Sulfuriferula thiophila sp. nov., a chemolithoautotrophic sulfur-oxidizing bacterium

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Running head: *Sulfuriferula thiophila* sp. nov.

Subject category: New taxa: *Proteobacteria*

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA gene, *sqr* and *soxB* of strain mst6^{T} are LC115045−LC115047.

Summary

A novel sulfur-oxidizing bacterium designated strain mst6^T was isolated from spring water of Masutomi Hot spring in Japan. The cells were rod-shaped (1.2−4.0 × 0.5−0.7 μm) and Gram-stain-negative. The G+C content of genomic DNA was around 52.6 mol%. The isolate possessed summed feature 3 ($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c), $C_{16:0}$ and $C_{12:0}$ as major cellular fatty acids. Strain mst6^T grew by inorganic carbon fixation and oxidation of inorganic sulfur compounds with oxygen as an electron acceptor. The isolate grew over a temperature range of 5−34°C, an NaCl concentration range of 0−110 mM, and a pH range of 4.6−8.1. Optimum growth occurred at 32°C, in the absence of NaCl and at pH 5.9−6.2. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain mst6^T belongs to the family *Sulfuricellaceae* in the class *Betaproteobacteria*. The closest cultured relative was *Sulfuriferula multivorans* TTN^T, with 16S rRNA gene sequence similarity of 97.0%. On the basis of the data obtained in this study, strain mst6^T represents a novel species of the genus *Sulfuriferula*, for which the name *Sulfuriferula thiophila* sp. nov. is proposed. The type strain is mst6^T (=NBRC 111150^T=DSM 101871^T). In addition we propose correcting the name *Sulfuriferula plumbophilus* (Watanabe, Kojima and Fukui 2015, 1507VP) to *Sulfuriferula plumbiphila* corrig. based on Rule 12c, Rule 61 and Appendix 9 of the International Code of Nomenclature of Prokaryotes.

Betaproteobacterial sulfur chemolithotrophs are recently recognized as one of the important constituents in freshwater lake ecosystems (Watanabe *et al*., 2013; Kojima *et al*., 2014). Within the class *Betaproteobacteria*, the family *Sulfuricellaceae* is comprised of only freshwater sulfur chemolithotrophs belonging to three genera – *Sulfuricella*, *Sulfurirhabdus* and *Sulfuriferula*. The genus *Sulfuricella* is composed of a facultatively anaerobic, obligately chemolithoautotrophic species (Kojima *et al*., 2010), and the genus *Sulfurirhabdus* consists of a strictly aerobic, chemolithoautotrophic species (Watanabe *et al*., 2015b). The genus *Sulfuriferula* contains a strictly chemolithoautotrophic species and a facultatively chemolithoautotrophic species, which able to grow autotrophically on inorganic sulfur compounds and heterotrophically on a number of organic substrates (including complex organic substrates, sugars, organic acids and an alcohol) in the absence of sulfur compounds as an electron donor (Drobner *et al*., 1992; Watanabe *et al*., 2015a). In this study, a novel sulfur-oxidizing bacterium phylogenetically related to the genus *Sulfuriferula* was isolated and characterized.

Strain mst6^{T} was isolated from a spring water sample obtained from a storage tank at Masutomi Hot spring located in Hokuto City, Yamanashi Prefecture, Japan (35°51'53"N, 138°31'49"E). The tank stores spring water pumped from 300 m depth below the ground for supplying water for public bath. The water sample was characterized as follows: temperature, 28.3°C; pH, 6.3; conductivity, 0.97 S m^{-1}; H_2S, 1.2 μM. Sampling was performed in June 2014. The water sample was transferred into a bicarbonate-buffered low-salt defined medium. The composition of the medium was as follows (l^{-1}): 0.2 g $MgCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g NH_4Cl, 0.1 g KH_2PO_4, 0.1 g KCl, 5g $Na_2S_2O_3 \cdot 5H_2O$, 1 ml trace element solution, 1 ml selenite-tungstate solution and 30 ml $NaHCO_3$ solution. All ingredients except stock solutions

