Marinibaculum pumilum gen. nov., sp. nov., isolated from seawater

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A Gram-stain-negative, facultatively anaerobic, motile and rod-shaped strain, designed H2^T, was isolated from the Western Pacific Ocean, and subjected to a taxonomic investigation using a polyphasic approach. Strain H2^T grew at 15–40 °C and pH 6.0–9.0 (optimum 37 °C and pH 6.5), and with 1–10 % (w/v) NaCl (optimum 2 %). The predominant respiratory quinone was ubiquinone-10 (Q-10) and the major fatty acids identified were $C_{19:0}$ cyclo ω8*c*, $C_{18:1}\omega$7*c*, $C_{18:0}$ and 11-methyl-$C_{18:1}\omega$7*c*. The polar lipids of strain H2^T consisted of phosphatidylglycerol, one unknown phospholipid, one unknown glycolipid and three unidentified aminolipids. The DNA G+C content was 75.0 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain H2^T formed a distinct clade belong to the family *Rhodospirillaceae* within the *Alphaproteobacteria*. On the basis of morphological, physiological and chemotaxonomic characteristics, together with the results of phylogenetic analysis, strain H2^T represents a novel species in a new genus in the family *Rhodospirillaceae*, for which the name *Marinibaculum pumilum* gen. nov., sp. nov. is proposed. The type strain of the type species is H2^T(=MCCC 1K02279^T=KCTC 42964^T).

The class *Alphaproteobacteria* comprises a large group of Gram-negative bacteria within the phylum *Proteobacteria* and is currently divided into eleven orders: *Caulobacterales*, *Kiloniellales*, *Kordiimonadales*, *Magnetococcales*, *Parvularculales*, *Rhodobacterales*, *Rhizobiales*, *Rickettsiales*, *Sneathiellales*, *Sphingomonadales* and *Rhodospirillales* (http://www.bacterio.net/alphaproteobacteria.html). These bacteria show a wide range of morphological, physiological and genetic characteristics (Batut *et al.*, 2004).

The family *Rhodospirillaceae*, belonging to the order *Rhodospirillales*, was established by Pfennig & Trüper (1971). At the time of writing, the family *Rhodospirillaceae* comprises 34 genera and 93 species with validly published names (http://www.bacterio.net/-classifgenerafamilies.html#Rhodospirillaceae), which show diverse morphological, metabolical and ecological features. Members of this family include chemo-organotrophs, chemolithotrophs and facultative photoheterotrophs, and some of them are also able to grow photoautotrophically (Garrity *et al.*, 2005). Species of genera in the family *Rhodospirillaceae* have been found in a wide variety of habitats, such as freshwater, activated sludge biomass, air, soil, roots, cystic fibrosis patients, Antarctic white rock, desert sand and saline environments (Amoozegar *et al.*, 2013). In this paper, we present a polyphasic study describing a novel bacterium, strain H2^T, which belongs to a new genus of the family *Rhodospirillaceae*.

Strain H2^T was isolated from a seawater sample of the Mariana Trench of Western Pacific Ocean (12.3508° N 145.2070° E) at the depth of 500 m. The water sample was diluted using the standard dilution-plating method (Williama & Davies, 1965) and was spread on modified marine agar 2216 medium (Pan *et al.*, 2014). A very small, circular and pale yellow colony of H2^T was collected after incubation at 28 °C for half a month. Purified strain H2^T was routinely cultured on marine agar (MA; BD Difco) or in marine broth 2216 (MB; Difco) at 37 °C and maintained at −80 °C with 30 % (v/v) glycerol, and was freeze-dried for long-term preservation. Three strains, *Taonella mepensis* CCTCC AB 2012861^T (Xi *et al.*, 2013), *Ferrovibrio denitrificans* LMG 25817^T (Sorokina *et al.*, 2012) and

Abbreviation: PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain H2^T is KT265740.

A supplementary table and two supplementary figures are available with the online Supplementary Material.

