Sulfurivermis fontis gen. nov., sp. nov., a novel sulfur-oxidizing autotroph, and proposal of *Thioprofundaceae* fam. nov.

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Summary

A novel Gram-stain-negative, chemolithoautotrophic sulfur oxidizer, strain JG42^T was isolated from a hot spring microbial mat. As an electron donor for autotrophic growth, strain JG42^T utilized sulfide, thiosulfate, tetrathionate and elemental sulfur. The cells of strain JG42^T were oxidase-positive and catalase-negative. The G+C content of genomic DNA was 65 mol%. The predominant cellular fatty acid was $C_{16:0}$ (47% of total). Phylogenetic analysis of the 16S rRNA gene indicated that strain JG42^T belonged to the order *Chromatiales*, but sequence similarities to the known species were less than 94%. On the basis of its properties, strain JG42^T (=DSM 104776^T = NBRC 112696^T) is proposed as type strain of a new species of a novel genus, *Sulfurivermis fontis* gen. nov., sp. nov., which belongs to the family *Thioalkalispiraceae*. A new family, *Thioprofundaceae* fam. nov. is also proposed to accommodate the genus *Thioprofundum*, transferred from the family *Thioalkalispiraceae*.

The order *Chromatiales* includes many phototrophic and chemolithotrophic sulfur oxidizers. Among 7 families in this order, the families *Halothiobacillaceae* and *Thioalkalispiraceae* consist of chemolithotrophic sulfur oxidizers [1, 2], whereas the family *Ectothiorhodospiraceae* encompasses both and phototrophic and chemotrophic sulfur-oxidizing bacteria [3]. In addition, a sulfur-oxidizing chemolithoautotroph was recently described as new member of the family *Granulosicoccaceae* [4], which consisted of only obligately chemoheterotrophic species before [5]. In this study, a novel sulfur-oxidizing chemolithoautotroph was isolated and characterized to be proposed as type species of a new genus is in the order *Chromatiales*.

The novel isolate, strain JG42^{T}, was isolated from an enrichment culture from which *Sulfuritorta calidifontis* J1A^{T} was isolated [6]. The enrichment culture was obtained with a medium containing elemental sulfur and nitrate as an electron donor and acceptor, respectively. A microbial mat from a microbial mat from Jozankei hot spring (42° 57' 53" N 141° 09' 47" E) in Japan was used as an inoculum of this enrichment culture established at 45°C. The basal medium of the following composition (hereafter referred to as S5 medium) was used throughout this study, unless otherwise specified (l^{-1}): 2.5 g $Na_2S_2O_3$. $5H_2O$, 0.5 g $MgSO_4$. $7H_2O$, 0.1 g $CaCl_2$. $2H_2O$, 0.1 g NH_4Cl, 0.1 g KH_2PO_4,

0.1 g KCl, 1 ml vitamin mixture solution (DSM 141), 1 ml trace element solution, 1 ml selenite-tungstate solution and 30 ml $NaHCO_3$ solution (1 M). Solutions of the last three components were prepared as described previously [7]. In order to induce changes in microbial community structure, a portion of the enrichment culture was transferred to the S5 medium supplemented with 10 mM nitrate and cultured at increased temperature. The cultivation was performed at 50°C under anoxic condition created by filling the headspace of the culturing bottles with mixed gas (N_2/CO_2; 80:20 v/v, 100 kPa in total pressure). After several times transfer under the same conditions, culturing medium was further changed to another one with higher salt concentration. The high-salt medium contained 20 g l^{-1} NaCl and 3 g l^{-1} MgCl . $6H_2O$, and the other composition was basically the same as that of the S5 medium (concentrations of $Na_2S_2O_3$. $5H_2O$ and $MgSO_4$. $7H_2O$ were changed to 5 g l^{-1} and 0.3 g l^{-1}, respectively). The culturing with this medium was performed at 45°C without shaking, in closed bottles with the headspace filled with air at atmospheric pressure. From the resulting enrichment culture, an isolate designated as strain JG42^T was obtained by repeated agar shake dilution under anoxic conditions [7], using the high-salt medium supplemented with 10 mM nitrate. The final concentration of agar was 1.1% w/v. Purity of the isolate was checked by microscopy and repeated sequencing of the 16S rRNA gene.

