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Sulfurifustis variabilis gen. nov., sp. nov., a novel sulfur oxidizer isolated from a lake, and proposal of *Acidiferrobacteraceae* fam. nov. and *Acidiferrobacterales* ord. nov.

Hisaya Kojima*, Arisa Shinohara, and Manabu Fukui

The Institute of Low Temperature Science, Hokkaido University. Kita-19, Nishi-8, Kita-ku, Sapporo 060-0819, Japan

*Corresponding author.

E-mail: kojimah@pop.lowtem.hokudai.ac.jp

Phone: +81-11-706-5460 Fax: +81-11-706-5460

Running head: *Sulfurifustis variabilis* gen. nov., sp. nov.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain is LC062790. The accession numbers for *aprA* and cbbL genes are LC062791 and LC062959, respectively.

Summary

A novel autotrophic bacterium, strain skN76^{T} was isolated from sediment of a lake in Japan. As sole electron donor to support chemolithoautotrophic growth, the strain oxidized thiosulfate, tetrathionate, and elemental sulfur. For growth, the optimum temperature was 42–45°C and the optimum pH was 6.8–8.2. The cells were Gram-stain-negative, catalase-positive and oxidase-positive. The strain exhibited changes in morphology depending on growing temperature. Cells grown at the optimum temperature were rod-shaped (0.9–3.0 μm long and 0.3–0.5 μm wide), whereas filamentous form was observed when the strain was cultured at the lowest growth temperatures. The G+C content of genomic DNA was 69 mol%. The major components in the fatty acid profile were $C_{16:0}$, summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) and summed feature 9 (iso$C_{17:1}\omega9c$ and/or $C_{16:0}$ 10-methyl). Phylogenetic analysis based on 16S rRNA gene indicated that the closest cultivated relative of strain skN76^{T} was *Acidiferrobacter thiooxydans* m-1^{T}, with the sequence similarity of 93%. On the basis of its phylogenetic and phenotypic properties, the strain skN76^{T} (= DSM 100313^{T} = NBRC 110942^{T}) is proposed as type strain of a new species of a novel genus, *Sulfurifustis variabilis* gen. nov., sp. nov. Novel taxa, *Acidiferrobacteraceae* fam. nov., and *Acidiferrobacterales* ord. nov. are also proposed to accommodate the genera

Acidiferrobacter and *Sulfurifustis* gen. nov.

There are phylogenetically diverse sulfur-oxidizing bacteria which were referred to as "*Thiobacillus*" in the past. After successive reclassifications, they are now distributed in 4 classes in the phylum *Proteobacteria* (Kelly & Wood, 2000; Kelly & Wood, 2013; Watanabe *et al*., 2015). One of them, *Acidiferrobacter thiooxydans* m-1^T corresponds to the organism known as "*Thiobacillus ferrooxidans* m-1" (Hallberg *et al.*, 2011). *A. thiooxydans* has been classified into the family *Ectothiorhodospiraceae* in the class *Gammaproteobacteria*, but detailed analysis of 16S rRNA gene sequences indicated that this bacterium is phylogenetically distinct from other members of the family (Oren, 2014). In the present study, a novel chemolithoautotrophic sulfur oxidizer related to this bacterium was isolated and characterized.

A sulfur-oxidizing enrichment culture was established from a freshwater sediment as described previously (Watanabe *et al.*, 2014). The basal medium used for enrichment and isolation was bicarbonate-buffered low-salt defined medium previously described (Kojima and Fukui, 2011). As electron donor and acceptor, elemental sulfur (ca. 0.5 g l^{-1}) and nitrate (20 mM) were added to the medium just before inoculation. From the enrichment culture, isolate was obtained by agar shake dilution (Widdel & Bak, 1992), using the basal medium supplemented with 20 mM thiosulfate and 20 mM nitrate. Head space of the agar tubes were filled with mixture gas of N_2/CO_2 (80 : 20, v/v), but no

58 reductant was added to the medium and thus dissolved oxygen was not eliminated.
59 Well-separated colonies were picked up in a slightly modified medium supplemented
60 with 20 mM sodium thiosulfate. Composition of the modified medium was almost
61 identical to the original medium, but vitamin solutions were replaced with a single
62 vitamin mixture (1 ml l^{-1}) which contained following constituents (l^{-1}); 2 mg biotin, 2
63 mg folic acid, 10 mg pyridoxine-HCl, 5 mg thiamine-HCl·$2H_2O$, 5 mg riboflavin, 5 mg
64 nicotinic acid, 5 mg calcium D(+) pantothenate, 5 mg 4-aminobenzoic acid, 5 mg lipoic
65 acid, and 0.1 mg cyanocobalamine. The picked up colonies were cultivated under oxic
66 conditions in closed bottles, and one of the resulting pure cultures was designated as
67 strain skN76^T. All cultivation for enrichment and isolation was carried at 45°C. Purity
68 of the isolate was checked by microscopy and sequencing of the 16S rRNA gene
69 fragments amplified with several universal PCR primer pairs.
70 For the characterization of the strain, the modified medium (altered vitamin
71 composition as described above) supplemented with 20 mM sodium thiosulfate was
72 used unless otherwise specified. All culturing experiments were performed in bottles
73 closed with rubber stoppers, and the bottles were incubated without shaking.
74 The Gram-stain test was conducted with a kit (Fluka). Catalase activity was assessed
75 by pouring 3% H_2O_2 solution onto a pellet obtained by centrifugation of culture.