Oceanibacterium hippocampi DSM 23444^T (Balcázar *et al.*, 2012) were selected as references for physiological and chemotaxonomic analyses. Unless otherwise stated, the reference strains *F. denitrificans* LMG 25817^T and *O. hippocampi* DSM 23444^T were cultured under the same conditions as strain H2^T, while strain *T. mepensis* CCTCC AB 2012861^T was incubated on a NaCl-free LB medium containing 10.0 g l^{-1} tryptone (BD Difco) and 5.0 g l^{-1} yeast extract (BD Difco).

Cell morphological characteristics were observed by light microscopy (BX40; Olympus) and transmission electron microscopy (JEM-1230; JEOL). Motility was determined by microscopic observation and inoculation in semi-solid agar medium. Limits for growth conditions were confirmed in MB over a period of 10 days. The temperature range for growth was determined at 4–50 °C (4, 10, 15, 20, 25, 28, 30, 35, 37, 40, 45 and 50 °C). The pH range for growth was measured from 4.5–10.0 with an interval of 0.5 using appropriate biological buffers (MES for pH 4.5–6.0, PIPES for pH 6.5–7.0, Tricine for pH 7.5–8.5 and CAPSO for pH 9.0–10.5) at a concentration of 50 mM. Tolerance of NaCl (0–15.0 %, w/v, at intervals of 1 %) was investigated by using NaCl-free MB (prepared according to the MB formula, but without NaCl). Anaerobic growth was determined in the modified MB with sodium thiosulfate (20 mM), sodium sulfite (5 mM), sodium sulfate (20 mM), sodium nitrite (5 mM), sodium nitrate (20 mM) and L-arginine (5 g l^{-1}) as electron acceptors, cysteine (1 g l^{-1}) as reductant and resazurin (1 mg l^{-1}) as oxygen indicator.

The Gram-staining reaction, nitrate reduction, urease activity and hydrolysis of starch, gelatin and casein were tested according to Dong & Cai (2001). Catalase and oxidase activities were individually determined by using 3 % (v/v) hydrogen peroxide solution and oxidase reagent (bioMérieux). Tweens 20, 40, 60 and 80 were examined as described by Sun *et al.* (2015). H_2S and indole production tests were assayed according to Zhang *et al.* (2013). The utilization of carbohydrates, enzyme activities and other biochemical properties were tested using GN2 MicroPlates (Biolog), and the API ZYM and API 20NE strips (bioMérieux) according to the manufacturer's instructions. Antibiotic susceptibility was tested on MA plates with antibiotic discs containing one of the following: ampicillin (10 μg), erythromycin (15 μg), streptomycin (10 μg), rifampicin (5 μg), chloramphenicol (30 μg), gentamycin (10 μg), bacitracin (0.04 U), carbenicillin (100 μg) and kanamycin (30 μg).

For chemotaxonomic studies, cell mass of strain H2^T and the three reference strains were grown in MB or NaCl-free LB medium at 37 °C for 3 days and then freeze-dried. Cellular fatty acid methyl esters were extracted as described by Kuykendall *et al.* (1998) and analysed according to the instructions of the Microbial Identification System (MIDI). Isoprenoid quinones were extracted as described by Komagata & Suzuki (1987) and analysed by LC-MS (Agilent) (Tindall *et al.*, 2007). The polar lipids were extracted and separated by two-dimensional TLC (silica-gel plates 60, cat. no. 5554; Merck), and identified using standard procedures (Minnikin *et al.*, 1984; Fang *et al.*, 2012).

Genomic DNA was extracted by a Quick Bacteria Genomic DNA Extraction kit (DongSheng Biotech) and the 16S rRNA gene of strain H2^T was amplified using universal primers 27F/1492R (Kim *et al.*, 1998). PCR products were cloned into pMD-19T vector (TaKaRa) and the positive clones were sequenced. The resulting almost-complete 16S rRNA gene sequence (1464 bp) was subjected to pairwise sequence alignment by the EzTaxon-e server (Kim *et al.*, 2012). Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1985) methods with the software package MEGA version 5.0 (Tamura *et al.*, 2011). Bootstrap analyses for all trees were based on 1000 replications. The G+C content of the genomic DNA was determined by HPLC according to Mesbah *et al.* (1989).

