chemotaxonomic and phylogenetic data, strain HD-43^T is considered to represent a novel species of the genus *Pseudoruegeria*, for which the name *Pseudoruegeria lutimaris* sp. nov. is proposed. The type strain is HD-43^T (=KCTC 22690^T =CCUG 57754^T).

The genus *Pseudoruegeria*, belonging to the *Alphaproteobacteria*, was first proposed by Yoon *et al.* (2007) with the description of *Pseudoruegeria aquimaris*, which is the sole recognized species of the genus to date. In this study, we describe a bacterial strain, designated HD-43^T, which was isolated from a tidal flat off the west coast of the Korean peninsula. Comparative 16S rRNA gene sequence analysis indicated that this strain was phylogenetically most closely related to the genus *Pseudoruegeria*. The aim of the present work was to investigate the possibility that strain HD-43^T represents a second species of the genus *Pseudoruegeria* by using a polyphasic taxonomic study. Here, we describe phenotypic and chemotaxonomic properties of strain HD-43^T and a detailed phylogenetic investigation based on 16S rRNA gene sequences.

Strain HD-43^T was isolated by means of the standard dilution plating technique at 25 °C on marine agar 2216 (MA; Difco). Strain HD-43^T was maintained on MA at 4 °C for short-term preservation and as a 20 % (w/v) glycerol suspension at −80 °C for long-term preservation.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *gyrB* sequences of strain HD-43^T are FJ374173 and FJ441076, respectively.

P. aquimaris SW-255^T, which was used as a reference strain for phenotypic characterization, was obtained in the study of Yoon *et al.* (2007). Strain HD-43^T was cultivated in marine broth 2216 (MB; Difco) for 3 days at 30 °C to obtain the cell biomass required for DNA extraction and for the analyses of isoprenoid quinones and polar lipids. Cell mass of *P. aquimaris* SW-255^T for polar lipid analysis was obtained from cultures grown in MB at 30 °C. The morphological, physiological and biochemical characteristics of strain HD-43^T were investigated using routine cultivation on MA at 30 °C. The cell morphology and flagellation were examined by using light microscopy (E600; Nikon) and transmission electron microscopy (CM-20; Philips) as described by Yoon *et al.* (2007). Gram reaction was investigated using the bioMérieux Gram-stain kit according to the manufacturer's instructions. Growth at 4, 10, 20, 25, 30, 37 and 40 °C was measured on MA. Growth in the absence of NaCl and in the presence of 0.5, 1.0, 2.0 and 3.0 % (w/v) NaCl was investigated by using trypticase soy broth, prepared according to the formula of the Difco medium except that the medium was supplemented with 0.45 % (w/v) $MgCl_2.6H_2O$ or 0.06 % (w/v) KCl and that NaCl was excluded as necessary. Growth at various NaCl concentra-

tions (2.0–10.0 %, w/v, in increments of 1.0 %) was investigated in MB. The pH range for growth was determined in MB that was adjusted to pH 4.5–9.5 (in increments of 0.5 pH units) by the addition of HCl or Na_2CO_3. Growth under anaerobic conditions was determined after incubation in a Forma anaerobic chamber on MA and on MA supplemented with potassium nitrate (0.1 %, w/v), both of which had been prepared anaerobically under a nitrogen atmosphere. Catalase and oxidase activities were determined as described by Cowan & Steel (1965). Hydrolysis of casein, starch, hypoxanthine, tyrosine and xanthine was tested on MA by using the substrate concentrations described by Cowan & Steel (1965). Hydrolysis of aesculin, gelatin, Tweens 20, 40, 60 and 80 and urea and reduction of nitrate were investigated as described by Lányí (1987) with the replacement of distilled water by artificial seawater [containing (l^{-1} distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g $MgCl_2.6H_2O$, 5.94 g $MgSO_4.7H_2O$ and 1.3 g $CaCl_2.2H_2O$; Bruns *et al.*, 2001]. H_2S production was tested as described by Bruns *et al.* (2001). Susceptibility to antibiotics was tested by spreading bacterial suspension on MA and applying paper discs impregnated with the following antibiotics (μg per disc unless otherwise stated): polymyxin B (100 U), streptomycin (50), penicillin G (20 U), chloramphenicol (100), ampicillin (10), cephalothin (30), gentamicin (30), novobiocin (5), tetracycline (30), kanamycin (30), lincomycin (15), oleandomycin (15), neomycin (30) and carbenicillin (100). Acid production from carbohydrates was tested as described by Leifson (1963). Utilization of various substrates for growth was determined as described by Yurkov *et al.* (1994). The API ZYM system (bioMérieux) was used to determine enzyme activities. Morphological, cultural, physiological and biochemical characteristics of strain HD-43^T are given in the species description and Table 1.

Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), with the exception that RNase T1 was used in combination with RNase A to minimize contamination by RNA. The 16S rRNA gene sequence was amplified by PCR using two universal primers as described previously (Yoon *et al.*, 1998). Sequencing of the amplified fragments and phylogenetic analysis were performed as described by Yoon *et al.* (2003). The almost-complete 16S rRNA gene sequence of strain HD-43^T comprised 1423 nucleotides. In the neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, strain HD-43^T joined *P. aquimaris* SW-255^T with a bootstrap resampling value of 73.2 % (Fig. 1). The relationship between strain HD-43^T and *P. aquimaris* SW-255^T was also maintained in the tree constructed using the maximum-parsimony algorithm (Fig. 1). Strain HD-43^T exhibited highest 16S rRNA gene sequence similarity (96.6 %) with *P. aquimaris* SW-255^T and sequence similarities of less than 95.0 % with other species used in the phylogenetic analysis. The DNA G+C content was determined by the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC. The DNA G+C content of strain HD-43^T was 73.5 mol%.

PCR amplification of the DNA gyrase B subunit gene (*gyrB*) was performed by using two primers, UP-1 and UP-2r, according to the method described by Yamamoto & Harayama (1995). The PCR product was purified with the QIAquick PCR purification kit (Qiagen). The amplified fragment was cloned into pGEM-T Easy vector (Promega) according to the manufacturer's instructions. The sequence of the amplified fragment was determined for both strands by extension from vector-specific priming sites (T7 and SP-6 primers from pGEM-T Easy vector). The *gyrB* sequence similarity between strain HD-43^T and *P. aquimaris* SW-255^T was 79.4 %.

Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987) using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. The predominant isoprenoid quinone detected in strain HD-43^T was ubiquinone-10, which is the same as that of *P. aquimaris* (Yoon *et al.*, 2007). For cellular fatty acid analysis, cell mass of strain HD-43^T and *P. aquimaris* SW-255^T was harvested from MA plates after cultivation for 3 days at 30 °C. The fatty acids were extracted and fatty acid methyl esters were prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The fatty acid profile of strain HD-43^T is shown in Table 2, together with that of *P. aquimaris* SW-255^T, which was also analysed in this study. These fatty acid profiles were similar in that the predominant fatty acid is $C_{18:1}\omega7c$, although there were differences in the proportions of some fatty acids in addition to that of $C_{18:1}\omega7c$, particularly $C_{16:0}$, $C_{10:0}$ 3-OH, $C_{18:0}$, $C_{12:0}$ 3-OH, 11-methyl $C_{18:1}\omega7c$ and an unknown fatty acid (equivalent chain-length 11.799). The fatty acid profile of *P. aquimaris* SW-255^T determined in this study shows some differences in the contents of some fatty acids from those reported in our previous study (Yoon *et al.*, 2007). This may result from differences in growth temperatures; *P. aquimaris* SW-255^T was grown at 37 °C to obtain the cell mass required for fatty acid analysis in Yoon *et al.* (2007). Polar lipids were extracted in accordance with the procedures described by Minnikin *et al.* (1984) and were identified by two-dimensional TLC followed by spraying with the appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). The position of phosphatidylcholine on TLC was confirmed by spraying with Dragendorff's reagent (Sigma). The major polar lipids detected in strain HD-43^T were phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminolipid, an unidentified glycolipid and an unidentified lipid; minor amounts of diphosphatidylglycerol and an unidentified phospholipid were also present (Fig. 2). The major polar lipids of *P. aquimaris* SW-255^T, which were analysed anew in this study, were phosphatidylglycerol, diphosphatidylglycerol, an unidentified aminolipid and an unidentified lipid

Table 1. Differential phenotypic characteristics of strain HD-43^T and *P. aquimaris* SW-255^T

Data for *P. aquimaris* SW-255^T were taken from this study (utilization of substrates) and from Yoon *et al.* (2007). Both strains are positive for oxidase, catalase, hydrolysis of hypoxanthine, utilization of acetate, L-arabinose, cellobiose, D-fructose, D-galactose (weak for strain HD-43^T), D-glucose, L-glutamate, L-malate, maltose, D-mannose, pyruvate (weak for strain HD-43^T), salicin, succinate, sucrose, trehalose and D-xylose (weak for strain HD-43^T), acid production from D-glucose, cellobiose, D-fructose, D-galactose, D-mannitol, D-mannose, trehalose, maltose, melibiose, L-arabinose, L-rhamnose and sucrose, susceptibility to ampicillin, carbenicillin, cephalothin, chloramphenicol, gentamicin, kanamycin, neomycin, novobiocin, oleandomycin, penicillin G, polymyxin B (weak for strain HD-43^T), streptomycin and tetracycline (weak for strain HD-43^T) and activity of alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase (weak for *P. aquimaris* SW-255^T), β-galactosidase and *N*-acetyl-β-glucosaminidase. Both strains are negative for motility, hydrolysis of casein, gelatin, starch, L-tyrosine, Tweens 20, 40, 60 and 80 and xanthine, production of H_2S, reduction of nitrate, acid production from melezitose and raffinose, susceptibility to lincomycin and activity of lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, β-glucuronidase, α-mannosidase, α-fucosidase and valine arylamidase. +, Positive; W, weakly positive; −, negative.

