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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A sulfur-oxidizing bacterium, strain TTNT, was isolated from a 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’ Gro7T. Cells were rods, motile, and Gram-stain-negative. The G+C content of the genomic DNA was approximately 66 mol%. Strain TTNT grew over a temperature range of 8–32 °C (optimum 22–25 °C), an 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 TTNT was facultatively anaerobic and could utilize nitrate as an electron acceptor. The isolate oxidized tetrathionate, thiosulfate and elemental sulfur as the sole energy sources for autotrophic growth, and could also grow heterotrophically on a number of organic substrates. Based on its phylogenetic and phenotypic properties, strain TTNT represents a novel species of a novel genus, for which the name Sulfuriferula multivorans gen. nov., sp. nov. is proposed. The type strain is TTNT (=NBRC 110683T=DSM 29343T). Along with this, the reclassification of ‘Thiobacillus plumbophilus’ as Sulfuriferula plumbophilus sp. nov. (type strain Gro7T=NBRC 107929T=DSM 6690T) 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 novel genus in the class Betaproteobacteria (Kojima & Fukui, 2010). To date, Sulfuricella 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. Recently, a novel family, Sulfuricellaceae, and order, Sulfuricellales, have been proposed to accommodate the genus Sulfuricella, based on the phylogenetic analyses of the 16S rRNA gene and another six conserved genes (Watanabe et al., 2014). However, the names of these taxa have not been validated at the time of writing. In this study, a novel sulfur oxidizer belonging to these taxa was obtained.

Strain TTNT was isolated from a Thioploca sample obtained from the sediment of a freshwater lake in Japan, Lake Okotanpe (Nemoto et al., 2011). The Thioploca sample was transferred into bicarbonate-buffered low-salt defined medium (Kojima & Fukui, 2014). The composition of the medium was as follows (l−1): 0.2 g MgCl2-6H2O, 0.1 g CaCl2, 2H2O, 0.1 g NH4Cl, 0.1 g KH2PO4, 0.1 g KCl, 1 ml trace element solution, 1 ml selenite-tungstate solution, 1 ml vitamin mixture solution, 1 ml thiamine solution, 30 ml NaHCO3 solution and 1.0 ml Na2S2O3 solution. All stock solutions were prepared as described previously (Widdel & Bak, 1992). As an electron donor and acceptor, Na2S2O3 solution and NaNO3 solution were added to the medium at the final concentrations of 10 mM and 20 mM, respectively. The headspace of the culture bottle was filled with N2/CO2 (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 conditions (3% vol. CO₂ was added to the head space) to enhance growth. The composition of the medium was as follows (l⁻¹): 3 g Na₂HPO₄.12H₂O, 1.8 g KH₂PO₄, 0.1 g MgSO₄.7H₂O, 0.1 g (NH₄)₂SO₄, 0.04 g CaCl₂.2H₂O, 0.03 g FeCl₃.6H₂O, 0.03 g MnSO₄.5H₂O and 10 g Na₂SO₄. Finally, strain TTNT was isolated in pure culture by serial dilution. The purity of the isolate was checked by phase-contrast light microscopy (Axioplan 2; Zeiss, Germany) and sequencing of the 16S rRNA gene fragments.

To determine the phylogenetic position of strain TTNT, the 16S rRNA gene fragment was amplified with the primer set, 27F/1492R (Lane, 1991), and then sequenced. Partial fragments of genes for sulfur oxidation (sqr encoding sulfide: quinone oxidoreductase, soxB encoding sulfate thioester-sulfate hydratase, dsrA encoding dissimilatory sulfite reductase and aprA encoding adenosine-5'-phosphosulfate reductase) were also amplified and sequenced. The fragments of the sqr, soxB and dsrA genes were amplified with the primer pairs, sqr 473F/982R, soxB 704F/1199R and dsrA 625F/877R, respectively (Luo et al., 2011). The aprA gene fragments were obtained using Apr-1-FW/Apr-5-RV (Meyer & Kuever, 2007). The amplification of sqr and dsrA genes was also tested with genomic DNA extracted from ‘Thiobacillus plumbophilus’ Gro7T purchased from DSMZ (DSM 6690T).

The morphological and physiological characteristics of the isolate were investigated. Throughout the characterization, ATCC 290 S6 medium (3% vol. CO₂ was added in the head space) was used unless otherwise specified. Motility and morphology were observed by phase-contrast microscopy. Tests for Gram staining and catalase activity and morphology were observed by phase-contrast light microscopy (Axioplan 2; Zeiss, Germany). The isolate grew over a temperature range of 8–32 °C, an 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 at pH 6.4–7.0, respectively.

Anaerobic growth of strain TTNT 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 TTNT, 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 TTNT grew heterotrophically on a number of organic substrates including complex organic substances, sugars, organic acids and an alcohol (Table 1). The following organic substances could not support growth of the isolate: formate, propionate, acetate, n-butyrate and benzoate. As to ‘Thiobacillus plumbophilus’ Gro7T, 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 strain TTNT was determined by using a Yamasa GC kit (Yamasa Shoyu, Choshi, Japan) with 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 TTNT belongs to the class Betaproteobacteria (Fig. 1). The closest relative of strain TTNT with a validly published name among the isolates was Sulfuricella denitrificans skB26T, but strain TTNT was more closely related to another obligately aerobic chemolithoautotroph ‘Thiobacillus plumbophilus’ Gro7T (Drobner et al., 1992) (93.9 and 97.1% 16S rRNA gene sequence similarities, respectively). Strains TTNT and Gro7T also formed a monophyletic cluster in the SoxB and AprA trees (Figs S1 and S2, available in the online Supplementary Material). Phylogenetic trees of Sqr and DsrA, including strains TTNT and skB26T, 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 Gro7T.

