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Reclassification of *Thiomicrospira* *hydrogeniphila* (Watsuji et al. 2016) to *Thiomicroorhabdus hydrogenophila* comb. nov., with emended description of *Thiomicroorhabdus* (Boden et al., 2017)

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Reclassification of Thiomicrospira hydrogeniphila (Watsuji et al. 2016) to
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Abstract:	The genus Thiomicrospira (Tmr) in the Piskirickettseaceae in the Thiotrichales of the Gammaproteobacteria contains four species of sulfur-oxidizing obligate chemolithoautotroph with validly published names, all previously classified as Thiomicrospira (Tms) species. Here we demonstrate that Thiomicrospira hydrogeniphila, a recently published hydrogen-utilizing chemolithoautotroph closely related to Thiomicrospira frisia (type species of Thiomicrospira) should be classified as a member of the genus Thiomicrospira and not Thiomicrospira, as Thiomicrospira hydrogeniphila comb. nov., on the basis of comparative physiology and morphology as well as 16S rRNA (rrs) gene identity of Tms. hydrogeniphila MAS2T being closer to that of Tmr. frisia JB-A2T (99.1 %) than to Tms. pelophila DSM 1534T (90.5 %) or Hydrogenovibrio marinus MH-110T (94.1 %), and on the basis of the topology of 16S rRNA gene maximum likelihood trees, which clearly place Tms. hydrogeniphila within the genus Thiomicrospira. It was also noted that thiosulfate-grown Thiomicrospira spp. can be distinguished from Thiomicrospira spp. or Hydrogenovibrio spp. on the basis of the 3 dominant fatty acids (C16:1, C18:1 and C16:0), and from other Thiomicrospira spp. on the basis of the 4th dominant fatty acid, which varies between the species of this genus - this could provide a useful diagnostic method. We provide an emended description of Thiomicrospira [1] to take into account the properties of Thiomicrospira hydrogeniphila comb. nov.

1 **Reclassification of *Thiomicrospira hydrogeniphila* (Watsuji *et al.* 2016) to**
2 ***Thiomicrohabdus hydrogeniphila* comb. nov., with emended description of**
3 ***Thiomicrohabdus* (Boden *et al.*, 2017)**

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13 **KEYWORDS:** Chemolithoautotroph, *Thiomicrospira*, *Thiomicrohabdus*, *Hydrogenovibrio*,
14 *Thioalkalimicrobium*, hydrogen

15 **RUNNING TITLE:** Reclassification of *Thiomicrospira hydrogeniphila* to *Thiomicrohabdus*
16 *hydrogeniphila* comb. nov.

17 **ABBREVIATIONS:** We have used 3-letter abbreviation convention recommended in Boden
18 *et al.* (2017) for the genera with similar names, *viz.* *Thiomicrospira* (*Tms.*),
19 *Thiomicrohabdus* (*Tmr.*) and *Thioalkalimicrobium* (*Tam.*), to avoid ambiguity particularly
20 given the recent reclassification.

21

22 **Abstract**

23 The genus *Thiomicroorhabdus* (*Tmr*) in the *Piskirickettsiaceae* in the *Thiotrichales* of the
24 *Gammaproteobacteria* contains four species of sulfur-oxidizing obligate chemolithoautotroph
25 with validly published names, all previously classified as *Thiomicrospira* (*Tms*) species. Here
26 we demonstrate that *Thiomicrospira hydrogeniphila*, a recently published hydrogen-utilizing
27 chemolithoautotroph closely related to *Thiomicroorhabdus frisia* (type species of
28 *Thiomicroorhabdus*) should be classified as a member of the genus *Thiomicroorhabdus* and not
29 *Thiomicrospira*, as *Thiomicroorhabdus hydrogeniphila* comb. nov., on the basis of
30 comparative physiology and morphology as well as 16S rRNA (*rrs*) gene identity of *Tms*.
31 *hydrogeniphila* MAS2^T being closer to that of *Tmr. frisia* JB-A2^T (99.1 %) than to *Tms*.
32 *pelophila* DSM 1534^T (90.5 %) or *Hydrogenovibrio marinus* MH-110^T (94.1 %), and on the
33 basis of the topology of 16S rRNA gene maximum likelihood trees, which clearly place *Tms*.
34 *hydrogeniphila* within the genus *Thiomicroorhabdus*. It was also noted that thiosulfate-grown
35 *Thiomicroorhabdus* spp. can be distinguished from *Thiomicrospira* spp. or *Hydrogenovibrio*
36 spp. on the basis of the 3 dominant fatty acids (C_{16:1}, C_{18:1} and C_{16:0}), and from other
37 *Thiomicroorhabdus* spp. on the basis of the 4th dominant fatty acid, which varies between the
38 species of this genus – this could provide a useful diagnostic method. We provide an
39 emended description of *Thiomicroorhabdus* [1] to take into account the properties of
40 *Thiomicroorhabdus hydrogeniphila* comb. nov.

