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3 ***Sulfurimonas aquatica* sp. nov., a sulfur-oxidizing bacterium**  
4 **isolated from water of a brackish lake**

5

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15 **Abstract**

16 A novel chemolithoautotrophic bacterium, strain H1576<sup>T</sup>, was isolated from  
17 water of a brackish lake. Strain H1576<sup>T</sup> grew aerobically on inorganic sulfur compounds.  
18 Hydrogen gas did not support autotrophic growth, and heterotrophic growth was not  
19 observed. Cells were rod-shaped, motile, 1.5–2.7 μm in length and 0.6–0.7 μm in width.  
20 Growth was observed at 3–22°C with an optimum growth temperature of 13–15°C. The  
21 pH range for growth was 6.0–7.4 with an optimum pH of 6.6–6.8. Major fatty acids were  
22 summed feature 3 (C<sub>16:1</sub>ω7c and/or C<sub>16:1</sub>ω6c). The complete genome of strain H1576<sup>T</sup>  
23 consists of a circular chromosome and a plasmid, with total length of 2.8 Mbp and G + C  
24 content of 46.4 mol%. Phylogenetic analyses indicated that strain H1576<sup>T</sup> belongs to the  
25 genus *Sulfurimonas* but distinct from representatives of existing species. On the basis of  
26 genomic and phenotypic characteristics, a new species named *Sulfurimonas aquatica* sp.  
27 nov. is proposed with the type strain of strain H1576<sup>T</sup> (= BCRC 81254<sup>T</sup> = JCM 35004<sup>T</sup>).

28

## 29 **Introduction**

30           According to the List of Prokaryotic Names with Standing in Nomenclature,  
31 LPSN (Parte et al., 2020), the genus *Sulfurimonas* belongs to the family  
32 *Helicobacteraceae* and currently includes eight species with validly published names (as  
33 of 26 July 2022). They grow chemolithoautotrophically by oxidizing inorganic sulfur  
34 compounds, with oxygen as electron acceptor. In some species, anaerobic growth and H<sub>2</sub>  
35 gas oxidation are observed. As chemotaxonomic feature, they share major fatty acids of  
36 C<sub>16</sub> : 1, C<sub>18</sub> : 1 and C<sub>16</sub> : 0. Besides these eight species, three other species and two  
37 *Candidatus* species have been proposed in this genus, on the basis of genomic and  
38 phenotypic characterizations of isolated strains (Table 1).

39           As reviewed previously (Han & Perner, 2015), members of the genus  
40 *Sulfurimonas* have been repeatedly detected by 16S rRNA gene sequence analysis, in  
41 various ecosystems represented by hydrothermal vents, marine sediments and water  
42 columns. In addition, *Sulfurimonas* is known to be a dominating bacterial genus in some  
43 engineered microbial systems, as shown in recent studies employing 16S rRNA gene  
44 amplicon sequencing (Fu et al., 2020; Wu et al., 2020; Haosagul et al, 2021). With the  
45 same approach, a dominance of *Sulfurimonas* species at specific water depths of a  
46 stratified brackish lake was recently reported (Watanabe et al., 2022). This shallow

47 eutrophic lake, Lake Harutori in Japan, is characterized by steep chemocline and high  
48 concentration of sulfide in bottom water (Kubo et al, 2014; Watanabe et al., 2022). In this  
49 study, a novel sulfur-oxidizing autotroph was isolated from anoxic water of Lake Harutori,  
50 and characterized as a representative of a new species in the genus *Sulfurimonas*.

51

## 52 **Materials and methods**

53         Sampling of water from Lake Harutori was conducted on 16 Feb 2016. A sample  
54 of anoxic bottom water was collected from 5 m depth, at a site where previous studies  
55 were conducted (Kubo et al., 2014; Watanabe et al., 2022). A portion of the sample (0.3  
56 ml) was inoculated into 30 ml of a medium for aerobic thiosulfate oxidizers. The medium  
57 (hereafter referred to as basal medium) was prepared as described below. First, the  
58 following salts ( $\text{g l}^{-1}$ ) were dissolved in distilled water and then sterilized by autoclaving:  
59 NaCl (20),  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  (5),  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (3),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.3),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$   
60 (0.1),  $\text{NH}_4\text{Cl}$  (0.1),  $\text{KH}_2\text{PO}_4$  (0.1) and KCl (0.1). To the autoclaved and cooled salt  
61 solution, the following stock solutions ( $\text{ml l}^{-1}$ ) were aseptically added: trace element  
62 solution (1), selenite-tungstate solution (1), vitamin mixture solution (1) and 1M  $\text{NaHCO}_3$   
63 solution (30). The vitamin mixture solution consisted of the followings ( $\text{mg l}^{-1}$ ): biotin  
64 (20), folic acid (20), pyridoxine-HCl (100), thiamine-HCl $\cdot 2\text{H}_2\text{O}$  (50), riboflavin (50),

