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

Subject category: New taxa: *Proteobacteria*

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

Summary

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

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

Strain TTN^{T} was isolated from *Thioploca* sample obtained from sediment of a freshwater lake in Japan, Lake Okotanpe (Nemoto *et al*., 2011). The sediment sample was transferred into bicarbonate-buffered low-salt defined medium (Kojima & Fukui, 2014) containing 10 mM $Na_2S_2O_3$ and 20 mM $NaNO_3$ as electron donor and acceptor, respectively. The headspace of the culture bottle was filled with N_2/CO_2 (80:20, v/v), and incubation was performed in the dark at 22°C. After several transfers in the same medium, subsequent subcultures were carried out in ATCC 290 S6 medium under aerobic condition (3 vol% CO_2 was added in the head space) to enhance the growth. The composition of the medium was as follows (l^{-1}): 3 g $Na_2HPO_4 \cdot 12H_2O$, 1.8 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.1 g $(NH_4)_2SO_4$, 0.04 g $CaCl_2 \cdot 2H_2O$, 0.03 g $FeCl_3 \cdot 6H_2O$, 0.03 g $MnSO_4 \cdot 5H_2O$, and 10 g $Na_2S_2O_3$. Finally, strain TTN^{T} was isolated in pure culture by serial dilution. Purity of the isolate was checked by phase-contrast 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% CO_2 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 $Na_2S_2O_3$, 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

l^{-1}), yeast extract (5 mg l^{-1}), peptone (0.1 g l^{-1}), arabinose, glucose, galactose, lactose, mannose, fumarate, pyruvate, malate, lactate, propionate, ethanol, formate, acetate, n-butyrate, and benzoate (each 5 mM). Growth on sulfide (1 mM) and hydrogen ($H_2/N_2/CO_2$ 50 : 40 : 10, v/v; 200 kPa total pressure) was tested anaerobically in the bicarbonate-buffered low-salt defined medium (Kojima & Fukui, 2014) containing 20 mM nitrate. Utilization of electron acceptors, including nitrate (20 mM) and arsenate (5 mM), was tested in the defined medium containing 10 mM thiosulfate as an electron donor. For comparison, aerobic growth of '*Thiobacillus plumbophilus*' Gro7^T on fumarate, malate, and ethanol (each 5 mM) was also tested in DSMZ 561 medium.

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

The 16S rRNA gene sequence analysis demonstrated that strain TTN^T belongs to the class *Betaproteobacteria* (Fig. 1). The closest relative of strain TTN^T among the isolates with validly published names was *Sulfuricella denitrificans* skB26^T, but strain TTN^T was more closely related to another obligately aerobic chemolithoautotroph '*Thiobacillus plumbophilus*' Gro7^T (Drobner *et al*., 1992) (93.9 and 97.1% 16S rRNA gene sequence similarities, respectively). Strains TTN^T and Gro7^T also formed a monophyletic cluster in the SoxB and AprA trees (Figs S1 and S2). Phylogenetic trees of Sqr and DsrA including strains TTN^T and

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

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

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

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

The G+C content of the strain TTN^T genomic DNA was 63.0 mol%, which is more similar to that of strain Gro7^T (66 mol%) than that of *Sulfuricella denitrificans* skB26^T (56 mol%). The cellular fatty acid profile of strain TTN^T was characterized by a high concentration of summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; 44.7%), $C_{16:0}$ (33.6%), and

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

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

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* (plum.bo´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 Gro7^T (=NBRC 107929^T=DSM 6690^T).
182
183 **Description of *Sulfuricellaceae* fam. nov.**
184 *Sulfuricellalceae* (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

Acknowledgements

We thank A. Shinohara for technical assistance. This study was supported by KAKENHI Grant Number 22370005 to M. Fukui, and a grant from the Institute for Fermentation, Osaka, to H. Kojima.