were dissolved in 20 mM MOPS-NaOH (pH 7.1), and the solution was autoclaved at 121°C for 20 min. All stock solutions were prepared as described previously (Widdel & Bak, 1992), and were aseptically added after autoclaving. Thereafter, 20 ml aliquots of the solution were transferred to 50 ml sterile glass bottles (Glasgerätebau Ochs, Bovenden, Germany). The headspace of the bottle was filled with air (100 kPa), and the bottle was sealed with a butyl rubber stopper. Incubation was performed in the dark at 25°C for 6 days. After repeated subcultures, the resulting culture was streaked on the medium solidified with 1.5 % agar. A colony was picked up and maintained in the liquid medium. Purity of the culture was checked by phase-contrast light microscopy (Axioplan 2; Zeiss, Germany), inoculation into media containing decreased concentration of thiosulfate (1 mM) and one of the various heterotrophic substrates (yeast extract, glucose, fumarate, formate and acetate), and direct sequencing of the 16S rRNA gene fragments amplified using various primer sets.

For the characterization of the isolate, the medium was modified as follows: MOPS-NaOH was excluded and vitamin solution DSM 141 was added (1 ml l^{-1}). Basically, the pH value of the medium was adjusted to 7.1 with HCl solution, and incubations were performed at 25°C under aerobic conditions (headspace of the bottles was filled with air, 100 kPa). Morphology was observed by phase-contrast microscopy. The Gram-stain test was conducted with a kit (Fluka). Test for oxidase activity was performed using an oxidase test reagent (bioMérieux). Catalase activity was tested by pouring 3% H_2O_2 solution onto a pellet obtained by centrifugation of culture. Effect of the temperature on growth was tested by culturing the isolate at various temperatures (0, 5, 8, 15, 18, 22, 25, 28, 32, 34, 36, 37, 42°C). To test the effect of NaCl concentration on growth, the isolate was inoculated into the media supplemented with varying concentrations of NaCl (0, 10, 15, 22, 33, 49, 74, 110, 170, 250,

500 mM). Effect of pH on growth was tested as described previously (Kojima et al., 2015). The buffering reagents and tested pH were as follows: MES (pH 3.7, 4.3, 4.6, 5.0, 5.1, 5.5, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.6), PIPES (pH 6.6, 6.8, 7.0), MOPS (pH 6.9, 7.1), Tricine (pH 7.6, 7.9, 8.1), or CHES (8.2, 8.9, 9.2, 9.4, 9.5). To adjust pH, NaOH solution was used, except for media adjusted to pH 3.7−5.7, which were prepared with HCl solution. Utilization of electron donors was tested in the media, each containing one of the substrates listed later. Utilization of nitrate, nitrite or arsenate as an electron acceptor was tested under anaerobic conditions (headspace of the bottles was filled with N_2/CO_2, 80:20, 100 kPa). Growth under different conditions was assessed after 2−8 weeks of incubation. All testes were performed at least twice with appropriate negative and/or positive controls.

The G+C content of the genomic DNA 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 was analyzed using the Sherlock Microbial Identification System (Version 6.0; database, TSBA6; MIDI) at Techno Suruga (Shizuoka, Japan).

The 16S rRNA gene fragment was amplified with the primer set, 27F/1492R (Lane, 1991), and then sequenced. The amplification of genes for sulfur oxidation was also tested. PCR amplification of genes encoding sulfide:quinone oxidoreductase (Sqr), component of the periplasmic thiosulfate-oxidizing Sox enzyme complex (SoxB), dissimilatory sulfite reductase alpha subunit (DsrA) and adenylylsulfate reductase alpha subunit (AprA) was performed using the primer pairs sqr 473F/982R, soxB 704F/ 1199R, dsrA 625F/877R and AprA-1-FW/AprA-5-RV, respectively (Luo *et al*., 2011; Meyer & Kuever, 2007b).