65 nicotinic acid (50), calcium D(+) pantothenate (50), 4-Aminobenzoic acid (50), lipoic  
66 acid (50) and cyanocobalamine (1). The other stock solutions were prepared as described  
67 previously (Widdel & Bak, 1992). Finally, pH of the medium was adjusted to 7.0–7.2  
68 with HCl. From the enrichment culture established, pure culture of strain H1576<sup>T</sup> was  
69 obtained by repeated serial dilution with the basal medium. The enrichment and isolation  
70 were performed at 15°C in the dark.

71 Phenotypic characteristics of strain H1576<sup>T</sup> were investigate by culturing the  
72 strain at 15°C in the basal medium, unless otherwise specified. Cell morphology was  
73 observed with phase-contrast light microscopy, and Gram-stain test was conducted with  
74 a kit (Fluka). Cellular fatty acid profile was obtained with the Sherlock Microbial  
75 Identification System (MIDI) version 6.0 (database; TSBA6).

76 To determine upper and lower limits of temperature for growth, strain H1576<sup>T</sup>  
77 was inoculated into the basal medium and incubated at 0, 3, 5, 8, 13, 15, 18, 22, 25, 28,  
78 30 and 32°C. Effect of NaCl concentration on growth was examined by using media  
79 modified from the basal medium, with lowered concentration of MgCl<sub>2</sub> · 6H<sub>2</sub>O (0.2 g l<sup>-1</sup>  
80 <sup>1</sup>) and varying concentrations of NaCl (0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 % and 6.0 w/v).  
81 Effect of pH on growth was tested with media of various pH which were prepared as  
82 below. The media commonly contained the following constituents (l<sup>-1</sup>): 20 g NaCl, 5 g

83  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ , 1 g  $\text{NaHCO}_3$ , 0.2 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.1 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.1 g  $\text{NH}_4\text{Cl}$ , 0.1 g  
84  $\text{KH}_2\text{PO}_4$ , 0.1 g KCl, 1 ml trace element solution, 1 ml selenite-tungstate solution and 1 ml  
85 vitamin mixture solution. Each medium of varying pH contained one of buffering  
86 reagents listed below (at a final concentration of 20 mM), along with NaOH for pH  
87 adjustment. Tested pH and buffering reagents were as follows; pH 5.8, 5.9, 6.0, 6.1, 6.2,  
88 6.3, 6.4, 6.5, 6.7, 6.8, 6.9, 7.1, 7.2, 7.4 and 7.7 with MES; pH 6.6, 6.9 and 7.2 with PIPES;  
89 pH 7.0, 7.2, 7.3 and 7.6 with MOPS. All ingredients were mixed and then sterilized by  
90 filtration.

91 Utilization of electron donors was tested with the basal medium, by replacing  
92 thiosulfate with one of the followings (mM); sulfide (2), pyruvate (5), lactate (5), acetate  
93 (5), propionate (2.5), succinate (2.5), fumarate (2.5), malate (2.5), butyrate (2.5), benzoate  
94 (2.5), isobutyrate (2.5), methanol (5), ethanol (2.5), formate (5), citrate (5), glucose (2.5),  
95 xylose (2.5), phenol (2), *m*-cresol (1). As insoluble substrates, elemental sulfur ( $0.5 \text{ g l}^{-1}$ )  
96 and hydrogen gas (air/ $\text{H}_2$ ; 2 : 1, v/v; 150 kPa total pressure) were also tested with the  
97 thiosulfate-free basal medium. Utilization of electron acceptors was tested with the basal  
98 medium supplemented with nitrite (2 mM) or nitrate (5, 10 mM), under atmosphere of  $\text{N}_2$   
99 and  $\text{CO}_2$  (80% and 20% in volume, respectively).