The cells of strain TTNT were motile, Gram-stain-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, an 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 at pH 6.4–7.0, respectively.

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The G+C content of the genomic DNA of strain TTNT was 63.0 mol%, which is closer to that of strain Gro7T (66 mol%) than that of Sulfuricella denitrificans skB26T (56 mol%) (Watanabe et al., 2014). The cellular fatty acid profile of
its close phylogenetic relatives.* Determined in the present study.

Kojima & Fukui (2010), respectively.

Table 1. Differential phenotypic properties of strain TTNT and its close phylogenetic relatives

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<tr>
<td>Lead sulfide</td>
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<td>+</td>
<td>ND</td>
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<tr>
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<td>+</td>
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<tr>
<td>Sulfide</td>
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<td>−</td>
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<tr>
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<tr>
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<td>+</td>
</tr>
<tr>
<td>Casamino acids</td>
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<tr>
<td>Peptone</td>
<td>+</td>
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<td>Mannose</td>
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<tr>
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<td>−*</td>
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</tr>
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<td>Malate</td>
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<tr>
<td>Electron acceptor:</td>
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<tr>
<td>Nitrate</td>
<td>+</td>
<td>−</td>
<td>+</td>
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</table>

Strains: 1, TTNT; 2, Gro7T; 3, Sulfuricella denitrificans skB26T. Data for strains Gro7T and skB26T were obtained from Drobner et al. (1992) and Kojima & Fukui (2010), respectively. +, Positive; −, negative; ND, not determined.

strain TTNT was characterized by having a high concentration of summed feature 3 (C₁₆:₁ω7c and/or C₁₆:₁ω6c; 44.7%), C₁₆:₀ (33.6%), and C₁₂:₀ (5.1%). The two major components were also reported as major fatty acids of the closely related strain, Sulfuricella denitrificans skB26T (Table 2). Differences in the levels of other minor fatty acids of these two strains were found (Table 2).

The name ‘Thiobacillus plumbophilus’ has never been validated, and the need to reclassify it has been pointed out (Kelly & Wood, 2000). In the phylogenetic trees of 16S rRNA, sexB and aprA genes, the monophyly of strain TTNT and ‘Thiobacillus plumbophilus’ Gro7T was demonstrated. However, utilization of electron donors and acceptors distinguished them from each other and from Sulfuricella denitrificans skB26T (Table 1). Therefore, strains TTNT and Gro7T represent different species of a single genus. On the basis of their phylogenetic and phenotypic properties, we propose a novel species of a novel genus, Sulfuriferula multivorans gen. nov., sp. nov., for strain TTNT, 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 gen nov. and Sulfuricella, they formed a cluster, which was distinct from other betaproteobacteria in the 16S rRNA gene tree (Fig. 1). Therefore, the genus Sulfuriferula gen nov. 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 novel family and order for the genus Sulfuricella (Watanabe et al., 2014), but the names of these taxa have not
**Description of Sulfuriferula gen. nov.**

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

Cells are motile, Gram-stain-negative and non-spore-forming. Grow autotrophically on inorganic sulfur compounds. Is a member of the family *Sulfuricellaceae* as determined by 16S rRNA gene sequence analysis. The type species is *Sulfuriferula multivorans* sp. nov.

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


Cells are motile, Gram-stain-negative rods, 1.0–2.2 μm in length, 0.3–0.5 μm in width and non-spore-forming, catalase- and oxidase-positive. Facultatively anaerobic and reduce nitrate with the generation of nitrite. Autotrophic growth occurs by the oxidation of tetrathionate, thiosulfate and elemental sulfur. Heterotrophic growth occurs on casamino acids, yeast extract, peptone, arabinose, glucose, galactose, lactose, mannose, fumarate, pyruvate, malate, lactate and ethanol. Grows at a temperature range of 8–32 °C, an NaCl concentration range of 0–133.3 mM and a pH range of 5.3–8.6. Optimum growth is observed at 22–25 °C, 0–3 mM NaCl and at pH 6.4–7.0.

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

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


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**Description of Sulfuriferula plumbo(ph)ilus sp. nov.**

*Sulfuriferula plumbo(ph)ilus* (plum.bo’phi.lus. L. n. *plumbum* lead; Gr. verb. *philein* to love; M.L. adj. *plumbophilus* loving lead, referring to its ability to grow with PbS as the sole energy source).

The description is as given for *‘Thiobacillus plumbophilus’* by Drobner *et al.* (1992).

The type strain is *Gro7T* (=NBRC 107929T=DSM 6690T).

**Description of Sulfuricellales fam. nov.**

*Sulfuricellales* (Sul.fu.ri.cell.‘la.cea.e. N.L. fem. n. *Sulfuricella* type genus of the family; -ceae ending to denote family; N.L. fem. pl. n. *Sulfuricellaceae* the family of the genus *Sulfuricella*).

Encompasses Gram-stain-negative bacteria isolated mainly from freshwater environments, within the class *Betaproteobacteria*. All utilize inorganic sulfur compounds as their energy source. At the time of writing, the family contains the genera *Sulfuricella* and *Sulfuriferula* gen. nov. Delineation of the family is determined primarily by phylogenetic information from 16S rRNA gene sequences. The type genus is *Sulfuricella* (Kojima & Fukui, 2010).

**Description of Sulfuricellales ord. nov.**

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

Contains the family *Sulfuricellaceae* fam. nov. Delineation is determined primarily by phylogenetic information from 16S rRNA gene sequences. The type genus is *Sulfuricella* (Kojima & Fukui, 2010).

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References