Cells of strain H2^T were Gram-stain-negative, facultatively anaerobic, motile and rod-shaped (0.4–0.8 μm in width and 2.0–3.0 μm in length) without flagella (Fig. S1, available in the online Supplementary Material). Colonies were 0.1–0.2 mm in diameter, circular, slightly convex and pale yellow with entire margins after growth on MA at 37 °C for 3 days. The temperature range for growth was 15–40 °C (optimum 37 °C). Growth occurred at pH 6.0–9.0 (optimum pH 6.5) and with 1–10 % (w/v) NaCl (optimum 2 %). No growth was observed without NaCl. Under the optimal growth conditions, cells reached the logarithmic phase on the third day and the stationary phase on the fifth day. Strain H2^T was susceptible to streptomycin (10 μg), rifampicin (5 μg), erythromycin (15 μg), kanamycin (30 μg), chloramphenicol (30 μg), and carbenicillin (100 μg), and was resistant to ampicillin (10 μg), gentamicin (10 μg), and bacitracin (0.04 U). Detailed physiological and biochemical characteristics are given in Tables 1 and S1, and in the species description.

On the basis of 16S rRNA gene sequence alignment, strain H2^T was related most closely to three single-species genera (http://www.bacterio.net/), namely *Taonella*, *Ferrovibrio* and *Oceanibacterium*, with similarities of 91.2, 90.1 and 90.0 %, respectively. The phylogenetic trees reconstructed with all three treeing methods showed that strain H2^T constituted an independent clade within the family *Rhodospirillaceae* (except for the genus *Oceanibacterium*), which was separate from other taxa with validly published names, and formed a cluster with *T. mepensis* and *F. denitrificans* (Fig. 1). The DNA G+C content of strain H2^T was calculated to be 75.0 mol%. This value corresponds closely to some recognized members of the family *Rhodospirillaceae* (Coenye *et al.*, 2002; Amoozegar *et al.*, 2013).

The chemotaxonomic data supported that strain H2^T was similar to the members within the class *Alphaproteobacteria*. Respiratory quinone composition analysis showed that Q-10 was the sole quinone in strain H2^T. This result

Table 1. Differential phenotypic characteristics of strain H2^T and the reference strains

Taxa: 1, H2^T; 2, *Taonella mepensis* CCTCC AB 2012861^T; 3, *Ferrovibrio denitrificans* LMG 25817^T; 4, *Oceanibacterium hippocampi* DSM 23444^T. All data are from this study unless otherwise indicated. +, Positive; −, negative; W, weakly positive; ND, no data available.

Characteristic	1	2	3	4
Source	Seawater	Activated sludge[a]	Redox zone of a low-salinity spring[b]	Cutaneous mucus of seahorse[c]
Flagella	Not found	Single polar[a]	Single polar[b]	Not found[c]
Cell size (width×length; μm)	0.4–0.8×2.0–8.0	1.2–1.4×1.5–1.7[a]	0.3×0.8–1.3[b]	0.4×1.0–1.6[c]
Temperature range (optimum)(°C)	15–40 (37)	25–40 (37)[a]	5–45 (35)[b]	10–35 (25)[c]
pH range (optimum)	6.0–9.0 (6.5)	5.0–8.0 (6.0–7.0)[a]	5.5–8.0 (6.2)[b]	5.0–9.0 (7.0)[c]
NaCl tolerance (optimum) (%, w/v)	1–10 (2)	0–4 (0.5)[a]	0–2.5 (ND)[b]	1–6 (2)[c]
Type of metabolism	Facultatively anaerobic	Strictly aerobic[a]	Facultatively anaerobic[b]	Aerobic[c]
Catalase activity	+	+	W	+
Hydrolysis of:				
Casein	−	+	−	−
Gelatin	−	+	−	−
Starch	−	W	−	−
Tween 40	+	+	−	+
Tween 80	−	+	+	−
Urea	W	+	+	+
Nitrate reduction	−	+	+	−
H_2S production	−	+	W	−
API ZYM tests				
Alkaline phosphatase	+	W	+	+
Esterase (C4)	+	W	−	−
Esterase lipase (C8)	+	W	W	W
Lipase (C14)	W	−	−	−
Leucine arylamidase	W	+	+	+
Valine arylamidase	+	W	W	+
Cysteine arylamidase	+	W	−	W
Trypsin	+	+	−	−
Chymotrypsin	+	W	+	−
API 20NE tests				
Hydrolysis of aesculin	−	−	+	−
β-Galactosidase	−	−	+	+
Assimilation of:				
L-Arabinose	−	+	W	W
D-Mannitol	−	W	W	W
Maltose	W	+	+	W
Potassium gluconate	−	+	−	W
Capric acid	W	+	−	W
Malic acid	W	W	+	+
Phenylacetic acid	−	−	W	−
DNA G+C content (mol%)	75.0	65.1[a]	64.2[b]	57.8[c]