100 The novel isolate was subjected to whole genome sequencing, with the PacBio

101 RS II platform. From linear contigs obtained, circular chromosome and plasmid were  
102 manually reconstructed based on sequence alignment. The resulting complete genome  
103 sequence was subjected to comparative analysis with the closest relatives, by the TYGS  
104 web server (<https://tygs.dsmz.de>). In the TYGS, the Type (Strain) Genome Server,  
105 relatives of the subjected genome were automatically identified for subsequent genome-  
106 based phylogenetic analysis and calculation of digital DNA-DNA hybridization (dDDH)  
107 values (Meier-Kolthoff & Göker, 2019). Phylogenetic analysis was also conducted with  
108 the 16S rRNA gene identified in the genome, by using MEGA version 11 (Tamura et al.,  
109 2021). The reference sequences of *Sulfurimonas* species were retrieved from LPSN  
110 (accessed on 06 July 2022). The sequences of strain H1576<sup>T</sup> and references were aligned  
111 with the MUSCLE algorithm. As an outgroup, *Sulfuricurvum kujiense* YK-1<sup>T</sup> was  
112 included in the alignment. The best substitution model with the lowest Bayesian  
113 Information Criterion score was selected by the model selection tool in MEGA.  
114 Phylogenetic tree was constructed with the selected model by excluding positions with gaps.  
115 Values of average nucleotide identity (ANI) between strain H1576<sup>T</sup> and type strains of  
116 *Sulfurimonas* species were computed by ANI calculator available in the EzBioCloud,  
117 based on the OrthoANIu algorithm (Yoon et al., 2017).

118



119 **Results**

120 Cells of the novel isolate, strain H1576<sup>T</sup>, were Gram-stain-negative, motile, rod-  
121 shaped, 1.5–2.7 μm in width, 0.6–0.7 μm in length. The strain grew at 3–22°C with  
122 optimum growth at 13–15°C. At 15°C, growth was observed at pH range of 6.0–7.4, with  
123 optimum growth at pH of 6.6–6.8. Growth was observed in the presence of 2–5% (w/v)  
124 NaCl. The cellular fatty acid profile of strain H1576<sup>T</sup> is shown in Table S1. In the profile,  
125 summed feature 3 (C<sub>16:1ω7c</sub> and/or C<sub>16:1ω6c</sub>) and C<sub>16:0</sub> were predominant, accounting  
126 for 65.5% and 21.9%, respectively.

127 Chemolithoautotrophic growth of strain H1576<sup>T</sup> was supported by thiosulfate,  
128 sulfide and elemental sulfur, but not by H<sub>2</sub> gas. None of the tested organic substrate  
129 supported aerobic growth of the strain. As sole electron acceptor for thiosulfate oxidation,  
130 nitrate and nitrite did not support anaerobic growth of strain H1576<sup>T</sup>.

131 The reconstructed genome of strain H1576<sup>T</sup> consists of a circular chromosome  
132 and a plasmid, with length of 2.76 Mbp and 81.9 kbp, respectively. The G+C contents of  
133 the chromosome and plasmid are 34.8% and 32.8%, respectively. By analyzing the  
134 genome with the TYGS platform, it was revealed that the closest relatives of strain  
135 H1576<sup>T</sup> are *Sulfurimonas* species. Genome-based phylogenetic analysis by the TYGS  
136 indicated that strain H1576<sup>T</sup> belongs to the genus *Sulfurimonas*, but not to any known

137 species (Fig. S1). The calculated values of dDDH and ANI indicated strain H1576<sup>T</sup> should  
138 not be affiliated to any *Sulfurimonas* species previously proposed (Table 1). Phylogenetic  
139 analysis was also conducted with the 16S rRNA gene identified in the genome. The  
140 generated phylogenetic tree indicated that strain H1576<sup>T</sup> is phylogenetically distinct from  
141 all type strains of the genus (Fig. 1). The genome of H1576<sup>T</sup> has been incorporated in the  
142 genome taxonomy database (GTDB), which provides genome-based taxonomy  
143 framework on the basis of conserved proteins (Parks et al., 2018). In the latest release of  
144 the GTDB (07-RS207), strain H1576<sup>T</sup> is classified into a *Sulfurimonas* species which  
145 encompasses no other organisms. All these analyses consistently indicate that strain  
146 H1576<sup>T</sup> is representative of a new species in the genus *Sulfurimonas*.

147

## 148 **Conclusion**

149 The genomic analyses of different approaches consistently indicated that strain  
150 H1576<sup>T</sup> should be classified into a new species of the genus *Sulfurimonas*. Within the  
151 genus, strain H1576<sup>T</sup> is differentiated from the type strains of the other species by a  
152 unique combination of phenotypic characteristics (Table 1). On the basis of these results,  
153 H1576<sup>T</sup> is proposed to be assigned to a new species, with the name *Sulfurimonas aquatica*  
154 sp. nov.

