Halioglobus japonicus gen. nov., sp. nov. and *Halioglobus pacificus* sp. nov., members of the class *Gammaproteobacteria* isolated from seawater

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Two coccoid, non-motile bacteria were isolated from seawater in the north-western Pacific Ocean near Japan. The two strains, designated S1-36^T and S1-72^T, were Gram-negative, obligately aerobic, heterotrophic and catalase-negative. They were able to reduce nitrate to nitrogen. Both strains required NaCl for growth, with optimum growth in 2% NaCl, and grew at 15–30 °C, with optimum growth at 20–25 °C. Genomic DNA G + C contents of strains S1-36^T and S1-72^T were 59.6 and 59.4 mol%, respectively. The predominant isoprenoid quinone was Q-8 and major cellular fatty acids were C_{16:1}ω7c, C_{18:1}ω7c and C_{17:1}ω8c. Analyses of 16S rRNA gene sequences revealed that strains S1-36^T and S1-72^T were related to each other (96.1% sequence similarity) and both strains showed 92.3–94.7% sequence similarity with members of the genus *Haliae*. On the basis of phenotypic and phylogenetic features, strains S1-36^T and S1-72^T should be classified as representatives of two novel species in a new genus, *Halioglobus* gen. nov., within the class *Gammaproteobacteria*. The names proposed are *Halioglobus japonicus* sp. nov., the type species of the genus, with S1-36^T (=NBRC 107739^T=KCTC 23429^T) as type strain, and *Halioglobus pacificus* sp. nov., with S1-72^T (=NBRC 107742^T=KCTC 23430^T) as type strain.

The genus *Haliae* in the family *Alteromonadaceae* was proposed by Urios *et al.* (2008b) and currently comprises *Haliae salexigens* (Urios *et al.*, 2008b), *Haliae rubra* (Urios *et al.*, 2009) and *Haliae mediterranea* (Lucena *et al.*, 2010). These organisms belong to a branch of marine *Gammaproteobacteria* that corresponds to the so-called NOR5/OM60 clade (Yan *et al.*, 2009). This clade also includes *Congregibacter litoralis*, the first cultured representative of marine aerobic anoxygenic phototrophic *Gammaproteobacteria*. The NOR5 clade includes more than 500 16S rRNA gene sequences from public databases but, until recently, it has contained very few cultured and taxonomically characterized representatives.

Two aerobic bacteria, strains S1-36^T and S1-72^T, isolated from seawater collected in the north-western Pacific Ocean

were found to belong to the NOR5/OM60 clade within the class *Gammaproteobacteria*. A polyphasic taxonomic analysis of these isolates demonstrated that they represent two novel species in a new genus.

These aerobic bacteria were isolated from seawater samples collected from the north-western Pacific Ocean (30° 11' N 145° 05' E; depth, 100 m) during the *R/V Mirai* [Japan Agency for Marine-Earth Science and Technology (JAMSTEC)] MR10-01 cruise on February 9, 2010. The seawater (200 µl) sample was inoculated on 1/10 strength ZoBell agar medium [0.5 g peptone, 0.1 g yeast extract, 15 g agar in 1 l 80% aged natural seawater (80% seawater + 20% water, aged for at least 6 months)] and incubated at 20 °C for 30 days. The isolated strains were maintained on marine agar 2216 (MA; Difco) at 25 °C. The temperature (5, 10, 15, 20, 25, 30, 37 and 45 °C) and pH (5–10) ranges for growth were determined on MA. The NaCl concentration for growth was determined on MA containing 0, 1, 2, 3, 5, 8, 10 and 15% (w/v) NaCl. Gram-staining was performed as described by Murray *et al.* (1994). Cell morphology and motility were observed using light microscopy (BX60; Olympus) and transmission electron

Abbreviations: ML, maximum-likelihood; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains S1-36^T and S1-72^T are AB602427 and AB602430, respectively.