Cells of the isolate were short rods (1.2−4.0 μm long and 0.5−0.7 μm width) and sometimes elongated without septa (more than 9 μm long). The cells were Gram-negative, and motility was observed. Oxidase and catalase activities were detected. The G+C content of the genomic DNA was around 52.6 mol%. The isolate possessed summed feature 3 ($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c; 55.5%), $C_{16:0}$ (26.7%) and $C_{12:0}$ (6.8%) as major cellular fatty acids (Table S1).

Autotrophic growth of the isolate was observed in the presence of thiosulfate (10 and 20 mM), tetrathionate (10 mM) and elemental sulfur (approximately 0.25−0.5 g l^{-1}). Tetrathionate seems to be the best substrate for growth of the isolate. Sulfide (2 mM), sulfite (5 mM) and hydrogen (air/H_2, 50:50 by vol.; 200 kPa total pressure) did not support autotrophic growth of the isolate. The following substrates could not support heterotrophic growth of strain mst6^T: casamino acids (50 mg l^{-1}), yeast extract (50 mg l^{-1}), peptone (100 mg l^{-1}), glucose, galactose, mannose, fumarate, malate, lactate, formate, acetate, and ethanol (all 5 mM). The utilization of organic compounds as an energy source was also tested using two different media. The composition of one medium was almost the same as that of the medium used for the first enrichment, but thiosulfate concentration was decreased to 1 mM. The other one was the ATCC 290 S6 medium (3 % vol. CO_2 was added to the headspace), which is used for the cultivation of its closest cultured relative *Sulfuriferula multivorans* TTN^T (discussed below) (Watanabe et al., 2015a). The isolate was inoculated into these two basal media, each containing one of the heterotrophic substrates described above, but no growth was observed. In the presence of thiosulfate as an electron donor, nitrate (20 mM), nitrite (2.5 mM) and arsenate (5 mM) did not support anaerobic growth of the isolate.

129 The range of temperature for growth was 5−34°C, and optimum temperature was 32°C.
130 The isolate grew optimally in medium without NaCl, and no growth occurred in the medium
131 with 170 mM NaCl. The isolate grew over an initial pH range of 4.6−8.1, and optimum
132 growth was observed pH 5.9−6.2. Under optimum growth conditions, growth of the isolate
133 was observed after 2 days of incubation.

134 Phylogenetic analysis based on the 16S rRNA gene sequences indicated that strain mst6^T
135 is a member of the family *Sulfuricellaceae* (Fig. 1) and is most closely related to *Sulfuriferula*
136 *multivorans* TTN^T (97.0%). Environmental clones sharing 98.5−99.7% sequence similarities
137 with the novel isolate have been reported from acid mine drainage samples (according to the
138 GenBank record). These sequences formed a phylogenetic cluster distinct from the cluster
139 comprising *Sulfuriferula* spp. with bootstrap confidence. From the extracted genomic DNA of
140 strain mst6^T, *sqr* and *soxB* were identified, but *dsrA* and *aprA* amplicons were not obtained.
141 Phylogenetic trees of Sqr and SoxB are shown in Figs S1 and S2. Considering the fact that the
142 primer sets used in this study have successfully generated PCR products from genomic DNAs
143 of members within the family *Sulfuricellaceae* (*Sulfuricella denitrificans*, *Sulfurirhabdus*
144 *autotrophica* and *Sulfuriferula multivorans*) (Table 1), the lack of *dsrA* and *aprA* amplicoms
145 most likely reflects the absence of these genes in strain mst6^T.