41 The taxonomy and systematics of the genera *Thiomicrospira* (*Tms*), *Hydrogenovibrio* (*H*) and
42 *Thioalkalimicrobium* (*Tam*) were recently revised [1], proposing the genus *Thiomicrorhabdus*
43 (*Tmr*) for a clade of rod-shaped organisms previously classified as *Thiomicrospira* spp., and
44 reclassifying one other clade of *Thiomicrospira* spp. as *Hydrogenovibrio* spp., leaving only
45 *Tms. pelophila* (type species) and *Tms. thyasirae* as *Thiomicrospira* spp., but all four
46 members of *Thioalkalimicrobium* were also reclassified to *Thiomicrospira*. This was based
47 on a polyphasic study of chemotaxonomic, physiological, genomic and phylogenetic
48 elements. Shortly before our study [1] was accepted, *Thiomicrospira hydrogeniphila* [2] was
49 published, based on a eurypsychrophilic hydrogen and sulfur-oxidizing chemolithoautotroph
50 isolated from enrichment cultures inoculated with the slurry of a 10-month old tank of
51 seawater from Toyko Bay, Japan, that had been incubated with a block of beef tallow at
52 10 °C. Here we present the short case that this species should also be circumscribed into the
53 genus *Thiomicrorhabdus* per all other members of the same clade of *Thiomicrospira* as
54 proposed by Boden *et al.* [1], as *Thiomicrorhabdus hydrogeniphila* comb. nov., and we
55 accordingly revise the description of *Thiomicrorhabdus* [1] to take into account the properties
56 of this species.

57 *Thiomicrorhabdus* [1] comprises four species with validly published names –
58 *Thiomicrorhabdus frisia* [3], *Thiomicrorhabdus chilensis* [4], *Thiomicrorhabdus arctica* [5]
59 and *Thiomicrorhabdus psychrophila* [5]. All were isolated from marine sediments and can
60 use thiosulfate and other reduced sulfur species as electron donors but none have been found
61 to use molecular hydrogen thus far. *Tmr. psychrophila* and *Tmr. arctica* are
62 stenopsychrophilic, whereas the other two species are eurypsychrophilic and have more
63 mesophilic temperature optima. All species are moderate halophiles requiring 40 – 100 mM
64 sodium chloride (2.3 – 5.8 g/L) for growth, with optima of 250 – 400 mM (14.6 – 23.4 g/L).
65 *Thiomicrorhabdus* spp. share the same 3 dominant fatty acids (palmitoleic (C_{16:1}), vaccenic

66 (C_{18:1}), palmitic (C_{16:0}) acids) when grown on thiosulfate agar plates, but can be easily
67 distinguished by their next-dominant fatty acid (*Tmr. chilensis*, stearic acid (C_{18:0}); *Tmr.*
68 *arctica*, myristoleic acid (C_{14:1}); *Tmr. psychrophila*, lauroleic acid (C_{12:1})). The G+C contents
69 of genomic DNA range from 39.6 to 49.9 mol%.