For the characterization of strain JG42^T, all culturing experiments were performed at 45°C under oxic conditions (as described above) using the S5 medium, unless otherwise specified.

Gram-stain test was performed with a staining kit (Fluka). Catalase activity was assessed with 3% H_2O_2 solution and oxidase activity was tested with a test reagent (bioMérieux). The G+C content of the genomic DNA was determined by HPLC method [8], using a kit (Yamasa Shoyu). The analysis of cellular fatty acids was carried out at the Techno Suruga Co. Ltd (Shizuoka, Japan), with cells grown at 45°C for 3 days. The fatty acid profile was analyzed by using the Sherlock Microbial Identification System version 6.0 (MIDI), with database of TSBA6.

Utilization of electron donors was tested under aerobic conditions, with a modified S5 medium without thiosulfate. Utilization of carbon sources was tested with S5 medium without bicarbonate, buffered with 20 mM MOPS. Anaerobic growth was tested with the S5 medium supplemented with nitrate or nitrite in closed bottles with headspace filled with N_2/CO_2 (80:20; v/v, 100 kPa in total pressure). Heterotrophic growth in complex liquid media was tested for the following media and those supplemented with 2% NaCl: R2A (Daigo), diluted (1/10) R2A, NB (Difco) and TSB (Oxoid). Utilization of nitrate as nitrogen source was tested by replacing NH_4Cl in S5 medium with $NaNO_3$

90 (0.2 g L^{-1}).

91 Effects of the temperature on growth were examined at 10, 13, 15, 18, 22, 25, 28, 30,
92 37, 45, 48, 50, 52 and 55°C. The effect of pH on the growth was tested at 37°C as
93 described previously [9]. The tested pH and buffering reagents used were as follows; pH
94 5.7, 5.8, 5.9, 6.1, 6.2, 6.3, 6.4 and 7.0 with MES; pH 6.9, 7.2 and 7.4 PIPES; pH 7.0, 7.2,
95 7.0, 7.7 and 7.9 with MOPS; pH 7.7, 8.2, 8.5 and 8.9 with Tricine; pH 8.0, 8.3, 9.4, 8.7,
96 9.1 and 9.7 with CHES. The effect of salt concentration was tested in S5 medium
97 supplemented with varying concentrations of NaCl (0, 1, 2, 3, 4, and 5% w/v).
98 Sensitivity to antibiotics was tested in S5 medium supplemented with 50 μg ml^{-1} of
99 kanamycin sulfate or ampicillin sodium salt.

100 The 16S rRNA gene of strain JG42^T was amplified by PCR using the primer pair 27F
101 and 1492R [10] and then directly sequenced by using a BigDye Terminator v3.1 Cycle
102 Sequencing kit (Applied Biosystems). The obtained gene sequence was aligned with
103 reference sequences retrieved from the GenBank/EMBL/DDBJ database, by using the
104 program CLUSTAL X version 2.1 [11]. The reference sequences included 51
105 representatives from all families of the order *Chromatiales* and 4 environmental clone
106 sequences which have 97% or higher sequence similarity with strain JG42^T, identified
107 by BLAST search. All positions with gaps were excluded from the calculation, and

1091 positions were used for the following analyses. The evolutionary distances were computed using the maximum composite likelihood method. Phylogenetic trees were reconstructed by using the methods of neighbor-joining and minimum evolution with the program MEGA version 7.0.20 [12].

Cells of strain JG42^{T} were curved rods, 0.4–0.6 µm in width and 1.5–12 µm in length (Fig. S1). The cells were motile, Gram-stain-negative, catalase-negative and oxidase-positive. The G+C content of the genomic DNA of was 65 mol% (HPLC).