Characteristic	Strain HD-43^T	*P. aquimaris* SW-255^T
Optimum temperature for growth (°C)	30	37
Growth at:		
10 °C	+	−
40 °C	−	+
Anaerobic growth	+	−
Hydrolysis of:		
Aesculin	−	+
Urea	−	W
Utilization of:		
Benzoate	W	−
Citrate	−	W
Formate	W	−
Acid production from:		
myo-Inositol	−	+
Lactose	+	−
D-Ribose	+	−
D-Sorbitol	+	−
D-Xylose	+	−
Enzyme activity (API ZYM)		
α-Galactosidase	+	−
α-Glucosidase	+	−
β-Glucosidase	+	−
Major polar lipids*	PC, PG, PE, AL, GL, L	PG, DPG, AL, L
DNA G+C content (mol%)	73.5	67.0

*AL, Unidentified aminolipid; DPG, diphosphatidylglycerol; GL, unidentified glycolipid; L, unidentified lipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, unidentified phospholipid.

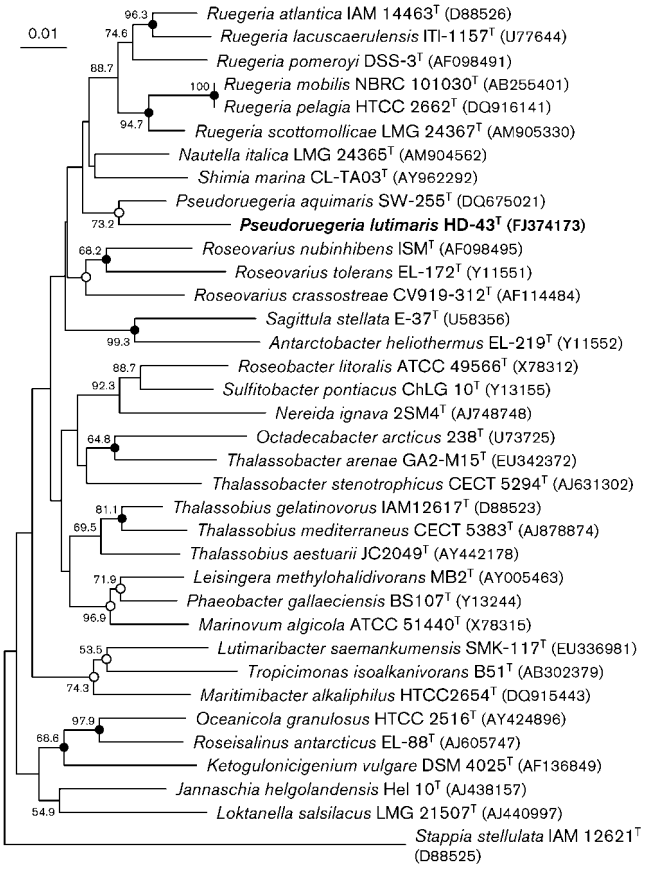

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of strain HD-43^T and some other related taxa. Bootstrap values (>50 %) based on 1000 replications are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. Open circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. *Stappia stellulata* IAM 12621^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

(Fig. 2). The polar lipid profile of *P. aquimaris* SW-255^T determined in this study differed from that given in our previous study (Yoon *et al.*, 2007). An unidentified aminolipid and an unidentified lipid were misinterpreted as phosphatidylethanolamine and an unidentified phospholipid, respectively, in the previous study (Yoon *et al.*, 2007).

Strain HD-43^T differed from *P. aquimaris* SW-255^T in that phosphatidylethanolamine and phosphatidylcholine are present and diphosphatidylglycerol is present as a minor polar lipid (Fig. 2). These intrageneric differences in polar lipid profiles have been reported for several genera (Kämpfer *et al.*, 1997; Yoon *et al.*, 2008). Although there are differences in the fatty acid profiles between the two strains, because of the presence or absence of some fatty acids, the differences are not enough to justify the

Table 2. Cellular fatty acid compositions of strain HD-43^T and *P. aquimaris* SW-255^T

Data were obtained in this study and are percentages of total fatty acids; fatty acids that represented <0.5 % in both strains were omitted. ECL, Equivalent chain-length.

