Pseudorhodobacter aquimaris sp. nov., isolated from seawater, and emended description of the genus *Pseudorhodobacter* Uchino *et al.* 2002

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A Gram-stain-negative, aerobic, motile, ovoid bacterial strain, designated HDW-19^T, was isolated from seawater of the west coast of Korea and subjected to a polyphasic taxonomic study. Strain HDW-19^T grew optimally at pH 7.0–8.0, at 30 °C and in the presence of 2–3% (w/v) NaCl. Bacteriochlorophyll *a* was not produced by strain HDW-19^T. Neighbour-joining, maximum-likelihood and maximum-parsimony phylogenetic trees based on 16S rRNA gene sequences showed that strain HDW-19^T clustered with *Pseudorhodobacter ferrugineus* IAM 12616^T, with which it shared 96.4% similarity. A neighbour-joining phylogenetic tree based on *gyrB* gene sequences showed that strain HDW-19^T also clustered with the type strain of *P. ferrugineus*, sharing 83.0% similarity. Strain HDW-19^T contained Q-10 as the predominant ubiquinone and C_{18:1}ω7c as the major fatty acid. The major polar lipids were phosphatidylcholine, phosphatidylglycerol, three unidentified aminophospholipids and two unidentified aminolipids. The DNA G + C content of strain HDW-19^T was 60.9 mol%. Differential phenotypic properties, together with phylogenetic distinctiveness, showed that strain HDW-19^T can be differentiated from *P. ferrugineus*. On this basis, strain HDW-19^T is considered to represent a novel species of the genus *Pseudorhodobacter*, for which the name *Pseudorhodobacter aquimaris* sp. nov. is proposed. The type strain is HDW-19^T (=KCTC 23043^T =CCUG 58879^T).

The genus *Pseudorhodobacter*, which belongs to the class *Alphaproteobacteria*, was proposed by Uchino *et al.* (2002) based on the reclassification of *Agrobacterium ferrugineum* (Rüger & Höfle, 1992) as *Pseudorhodobacter ferrugineus*, which, at the time of writing, is the sole recognized species of the genus. The genus *Pseudorhodobacter* was differentiated from species of the genus *Rhodobacter* by lower DNA G + C content, an insertion in its 16S rRNA gene sequence, and the lack of photosynthetic abilities and bacteriochlorophyll *a* (BChl *a*; Uchino *et al.*, 2002). In this study, we describe a bacterial strain, designated HDW-19^T, isolated from seawater of Hwang-do of the Yellow Sea, Korea. Comparative 16S rRNA gene sequence analysis indicated that this strain was phylogenetically related most closely to *P. ferrugineus*. The aim of the present work was to investigate the possibility that

strain HDW-19^T represents a second species of the genus *Pseudorhodobacter* by using a polyphasic taxonomic study. Here we describe the phenotypic and chemotaxonomic properties of strain HDW-19^T and detailed phylogenetic investigations based on 16S rRNA and *gyrB* gene sequences.

Strain HDW-19^T was isolated by means of the standard dilution plating technique at 25 °C on marine agar 2216 (MA; Difco). *P. ferrugineus* JCM 20687^T, which was used as a reference strain for phenotypic characterization and fatty acid analysis, was obtained from the Japan Collection of Microorganisms (JCM), Saitama, Japan. The morphological, physiological and biochemical characteristics of strain HDW-19^T were investigated by using routine cultivation on MA at 30 °C. Cell morphology was examined by using light microscopy (E600; Nikon) and transmission electron microscopy. Flagellation was determined with a Philips CM-20 transmission electron microscope with cells from an exponentially growing culture. For this purpose, cells were negatively stained with 1% (w/v) phosphotungstic acid and the grids were examined after being air-dried. The

Abbreviation: BChl *a*, bacteriochlorophyll *a*.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrB* gene sequences of strain HDW-19^T are GU086365 and GU086366, respectively.