70 We have shown previously [1] that the 16S rRNA gene phylogeny reliably supports those of
71 the *recA* and *gyrB* genes and of more complex analyses using 53 ribosomal protein genes
72 (rMLST, [6]). In the absence of a genome sequence for *Tms. hydrogeniphila*, we are limited
73 to the 16S rRNA gene here for phylogenetic analyses, but are confident from our previous
74 study of this group of genera that this gene indeed accurately reflects the phylogeny using
75 more genes. Gene sequences of this clade of organisms were curated from the GenBank™
76 database and aligned using the MUSCLE algorithm [7] in MEGA 7.0.2 [8]. A maximum-
77 likelihood tree (Figure 1) was built using the Tamura-Nei model [9]. Bootstrap values at
78 nodes represent 5,000 resamplings of the tree, and are shown where greater than or equal to
79 70 %. It can be seen from Figure 1 that *Tms. hydrogeniphila* falls within the genus
80 *Thiomicrohabdus*, which is further supported on the basis of the 16S rRNA gene identity to
81 *Tmr. frisia* (type species) of 99.1 %, versus 90.5 % to *Tms. pelophila* and 94.1 % to *H.*
82 *marinus*. These data indicate that *Tms. hydrogeniphila* is not the same genus as either *Tms.*
83 *pelophila* or *H. marinus* on the basis of the proposed genus cut-off of 94.5 % identity (“the
84 Yarza cut-off” [10]), and on the basis of tree topology. It is worth noting that *Tms.*
85 *hydrogeniphilus* was robustly demonstrated by Watsuji *et al.* [2] to be distinct from *Tmr.*
86 *frisnia* on the basis of DNA-DNA hybridisation, thus we regard it as a distinct species. We
87 have also previously demonstrated [1] that the other species of *Thiomicrohabdus* (and
88 *Hydrogenovibrio* and *Thiomicrospira*) are also correctly circumscribed on the basis of *in*
89 *silico* DNA-DNA hybridisation scores.

90 Differential properties of *Tms. hydrogeniphila* versus *Thiomicrospira sensu stricto*,
91 *Hydrogenovibrio* and *Thiomicrohabdus* spp. are curated in Table 1. It can be seen that the
92 cell size, morphology, maximum specific growth rate on thiosulfate, motility and flagellation
93 are more similar to *Thiomicrohabdus* spp. rather than to *Thiomicrospira* spp. or
94 *Hydrogenovibrio* spp. The temperature range and optimum for *Tms. hydrogeniphila* are very
95 similar to the nearest neighbour in terms of the 16S rRNA gene, *Thiomicrohabdus frisia*
96 (type species). The dominant three fatty acids of *Tms. hydrogeniphila* are identical to all
97 *Thiomicrohabdus* spp. but fall in a different order to *Thiomicrospira* and *Hydrogenovibrio*
98 spp. The fourth-dominant fatty acid is lauric acid (C_{12:0}), which is distinct from the other
99 *Thiomicrohabdus* spp. and could provide a means to diagnose speciation with rapidity since
100 all species differ in this fourth dominant acid. The G+C content of genomic DNA is identical
101 to *Tmr. frisia*, but as already stated, it has been distinguished from this species on the basis of
102 DNA-DNA hybridisation previously, however, future *in silico* DNA-DNA hybridisation
103 studies once the genome sequence is complete will be needed to confirm this since both the
104 G+C content and 16S rRNA gene identity with *Tmr. frisia* are very high, yet the DNA-DNA
105 hybridisation percentage of Watsuji *et al.* [2] is still below 20 %, which appears rather low.
106 *Tms. hydrogeniphila* can use molecular hydrogen as an electron donor, which is distinct from
107 all *Thiomicrohabdus* spp., and this warrants the emending of the genus description to add
108 this property, which is found in some *Hydrogenovibrio* spp., but not in this genus.

109 We conclude that *Thiomicrospira hydrogeniphila* actually represents a member of the
110 recently defined genus *Thiomicrohabdus* (composed entirely of former *Thiomicrospira* spp.)
111 and propose the name *Thiomicrohabdus hydrogeniphila* comb. nov. to reflect this. We also
112 propose some minor revisions to the Boden *et al.* [1] description of *Thiomicrohabdus* to take
113 into account the properties of *Tmr. hydrogeniphila* comb. nov. and other data from Watsuji *et*
114 *al.* [2].