Two supplementary figures and a supplementary table are available with the online version of this paper.

microscopy. Growth under anaerobic conditions was determined after incubation for 4 weeks in an AnaeroPack (Mitsubishi Gas Chemical) on MA. Catalase activity was determined by bubble formation in a 3 % H₂O₂ solution. Oxidase activity was determined using cytochrome oxidase test paper (Nissui Pharmaceutical). API 20E, API 20NE, API 50CH and API ZYM strips (bioMérieux) were used to determine physiological and biochemical characteristics. All suspension media for the API test strips were supplemented with 2 % (w/v) NaCl solution (final concentration). API 20E, API 20NE and API 50CH tests were read after incubation for 5 days and API ZYM test strips were read after incubation for 2 days at 25 °C. Respiratory quinone determination was carried out as described previously (Xie & Yokota, 2003). The two novel bacterial strains were grown on MA for 3 weeks at 25 °C for analysis of fatty acid methyl esters. Fatty acid methyl esters were extracted and prepared according to standard protocols provided by the MIDI/Hewlett Packard Microbial Identification system Sherlock Version 4.0 TSBA 40 (Sasser, 1990). Polar lipids were extracted according to the procedures described by Minnikin *et al.* (1984). They were identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). Phospholipids were detected with the Zinzadze reagent of Dittmer & Lester (1964). Whole lipid profiles were detected by spraying with molybdato-phosphoric acid (10 g molybdato-phosphoric acid hydrate in 100 ml ethanol) followed by heating at 120–160 °C (Worliczek *et al.*, 2007). DNA was prepared according to the method of Marmur (1961) from cells grown on MA and the DNA G + C content was determined by using the HPLC method of Mesbah *et al.* (1989). A fragment of approximately 1400 bp from the 16S rRNA gene was amplified from the extracted DNA by using bacterial universal primers specific to the 16S rRNA gene: 27F and 1492R (Lane, 1991; Weisburg *et al.*, 1991). To ascertain the phylogenetic position of the novel isolates, the 16S rRNA gene sequences of strains S1-36^T and S1-72^T were compared with sequences obtained from GenBank (<http://www.ncbi.nlm.nih.gov>). Multiple alignments of sequences were performed using CLUSTAL_X (version 1.83) (Thompson *et al.*, 1997). Alignment gaps and ambiguous bases were not taken into consideration when the 1282 bases of 16S rRNA gene nucleotides were compared. Aligned sequences were analysed using MEGA4 (Tamura *et al.*, 2007). Evolutionary distances (Kimura, 1983), and clustering with the neighbour-joining (NJ; Saitou & Nei, 1987) and maximum-likelihood (ML; Felsenstein, 1981) methods using PhyML program version 2.4.4 (Guindon & Gascuel, 2003) were determined by using bootstrap values based on 1000 replications (Felsenstein, 1985). Similarities were calculated using the same software values.

Cells of strain S1-36^T and S1-72^T grown on MA at 25 °C for 3 weeks were coccoid-shaped, approximately 0.4–0.6 µm diameter. Flagella and spores were not observed microscopically (Fig. S1, available in IJSEM Online). No

growth was observed under anaerobic conditions on MA. Growth occurred only under aerobic conditions between 15 and 30 °C. Optimal temperature and pH ranges for growth were 20–25 °C and pH 7–8, respectively. Both strains required NaCl for growth, but could not grow in NaCl concentrations above 8 % (Table 1). The major cellular fatty acids of strains S1-36^T and S1-72^T were C_{16:1}ω7c (24.6 and 25.4 %, respectively), C_{18:1}ω7c (18.9 and 16.6 %) and C_{17:1}ω8c (9.0 and 11.1 %) (Table S1).

The polar lipids of strain S1-36^T comprised phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and an unidentified phospholipid, whereas those of strain S1-72^T were phosphatidylglycerol, two diphosphatidylglycerols and an unidentified phospholipid (Fig. S2).

Physiological and biochemical characteristics of strains S1-36^T and S1-72^T were compared with those of members of related genera, i.e. *Haliea* (Urios *et al.*, 2008b), *Congregibacter* (Spring *et al.*, 2009), *Dasania* (Lee *et al.*, 2007), ‘*Oceanicoccus*’ (Park *et al.*, 2011), *Spongiibacter* (Graeber *et al.*, 2008), *Melitea* (Urios *et al.*, 2008a) and *Zhongshania* (Li *et al.*, 2011), in the class *Gammaproteobacteria* (Table 1).

Almost complete 16S rRNA gene sequences for strain S1-36^T and S1-72^T were determined and a BLAST search in the GenBank showed that the strains belonged to the class *Gammaproteobacteria*. Strains S1-36^T and S1-72^T showed 96.1 % sequence similarity to each other and high 16S rRNA gene sequence similarities to *H. salexigens* 3X/A02/235^T (93.3 and 94.3 %, respectively), *H. mediterranea* 7SM29^T (92.7 and 94.7 %, respectively) and *H. rubra* CM41_15a^T (92.7 and 94.0 %, respectively). Phylogenetic trees obtained by the NJ (Fig. 1) and ML methods revealed clear affiliations of the novel isolates S1-36^T and S1-72^T to the cluster comprising *H. salexigens* 3X/A02/235^T, *H. mediterranea* 7SM29^T, *H. rubra* CM41_15a^T, *C. litoralis* KT71^T and six uncultured clones.