Gram reaction was investigated by using the bioMérieux Gram stain kit according to the manufacturer's instructions. 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 nitrogen atmosphere. Growth at 4, 10, 20, 25, 30, 32, 33, 35 and 37 °C was measured on MA. The pH range for growth was determined in marine broth 2216 (MB; Difco) that was adjusted to pH 4.5–9.5 (increments of 0.5 pH units) by the addition of HCl or Na₂CO₃. Growth in the presence of 2.0–15.0 % (w/v) NaCl (increments of 1.0 %) was investigated in MB. Growth in the absence of NaCl and in the presence of 0.5, 1.0 and 2.0 (w/v) NaCl was investigated by using trypticase soy broth, supplemented with 0.45 % (w/v) MgCl₂·6H₂O or 0.06 % (w/v) KCl, prepared according to the formula of the Difco medium except that NaCl was excluded from the medium formula. Catalase and oxidase activities and hydrolysis of casein, starch and Tweens 20, 40, 60 and 80 were determined as described by Cowan & Steel (1965). Hydrolysis of hypoxanthine, tyrosine and xanthine was tested on MA, by using the substrate concentrations given by Cowan & Steel (1965). Nitrate reduction and hydrolysis of aesculin, gelatin and urea were investigated as described by Lányi (1987) but with the modification that artificial seawater was used for the preparation of media. The artificial seawater contained (per litre distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O and 1.3 g CaCl₂·2H₂O (Bruns *et al.*, 2001). H₂S production was tested as described by Bruns *et al.* (2001). Susceptibility to antibiotics was investigated by spreading a bacterial suspension on MA and applying paper discs impregnated with the following compounds: polymyxin B (100 U), streptomycin (50 µg), penicillin G (20 U), chloramphenicol (100 µg), ampicillin (10 µg), cephalothin (30 µg), gentamicin (30 µg), novobiocin (5 µg), tetracycline (30 µg), kanamycin (30 µg), lincomycin (15 µg), oleandomycin (15 µg), neomycin (30 µg) and carbenicillin (100 µg). Acid production from carbohydrates was tested as described by Leifson (1963). Utilization of substrates as sole carbon and energy sources was tested according to the method of Yurkov *et al.* (1994). The API ZYM system (bioMérieux) was used to determine enzyme activities. For spectral analysis of *in vivo* pigment absorption, strain HDW-19^T was cultivated aerobically in the dark at 30 °C in MB. The culture was washed twice via centrifugation with a MOPS buffer (MOPS/NaOH, 0.01 M; KCl, 0.1 M; MgCl₂, 0.001 M; pH 7.5) and disrupted by means of sonication (450 sonifier; Branson). After removal of cell debris by centrifugation, the absorption spectrum of the supernatant was examined on a Beckman Coulter DU800 spectrophotometer.

Cell biomass for DNA extraction and for isoprenoid quinone analysis was obtained from cultures grown in MB at 30 °C. Chromosomal DNA was extracted 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 the contamination of RNA. The

16S rRNA gene was amplified by PCR with two universal primers (5'-GAGTTTGATCCTGGCTCAG-3' and 5'-AG-AAAGGAGGTGATCCAGCC-3') as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). PCR amplification of the DNA gyrase B subunit (*gyrB*) gene 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 a QIAquick PCR purification kit (Qiagen). The amplified *gyrB* gene was cloned into pGEM T-easy vector (Promega) according to the manufacturer's instructions. Sequences of the *gyrB* gene were determined for both strands by extension from vector-specific priming sites (T7 and SP-6 primers from pGEM T-easy vector). Phylogenetic analysis of the *gyrB* gene sequence was performed as described by Yoon *et al.* (2007). The DNA G+C content was determined according to the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC. Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987), by using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. For cellular fatty acid analysis, cell mass of strain HDW-19^T and *P. ferrugineus* JCM 20687^T was harvested from MA plates after cultivation for 5 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). 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).

Morphological, cultural, physiological and biochemical characteristics of strain HDW-19^T are given in the species description below and in Table 1. Sonicated *in vivo* cell extracts of strain HDW-19^T showed no absorption maximum, indicating that it does not produce BChl *a* aerobically in the dark.

The almost-complete 16S rRNA gene sequence of strain HDW-19^T comprised 1420 nt. In the neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, strain HDW-19^T joined *P. ferrugineus* IAM 12616^T at a bootstrap resampling value of 98.7 % (Fig. 1). The relationship between strain HDW-19^T and *P. ferrugineus* IAM 12616^T was also found in the trees reconstructed with the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). Strain HDW-19^T showed highest 16S rRNA gene sequence similarity (96.4 %) to *P. ferrugineus* IAM 12616^T and less than 95.4 % similarity to the type strains of other species used in the phylogenetic analysis. An insertion in the 16S rRNA gene sequence found in *P. ferrugineus* IAM 12616^T was not detected in strain HDW-19^T (data not shown). The *gyrB* gene sequence of strain HDW-19^T determined in this study comprised 1210 nt. The neighbour-joining phylogenetic tree based on *gyrB*

