Pseudoruegeria haliotis sp. nov., isolated from the gut of the abalone *Haliotis discus hannai*

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A novel Gram-negative, aerobic, non-motile and rod-shaped bacterium, designated strain WM67^T, was isolated from the gut of an abalone (*Haliotis discus hannai*) collected from the northern coast of Jeju Island in Korea. Phylogenetic analyses based on the 16S rRNA gene sequence indicated that strain WM67^T clustered in the genus *Pseudoruegeria*, and the highest sequence similarity was shared with *Pseudoruegeria lutimaris* (98.0 % similarity to the type strain). Optimal growth of the isolate occurred at 30 °C, pH 7–8 and with 1 % (w/v) NaCl. The major cellular fatty acids were summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) and $C_{16:0}$. Ubiquinone Q-10 was the major respiratory quinone. The polar lipids of strain WM67^T comprised phosphatidylserine, phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, an unidentified glycolipid and three unidentified lipids. The genomic DNA G+C content was 66.5 mol%. DNA–DNA hybridization indicated <17 % genomic relatedness to other members of the genus *Pseudoruegeria*. The physiological, biochemical, chemotaxonomic and genotypic analyses indicated that strain WM67^T represents a novel species of *Pseudoruegeria*, for which the name *Pseudoruegeria haliotis* sp. nov. is proposed. The type strain is WM67^T (=KACC 17214^T=JCM 18872^T).

The abalone *Haliotis discus hannai*, known as Pacific abalone or Japanese abalone, is distributed throughout coastal waters of East Asia. *H. discus hannai* is recognized as a valuable marine resource, and its aquaculture has been developed widely in East Asia (Oakes & Ponte, 1996). Microbes in the intestinal tract of abalone have important roles in the host physiology, and they can affect the efficiency of aquaculture (Sawabe *et al.*, 2007). Most bacteria isolated from *H. discus hannai* have been identified as alginate-degrading bacteria (Sawabe *et al.*, 1995). Alginate-degrading bacteria such as *Vibrio halioticoli* can enhance the performance of the host digestive system by facilitating algal polysaccharide degradation (Tanaka *et al.*, 2003). However, infections or blooms of bacterial pathogens can cause mass mortality in abalone. For example, *Vibrio harveyi* caused a mass mortality event that affected *H. discus hannai* in August 2002 by generating haemolymphatic oedema in the major circulatory system (Sawabe *et al.*, 2007). A recent study using culture-independent methods showed that members of the *Alphaproteobacteria*, *Epsilonproteobacteria*, *Mollicutes* and *Fusobacteria* were present in the intestine of *H. discus hannai*, while the *Vibrio* group in the class *Gammaproteobacteria* was the predominant taxon (Tanaka *et al.*, 2004).

The genus *Pseudoruegeria* (family *Rhodobacteraceae*) was proposed by Yoon *et al.* (2007), and its members are defined as aerobic, Gram-negative and rod-shaped bacteria that contain ubiquinone-10 (Q-10) as the predominant quinone and $C_{18:1}\omega7c$ as the predominant fatty acid. At the time of writing, *Pseudoruegeria aquimaris* (Yoon *et al.*, 2007) and *Pseudoruegeria lutimaris* (Jung *et al.*, 2010) were recognized as species in the genus *Pseudoruegeria*. Both species were isolated from marine environments: *P. aquimaris* was isolated from seawater (Yoon *et al.*, 2007) and *P. lutimaris* was isolated from a tidal flat sediment (Jung *et al.*, 2010). In a study of the intestinal bacterial diversity of an abalone (*H. discus hannai*), a novel bacterial strain was isolated and subjected to taxonomic investigation. Thus, the present study describes a novel *Pseudoruegeria*-like strain, designated WM67^T, based on physiological, biochemical, chemotaxonomic and genotypic analyses.

The intestinal tract was sampled from an abalone sample collected on the northern coast of Jeju Island in Korea. Intestinal bacteria were isolated by homogenizing the intestinal tract, preparing serial dilutions using 0.22 μm-filtered PBS and spreading on marine agar 2216 (MA; Difco) plates. Strain WM67^T was isolated from a 10^{-2}-diluted

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrB* gene sequences of strain WM67^T are KC196070 and KC855268, respectively.