The growth of strain JG42^{T} was observed over a temperature range between 25°C and 50°C, with an optimum growth at 42–48°C. The range of pH for growth was 6.1–8.9, and the optimum pH was 7.2–7.9. Strain JG42^{T} exhibited an optimum growth in the presence of 0‑1% NaCl, and did not grow in the presence of 3% or more NaCl.

In the cellular fatty acid profile of the strain, $C_{16:0}$ was the predominant fatty acid accounting for 47% of total. The other fatty acids detected were summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; 23.9 %), summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$; 13.0 %),. summed feature 9 (iso$C_{17:1}\omega7c$ and/or $C_{16:0}$ 10-methyl; 6.2 %), $C_{14:0}$ (4.7%), $C_{10:0}$ 3-OH (1.5%), $C_{12:0}$ 3-OH (1.2%), $C_{12:0}$ (0.5%), $C_{18:1}\omega9c$ (0.5 %), $C_{17:0}$ (0.4%), $C_{16:1}\omega5c$ (0.3 %) and $C_{11:0}$ 3-OH (0.1%).

126 Strain JG42^T was facultative anaerobe which can use nitrate (10 mM) as terminal
127 electron acceptor to support growth, but not nitrite (1 and 5 mM).
128 Chemolithoautotrophic growth of strain JG42^T was supported by thiosulfate (10 mM),
129 tetrathionate (10 mM), sulfide (2 mM) and elemental sulfur (0.5 g l^{-1}), but sulfite (5
130 mM) and hydrogen (air/H_2, 50 : 50 v/v; 200 kPa total pressure) did not support the
131 growth. The following substrates did not support heterotrophic growth of strain JG42^T:
132 acetate, lactate, fumarate, succinate, malate, benzoate, butyrate, isobutyrate, formate,
133 D-glucose, D-sorbitol, mannose, D-xylose, L-arabinose, and *N*-acetylglucosamine (all 5
134 mM). Strain JG42^T exhibited no growth on R2A, diluted R2A, NB, or TSB, and
135 addition of 2% NaCl did not affect the results. In the presence of thiosulfate, aerobic
136 growth was observed in the medium containing bicarbonate, but the followings did not
137 serve as carbon source to support growth under the same conditions: acetate, lactate,
138 succinate, malate, formate (all 5 mM), and yeast extract (0.05% w/v). Growth of strain
139 JG42^T was observed in the media containing nitrate or ammonium as sole nitrogen
140 source, and inhibited by kanamycin and ampicillin.
141 Analysis of the 16S rRNA gene revealed that strain JG42^T is a relative of species in the
142 order *Chromatiales*, but sequence similarities with these species were less than 94%.
143 Uncultured bacterial clones with the highest sequence similarity to strain JG42^T were

144 detected in a bioreactor for wastewater treatment [13] and water sample from an oil well
145 [14]. By constructing phylogenetic trees, it was confirmed that strain JG42^T is a member
146 of the order *Chromatiales* (Fig. 1, Fig. S2). In the trees of identical topology obtained
147 with the neighbour-joining and minimum evolution methods (Fig. 1), strain JG42^T and
148 related clones formed a cluster with the genera *Thioalkalispira* [15] and *Thiohalophilus*
149 [16]. These genera belong to the family *Thioalkalispiraceae*, but the other members of
150 this family (*Thioprofundum* species) were positioned apart from strain JG42^T in the
151 phylogenetic trees (Fig. 1). A different tree was obtained with the maximum-likelihood
152 method (Fig. S2), but strain JG42^T was also grouped with the genera *Thioalkalispira*
153 and *Thiohalophilus* in this tree.
154
155 All phylogenetic trees constructed suggested that strain JG42^T should be classified into
156 the family *Thioalkalispiraceae*, although it was not fully supported by the boot strap
157 analyses (between 40% and 50% in all trees). At this point, strain JG42^T can only be
158 regard as a member of the family *Thioalkalispiraceae*. On the other hand, phylogenetic
159 isolation of the genus *Thioprofundum* in this family was also indicated (Fig. 1). In fact,
160 the polyphyly of the family *Thioalkalispiraceae* has been repeatedly observed in
161 previous phylogenetic trees [4, 17, 18]. To solve this problem, a new family,