115 **Emended description of *Thiomicrorhabdus* (Boden *et al.* 2017)**

116 *Thiomicrorhabdus* (Thi.o.mi.cro.rhab'dus. Gr. n. *theion*, L. transliteration *thium*, sulfur; Gr.
117 adj. *mikrós*, small; Gr. fem. n. *rhabdos*, N.L. transliteration *rhabdus*, rod or wand. N.L. fem.
118 n. *Thiomicrorhabdus*, small sulfur-oxidising rod).

119 Gram-stain-negative. Cells when grown in liquid media are rod-shaped. Typical cell lengths
120 are 0.8 – 2.7 μm and diameters are 0.3 – 0.6 μm , wider than *Thiomicrospira* spp. Do not form
121 endospores or exospores. Use molecular oxygen as the sole terminal electron acceptor. Have
122 a *cbb₃*-type cytochrome *c* oxidase (EC 1.9.3.1). Form white to yellow, entire colonies on
123 thiosulfate-agar, coated in small granules of elementary sulfur. Motile, cells are monotrichous
124 when grown in liquid media. Obligately chemolithoautotrophic with heterotrophy never
125 observed. Can use thiosulfate, tetrathionate or sulfide as sole electron donors and at least one
126 species uses molecular hydrogen, but none are known to use thiocyanate, sulfite, iron or
127 manganese. Some species can use elementary sulfur as a sole electron donors. Fix carbon
128 dioxide *via* the transaldolase-variant Calvin-Benson-Bassham cycle. All species use
129 ammonium as a nitrogen source. Does not fix dinitrogen. No nitrogenase genes observed in
130 genome sequences. Has form IAc and/or form IAq, and form II RuBisCo.

131 All species produce elementary sulfur when growing on thiosulfate at neutrality, but at
132 varying degrees. Never auxotrophic for vitamin B₁₂. Growth occurs from pH 4.2 to pH 9.0
133 but range varies with species – pH optima are pH 6.5 to 8.5. Grows from -2 °C to 42 °C with
134 optima of 11.5 °C to 35 °C, varying by species, most of which are stenopsychrophilic or
135 eurypsychrophilic. NaCl is required for growth, with minima of 40 – 100 mM, maxima of
136 1,240 mM across the genus and optima of 250 – 470 mM. Does not reduce nitrate to nitrite.
137 G+C fractions of genomic DNA are 39.6 – 49.9 mol%. Dominant respiratory quinone is
138 ubiquinone-8. Dominant fatty acids are palmitoleic (C_{16:1}), vaccenic (C_{18:1}) and palmitic

139 (C_{16:0}) acids, in that order, which is distinct from *Thiomicrospira* spp. and *Hydrogenovibrio*
140 spp., but consistent with other *Thiomicrohabdus* spp., followed by stearic (C_{18:0}) and
141 myristoleic (C_{14:1}) acids. Members of the *Piskirickettsiaceae* in the *Thiotrichales* of the
142 *Gammaproteobacteria*.

143 Type species: *Thiomicrohabdus frisia* (Basonym: *Thiomicrospira frisia*) [3]

144 **Description of *Thiomicrohabdus hydrogeniphila* comb. nov.**

145 *Thiomicrohabdus hydrogeniphila* (hy.dro.ge.ni'phi.la. Gr. n. *hydôr*, water; Gr. v. *gennaô*, to
146 beget, to bring forth, to produce; N.L. n. *hydrogenum*, hydrogen, *i.e.* that which produces
147 water; N.L. adj. *philus* from Gr. adj. *philos*, friend, someone dearly loved; N.L. fem. adj.
148 *hydrogeniphila*, hydrogen-loving).