A supplementary figure and a supplementary table are available with the online version of this paper.

sample after incubation at 30 °C for 72 h. The isolate was purified by repeated subculture. The isolate was stored as a suspension at −80 °C in marine broth 2216 (MB; Difco) containing 40 % (v/v) glycerol. Physiological, biochemical, chemotaxonomic and genotypic analyses were performed at least three times.

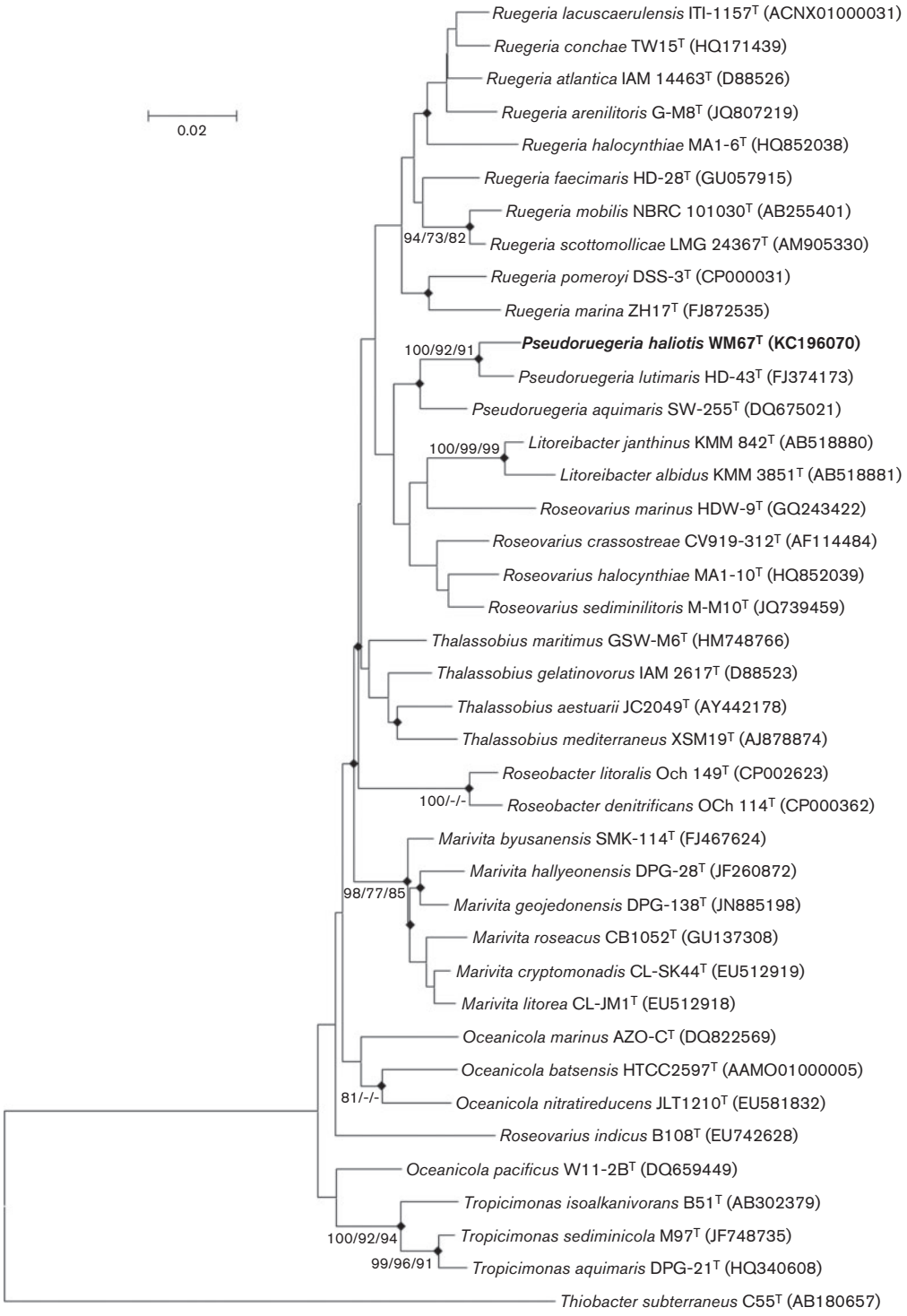
A phylogenetic analysis was performed based on 16S rRNA gene sequences. The 16S rRNA gene of the isolate was amplified by colony PCR using a PCR Premix (*i*NtRon Biotechnology) with two universal bacterial primers: forward primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-GGYTACCTTGTTACGACTT-3′) (Lane, 1991). The 16S rRNA gene amplicon was sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems), according to the manufacturer's instructions. The reaction mixtures were analysed using an automated DNA analyser (Applied Biosystems 3730xl DNA Analyzer). The 16S rRNA gene sequence was assembled using SeqMan (DNASTAR) and the assembled sequence from the isolate was compared with those of type strains in the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). The comparison showed that strain WM67^T was closely related to the genus *Pseudoruegeria* in the class *Alphaproteobacteria*. The closest relatives of the isolate were *P. lutimaris* HD-43^T (98.0 % similarity) and *P. aquimaris* SW-255^T (96.0 %). To determine the phylogenetic relationships among strain WM67^T and members of closely related species, their sequences were aligned using the multiple sequence alignment program CLUSTAL W (Thompson *et al.*, 1994). A phylogenetic consensus trees was reconstructed based on the aligned sequences using the MEGA 5 program (Tamura *et al.*, 2011) with the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Kluge & Farris, 1969) and maximum-likelihood (Felsenstein, 1981) algorithms based on 1000 bootstrap replicates. The phylogenetic consensus tree based on the 16S rRNA gene sequence indicated that strain WM67^T formed a cluster with other members of the genus *Pseudoruegeria* (Fig. 1). Sequences of the DNA gyrase subunit *β* (*gyrB*) genes of strain WM67^T, *P. lutimaris* HD-43^T and *P. aquimaris* SW-255^T were compared to obtain a detailed phylogenetic description. The *gyrB* gene was amplified using primers UP-1 and UP-2R, according to the method described by Yamamoto & Harayama (1995). The isolate shared 80.0 % *gyrB* sequence similarity with *P. lutimaris* HD-43^T and 80.3 % *gyrB* sequence similarity with *P. aquimaris* SW-255^T. To characterize strain WM67^T more comprehensively, *P. lutimaris* HD-43^T (=KCTC 22690^T) and *P. aquimaris* SW-255^T (=KCTC 12737^T) were obtained from the Korean Collection for Type Cultures and used as reference strains.