*Data from: *a*, Xi *et al.* (2013); *b*, Sorokina *et al.* (2012); *c*, Balcázar *et al.* (2012).

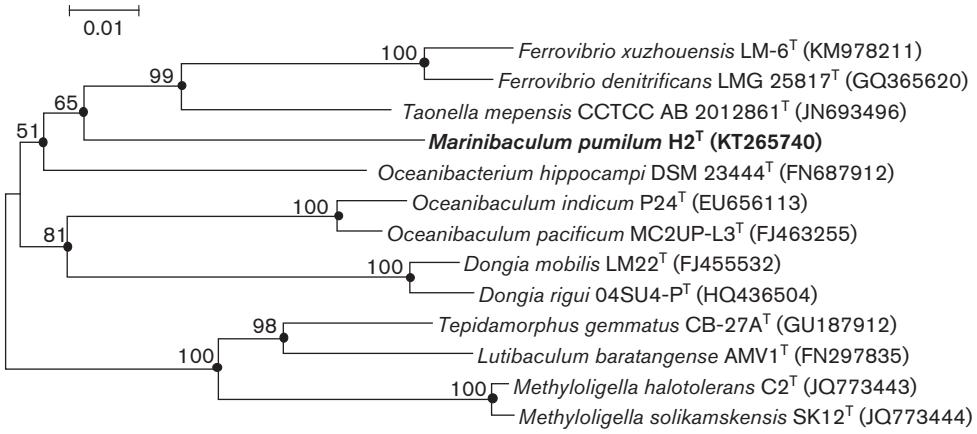

Fig. 1. Neighbour-joining phylogenetic tree of strain H2^T and some closely related taxa, based on 16S rRNA gene sequences (1464 bp). Numbers at branching points represent bootstrap values (%) from 1000 replicates; only values ≥50 % are shown. Filled circles indicate that the corresponding nodes were also recovered in maximum-likelihood and maximum-parsimony trees. Bar, 0.01 substitutions per nucleotide position.

supported the affiliation of strain H2^T to the class *Alphaproteobacteria* because the possession of Q-10 as the major quinone is a characteristic of this group (Xie & Yokota, 2005). Fatty acid analysis of strain H2^T revealed that $C_{19:0}$ cyclo $\omega 8c$ (34.8 %), $C_{18:1}\omega 7c$ (33.7 %), $C_{18:0}$ (7.6 %) and 11-methyl-$C_{18:1}\omega 7c$ (6.0 %) were the major cellular fatty acids (≥5 %) (Table 2). These data were similar to the other three reference strains, and all four strains possessed $C_{18:1}\omega 7c$ as one of the most abundant fatty acids; most members of the class *Alphaproteobacteria* have $C_{18:1}\omega 7c$ as their predominant fatty acid (Labrenz *et al.*, 2000). The polar lipids detected in strain H2^T were phosphatidylglycerol (PG), one unknown phospholipid (PL1), one unknown glycolipid (GL1) and three unidentified aminolipids (AL1–3) (Fig. S2). Among these, four polar lipids including PG, GL1 and two unknown aminolipids (AL1 and AL2), were also present in the three reference species as major polar lipids.