149 Gram-stain-negative. Cells grown in liquid media are rod shaped and motile by means of a
150 single polar flagellum. 0.9 – 1.8 µm in length and 0.3 – 0.5 µm in diameter. Cells grown in
151 MMJS broth [2] were obligately aerobic, using only molecular oxygen as their terminal
152 electron acceptor and tolerating up to 40 % (v/v) oxygen in a gas phase of nitrogen
153 supplemented with 5 % (v/v) carbon dioxide as sole carbon source, at atmospheric pressure.
154 Nitrate, nitrite, ferric iron, ferrihydrite, selenate and fumarate are not used as terminal
155 electron acceptors. Obligately chemolithoheterotrophic, electron donors supporting growth
156 are: thiosulfate, tetrathionate, elementary sulfur, sulfide and molecular hydrogen. Sulfite was
157 not used. Oxidation of molecular hydrogen is repressed at high oxygen partial pressures.
158 Ammonium was the sole nitrogen source, and nitrate, nitrite and molecular nitrogen were not
159 used. Selenate, tungstate and vitamins (*viz.* biotin, folate, pyridoxine, thiamine, riboflavin,
160 nicotinate, pantothenate, cyanocobalamin, *p*-aminobenzoate or lipoate) were not required for
161 growth. Growth occurs between 2 °C and 40 °C with an optimum of 30 °C, and between pH
162 5.0 and pH 8.0 with an optimum of pH 6.0, in MMJS medium incubated under air. Growth

163 occurs from 30 – 1,380 mM Na⁺, with optimal growth at 270 mM Na⁺. Sensitive to
164 ampicillin (50 µg/mL, 143 µM), chloramphenicol (50 µg/mL, 155 µM), , kanamycin A (50
165 µg/mL, 103 µM), rifampicin (50 µg/mL, 60 µM), streptomycin (50 µg/mL, 86 µM); resistant
166 to vancomycin (50 µg/mL, 35 µM). Heterotrophic or chemolithoheterotrophic growth (the
167 latter with thiosulfate) is not observed on any carbon source tested, *viz.* yeast extract, peptone,
168 tryptone, casein, starch, carboxymethylcellulose or casamino acids (each at 0.1% (w/v));
169 formate, acetate, glycerol, citrate, tartarate, fumarate, malate, succinate, propionate, lactate,
170 oxalate, pyruvate or the 20 structural amino acids (each at 5 mM); glucose, galactose,
171 fructose (each at 1.1 mM); sucrose, lactose, maltose or trehalose (each at 0.6 mM). Maximum
172 specific growth rate on thiosulfate under optimal conditions was 0.4 h⁻¹. Dominant fatty acids
173 in thiosulfate-grown cells are palmitic acid (C_{16:0}), palmitoleic acid (C_{16:1}) and vaccenic acid
174 (C_{18:1}). Of the hydroxylated fatty acids, only 3-hydroxycaprylic acid (C_{10:0} 3-OH) is found.
175 Lauroleic (C_{12:1}) and myristoleic (C_{14:1}) acids are not found, but lauric (C_{12:0}) and myristic
176 (C_{14:0}) acids are in minor amounts – these 4 acids are diagnostic *versus* other
177 *Thiomicrothabodus* spp. (*cf.* Table 1). The G+C content of genomic DNA of the type strain is
178 39.6 mol% (HPLC).

179 Basonym: *Thiomicrospira hydrogeniphila* Watsuji *et al.* 2016.

180 Type strain is MAS2^T = JCM 30760^T = DSM 100274^T, isolated from enrichment cultures
181 using molecular hydrogen as the sole energy source, inoculated with slurry obtained from a
182 sulfidic tank containing surface seawater from Tokyo Bay, supplemented with a block of beef
183 tallow (*c.* 350g/L), held at 10 °C for 10 months.

