



Title	Sulfuriferula multivorans gen. nov., sp nov., isolated from a freshwater lake, reclassification of 'Thiobacillus plumbophilus' as Sulfuriferula plumbophilus sp nov., and description of Sulfuricellaceae fam. nov and Sulfuricellales ord. nov.
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4 ***Sulfuricellaceae fam. nov. and Sulfuricellales ord. nov.***

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16 Running head: *Sulfuriferula multivorans* gen. nov., sp. nov.

17 Subject category: New taxa: *Proteobacteria*

18

19 The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of strain
20 TTN^T is LC005593. The accession numbers for the *sqr*, *soxB*, *dsrA*, and *aprA* sequences of
21 strain TTN^T are LC005597, LC005595, LC005596, and LC005594, respectively.

22

23 **Summary**

24 A sulfur-oxidizing bacterium, strain TTN^T, was isolated from *Thioploca* sample obtained
25 from a freshwater lake in Japan. The isolate shared 97.1% 16S rRNA gene sequence
26 similarity with an obligately aerobic chemolithoautotroph ‘*Thiobacillus plumbophilus*’ Gro7^T.
27 Cells were rods, motile, and Gram-reaction negative. The G+C content of genomic DNA was
28 around 66 mol%. Strain TTN^T grew over a temperature range of 8–32°C (optimum 22–25°C),
29 a NaCl concentration range of 0–133.3 mM (optimum 0–3.3 mM), and a pH range of 5.3–8.6
30 (optimum pH 6.4–7.0). Strain TTN^T was facultatively anaerobic and could utilize nitrate as an
31 electron acceptor. The isolate oxidized tetrathionate, thiosulfate, and elemental sulfur as sole
32 energy source for autotrophic growth, and could also grow heterotrophically on a number of
33 organic substrates. Based on its phylogenetic and phenotypic properties, strain TTN^T
34 represents a novel species of a new genus, for which the name *Sulfuriferula multivorans* gen.
35 nov., sp. nov. is proposed. The type strain is TTN^T (=NBRC 110683^T=DSM 29343^T). Along
36 with it, the reclassification of ‘*Thiobacillus plumbophilus*’ as *Sulfuriferula plumbophilus* sp.
37 nov. (type strain Gro7^T=NBRC 107929^T=DSM 6690^T) is proposed. Based on the data
38 obtained in this study, we describe the designations *Sulfuricellaceae* fam. nov. and
39 *Sulfuricellales* ord. nov.

40

41 An obligately chemolithoautotrophic sulfur oxidizer *Sulfuricella denitrificans* was
42 isolated from a stratified freshwater lake, as a representative of a new genus in the class
43 *Betaproteobacteria* (Kojima & Fukui, 2010). To date, *S. denitrificans* has been placed in the
44 family *Hydrogenophilaceae* in the List of Prokaryotic Names with Standing in Nomenclature
45 (Euzéby, 1997; Parte, 2014), but this classification is not supported by 16S rRNA-based
46 phylogeny. On the other hand, a new family *Sulfuricellaceae* and order *Sulfuricellales* have
47 been proposed to accommodate the genus *Sulfuricella*, based on the phylogenetic analyses of
48 16S rRNA gene and other six conserved genes (Watanabe *et al.*, 2014). However, the names
49 of these taxa have not been validated at this time. In this study, a novel sulfur oxidizer
50 belonging to these taxa was obtained.