In summary, their coccoid shape, catalase-negative activity, esterase activity, and ability to utilize arabinose, ribose and fructose distinguish strains S1-36^T and S1-72^T from members of the most closely related genus *Haliea*. Also, strain S1-36^T differed from S1-72^T in *N*-acetyl-β-glucosaminidase and naphthol-AS-BI-phosphohydrolase activities, hydrolysis of aesculin, utilization of glycerol, maltose and trehalose, and polar lipid profiles.

Differential physiological and chemotaxonomic properties of strains S1-36^T and S1-72^T and related genera are shown in Table 1. The data presented show that strains S1-36^T and S1-72^T are sufficiently different from related genera and that they represent two novel species, *Halioglobus japonicus* gen. nov., sp. nov. and *Halioglobus pacificus* sp. nov., in a new genus in the class *Gammaproteobacteria*.

Description of *Halioglobus* gen. nov.

Halioglobus (Ha.li.o.glo'bus. Gr. adj. *halios* belonging to the sea or marine; L. masc. n. *globus* a ball, sphere, globe; N.L. masc. n. *Halioglobus* a marine coccus).

Table 1. Differential characteristics of strains S1-36^T and S1-72^T and related genera in the class *Gammaproteobacteria*

Taxa: 1, S1-36^T; 2, S1-72^T; 3, *Haliea* (Urios *et al.*, 2008b, 2009; Lucena *et al.*, 2010); 4, *Congregibacter* (Spring *et al.*, 2009); 5, *Dasania* (Lee *et al.*, 2007); 6, '*Oceanicoccus*' (Park *et al.*, 2011); 7, *Spongiibacter* (Graeber *et al.*, 2008; Hwang & Cho, 2009); 8, *Melitea* (Urios *et al.*, 2008a); 9, *Zhongshania* (Li *et al.*, 2011). –, Negative or none; v, variable; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9
Shape	Coccus	Coccus	Rod	Pleomorphic	Irregular rod	Coccus/ amorphous	Rod	Rod	Rod
Flagellation	–	–	v	+	+	+	+	+	+
Growth temperature (°C)									
Range	10–30	10–30	10–44	9–33	4–30	10–30	10–40	15–37	4–35
Optimum	20–25	20–25	25–30	28	17–22	15–20	20–35	30	30
NaCl tolerance (%)	1–4	1–5	0.4–15	1–7	1–9	1–5	1–9	0.7–7	0–9
Catalase	–	–	+	+	+	–	+	+	+
Esterase	+	+	–	ND	–	+	+	–	ND
<i>N</i> -Acetyl- β -glucosaminidase	–	+	v	ND	–	–	v	–	ND
Utilization of:									
Arabinose	–	–	–	ND	–	+	v	–	–
Glucose	+	+	v	–	–	+	ND	+	–
Ribose	+	+	–	ND	–	+	ND	ND	ND
Fructose	+	+	–	–	–	–	–	+	ND
Maltose	+	–	v	ND	–	–	ND	+	–
Polar lipids*	PG, DPG, PE, PL	PG, 2DPG, PL	PG, DPG, PE, AL, GL	PC, PE, PL	ND	ND	ND	ND	ND
DNA G + C content (mol%)	59.6	59.4	61.4–64.8	57.8	37	49.8	57.7–69.1	57	51.5–51.8

*DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; AL, unidentified aminolipid; GL, atypical glycolipid; PL, unidentified phospholipid.

Fig. 1. NJ phylogenetic tree based on the 16S rRNA gene sequences showing the position of strains S1-36^T and S1-72^T, among the currently known and related species of the family *Alteromonadaceae*. Numbers at nodes are bootstrap percentages derived from 1000 replications (NJ/ML). The sequence of *Simidiua agarivorans* SA1^T (EF617350) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position. Asterisks indicate nodes recovered with nodes with bootstrap values <50 % in the ML tree.