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185

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190 **Conflicts of interest**

191 The authors declare that they have no competing interests.

192 **Ethical Statement**

193 No human or animal experiments were conducted in this study.

194

195

196

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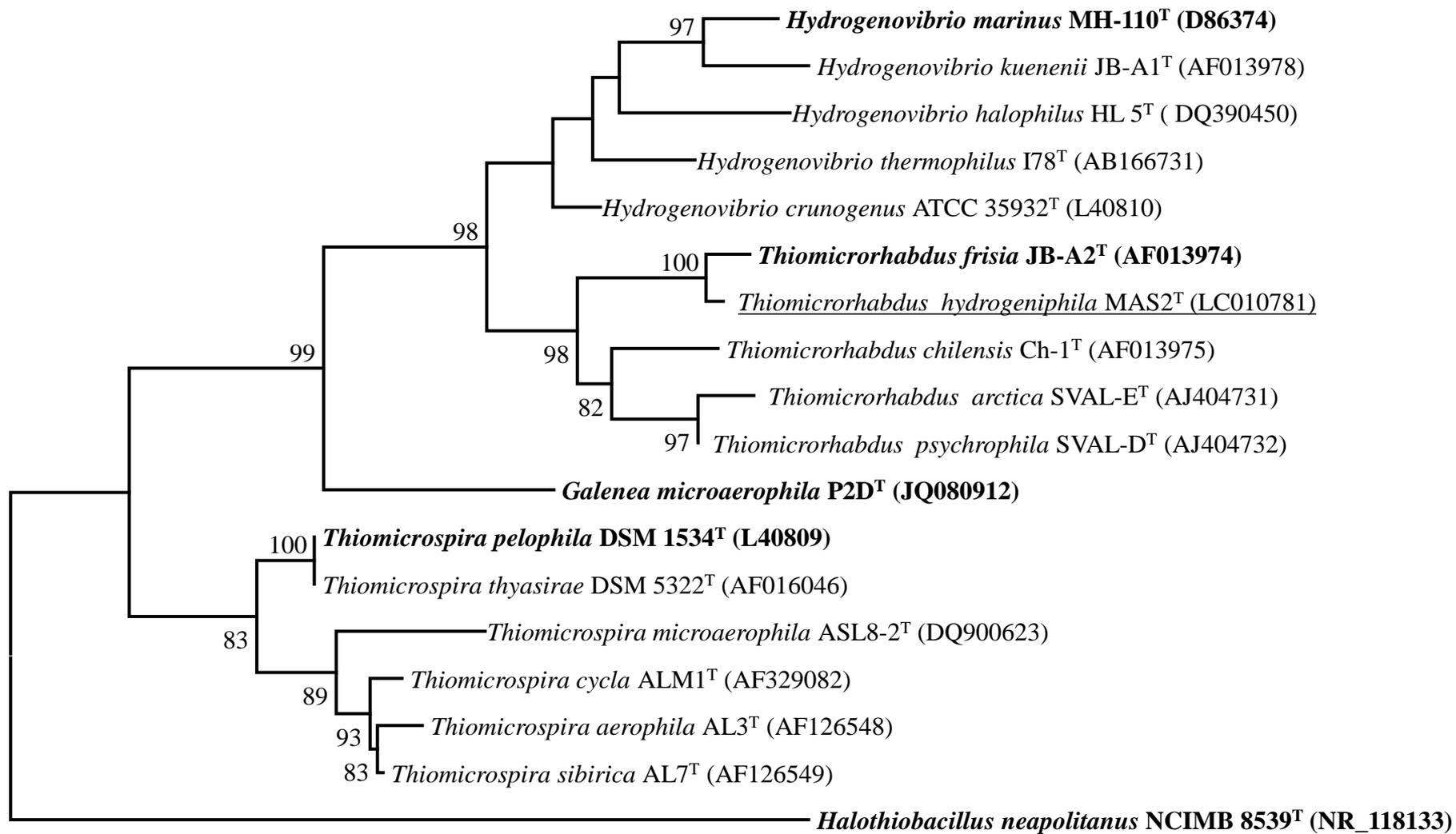
231