Gram-staining, cell morphology and colony appearance of the isolate were tested after incubation at 30 °C for 48 h. Gram-staining was performed using a Gram-staining kit (bioMérieux), according to the manufacturer's instructions. Gram-staining and cell morphology were observed by light microscopy (Eclipse 50*i*; Nikon). The motility of the isolate was determined using semi-solid MA (containing 0.4 % agar) (Tittsler & Sandholzer, 1936) after incubation at 30 °C for 1 week. Strain WM67^T produced circular, grey–yellow colonies, 0.6–1.2 mm in diameter, that were convex with an entire margin. The cells were Gram-negative, non-motile and rod-shaped (0.5–0.8 μm wide and 1.0–4.6 μm long). Growth tests of strain WM67^T were conducted in MB under different conditions. The temperature range and optimum were determined at 4, 10, 15, 20, 25, 30, 37, 45, 55 and 65 °C. Growth was tested in the presence of 0, 0.5, 1, 1.5, 2, 3, 4, 5, 8, 10, 12 and 15 % (w/v) NaCl in a medium that contained all of the ingredients of MB except NaCl. The pH tolerance range and optimum pH for growth were tested at pH 4–11 (at intervals of 1.0 pH unit) by adjusting the pH of MB using 10 mM MES for pH 4–6, 10 mM TAPS for pH 7–8 and 10 mM Na_2HPO_4 for pH 9–11. Growth under each condition was determined by measuring the OD_{600} using a spectrophotometer (SYNERGY MX; BioTek) after 24 and 48 h and 7 days of incubation. Anaerobic growth was examined after 7 days of cultivation at 37 °C on MA plates in an anaerobic chamber filled with an $N_2/CO_2/H_2$ (90 : 5 : 5) atmosphere. The isolate grew at 10–37 °C, at pH 5–9 and in the presence of 0.5–5 % (w/v) NaCl. Optimal growth occurred at 30 °C, pH 7–8 and with 1 % (w/v) NaCl. Anaerobic growth of strain WM67^T was not observed.