Table 1. Differential phenotypic characteristics between strain HDW-19^T and *Pseudorhodobacter ferrugineus* IAM 12616^T

Strains: 1, HDW-19^T; 2, *P. ferrugineus* IAM 12616^T. Data are from Rüger & Höfle (1992), Uchino *et al.* (2002) and this study. Both strains are positive for: catalase and oxidase activities, susceptibility to* streptomycin, chloramphenicol, cephalothin, gentamicin, kanamycin, oleandomycin, neomycin and carbenicillin, and activity of* leucine arylamidase. Both strains are negative for: Gram stain, anaerobic growth, nitrate reduction, BChl *a* production, hydrolysis of aesculin, urea, xanthine, L-tyrosine, starch, gelatin, and Tweens 20, 40, 60 and 80, utilization of* D-glucose, D-fructose, D-galactose, cellobiose, D-mannose, trehalose, D-xylose, L-arabinose, sucrose, maltose, benzoate, pyruvate, salicin, formate and L-glutamate, acid production from* D-ribose, D-fructose, L-arabinose, melezitose, D-glucose, D-galactose, D-rhamnose, cellobiose, lactose, sucrose, maltose, trehalose and raffinose, susceptibility to* ampicillin, novobiocin and lincomycin, and activity of* lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. w, Weakly positive.

Characteristic	1	2
Optimum temperature for growth (°C)	30	25
Motility	+	–
Hydrolysis of:		
Hypoxanthine	+	–*
Casein	w	–
Utilization of:*		
Acetate	+	–
Citrate	+	–
Succinate	+	–
L-Malate	+	–
Acid production from:*		
D-Sorbitol	w	–
myo-Inositol	+	–
D-Xylose	–	w
D-Mannitol	+	–
Melibiose	–	w
D-Mannose	–	w
Susceptibility to:*		
Polymycin B	+	–
Penicillin G	+	–
Tetracycline	–	+
Enzyme activity (API ZYM)*		
Alkaline phosphatase	–	+
Esterase (C4)	+	w
Esterase lipase (C8)	w	–
Acid phosphatase	–	w
DNA G + C content (mol%)	60.9	58

*Data for *P. ferrugineus* IAM 12616^T from this study.

gene sequences showed that strain HDW-19^T clustered with *P. ferrugineus* IAM 12616^T, with which it shared 83.0 % similarity (Fig. 2). Strain HDW-19^T exhibited *gyrB* gene sequence similarity values of less than 77.5 % to the

type strains of other species used in this study. The DNA G + C content of strain HDW-19^T was 60.9 mol%. The predominant isoprenoid quinone detected in strain HDW-19^T was ubiquinone-10 (Q-10), at a peak area ratio of approximately 82 %, consistent with data for *P. ferrugineus* (Uchino *et al.*, 2002); a minor amount of Q-9 (approx. 9 %) was also found. The fatty acid profile of strain HDW-19^T is shown in Table 2, together with that of *P. ferrugineus* JCM 20687^T, which was also analysed under the same conditions in this study. The major fatty acid found in the two strains was C_{18:1} ω 7c (Table 2). These fatty acid profiles were similar, although there were differences in the proportions of some components (Table 2). C_{14:1} 3-OH was detected in *P. ferrugineus* from the study of Uchino *et al.* (2002), but it was not detected in strain HDW-19^T nor in *P. ferrugineus* JCM 20687^T here (Table 2). The major polar lipids detected in strain HDW-19^T were phosphatidylcholine, phosphatidylglycerol, three unidentified aminophospholipids and two unidentified aminolipids (Fig. 3). The major polar lipids of *P. ferrugineus* JCM 20687^T were the same as those of strain HDW-19^T except that two unidentified aminophospholipids were not detected (Fig. 3). The results obtained from chemotaxonomic analyses showed the properties that are shared by *P. ferrugineus* and thereby support the result of the phylogenetic analysis, i.e. that strain HDW-19^T is a member of the genus *Pseudorhodobacter*. Lack of BChl *a*, which is characteristic of *P. ferrugineus*, was also found for strain HDW-19^T.