51 Strain TTN^T was isolated from *Thioploca* sample obtained from sediment of a freshwater
52 lake in Japan, Lake Okotanpe (Nemoto *et al.*, 2011). The sediment sample was transferred
53 into bicarbonate-buffered low-salt defined medium (Kojima & Fukui, 2014) containing 10
54 mM Na₂S₂O₃ and 20 mM NaNO₃ as electron donor and acceptor, respectively. The headspace
55 of the culture bottle was filled with N₂/CO₂ (80:20, v/v), and incubation was performed in the
56 dark at 22°C. After several transfers in the same medium, subsequent subcultures were carried
57 out in ATCC 290 S6 medium under aerobic condition (3 vol% CO₂ was added in the head
58 space) to enhance the growth. The composition of the medium was as follows (l⁻¹): 3 g
59 Na₂HPO₄·12H₂O, 1.8 g KH₂PO₄, 0.1 g MgSO₄·7H₂O, 0.1 g (NH₄)₂SO₄, 0.04 g CaCl₂·2H₂O,
60 0.03 g FeCl₃·6H₂O, 0.03 g MnSO₄·5H₂O, and 10 g Na₂S₂O₃. Finally, strain TTN^T was
61 isolated in pure culture by serial dilution. Purity of the isolate was checked by phase-contrast
62 light microscopy and sequencing of the 16S rRNA gene fragments.

63 To determine the phylogenetic position of strain TTN^T, the 16S rRNA gene fragment
64 was amplified with the primer set 27F/1492R (Lane, 1991) and then sequenced. Partial
65 fragments of genes for sulfur oxidation (*sqr* encoding sulfide:quinone oxidoreductase, *soxB*
66 encoding sulfate thioesterase/sulfate thiohydrolase, *dsrA* encoding dissimilatory sulfite
67 reductase, and *aprA* encoding adenosine-5'-phosphosulfate reductase) were also amplified
68 and sequenced. The fragments of *sqr*, *soxB*, and *dsrA* genes were amplified with the primer
69 pairs, *sqr* 473F/982R, *soxB* 704F/1199R, and *dsrA* 625F/877R, respectively (Luo *et al.*, 2011).
70 The *aprA* gene fragments were obtained using Apr-1-FW/Apr-5-RV (Meyer & Kuever, 2007).
71 The amplification of *sqr* and *dsrA* genes was also tested with genomic DNA extracted from
72 '*Thiobacillus plumbophilus*' Gro7^T purchased from DSMZ (DSM 6690^T).

73 Morphological and physiological characteristics of the isolate were investigated.
74 Throughout the characterization, ATCC 290 S6 medium (3 vol% CO₂ was added in the head
75 space) was used unless otherwise specified. Motility and morphology were observed by phase
76 contrast microscopy. Tests of Gram staining and catalase activity were performed as
77 described previously (Kojima & Fukui, 2010). Oxidase activity was tested using oxidase
78 reagent (bioMérieux, France). Effects of temperature (5, 8, 13, 18, 20, 22, 25, 28, 30, 32, 35,
79 37, and 42°C), NaCl concentration (0, 3.3, 6.7, 13.3, 26.7, 66.7, 106.7, 133.3, 160, and 180
80 mM), and initial pH (4.8, 5.3, 5.7, 5.9, 6.2, 6.4, 6.7, 7.0, 7.2, 7.5, 8.1, 8.6, 8.7, 8.8, 8.9, 9.0,
81 9.1, 9.2, 9.3, 9.4, 9.5, and 9.6) on growth were tested. The pH was increased by the addition
82 of KOH, and gradually decreased by the addition of HCl. Utilization of electron donors was
83 tested in ATCC 290 S6 media without Na₂S₂O₃, each containing one of the following
84 substrates: hydrogen (100 kPa in the head space), lead sulfide (0.5 g l⁻¹), tetrathionate (10
85 mM), thiosulfate (63 mM), elemental sulfur (1.5 g l⁻¹), sulfite (5 mM), casamino acids (5 mg

86 l^{-1}), yeast extract (5 mg l^{-1}), peptone (0.1 g l^{-1}), arabinose, glucose, galactose, lactose,
87 mannose, fumarate, pyruvate, malate, lactate, propionate, ethanol, formate, acetate, n-butyrate,
88 and benzoate (each 5 mM). Growth on sulfide (1 mM) and hydrogen ($\text{H}_2/\text{N}_2/\text{CO}_2$ 50 : 40 : 10,
89 v/v; 200 kPa total pressure) was tested anaerobically in the bicarbonate-buffered low-salt
90 defined medium (Kojima & Fukui, 2014) containing 20 mM nitrate. Utilization of electron
91 acceptors, including nitrate (20 mM) and arsenate (5 mM), was tested in the defined medium
92 containing 10 mM thiosulfate as an electron donor. For comparison, aerobic growth of
93 '*Thiobacillus plumbophilus*' Gro 7^T on fumarate, malate, and ethanol (each 5 mM) was also
94 tested in DSMZ 561 medium.