146 Based on the high 16S rRNA gene similarity of the novel isolate with *Sulfuriferula*
147 *multivorans* TTN^T (97%), it may be reasonable to place strain mst6^T in the genus
148 *Sulfuriferula*. This value is lower than the theoretical threshold (98.7−99.0%) for the
149 delineation of bacterial species based on 16S rRNA gene sequence similarity (Stackebrandt &
150 Ebers, 2006), and thus the isolate seems to represent a novel species within the genus
151 *Sulfuriferula*. The genus *Sulfuriferula* currently contains two sulfur-oxidizing species, *S.*

multivorans and *S. plumbiphila* corrig. *S. multivorans* utilize a wide spectrum of electron donors (tetrathionate, thiosulfate, elemental sulfur and various organic substrates) and acceptors (oxygen and nitrate). *S. plumbiphila* corrig. is an obligate chemolithoautotroph grows with H_2, lead sulfide and hydrogen sulfide as electron donors coupled with aerobic respiration (Drobner *et al*., 1992). In common with *S. plumbiphila* corrig., the novel isolate was characterized as an autotrophic, aerobic sulfur-oxidizing bacterium, but their utilization patterns of electron donors are completely different (Table 1). Strain mst6^T was also distinguished from known *Sulfuriferula* spp. by its lower genomic DNA G+C content (Table 1). Compared to the closest cultured relative *Sulfuriferula multivorans*, the cell length of the isolate was longer, and its optimum pH was more acidic. Also, differences in the components of minor fatty acids between strain mst6^T and *S. multivorans* were apparent in Table 1. Although all known members of the family *Sulfuricellaceae* harbor the *aprA* gene, PCR amplification results suggest that the novel isolate lacks *dsrA* and *aprA* (Table 1). Strain mst6^T may possess a genetic machinery for sulfur oxidation distinct from them. Based on the data obtained in this study, we propose a novel species, *Sulfuriferula thiophila* sp. nov., for strain mst6^T.

Description of *Sulfuriferula thiophila* sp. nov.

Sulfuriferula thiophila (thi.o'phi.la. Gr. n. *theion* (Latin transliteration *thium*) sulfur; N.L. adj. *philus –a –um* (from Gr. adj. *philos –ê –on*), friend, loving; N.L. fem. n. *thiophila* sulfur-loving).

Cells are Gram-stain-negative rods. Catalase- and oxidase-positive. The major cellular fatty acids are summed feature 3 ($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c), $C_{16:0}$ and $C_{10:0}$ 3-OH. The G+C

content of genomic DNA is around 52.6 mol%. Obligate chemolithoautotroph grows using tetrathionate, thiosulfate and elemental sulfur as electron donors and O_2 as an electron acceptor. Grows a temperature range of 5−34°C, a NaCl concentration range of 0−110 mM, and a pH range of 4.6−8.1. Optimum growth was observed at 32°C, in the absence of NaCl, and pH 5.9−6.2. The type strain, mst6^T (=NBRC 111150^T=DSM 101871^T), was isolated from spring water of Masutomi Hot spring in Japan.

In addition we propose correcting the name *Sulfuriferula plumbophilus* (Watanabe, Kojima and Fukui 2015, 1507VP) to *Sulfuriferula plumbiphila* corrig. based on Rule 12c, Rule 61 and Appendix 9 of the International Code of Nomenclature of Prokaryotes.

Description of *Sulfuriferula plumbiphila* corrig.

Sulfuriferula plumbiphila (plum.bi'phi.la. L. neut. n. *plumbum* lead; N.L. adj. *philus –a –um* (from Gr. adj. *philos –ê –on*), friend, loving; N.L. fem. adj. *plumbiphila*, 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 Gro7^T (= NBRC 107292^T = DSM 6690^T).

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Table 1. Differential characteristics among all type strains of the family *Sulfuricellaceae*. Strains: 1, mst6^T; 2, *Sulfuriferula multivorans* TTN^T (Watanabe *et al*., 2015a); 3, *Sulfuriferula plumbiphila* corrig. Gro7^T (Drobner *et al*., 1992); 4, *Sulfuricella denitrificans* skB26^T (Kojima & Fukui, 2010); 5, *Sulfurirhabdus autotrophica* BiS0^T (Watanabe *et al*., 2015b). Data for fatty acid composition and genomic DNA G+C content of strain skB26^T were obtained from Watanabe *et al*. (2014). Data for PCR amplifications of strain Gro7^T were obtained from Meyer *et al*. (2007a) (for *soxB*), Meyer and Kuever (2007c) (for *aprA*), and Watanabe *et al*. (2015a) (*sqr* and *dsrA*). +, Positive; −, negative; ND, not determined.