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206

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-C_{15} 2-OH

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

216

Legends to figure

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

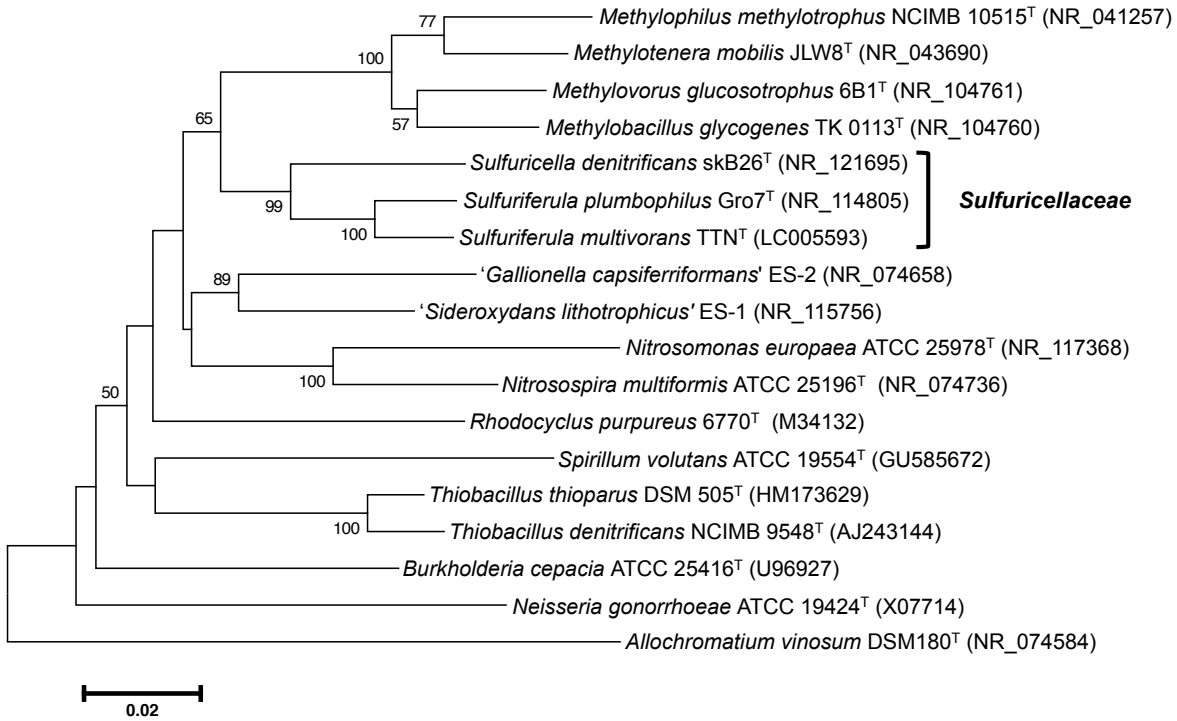
Methylophilus methylotrophus NCIMB 10515T (NR_041257)
Methylotenera mobilis JLW8T (NR_043690)
Methylovorus glucosotrophus 6B1T (NR_104761)
Methylobacillus glycogenes TK 0113T (NR_104760)
Sulfuricella denitrificans skB26T (NR_121695)
Sulfuriferula plumbophilus Gro7T (NR_114805)
Sulfuriferula multivorans TTNT (LC005593)
Sulfuricellaceae
'Gallionella capsiferriformans' ES-2 (NR_074658)
'Sideroxydans lithotrophicus' ES-1 (NR_115756)
Nitrosomonas europaea ATCC 25978T (NR_117368)
Nitrosospira multiformis ATCC 25196T (NR_074736)
Rhodocyclus purpureus 6770T (M34132)
Spirillum volutans ATCC 19554T (GU585672)
Thiobacillus thioparus DSM 505T (HM173629)
Thiobacillus denitrificans NCIMB 9548T (AJ243144)
Burkholderia cepacia ATCC 25416T (U96927)
Neisseria gonorrhoeae ATCC 19424T (X07714)
Allochromatium vinosum DSM180T (NR_074584)
77
100
65
57
99
100
89
100
50
100
0.02

Fig. 1