95 The G+C content of strain TTN T was determined by using a Yamasa GC kit (Yamasa
96 shoyu, Chosu, Japan) with the HPLC methods as described previously (Katayama-Fujimura *et*
97 *al.*, 1984). The cellular fatty acid profile of the isolate was constructed using cells aerobically
98 grown in ATCC 290 S6 medium. The fatty acid analysis was performed at Techno Suruga
99 (Sizuoka, Japan), by using the Sherlock Microbial Identification System (Version 6.0;
100 database, TSBA6; MIDI).

101 The 16S rRNA gene sequence analysis demonstrated that strain TTN T belongs to the
102 class *Betaproteobacteria* (Fig. 1). The closest relative of strain TTN T among the isolates with
103 validly published names was *Sulfuricella denitrificans* skB26 T , but strain TTN T was more
104 closely related to another obligately aerobic chemolithoautotroph '*Thiobacillus plumbophilus*'
105 Gro 7^T (Drobner *et al.*, 1992) (93.9 and 97.1% 16S rRNA gene sequence similarities,
106 respectively). Strains TTN T and Gro 7^T also formed a monophyletic cluster in the SoxB and
107 AprA trees (Figs S1 and S2). Phylogenetic trees of Sqr and DsrA including strains TTN T and

108 skB26^T are shown in Figs S3 and S4. The primer sets sqr 473F/982R and dsrA 625F/877F
109 generated no PCR products from the extracted genomic DNA of strain Gro7^T.

110 The cells of strain TTN^T were motile and Gram-negative rods (1.0–2.2 µm long and 0.3–
111 0.5 µm wide). Spore formation was not observed. Catalase and oxidase tests were positive.

112 The isolate grew over a temperature range of 8–32°C, a NaCl concentration range of
113 0–133.3 mM, and a pH range of 5.3–8.6. Optimum growth was observed at 22–25°C, 0–3.3
114 mM NaCl, and pH 6.4–7.0, respectively.

115 Anaerobic growth of strain TTN^T was observed when nitrate was used as an electron
116 acceptor, and accumulation of nitrite was observed. Arsenate was also tested as a possible
117 electron acceptor for strain TTN^T, but no growth was observed. Autotrophic growth of the
118 isolate was observed in the presence of tetrathionate, thiosulfate, and elemental sulfur. The
119 end product of sulfur oxidation was sulfate. Sulfite, lead sulfide, or hydrogen did not support
120 aerobic growth of the isolate. Under anaerobic conditions, growth on sulfide and hydrogen
121 was tested, but they did not support growth. Strain TTN^T could grow heterotrophically on a
122 number of organic substrates including complex organic substrates, sugars, organic acids, and
123 an alcohol (Table 1). The following organic substrates could not support growth of the isolate:
124 formate, propionate, acetate, n-butyrate, and benzoate. As to '*Thiobacillus plumbophilus*'
125 Gro7^T, fumarate, malate, and ethanol were tested as possible electron donors, but all of them
126 could not support growth (Table 1).

127 The G+C content of the strain TTN^T genomic DNA was 63.0 mol%, which is more
128 similar to that of strain Gro7^T (66 mol%) than that of *Sulfuricella denitrificans* skB26^T (56
129 mol%). The cellular fatty acid profile of strain TTN^T was characterized by a high
130 concentration of summed feature 3 (C_{16:1}ω7c and/or C_{16:1}ω6c; 44.7%), C_{16:0} (33.6%), and

131 C_{12:0} (5.1%). The two most major components were also reported as major fatty acids for the
132 closely related strain *S. denitrificans* skB26^T (Table 2). On the other hand, differences
133 between these two strains were found in the levels of other minor fatty acids (Table 2).