	Sulfuriferula			*Sulfuricella*	*Sulfurirhabdus*
	1	**2**	**3**	**4**	**5**
16S rRNA gene similarity to the novel isolate	−	97.0	95.4	93.8	93.1
Cell length (μm)	1.2−4.0	1.0–2.2	3≥	0.8−2.0	1.4−4.6
Inorganic electron donors:					
Hydrogen	−	−	+	−	−
Tetrathionate	+	+	−	−	+
Thiosulfate	+	+	+	+	+
Elemental sulfur	+	+	−	+	+
Lead sulfide	−	−	+	−	−
Nitrate reduction	−	+	−	+	−
Heterotrophic growth	−	+	−	−	−
Optimal growth conditions (limits):					
Temperature (°C)	32 (5−34)	22−25 (8−32)	27 (9−41)	22 (0−28)	15−22 (0−32)
pH	5.9−6.2 (4.6−8.1)	6.4−7.0 (5.3−8.6)	ND (4.5−6.5)	7.5−8.0 (6.0−9.0)	6.1−6.3 (5.2−8.1)

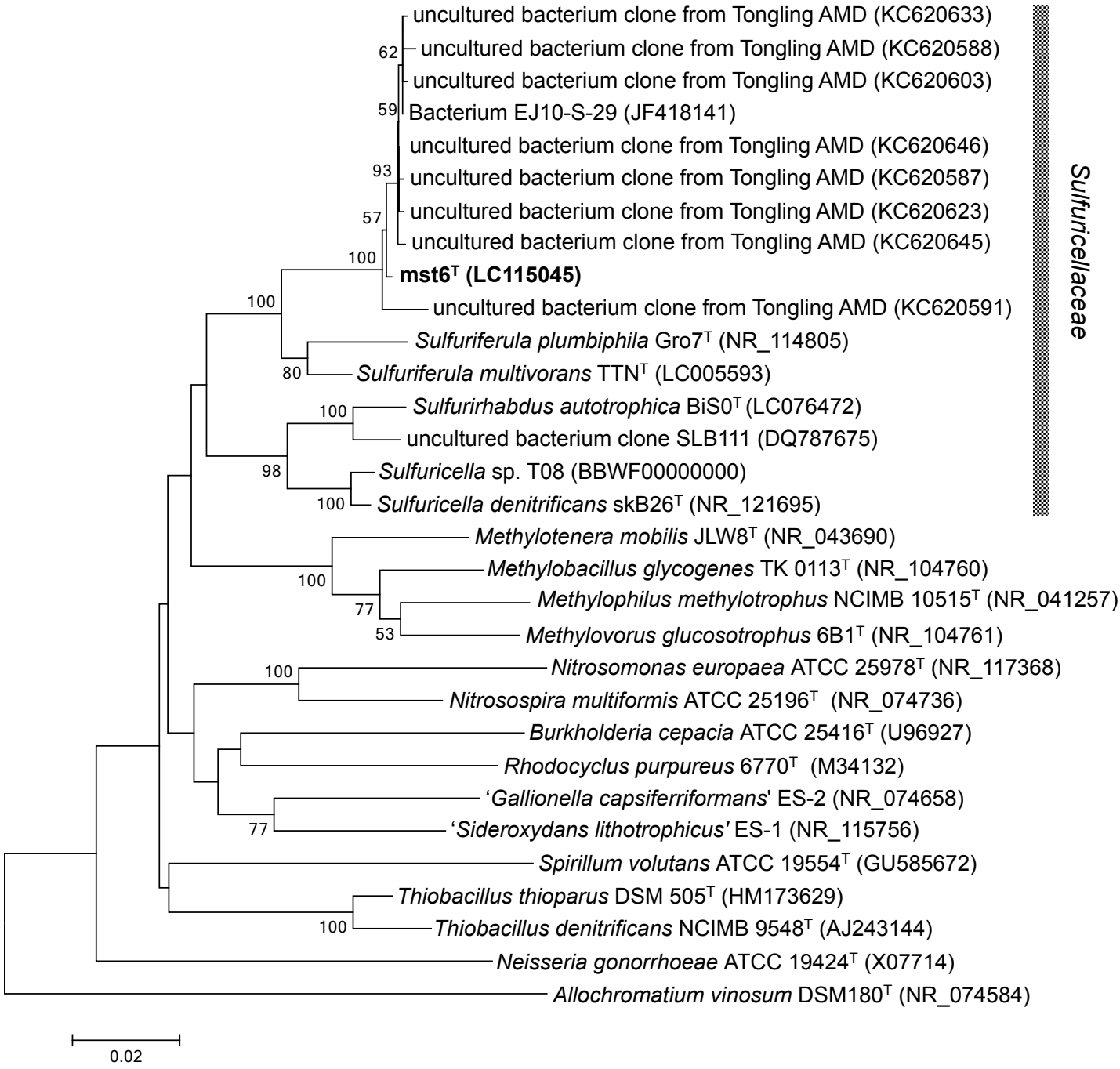
NaCl (mM)	0 (0−110)	0−3.3 (0−133.3)	ND	0 (0−220)	0−66.7 (0−546.4)
Major fatty acid (%):					
Summed feature 3	56	45	ND	62	62
$C_{16:0}$	27	34	ND	24	26
$C_{10:0}$ 3-OH	1.4	1.5	ND	−	5.4
Summed feature 8	3.3	3.8	ND	8.4	2.0
$C_{12:0}$	6.8	5.1	ND	−	0.5
PCR product obtained with primer set					
sqr 473F/982R	+	+	−	+	+
soxB 704F/1199R	+	+	+	+	+
AprA-1-FW/AprA-5-RV	−	+	+	+	+
dsrA 625F/877R	−	+	−	+	+
DNA G+C content (mol%)	52.6	63.0	66	56.1	43.7
Isolation source	Spring water	*Thioploca* sample of freshwater lake	Uranium mine	Freshwater lake water	Freshwater lake sediment