155

156 **Description of *Sulfurimonas aquatica* sp. nov.**

157 *Sulfurimonas aquatica* (a.qua'ti.ca. L. fem. adj. *aquatica*, aquatic).

158 Cells and rod-shaped, motile, 1.5–2.7  $\mu\text{m}$  in length and 0.6–0.7  $\mu\text{m}$  in width. Gram-stain

159 -negative. Grows chemolithoautotrophically by oxidizing thiosulfate, sulfide and

160 elemental sulfur. Hydrogen gas is not used as electron donor. Aerobic. Nitrate and nitrite

161 do not support anaerobic growth when thiosulfate is provided as the sole electron donor.

162 Grows at 3–22°C with an optimum growth at 13–15°C. The pH range for growth is 6.0–

163 7.4, with an optimum pH range of 6.6–6.8. Grows with 2–5% NaCl (optimum 2–3%).

164 Predominant fatty acid is C<sub>16:1</sub>. G + C content of genomic DNA of the type strain is 34.7

165 mol%.

166 The type strain H1576<sup>T</sup> (= BCRC 81254<sup>T</sup> = JCM 35004<sup>T</sup>) was isolated from

167 water of a brackish lake in Japan.

168 The GenBank/EMBL/DDBJ accession numbers for the chromosome and

169 plasmid of type strain are CP046072 and CP046073, respectively.

170

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262

263 **Statements and Declarations**

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267 Government of Japan.

268

269 **Figure legends**

270 Fig. 1. Phylogenetic position of strain H1576<sup>T</sup> within the genus *Sulfurimonas*, based on  
271 the 16S rRNA gene sequences. This maximum likelihood tree was constructed based on  
272 the Kimura 2-parameter model. All positions containing gaps and missing data were  
273 eliminated, leaving 1099 positions in the final dataset. A discrete gamma distribution was  
274 used to model differences in evolutionary rates among sites (5 categories, parameter =  
275 0.3206). The rate variation model allowed for some sites to be invariable (68.83% sites).  
276 Bar, substitutions per site. Numbers on nodes represent percentage values of 1000  
277 bootstrap resampling.