134 The name of '*Thiobacillus plumbophilus*' has never been validated, and the need to
135 reclassify has been pointed out (Kelly & Wood, 2000). In the phylogenetic trees of 16S rRNA,
136 *soxB*, and *aprA* genes, the monophyly of strain TTN^T and '*Thiobacillus plumbophilus*' Gro7^T
137 was demonstrated. On the other hand, their utilization of electron donors and acceptors were
138 distinguished from each other and from *Sulfuricella denitrificans* skB26^T (Table 1). Therefore,
139 strains TTN^T and Gro7^T represent different species of a single genus. On the basis of their
140 phylogenetic and phenotypic properties, we propose a novel species of a new genus,
141 *Sulfuriferula multivorans* gen. nov., sp. nov., for strain TTN^T, and propose the reclassification
142 of '*Thiobacillus plumbophilus*' as *Sulfuriferula plumbophilus* sp. nov. Despite different
143 physiological characteristics and relatively low 16S rRNA gene similarity (93.9%) between
144 the genera *Sulfuriferula* and *Sulfuricella*, they formed a cluster distinct from other
145 betaproteobacteria in the 16S rRNA gene tree (Fig. 1). Therefore, the genus *Sulfuriferula*
146 should be classified in a single family with the genus *Sulfuricella*. However, the genus
147 *Sulfuricella* was not classified in any family or order in the original description (Kojima &
148 Fukui, 2010). Previously, we proposed a new family and order for the genus *Sulfuricella*
149 (Watanabe *et. al.*, 2014), but the names of these taxa have not been validated. Here, we
150 propose the designations *Sulfuricellaceae* fam. nov. and *Sulfuricellales* ord. nov., to
151 accommodate the genera *Sulfuricella* and *Sulfuriferula*.

152

153 **Description of *Sulfuriferula* gen. nov.**

154 *Sulfuriferula* (Sul.fu.ri.fe’ru.la. L. neut. n. *sulfur* sulfur; L. fem. n. *ferula* a stick, cane;
155 N.L. fem. n. *Sulfuriferula*, sulfur-oxidizing stick).

156 Cells are motile, Gram-stain-negative, and non-spore-forming. Can grow autotrophically
157 on inorganic sulfur compounds. As determined by 16S rRNA gene sequence analysis, the
158 genus *Sulfuriferula* is a member of the family *Sulfuricellaceae*. The type species is
159 *Sulfuriferula multivorans*.

160

161 **Description of *Sulfuriferula multivorans* sp. nov.**

162 *Sulfuriferula multivorans* (mul.ti.vo’rans. L. adj. *multus* many; L. part. adj. *vorans*
163 devouring, eating; N.L. part. adj. *multivorans* devouring various substrates).

164 Cells are motile, Gram-stain-negative rods, 1.0–2.2 µm in length and 0.3–0.5 µm in
165 width, and non-spore-forming. Catalase-positive and oxidase-positive. Facultatively
166 anaerobic and reduce nitrate with generation of nitrite. Autotrophic growth occurs by the
167 oxidation of tetrathionate, thiosulfate, and elemental sulfur. Heterotrophic growth occurs on
168 casamino acids, yeast extract, peptone, arabinose, glucose, galactose, lactose, mannose,
169 fumarate, pyruvate, malate, lactate, and ethanol. Grows a temperature range of 8–32°C, a
170 NaCl concentration range of 0–133.3 mM, and a pH range of 5.3–8.6. Optimum growth was
171 observed at 22–25°C, 0–3.3 mM NaCl, and pH 6.4–7.0, respectively.

172 The type strain, TTN^T (=NBRC 110683^T =DSM 29343^T), was isolated from *Thioploca*
173 sample picked up from sediment of a freshwater lake in Japan. The G+C content of genomic
174 DNA is 63 mol%.

175

176 **Description of *Sulfuriferula plumbophilus*. sp. nov.**

177 *Sulfuriferula plumbophilus* (plumbo'phi.lus. L. n. *plumbum* lead; Gr. verb. *philein* to
178 love; M.L. adj. *plumbophilus* loving lead, referring to its ability to grow with PbS as sole
179 energy source).