Thioprofundaceae fam. nov. is proposed to accommodate genus *Thioprofundum*. These taxonomic assignments are not supported solid evidence at present, but seem to be the best options to avoid further confusion in classification within the order *Chromatiales*. The phylogenetic trees of neighbour-joining and minimum evolution also indicated that the existing genera in the family *Thioalkalispiraceae* cannot accommodate the strain JG42^T without disrupting the monophyly (Fig. 1). In addition to the low sequence similarities (<94%), phenotypic properties of the novel strain were also distinct from those of the strains representing *Thioalkalispira* and *Thiohalophilus* (Table 1). On the basis of these characteristics, strain JG42^T is proposed to be assigned to a new species of a novel genus in the family *Thioalkalispiraceae*, with the name *Sulfurivermis fontis* gen. nov., sp. nov.

Description of *Thioprofundaceae* fam. nov.

Thioprofundaceae (Thi.o.pro.fun.da.ce'ae. N.L. neut. n. *Thioprofundum* the type genus of the family; suff. -aceae ending to denote a family; N.L. fem. pl. n. *Thioprofundaceae* the family of the genus *Thioprofundum*).

The cell wall is Gram-negative type. The phylogenetic position is in the order *Chromatiales* within the class *Gammaproteobacteria* of the phylum *Proteobacteria*. The

type genus of the family is *Thioprofundum*.

Description of *Sulfurivermis* gen. nov.

Sulfurivermis (Sul.fu.ri.ver'mis. L. neut. n. *sulfur* sulfur; L. masc. n. *vermis* worm. N.L. masc. n. *Sulfurivermis* sulfur-oxidizing worm)

Grow chemolithoautotrophically by the oxidation of inorganic sulfur compounds. Gram-stain-negative. Major cellular fatty acid is $C_{16:0}$. Based on 16S rRNA gene sequence analysis, affiliated to the family *Thioalkalispiraceae* in the order *Chromatiales*. The type species is *Sulfurivermis fontis*.

Description of *Sulfurivermis fontis* sp. nov.

Sulfurivermis fontis (fon'tis. L. masc. gen. n. *fontis* of a spring).

Cells are motile, 0.4–0.6 μm wide and 1.5–12 μm long. Facultatively anaerobic and reduce nitrate as electron acceptor to support growth. Chemolithoautotrophic growth occurs with oxidation of sulfide, thiosulfate, tetrathionate and elemental sulfur. Nitrate and ammonium are utilized as nitrogen source. Oxidase-positive and catalase-negative. The temperature range for growth is 25–50°C, with an optimum of 42–48°C. The pH range for growth is 6.1–8.9, with an optimum of pH 7.2–7.9. No growth occurs in the

198 presence of 3% NaCl. The G+C content of genomic DNA is 65 mol% (HPLC). The type
199 strain JG42^{T} (= DSM 104776^{T} = NBRC 112696^{T}) was isolated from microbial mat of a
200 hot spring in Japan.
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209 **Conflicts of interest statement**
210 The authors declare that there is no conflict of interest.
211
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Table 1. Differential properties of strain JG42^T and strains representing related genera. Strains: 1, JG42^T; 2, *Thioalkalispira microaerophila* ALEN 1^T [15]; 3, *Thiohalophilus thiocyanoxidans* HRhD 2^T [16].

Characteristics	1	2	3
DNA G+C content (%)	65	58.9	58.2
Growth at			
pH 7	+	-	+
pH 10	-	+	-
1 M NaCl	-	+	+
Growth on nitrate reduction	+	-	-

Figure legend

Fig. 1 Neighbor-joining tree based on the 16S rRNA gene sequences of strain JG42^{T}, related environmental clones and representatives of all families of the order *Chromatiales*. Numbers following the family names represent the number of strains in each collapsed branch (all strains are shown in Fig. S2). Bootstrap values based on 1,000 resampling (%) are shown at nodes. Tree of identical topology was obtained with the minimum evolution method.