232 **Figure 1.** Maximum likelihood tree based on the 16S rRNA (*rrs*) gene sequences from
233 *Thiomicrospira hydrogeniphila* MAS2^T and all members of the genera *Thiomicrospira*,
234 *Thiomicrospira*, *Galenea* and *Hydrogenovibrio* with validly published names. The
235 outgroup is the gene sequence from *Halothiobacillus neapolitanus* NCIMB 8539^T. Genes
236 were aligned using the MUSCLE algorithm in MEGA 7.0.20 and trees built using the
237 Tamura-Nei model with the nearest-neighbour interchange (NNI) heuristic method and
238 partial deletion of gaps. Topology with the superior log-likelihood is shown, with numbers at
239 nodes representing the percentage of 5,000 bootstrap replicates for which that topology was
240 preserved (values < 70 % are omitted). GenBankTM gene accession numbers are given in
241 parentheses. Scale bar represents the number of substitutions per site. 1,270 nt were used in
242 the analysis. Type species of genera are emboldened and the test sequence (*Tms.*
243 *hydrogeniphila* MAS2^T) is underlined.

244 **Table 1.** Comparative properties of *Thiomicrospira hydrogeniphila* MAS2^T with all species
245 of *Thiomicrospira* with validly published names, and conglomerated properties of
246 *Thiomicrospira* and *Hydrogenovibrio*. Data are from [1] or [2].
247 Values are positive (+), weak positive (\pm), negative (-) or not determined (ND).
248

	<i>Thiomicrobacterium Hydrogeniphila</i> MAS2 ^T	<i>Thiomicrobacterium frisia</i> JB-A2 ^T	<i>Thiomicrobacterium chilensis</i> Ch-1 ^T	<i>Thiomicrobacterium arctica</i> SVAL-E ^T	<i>Thiomicrobacterium psychrophila</i> SVAL-D ^T	<i>Thiomicrospira</i> spp.	<i>Hydrogenovibrio</i> spp.
16S rRNA gene sequence identity to (%):							
<i>Tms. pelophila</i> DSM 1534 ^T	90.5	91.6	92.0	92.9	92.9	95.9-100.0	92.1-94.4
<i>Tmr. frisia</i> JB-A2 ^T	99.1	100.0	96.0	96.0	96.0	90.3-91.4	94.2-95.5
<i>H. marinus</i> MH-110 ^T	94.1	94.2	94.8	94.0	94.1	91.4-92.9	95.7-100.0
Properties							
Colony colour on thiosulfate agar grown under air	White, cream	White, yellow	ND	ND	ND	White, pink or red	White, cream or yellow
Heterotrophic	-	-	-	-	-	-	±
G+C fraction (mol%)	39.6	39.6	49.9	42.4	42.5	45.6 – 49.3	44.1 – 56.6
Maximum specific growth rate on thiosulfate under optimal conditions (h ⁻¹)	0.4	0.45	0.4	0.14	0.2	0.07 – 0.33	0.25 – 0.8
Temperature range (°C)	2.0 – 40.0	3.5 – 39.0	3.5 – 42.0	-2.0 – 20.8	-2.0 – 20.8	3.5 – 41.0	3.5 – 55.0
Temperature optimum (°C)	30	32 – 35	32 – 37	11.5 – 13.2	14.6 – 15.4	25 – 40	28 – 37
Dominant fatty acids in thiosulfate-grown cells	C _{16:1} C _{18:1} C _{16:0} C _{12:0}	ND	C _{16:1} C _{18:1} C _{16:0} C _{18:0}	C _{16:1} C _{18:1} C _{16:0} C _{14:1}	C _{16:1} C _{18:1} C _{16:0} C _{12:1}	C _{18:1} C _{16:1} C _{16:0} C _{14:0}	C _{16:1} C _{16:0} C _{18:0} C _{18:1}
Molecular hydrogen as an electron donor	+	-	-	-	-	-	±
Cell length (µm)	0.9-1.8	1.0-2.7	0.8-2.0	1.2-1.5	1.3-1.7	0.8 – 5.0	0.8 – 3.0
Cell width (µm)	0.3-0.5	0.3-0.5	0.3-0.5	0.5-0.6	0.5-0.6	0.2 – 2.0	0.2 – 0.7
Morphology	Rod	Rod	Rod	Rod	Rod	Vibrio, ring, spiral	Vibrio, curved rod
Motility	+	±	+	±	+	±	+
Flagella	1	ND	ND	1	1	0-3	1

Figure 1



0.02