180 The description is as given for '*Thiobacillus plumbophilus*' by Droner *et al.* (1992).

181 The type strain is Gro^{7T} (=NBRC 107929^T=DSM 6690^T).

182

183 **Description of *Sulfuricellaceae* fam. nov.**

184 *Sulfuricellaceae* (Sul.fu.ri.cel.la'ce.ae. N.L. fem. n. *Sulfuricella* type genus of the
185 family; -aceae ending to denote family; N.L. fem. pl. n. *Sulfuricellaceae* the family of the
186 genus *Sulfuricella*).

187 Encompasses Gram-stain-negative bacteria isolated mainly from freshwater
188 environments, within the class *Betaproteobacteria*. All of them utilize inorganic sulfur
189 compounds as their energy source. Currently, the family contains the genera *Sulfuricella* and
190 *Sulfuriferula*. Delineation of the family is determined primarily by phylogenetic information
191 from 16S rRNA gene sequences. The type genus is *Sulfuricella* Kojima and Fukui 2010.

192

193 **Description of *Sulfuricellales* ord. nov.**

194 *Sulfuricellales* (Sul.fu.ri.cel.la'les. N.L. fem. n. *Sulfuricella* type genus of the order; -ales
195 ending to denote order; N.L. fem. pl. n. *Sulfuricellales* the order of the genus *Sulfuricella*)

196 The order contains the family *Sulfuricellaceae*. Delineation of the order is determined
197 primarily by phylogenetic information from 16S rRNA gene sequences. The type genus is
198 *Sulfuricella* Kojima and Fukui 2010.

199

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204

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Watanabe, T., Kojima, H. & Fukui, M. (2014). Complete genomes of freshwater sulfur oxidizers *Sulfuricella denitrificans* skB26 and *Sulfuritalea hydrogenivorans* sk43H: Genetic insights into the sulfur oxidation pathway of betaproteobacteria. *Syst Appl Microbiol* **37**, 387–395.

207 Table 1. Differential phenotypic properties of strain TTN^T and its close relatives.
 208 Strains: 1, TTN^T; 2, Gro7^T; 3, *Sulfuricella denitrificans* skB26^T. Data for strains Gro7^T and
 209 skB26^T were obtained from Drobner *et al.* (1992) and Kojima and Fukui (2010), respectively.

Characteristic	1	2	3
Electron donor			
Lead sulfide	–	+	ND
Hydrogen	–	+	–
Sulfide	–	+	–
Tetrathionate	+	–	–
Thiosulfate	+	–	+
Elemental sulfur	+	–	+
Casamino acids	+	–	–
Yeast extract	+	–	–
Peptone	+	–	ND
Arabinose	+	–	ND
Glucose	+	–	–
Galactose	+	–	ND
Lactose	+	–	–
Mannose	+	–	ND
Fumarate	+	–*	–
Pyruvate	+	–	–
Malate	+	–*	ND
Lactate	+	–	–
Ethanol	+	–*	ND
Electron acceptor			
Nitrate	+	–	+

210 * Determined in this study.

211 Table 2. Cellular fatty acid content (percentage of total) of strain TTN^T (1) and closely related
 212 strain *Sulfuricella denitrificans* skB26^T (2, data from Watanabe *et al.* 2014).

Fatty acid	1	2
C _{8:0} 3-OH	-	2.4
C _{10:0}	0.38	-
C _{10:0} 3-OH	1.46	-
C _{12:0}	5.14	-
C _{12:0} 2-OH	0.15	-
C _{12:0} 3-OH	2.68	-
C _{13:1} at 12-13	-	0.12
C _{14:0}	0.17	0.36
C _{15:1} ω6c	0.18	-
Summed feature 3	44.69	62.24
Summed feature 4	3.7	-
C _{16:0}	33.63	23.52
C _{16:0} 3-OH	0.15	0.25
C _{16:1} 2-OH	-	2.93
C _{16:1} ω5c	-	1.4
C _{17:0}	0.56	-
cyclo-C _{17:0}	2.94	-
C _{18:0}	1.11	-
C _{18:1} ω9c	-	0.89
Summed feature 8	3.82	8.35
C _{18:1} ω5c	-	0.13
C _{18:0}	-	0.35