248

249 **Legend to figure**

250

251 Fig. 1. Phylogenetic position of strain mst6^T within the class *Betaproteobacteria*, based on
252 16S rRNA gene sequences aligned by ClustalW. *Allochromatium vinosum* was used as an
253 outgroup. Tree was constructed by the neighbor-joining method with 1000 bootstrap
254 resamplings (bootstrap values ≥ 50% are shown at the nodes). Bar, 0.02 substitutions per
255 nucleotide position.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Sulfuriferula plumbiphila Gro7T (NR_114805)
Sulfuriferula multivorans TTNT (LC005593)
Sulfurirhabdus autotrophica BiS0T (LC076472)
uncultured bacterium clone SLB111 (DQ787675)
Sulfuricella sp. T08 (BBWF00000000)
Sulfuricella denitrificans skB26T (NR_121695)
Methylotenera mobilis JLW8T (NR_043690)
Methylobacillus glycogenes TK 0113T (NR_104760)
Methylophilus methylotrophus NCIMB 10515T (NR_041257)
Methylovorus glucosotrophus 6B1T (NR_104761)
Nitrosomonas europaea ATCC 25978T (NR_117368)
Nitrosospira multiformis ATCC 25196T (NR_074736)
Burkholderia cepacia ATCC 25416T (U96927)
Rhodocyclus purpureus 6770T (M34132)
'Gallionella capsiferriformans' ES-2 (NR_074658)
'Sideroxydans lithotrophicus' ES-1 (NR_115756)
Spirillum volutans ATCC 19554T (GU585672)
Thiobacillus thioparus DSM 505T (HM173629)
Thiobacillus denitrificans NCIMB 9548T (AJ243144)
Neisseria gonorrhoeae ATCC 19424T (X07714)
Allochromatium vinosum DSM180T (NR_074584)
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Sulfuricellaceae
0.02