The chemotaxonomic results also showed clear differences in fatty acid compositions and polar lipid profiles between strain H2^T and closely related genera. The fatty acids $C_{18:1}\omega 7c$ and $C_{16:0}$ were the major fatty acids of *T. mepensis* CCTCC AB 2012861^T, *F. denitrificans* LMG 25817^T and *O. hippocampi* DSM 23444^T, but in strain H2^T, $C_{19:0}$ cyclo $\omega 8c$ and $C_{18:1}\omega 7c$ were the major fatty acids. Meanwhile, the amount of $C_{16:0}$ (2.9 %) in strain H2^T was strikingly lower than that of *T. mepensis* CCTCC AB 2012861^T (20.9 %), *F. denitrificans* LMG 25817^T (22.2 %) and *O. hippocampi* DSM 23444^T (20.3 %). In addition, fatty acids $C_{14:0}$, $C_{17:0}$ and $C_{16:0}$ 3-OH were present in three reference strains but absent in strain H2^T, and one unsaturated fatty acid, $C_{20:1}\omega 9c$, and unknown 18.814 were found in strain H2^T (1.6 and 1.1 %, respectively) but were not detected in other reference strains (Table 2). For the polar lipid profiles, the most conspicuous difference was that all three related genera *Taonella*, *Ferrovibrio* and *Oceanibacterium* had phosphatidylethanolamine (PE) as one of the major polar lipids, but it was not found in strain H2^T (Fig. S2).

Not only the chemotaxonomic analysis above, but also phylogenetic, genomic, phenotypic, physiological and biochemical characteristics clearly indicated that strain H2^{T}could also be distinguished from its closest relatives. For example, the DNA G+C content of strain H2^T (75.0 mol%) was obviously higher than that of the genera *Taonella*, *Ferrovibrio* and *Oceanibacterium* (65.1, 64.2 and 57.8 mol%, respectively). Strain H2^T could not grow without NaCl and had a wide tolerant range with 1–10 % (w/v) NaCl, which differed from the reference strains *T. mepensis* CCTCC AB 2012861^T and *F. denitrificans* LMG 25817^{T}that grew with 0–4 % and 0–2.5 % (w/v) NaCl, respectively. More detailed differences including physiological and biochemical characteristics are shown in Table 1. The results of the GN2 MicroPlate system are shown in Table S1. The low level of 16S rRNA gene sequence similarity between strain H2^T and all other members of the class *Alphaproteobacteria*, together with the differential properties described above, suggest that the isolate represents a novel species of a new genus within the family *Rhodospirillaceae*, for which the name *Marinibaculum pumilum* gen. nov., sp. nov. is proposed.

Description of *Marinibaculum* gen. nov.

Marinibaculum (Ma.ri.ni.ba′cu.lum.L. adj. *marinus* of the sea; L. neut. n. *baculum* stick; N.L. neut. n. *Marinibaculum* a rod from the sea).

Cells are Gram-stain negative, facultatively anaerobic, motile, non-spore-forming and rod-shaped. Growth occurs in a wide range of NaCl concentrations (1–10 %). Catalase- and oxidase-positive. The major isoprenoid quinone is ubiquinone-10 (Q-10). The principal cellular fatty acids

Table 2. Cellular fatty acid contents (%) of strain H2^T and the type strains of related species

Taxa: 1, H2^T; 2, *T. mepensis* CCTCC AB 2012861^T; 3, *F. denitrificans* LMG 25817^T; 4, *O. hippocampi* DSM 23444^T. All data are from this study. Fatty acids that represented <1.0 % in all strains are not shown. TR, Trace (<1.0 %); –, not detected.