Strain HDW-19^T was distinguishable from *P. ferrugineus* based on differences in several phenotypic characteristics, including motility and hydrolysis of, utilization of and acid production from some substrates (Table 1). The phylogenetic distinctiveness and differential phenotypic properties of strain HDW-19^T were sufficient to categorize it as a member of a species that is separate from *P. ferrugineus* (Stackebrandt & Goebel, 1994). Therefore, on the basis of the data presented, strain HDW-19^T is considered to represent a novel species of the genus *Pseudorhodobacter*, for which the name *Pseudorhodobacter aquimaris* sp. nov. is proposed.

Emended description of the genus *Pseudorhodobacter* Uchino *et al.* 2002

The description of the genus *Pseudorhodobacter* is as given by Uchino *et al.* (2002) with the following amendments. Motile or non-motile. Optimal growth temperature is 25–30 °C. Common major polar lipids are phosphatidylcholine, phosphatidylglycerol, two unidentified aminolipids and an unidentified aminophospholipid. The DNA G + C content is 58.0–60.9 mol%.

Description of *Pseudorhodobacter aquimaris* sp. nov.

Pseudorhodobacter aquimaris (a.qui.ma'ris. L. n. *aqua* -ae water; L. n. *mare* -is the sea; N.L. gen. n. *aquimaris* of seawater).

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain HDW-19^T among related taxa. Bootstrap values (expressed as percentages of 1000 replications) of >50% are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Rhodospirillum rubrum* ATCC 11170^T (GenBank accession no. D30778) was used as an outgroup (not shown). Bar, 0.01 substitutions per nucleotide position.

Fig. 2. Neighbour-joining phylogenetic tree based on *gyrB* gene sequences showing the position of strain HDW-19^T among related taxa. Bootstrap values (expressed as percentages of 1000 replications) of >50% are shown at branch points. *Oceanospirillum linum* ATCC 11336^T was used as an outgroup. Bar, 0.1 substitutions per nucleotide position.

Table 2. Fatty acid compositions (%) of strain HDW-19^T and *Pseudorhodobacter ferrugineus* JCM 20687^T

Strains: 1, HDW-19^T; 2, *P. ferrugineus* IAM 12616^T. Data are from this study. Fatty acids that represented <0.5% in both strains were omitted. —, Not detected.

Fatty acid	1	2
Straight-chain		
C _{16:0}	1.1	2.0
C _{17:0}	—	0.7
C _{18:0}	3.2	4.3
Unsaturated		
C _{18:1} ω7c	86.4	81.2
C _{18:1} ω9c	—	1.5
Hydroxy		
C _{10:0} 3-OH	2.8	4.0
Summed features*		
3	2.8	0.8
7	0.2	1.6
Unknown†		
ECL 11.799	2.7	3.4

*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. Summed feature 7 contained C_{19:1}ω6c, C_{19:0}ω10c cyclo and/or unknown fatty acid ECL 18.846.

†ECL, equivalent chain-length.

Cells are Gram-stain-negative, ovoid, 0.5–1.0 µm in diameter and 1.0–2.0 µm long. Motile by means of a single polar flagellum. Colonies on MA are circular, convex, smooth, glistening, cream-coloured and 1.0–2.0 mm in diameter after incubation for 5 days at 30 °C. Anaerobic growth does not occur on MA or on MA supplemented with nitrate. The optimal growth temperature is 30 °C; growth occurs at 4 and 35 °C but not at 37 °C. The optimal pH for growth is between 7.0 and 8.0; growth occurs at pH 5.0 but not at pH 4.5. Optimal growth occurs in the

presence of 2–3% (w/v) NaCl; growth occurs in the presence of 12.0% (w/v) NaCl but not in the absence of NaCl or in the presence of more than 13.0% (w/v) NaCl. Mg²⁺ ions are required for growth. BChl *a* is not produced. H₂S is not produced. The predominant ubiquinone is Q-10. The major fatty acid (>10% of the total) is C_{18:1}ω7c. The major polar lipids are phosphatidylcholine, phosphatidylglycerol, three unidentified aminophospholipids and two unidentified aminolipids. The DNA G+C content of the type strain is 60.9 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, HDW-19^T (=KCTC 23043^T =CCUG 58879^T), was isolated from sea water of Hwang-do, an island of Korea.

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Fig. 3. Thin-layer chromatograms of polar lipids of strain HDW-19^T (a) and *Pseudorhodobacter ferrugineus* JCM 20687^T (b). PC, phosphatidylcholine; PG, phosphatidylglycerol; AL1 and 2, unidentified aminolipids; PN1–3, unidentified aminophospholipids.

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