278

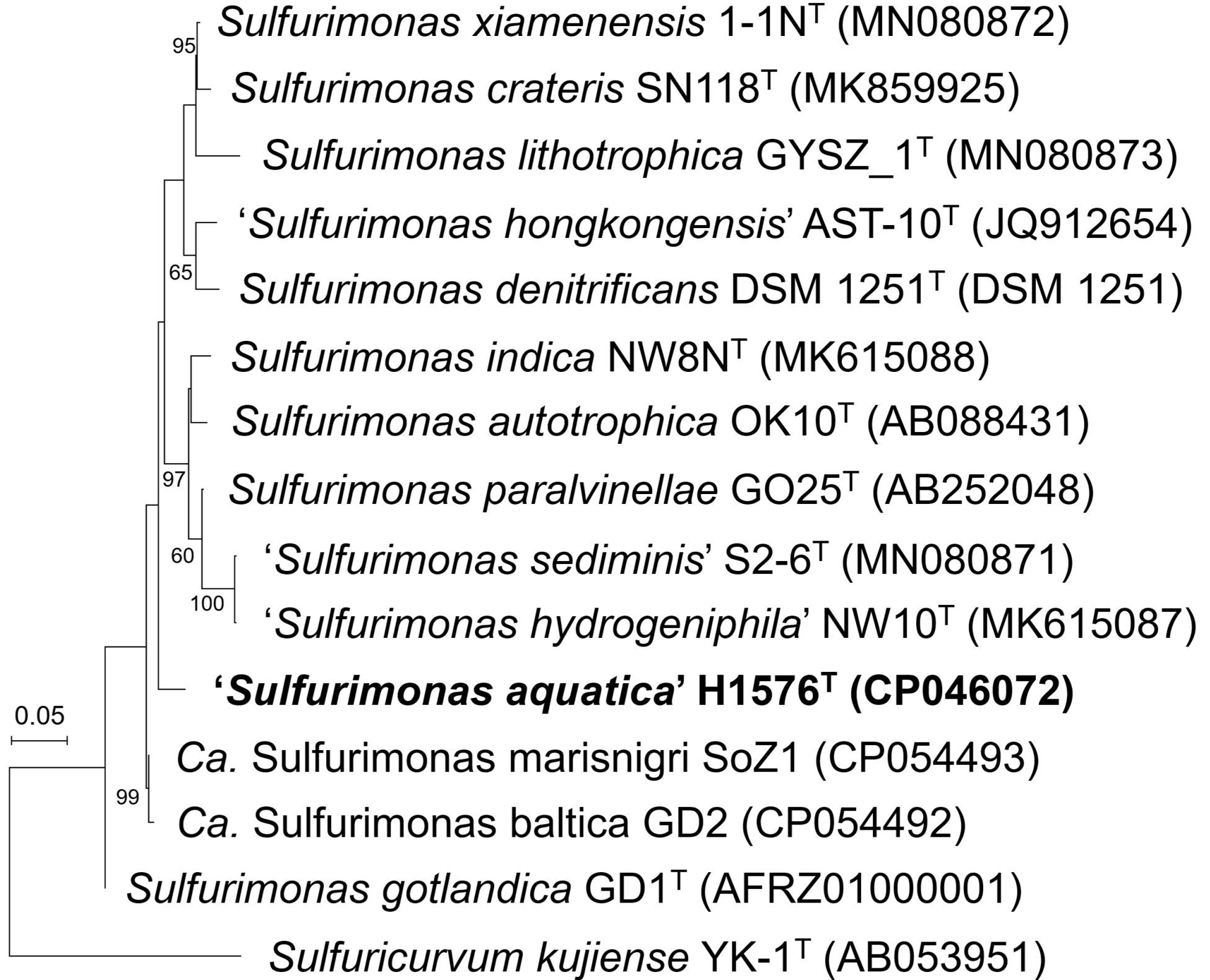


Table 1. Differential properties of strain H1576<sup>T</sup> and strains representing *Sulfurimonas* species. Strains: 1, H1576<sup>T</sup>; 2, *S. autotrophica* OK10<sup>T</sup> (Inagaki et al., 2003); 3, *S. paralvinellae* GO25<sup>T</sup> (Takai et al., 2006); 4, *S. denitrificans* DSM 1251<sup>T</sup> (Timmer-Ten Hoor, 1975); 5, *S. gotlandica* GD1<sup>T</sup> (Labrenz et al., 2013); 6, *S. crateris* SN118<sup>T</sup> (Ratnikova et al., 2020); 7, *S. xiamenensis* 1-1N<sup>T</sup> (Wang et al., 2020); 8, *S. lithotrophica* GYSG\_1<sup>T</sup> (Wang et al., 2020); 9, *S. indica* NW8N<sup>T</sup> (Hu et al., 2021); 10, ‘*S. hongkongensis*’ AST-10<sup>T</sup> (Cai et al., 2014); 11, ‘*S. hydrogeniphila*’ NW10<sup>T</sup> (Wang et al., 2021a); 12, ‘*S. sediminis*’ S2-6<sup>T</sup> (Wang et al., 2021b); 13, *Ca. S. marisnigri* SoZ1 (Henkel et al., 2021); 14, *Ca. S. baltica* GD2 (Henkel et al., 2021). NR, not reported.

Strain:	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Optimum temperature for growth (°C)	13-15	23-26	30	22	15	30	30	33	33	30	33	33	20	15
Growth at 5°C or lower	+	-	+	-	+	+	-	+	+	-	+	-	+	+
Growth at 35°C or higher	-	+	+	-	-	+	+	+	+	+	+	+	-	-
Optimum pH for growth	6.6-6.8	6.5	6.1	7.0	6.7-8.0	8.0	7.0	6.5	5.5	7.0-7.5	6.0-6.5	7.0	7.5-8.0	7.0-7.5
Growth at 8.5 or higher pH	-	+	+	NR	-	+	-	+	-	-	-	-	-	-
Growth at 5.5 or lower pH	-	+	+	NR	-	-	+	+	+	-	-	+	-	-
Growth by H <sub>2</sub> oxidation	-	-	+	-	+	-	+	+	+	+	+	+	+	+
Nitrate respiration	-	-	+	+	+	+	+	+	-	+	+	+	+	+
Nitrite respiration	-	-	-	+	+	+	-	-	-	-	-	-	-	-
dDDH with strain H1576 <sup>T</sup> (%)	100	19.4	18.5	19.1	20.1	18.2	21.1	18.6	18.9	19.0	18.7	19.1	20.2	20.4
ANI with strain H1576 <sup>T</sup> (%)	100	73.5	72.2	71.9	73.5	71.3	72.2	71.7	72.9	72.4	72.2	72.5	73.3	72.7