Fatty acid	1	2	3	4
Straight-chain				
$C_{14:0}$	–	TR	1.3	TR
$C_{16:0}$	2.9	20.9	22.2	20.3
$C_{17:0}$	–	TR	TR	2.0
$C_{18:0}$	7.6	1.0	8.0	4.3
Unsaturated				
$C_{16:1}\omega 11c$	–	–	–	3.8
$C_{18:1}\omega 9c$	TR	–	–	1.3
$C_{18:1}\omega 7c$	33.7	43.6	26.4	21.5
11-methyl-$C_{18:1}\omega 7c$	6.0	TR	TR	4.3
$C_{19:0}$ cyclo $\omega 8c$	34.8	17.1	13.8	33.1
$C_{20:1}\omega 9c$	1.6	–	–	–
Hydroxy				
$C_{16:0}$ 3-OH	–	3.1	4.9	TR
$C_{18:1}$ 2-OH	2.8	1.3	9.9	2.5
$C_{18:0}$ 3-OH	4.4	1.3	TR	2.8
Summed features*				
2	1.2	3.3	1.7	TR
3	TR	4.0	7.4	TR
Unknown				
14.502	1.0	TR	TR	–
18.814	1.1	–	–	–

*Summed features represent one or more fatty acids that cannot be separated by GLC with the MIDI system; summed feature 2 comprises iso-$C_{16:1}$ I and/or $C_{14:0}$ 3OH; summed feature 3 comprises $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH.

(>10 %) are $C_{19:0}$ cyclo $\omega 8c$ and $C_{18:1}\omega 7c$. The major polar lipids include PG, aminolipids, phospholipid and glycolipid. The type species is *Marinibaculum pumilum*.

Description of *Marinibaculum pumilum* sp. nov.

Marinibaculum pumilum (pu′mi.lum. L. neut. adj. *pumilum* small or tiny, referring to the tiny colonies formed by this organism).

Cells are approximately 0.4–0.8 μm in width and 2.0–8.0 μm in length without flagella. Colonies are 0.1–0.2 mm in diameter, circular, slightly convex and pale yellow with entire margins after growth on MA at 37 °C for 3 days. Growth occurs at 15–40 °C (optimum 37 °C), pH 6.0–9.0 (optimum pH 6.5) and with 1–10 % (w/v) NaCl (optimum 2 %). NaCl is necessary for growth. Positive results in tests for hydrolysis of gelatin, Tween 20, Tween 40 and Tween 60. Negative results in tests for hydrolysis of casein, starch and Tween 80, H_2S production and indole production. Nitrate is not reduced. Weakly positive result occurs in test for the hydrolysis of urea. In API ZYM tests, alkaline phosphatase, esterase (C4), esterase lipase (C8), valine arylamidase, cysteine arylamidase, trypsin and chymotrypsin activities are positive; lipase (C14), leucine arylamidase, acid phosphatase and napthol-AS-BI-phosphohydrase activities are weakly positive; and α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are negative. In API 20NE tests, assimilation of glucose and hydrolysis of gelatin are positive, but negative results are observed for production of indole, fermentation of glucose, activity of arginine dihydrolase and β-galactosidase, hydrolysis of aesculin, and assimilation of L-arabinose, D-mannitol, potassium gluconate and phenylacetic acid; weakly positive results are obtained for hydrolysis of urea, assimilation of D-mannose, maltose, *N*-acetylglucosamine, capric acid, adipic acid, malic acid and trisodium citrate. In GN2 MicroPlates, utilization of Tween 40, Tween 80, trehalose, acetic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, DL-lactic acid, succinic acid, succinamic acid, L-alaninamide and L-glutamic acid are positive; utilization of the other substrates are negative. The sole respiratory quinone is Q-10. Dominant cellular fatty acids (>5 %) are $C_{19:0}$ cyclo $\omega 8c$, $C_{18:1}\omega 7c$, $C_{18:0}$ and 11-methyl-$C_{18:1}\omega 7c$. The polar lipids are PG, one unknown phospholipid (PL1), one unknown glycolipid (GL1) and three unidentified aminolipids (AL1, AL2, AL3).

The type strain, H2^T (=MCCC 1K02279^T=KCTC 42964^T), was isolated from seawater of the Mariana Trench at a depth of 500 m in the Western Pacific Ocean. The DNA G+C content of the type strain is 75.0 mol%.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (no. 31470005). Cong Sun was also supported by the Science Foundation of Zhejiang Sci-Tech University (16042186-Y).

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