Cells are coccoid shaped, non-motile, Gram-negative and obligately aerobic. Catalase-negative and oxidase-positive. NaCl is need for growth. Nitrate is reduced to N₂. The respiratory quinone is Q-8. Predominant cellular fatty acids are C_{16:1}ω7c, C_{18:1}ω7c and C_{17:1}ω8c. The polar lipids present are phosphatidylglycerol, diphosphatidylglycerol and an undefined phospholipid. The type species is *Halioglobus japonicus*.

Description of *Halioglobus japonicus* sp. nov.

Halioglobus japonicus (ja.po'ni.cus. N.L. masc. adj. *japonicus* pertaining to Japan, from where the isolate originated).

Exhibits the following properties in addition to those given in the genus description. Cells are 0.3–0.5 μm in diameter. Temperature range for growth is 15–30 °C. Optimal temperature for growth is 20–25 °C. No growth occurs above 37 °C. pH range for growth is 6.0–9.0; optimum is pH 7.0–8.0. NaCl is required for growth. Optimum NaCl concentration for growth is 2 %; tolerates up to 4 % (w/v) NaCl. Catalase-negative but oxidase-positive. Production

of acetoin and reduction of nitrate to N₂ are observed. Gelatin and aesculin are hydrolysed but agar and starch are not. Alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase are positive but leucine arylamidase, β-glucosidase α-galactosidase, β-galactosidase, α-glucosidase, β-glucuronidase, α-mannosidase and α-fucosidase are negative. Acid is produced from ribose, glucose, fructose, aesculin, maltose, sucrose, trehalose and glycogen but not from glycerol, galactose, *N*-acetylglucosamine, melibiose, mannose, turanose, D-lyxose, 5-ketogluconate, D-arabinose, L-arabinose, D-tagatose, starch, xylitol, D-xylose, L-xylose, methyl β-D-xylopyranoside, rhamnose, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, salicin, cellobiose, lactose, melezitose, inulin, raffinose, gentiobiose, D-fucose, L-fucose, erythritol, adonitol, sorbose, dulcitol, inositol, mannitol, sorbitol, D-arabitol, L-arabitol, gluconate or 2-ketogluconate. Major cellular fatty acids (≥5 %) are C_{16:1}ω7c, C_{18:1}ω7c, C_{17:1}ω8c, C_{11:0}, C_{10:0} and C_{17:0}.

The type strain, S1-36^T (=NBRC 107739^T=KCTC 23429^T), was isolated from the north-western Pacific Ocean near Japan. The DNA G+C content of the type strain is 59.6 mol%.

Description of *Halioglobus pacificus* sp. nov.

Halioglobus pacificus (pa.ci'fi.cus. L. masc. adj. *pacificus* peaceful, pertaining to the Pacific Ocean).

Exhibits the following properties in addition to those given in the genus description. Cells are 0.3–0.5 µm in diameter. Temperature range for growth is 15–30 °C. Optimal temperature for growth is 20–25 °C. No growth occurs above 37 °C. pH range for growth is 6.0–10.0; optimum is pH 7.0–8.0. NaCl is required for growth. Optimum NaCl concentration is 2%; tolerates up to 5% (w/v) NaCl. Catalase-negative but oxidase-positive. Production of acetoin and reduction of nitrate to N₂ are observed. Gelatin is hydrolysed but agar, starch, aesculin are not hydrolysed. Alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin and acid phosphatase are positive but leucine arylamidase, β-glucosidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucuronidase, α-mannosidase, naphthol-AS-BI-phosphohydrolase, N-acetyl-β-glucosaminidase and α-fucosidase are negative. Acid is produced from glycerol, ribose, glucose, fructose, sucrose, arbutin and glycogen but not from galactose, N-acetylglucosamine, melibiose, mannose, turanose, 5-ketogluconate, D-arabinose, maltose, D-tagatose, D-xylose, L-arabinose, L-xylose, methyl β-D-xylopyranoside, rhamnose, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, salicin, cellobiose, lactose, melezitose, inulin, raffinose, trehalose, gentiobiose, D-fucose, L-fucose, erythritol, adonitol, sorbose, dulcitol, inositol, mannitol, sorbitol, starch, aesculin, xylitol, D-arabitol, L-arabitol, gluconate or 2-ketogluconate. Major cellular fatty acids (≥5%) are C_{16:1}ω7c, C_{18:1}ω7c, C_{17:1}ω8c and C_{11:0}.

The type strain, S1-72^T (=NBRC 107742^T=KCTC 23430^T), was isolated from the north-western Pacific Ocean near Japan. The DNA G+C content of the type strain is 59.4 mol%.

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