Catalase and oxidase activities were tested by bubble production with 3 % (v/v) hydrogen peroxide solution and indophenol blue production with 1 % (w/v) tetramethyl *p*-phenylenediamine (bioMérieux), respectively. Hydrolysis of starch and casein was tested by incubating the isolate on MA supplemented with 0.5 % soluble starch (Junsei) and 5 % skimmed milk (Difco) (Benson, 1994), respectively. Hydrolysis of Tweens 20, 40, 60 and 80 was determined according to the method proposed by Goszczynska & Serfontein (1998). Utilization of various sole carbon sources was tested using GN2 MicroPlates (Biolog) with GN/GP inoculating fluid (Biolog) supplemented with 1 % (w/v) NaCl for strain WM67^T, *P. lutimaris* KCTC 22690^T and *P. aquimaris* KCTC 12737^T. Production of acid from carbohydrates and enzyme activities was determined using API 50 CH test strips (bioMérieux) with 50 CHB/E medium and API ZYM test strips (bioMérieux), respectively. In addition, nitrate reduction, production of indole, fermentation of D-glucose and activities of L-arginine dihydrolase, urease, *β*-glucosidase, protease and *β*-galactosidase were tested using API 20NE test strips (bioMérieux).

The isolate could be distinguished from the reference strains by utilization of various carbon sources, enzyme activities and acid production from various carbohydrates. The complete results of the biochemical tests are given in the species description, and differences in biochemical characteristics of the isolate and the reference strains are shown in Table 1.

Chemotaxonomic characterization of strain WM67^T was achieved by comparing its cellular fatty acid profile and isoprenoid quinone composition with those of the type



strains of *P. lutimaris* and *P. aquimaris*. Chemotaxonomic analyses were performed using cell biomass of the isolate and the reference strains after growth on MA plates at 30 °C for 48 h. Cellular fatty acids were extracted according to the protocol used by the Sherlock Microbial Identification Systems (MIDI, 1999). The cellular fatty acid

Fig. 1. Phylogenetic consensus tree based on 16S rRNA gene sequences, reconstructed using the neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) methods, showing the phylogenetic position of strain WM67^T with respect to recognized *Pseudoruegeria* species. Filled diamonds indicate identical branches generated using all three methods. Numbers at nodes represent bootstrap values (NJ/MP/ML) as percentages of 1000 replicates; only values >70 % are shown (–, <70 %). *Thiobacter subterraneus* C55^T was used as an outgroup. Bar, 0.02 accumulated changes per nucleotide.

compositions of the isolate and reference strains were determined by GC (Agilent 6890; Agilent Technologies) and the Microbial Identification System software package (Sherlock version 6.2), based on the TSBA6 database (Sasser, 1990). The major cellular fatty acids (>5 % of the total) in strain WM67^T were summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$; 86.2 %) and $C_{16:0}$ (6.3 %). The fatty acid profile of the isolate was similar to those of both reference strains (Jung *et al.*, 2010; Yoon *et al.*, 2007) (Table 2). Isoprenoid quinones were extracted according to the

Table 1. Differential characteristics of strain WM67^T and its closest phylogenetic relatives in the genus *Pseudoruegeria*

Strains: 1, WM67^T; 2, *P. lutimaris* KCTC 22690^T; 3, *P. aquimaris* KCTC 12737^T. All data were obtained from the current study. +, Positive or weakly positive; −, negative. All strains are positive for the following: catalase and oxidase activities; utilization of dextrin, cellobiose, D-fructose, α-D-glucose, maltose, D-mannose, melibiose, sucrose, turanose, pyruvic acid methyl ester, β-hydroxybutyric acid, α-ketobutyric acid, DL-lactic acid, bromosuccinic acid, L-alanine, L-serine, inosine, uridine and glycerol; acid production from D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, aesculin, salicin, cellobiose, maltose, sucrose, trehalose, turanose, D-lyxose, gluconate and 5-ketogluconate; and activity of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase and β-glucosidase. All strains are negative for the following: utilization of α-cyclodextrin, Tweens 40 and 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, i-erythritol, L-fucose, *myo*-inositol, lactulose, D-mannitol, D-psicose, raffinose, L-rhamnose, D-sorbitol, xylitol, formic acid, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, malonic acid, quinic acid, D-saccharic acid, sebacic acid, glucuronamide, D-alanine, L-aspartic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, DL-carnitine, urocanic acid, thymidine, phenylethylamine, putrescine, 2-aminoethanol and 2,3-butanediol; acid production from glycerol, L-xylose, D-adonitol, methyl β-D-xyloside, L-sorbose, dulcitol, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, inulin, melezitose, starch, glycogen, xylitol, L-fucose, D- and L-arabitol and 2-ketogluconate; activity of lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, β-glucuronidase, α-mannosidase, α-fucosidase, L-arginine dihydrolase and protease; reduction of nitrates to nitrogen; production of indole; and hydrolysis of starch, casein and Tween 60.