213 Summed feature 3: C_{16:1}ω7c and/or C_{16:1}ω6c

214 Summed feature 4: C_{16:1}ω7c and/or iso-C₁₅ 2-OH

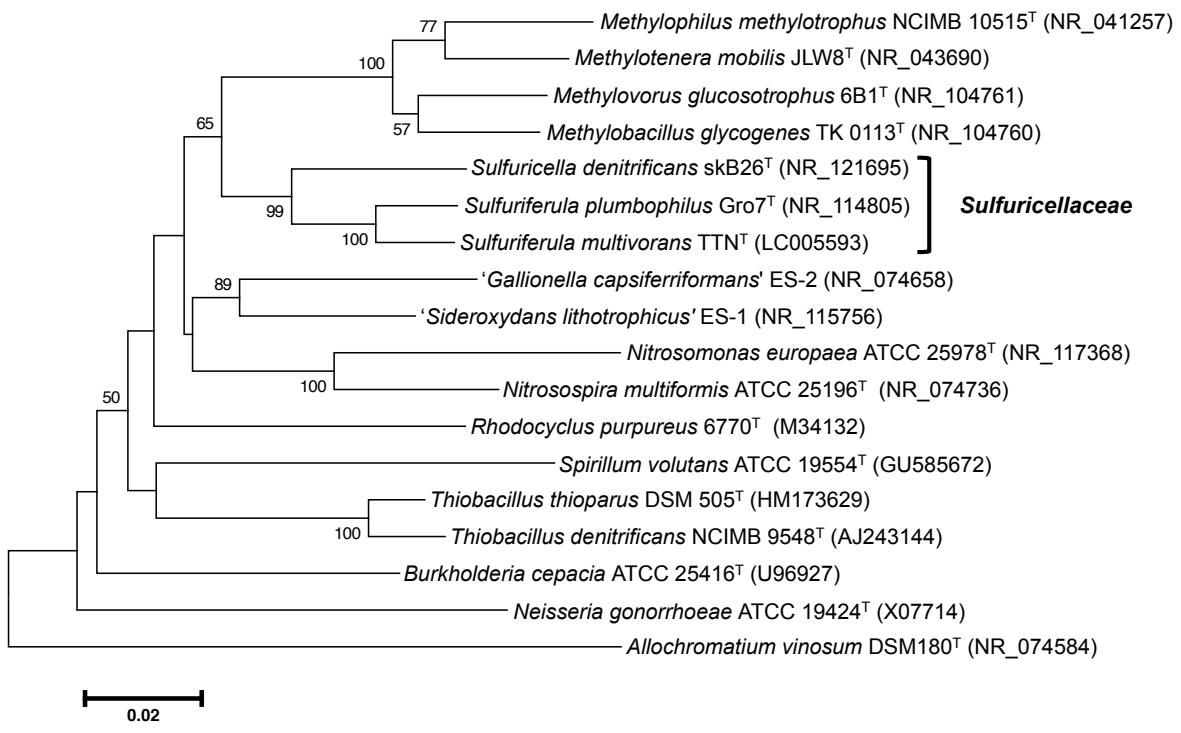
215 Summed feature 8: C_{18:1}ω7c and/or C_{18:1}ω6c

216

217 **Legends to figure**

218

219 Fig. 1. Phylogenetic position of strain TTN^T within the class *Betaproteobacteria*, based on
220 16S rRNA gene sequences aligned by ClustalW. *Allochromatium vinosum* was used as an
221 outgroup. Tree was constructed by the neighbor-joining method with 1000 bootstrap
222 resamplings (bootstrap values $\geq 50\%$ are shown at the nodes). Bar, 0.02 substitutions per
223 nucleotide position.



224

225 Fig. 1