Fatty acid	Strain HD-43^T	*P. aquimaris* SW-255^T
Straight-chain		
$C_{16:0}$	8.9	–
$C_{17:0}$	–	0.8
$C_{18:0}$	–	2.9
Unsaturated		
$C_{18:1}\omega7c$	88.6	83.4
$C_{20:1}\omega7c$	–	0.9
Hydroxy		
$C_{10:0}$ 3-OH	–	3.7
$C_{12:0}$ 3-OH	2.5	–
11-Methyl $C_{18:1}\omega7c$	–	1.8
$C_{19:0}$ cyclo $\omega8c$	–	0.8
Summed feature 3*	–	0.8
Unknown ECL 11.799	–	3.7

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH.

placement of strain HD-43^T and *P. aquimaris* SW-255^T as members of two different genera. Moreover, 16S rRNA gene sequence similarity between strain HD-43^T and *P. aquimaris* SW-255^T appears to be too high to classify them as members of different genera. Accordingly, it is appropriate to classify strain HD-43^T as a member of the genus *Pseudoruegeria*.

DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. DNA from strain HD-43^T and *P. aquimaris* SW-255^T was used individually as labelled DNA probes for cross-hybridization. The highest and lowest values obtained in each sample were excluded and the means of the remaining three values are quoted as DNA–DNA relatedness values. The mean DNA–DNA relatedness between strain HD-43^T and *P. aquimaris* SW-255^T was 5 %, indicating that the two strains are members of different genomic species (Wayne *et al.*, 1987).

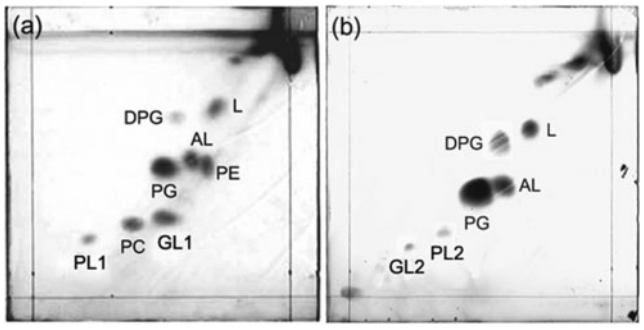

Fig. 2. Thin-layer chromatograms of polar lipids of strain HD-43^T (a) and *P. aquimaris* SW-255^T (b). AL, Unidentified aminolipid; DPG, diphosphatidylglycerol; GL, unidentified glycolipid; L, unidentified lipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, unidentified phospholipid.

Strain HD-43^T was clearly distinguishable from *P. aquimaris* by differences in several phenotypic characteristics, DNA G+C contents, fatty acid compositions and polar lipid profiles (Tables 1 and 2). The phylogenetic and genetic distinctiveness and differential phenotypic properties of strain HD-43^T are sufficient to show that it is separate from *P. aquimaris* (Stackebrandt & Goebel, 1994). On the basis of the data presented, strain HD-43^T is considered to represent a novel species within the genus *Pseudoruegeria*, for which the name *Pseudoruegeria lutimaris* sp. nov. is proposed.

Emended description of the genus *Pseudoruegeria* Yoon *et al.* 2007

The description of the genus *Pseudoruegeria* is as given by Yoon *et al.* (2007) with the following amendments. Common major polar lipids are phosphatidylglycerol, an unidentified aminolipid and an unidentified lipid. The DNA G+C content is 67.0–73.5 mol%.

Description of *Pseudoruegeria lutimaris* sp. nov.

Pseudoruegeria lutimaris (lu.ti.ma′ris. L. n. *lutum* mud; L. gen. n. *maris* of the sea, marine; N.L. gen. n. *lutimaris* of a marine mud).

Cells are non-motile, Gram-negative-staining rods, 0.4–0.6 μm in diameter and 1.0–8.0 μm long. Colonies on MA are circular, convex, smooth, glistening, greyish yellow in colour and 1.0–1.5 mm after incubation for 5 days at 30 °C. Anaerobic growth occurs on MA and on MA supplemented with nitrate. Growth occurs at 10 and 37 °C, but not at 4 or 40 °C. The optimal pH for growth is between pH 7.0 and 8.0; growth occurs at pH 5.0, but not at pH 4.5. Optimal NaCl concentration for growth is approximately 2.0 % (w/v) NaCl. Growth does not occur in the absence of NaCl or with >9.0 % (w/v) NaCl. Na^+ and Mg^{2+} ions are required for growth. The predominant ubiquinone is Q-10. The major fatty acid is $C_{18:1}\omega7c$. Fatty acids $C_{16:0}$ and $C_{12:0}$ 3-OH are also detected. The major polar lipids are phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminolipid, an unidentified glycolipid and an unidentified lipid. Other phenotypic characteristics are given in Table 1. The DNA G+C content of the type strain is 73.5 mol% (HPLC).

The type strain, HD-43^T (=KCTC 22690^T =CCUG 57754^T), was isolated from a tidal flat sediment from Hwang-do, an island of Korea.

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