Characteristic	1	2	3
Assimilation of:			
Trehalose	+	+	−
D-Galactose, gentiobiose, methyl β-D-glucoside, succinic acid monomethyl ester, γ-hydroxybutyric acid, α-ketoglutaric acid, succinic acid, succinamic acid, L-glutamic acid	+	−	+
α-Hydroxybutyric acid, L-alanyl glycine, glycyl L-glutamic acid, D-glucose 6-phosphate	−	+	+
Lactose, *p*-hydroxyphenylacetic acid, L-alaninamide, L-asparagine	+	−	−
D-Arabitol, α-ketovaleric acid, glycyl L-aspartic acid, DL-α-glycerol phosphate, α-D-glucose 1-phosphate	−	+	−
Glycogen, L-arabinose, acetic acid, *cis*-aconitic acid, citric acid, D-galactonic acid lactone, itaconic acid, propionic acid, L-threonine, γ-aminobutyric acid	−	−	+
Acid production from:			
D-Arabinose, lactose	+	+	−
D-Xylose, melibiose, gentiobiose	+	−	+
L-Arabinose	−	+	+
Erythritol, D-mannitol, D-sorbitol, D-tagatose	−	+	−
L-Rhamnose, inositol, arbutin, raffinose, D-fucose	−	−	+
Enzyme activities			
Valine arylamidase, urease	+	−	+
α-Glucosidase	−	+	+
N-Acetyl-β-glucosaminidase	−	−	+
Reduction of nitrates to nitrites	+	+	−
D-Glucose fermentation	+	−	−
Hydrolysis of:			
Tween 20	+	−	+
Tween 40, Tween 80	+	−	−

method proposed by Collins & Jones (1981a). Isoprenoid quinone extracts were purified by one-dimensional TLC on a silica gel 60 F_{254} plate (Merck) and analysed by reversed-phase HPLC (Collins & Jones, 1981b) using a reversed-phase Hydrosphere C18 column (150 × 2.0 mm; YMC). The major respiratory quinone in the isolate was ubiquinone 10 (Q-10), which is also the main quinone in the two recognized *Pseudoruegeria* species (Jung *et al.*, 2010; Yoon *et al.*, 2007). To determine the polar lipid composition of strain WM67^T, polar lipids were extracted according to the procedures described by Xin *et al.* (2000). The polar lipid extract was separated by two-dimensional TLC on a silica gel 60 F_{254} plate (Merck). Two solvent systems were used for separation: chloroform/methanol/water (65 : 25 : 4, by vol.) for the first dimension and chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by vol.) for the second dimension. Four spray reagents were applied to detect polar lipids (Tindall, 1990): 10 % ethanolic molybdatophosphoric acid reagent for total lipids, ninhydrin reagent for amino-group-containing lipids, Zinzadze reagent for phospholipids and α-naphthol reagent for glycolipids. The phospholipids were identified by one-dimensional TLC using four standard compounds (Sigma): phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine and diphosphatidylglycerol. The polar lipids of strain WM67^T comprised phosphatidylserine, phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, an unidentified glycolipid and three unidentified lipids (Fig. S1, available in IJSEM Online). The polar lipid composition of the isolate was compared with those of the two recognized *Pseudoruegeria* species based on results reported in a previous study (Jung *et al.*, 2010). Strain WM67^T and the reference strains all contained phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine and an unidentified glycolipid, but strain WM67^T lacked diphosphatidylglycerol.

Table 2. Cellular fatty acid contents of strain WM67^T and the type strains of *Pseudoruegeria* species

Strains: 1, WM67^T; 2, *P. lutimaris* KCTC 22690^T; 3, *P. aquimaris* KCTC 12737^T. All data were obtained from the current study. Values are percentages of total fatty acids. TR, Trace (<0.5 %); −, not detected.

Fatty acid	1	2	3
Saturated			
$C_{12:0}$	TR	−	TR
$C_{14:0}$	TR	TR	−
$C_{16:0}$	6.3	5.5	TR
$C_{17:0}$	1.1	3.8	TR
$C_{18:0}$	1.8	1.6	2.6
Unsaturated			
$C_{15:1}\omega8c$	−	TR	−
$C_{17:1}\omega6c$	−	0.6	TR
11-Methyl $C_{18:1}\omega7c$	0.7	TR	5.9
$C_{20:1}\omega7c$	0.8	0.5	1.4
Hydroxy			
$C_{10:0}$ 3-OH	TR	TR	TR
$C_{12:0}$ 3-OH	TR	1.2	−
Cyclopropane acids			
$C_{19:0}$ cyclo $\omega8c$	0.9	TR	3.0
Summed features*			
3	TR	TR	0.6
7	0.9	1.1	TR
8	86.2	83.6	83.5

*Summed features represent groups of two or three fatty acids that could not be separated by the Microbial Identification System. Summed feature 3 comprised $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; summed feature 7 comprised $C_{19:1}\omega6c$ and/or unknown ECL 18.446; summed feature 8 comprised $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$.

Genomic DNA of strain WM67^T, *P. lutimaris* KCTC 22690^T and *P. aquimaris* KCTC 12737^T was extracted and used for genotypic characterization of strain WM67^T, as described by Rochelle *et al.* (1992). The DNA G+C content of the isolate was measured by a fluorometric method with SYBR Gold I using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories) (Gonzalez & Saiz-Jimenez, 2002). Genomic DNA from *Bacteroides thetaiotaomicron* VPI-5482^T, *Escherichia coli* K-12, *Ruegeria pomeroyi* DSS-3^T and *Bacteroides fragilis* NCTC 9343^T was used as calibration references in the analysis. The DNA G+C content of strain WM67^T was 66.5 mol%. To clarify the genetic relatedness between the isolate and the reference strains, DNA–DNA hybridization was performed using a genome-probing microarray (Bae *et al.*, 2005; Chang *et al.*, 2008). DNA–DNA relatedness between strain WM67^T and the type strains of *P. lutimaris* and *P. aquimaris* was 16.5 ± 1.1 % (14.5 ± 0.9 % in the reciprocal analysis) and 6.9 ± 0.1 % (6.1 ± 1.2 % in the reciprocal analysis), respectively (Table S1). These DNA–DNA hybridization values show that strain WM67^T represents a distinct genospecies (Wayne *et al.*, 1987).

Based on the physiological, biochemical, chemotaxonomic and genotypic analyses, we suggest that strain WM67^T represents a novel species in the genus *Pseudoruegeria*, for which the name *Pseudoruegeria haliotis* sp. nov. is proposed.

Description of *Pseudoruegeria haliotis* sp. nov.

Pseudoruegeria haliotis (ha.li.o′tis. N.L. gen. n. *haliotis* of *Haliotis*, the systematic name of a genus of abalone, referring to the isolation of the type strain from *Haliotis discus hannai*).

Cells are Gram-negative, aerobic, rod-shaped (0.5–0.8 × 1.0–4.6 μm), non-motile and positive for catalase and oxidase. Colonies on MA are circular, convex with an entire margin and grey–yellow, 0.6–1.2 mm in diameter after 48 h of incubation at 30 °C. Growth occurs at 10–37 °C (optimum 30 °C), at pH 5–9 (optimum pH 7–8) and with 0.5–5 % (w/v) NaCl (optimum 1 %). Tweens 20, 40 and 80 are hydrolysed, whereas starch, casein and Tween 60 are not. Assimilates dextrin, cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, lactose, maltose,

D-mannose, melibiose, methyl β-D-glucoside, sucrose, trehalose, turanose, pyruvic acid methyl ester, succinic acid monomethyl ester, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketobutyric acid, α-ketoglutaric acid, DL-lactic acid, succinic acid, L-alaninamide, L-alanine, L-asparagine, L-glutamic acid, L-serine, inosine, uridine and glycerol (Biolog GN2). Acid is produced from D-arabinose, ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, gentiobiose, turanose, D-lyxose, gluconate and 5-ketogluconate (API 50 CHB). Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucosidase and urease, reduction of nitrates to nitrites and D-glucose fermentation. The major fatty acids are summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) and $C_{16:0}$. The predominant ubiquinone is Q-10. The polar lipids comprise phosphatidylserine, phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, an unidentified glycolipid and three unidentified lipids.

The type strain, WM67^T (=KACC 17214^T=JCM 18872^T), was isolated from the intestinal tract of an abalone, *Haliotis discus hannai*, which was collected from the northern coast of Jeju Island in the Republic of Korea. The DNA G+C content of the type strain is 66.5 mol%.

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