Oxidase activity was tested with the pellet of cells, by using an oxidase test reagent (bioMérieux). The genomic G+C content of the DNA was determined with the HPLC methods (Katayama-Fujimura et al., 1984). Fatty acids were extracted from cells grown with thiosulfate at 45°C. The fatty acid profile of the strain was analyzed at the Techno Suruga Co. Ltd (Shizuoka, Japan), by using the Sherlock Microbial Identification System (Version 6.0; database, TSBA6; MIDI).

The utilization of electron acceptor was tested at in the medium amended with 20 mM sodium thiosulfate under anoxic conditions (headspace of the bottles was filled with N_2/CO_2). Utilization of growth substrate was tested in the medium with lowered thiosulfate concentration (0.4 mM), supplemented with one of the substrates listed later. Aerobic growth in ordinary complex liquid media was tested for R2A, NB, LB, and TSB at 45°C.

Effects of the temperature on growth were examined by culturing the isolate at various temperatures (25, 28, 30, 32, 37, 42, 45, 46, 47, 48, and 50°C). Effect of salt concentration was tested by culturing the strain in medium supplemented with varying concentrations of NaCl (0–500 mM, 50 mM intervals). The effect of pH on the growth was tested at 42°C, with media of various pH prepared as below. The basal composition of the media was as follows (l^{-1}): 5 g $Na_2S_2O_3$. $5H_2O$, 1 g $NaHCO_3$, 0.2 g $MgCl_2$.

94 $6H_2O$, 0.1 g $CaCl_2$, 0.1 g NH_4Cl, 0.1 g KH_2PO_4, 0.1 g KCl, 1 ml trace element solution,
95 1 ml selenite-tungstate solution, and 1 ml vitamin mixture described above. Depending
96 on the final pH, one of the buffering reagents listed below was added to each medium at
97 the final concentration of 20 mM. All ingredients were mixed and then sterilized by
98 filtration after pH adjustment. To adjust pH, NaOH solution was used except media of
99 the lowest pH (5.4–5.8) prepared with HCl. The tested pH and buffering reagents were
100 as follows; pH 5.4, 5.7, 5.8, 6.1, 6.2, 6.3, 6.4, 6.7 with MES; pH 6.8 and 7.0 with
101 PIPES; pH 7.0, 7.1, 7.2, and 7.5 with MOPS; pH 7.7, 7.9, 8.1, 8.2, 8.5 and 8.7 with
102 Tricine; pH 8.7, 8.9, 9.1, 9.4, and 9.6 with CHES.
103 Fragment of 16S rRNA gene was amplified with the primer pair 27F and 1492R (Lane,
104 1991), and the resulting PCR product was directly sequenced. Phylogenetic analysis
105 was performed with the program MEGA version 5.05 (Tamura *et al.*, 2011). Fragments
106 of the *aprA* gene (encoding adenosine-5'-phosphosulfate reductase) were amplified and
107 sequenced with the primers Apr-1-FW and Apr-5-RV (Meyer & Kuever, 2007a). The
108 *cbbL* gene encoding form I ribulose-1,5-bisphosphate carboxylase/oxygenase was
109 amplified with primers cbbLG1F (Selesi *et al.*, 2005) and 898E (Boschker *et al*, 2014),
110 and then directly sequenced.
111

Cells of strain skN76^T grown at 45°C were motile Gram-stain-negative rods (0.9–3.0 μm long and 0.3–0.5 μm wide). As shown in Fig 1, strain skN76^T exhibited filamentous morphology when it was grown at 28°C or 30°C. The tests of catalase and oxidase both resulted in positive (cells grown at 45°C). The G+C content of the genomic DNA of was 69 mol%. Major components in the fatty acid profile of strain skN76^T grown at 45°C were $C_{16:0}$ (43.6%), summed feature 9 (iso$C_{17:1}$ω9c and/or $C_{16:0}$ 10-methyl; 21.1 %), and summed feature 3 ($C_{16:1}$ω7c and/or $C_{16:1}$ω6c; 17.2 %). The other fatty acids detected were $C_{10:0}$ (9.2%), summed feature 8 ($C_{18:1}$ω7c and/or $C_{18:1}$ω6c; 3.8 %), $C_{18:0}$ (1.8%), iso$C_{17:0}$ (0.8%), $C_{12:0}$ 3-OH (0.6%), $C_{17:0}$ (0.5%), $C_{14:0}$ (0.4%), iso$C_{10:0}$ (0.4%).

The growth of the strain skN76^T was observed over a temperature range between 28°C and 46°C, with an optimum at 42–45°C. The range of pH for growth was 6.3–8.9, and the optimum pH was 6.8–8.2. Optimum growth was observed in medium containing 0-50 mM NaCl. Very slow growth was observed in the medium containing 450 mM NaCl, but not in the medium of 500 mM NaCl.

The isolate grew chemolithoautotrophically on thiosulfate (10, 20 mM), tetrathionate (20 mM), and elemental sulfur (0.5 g l^{-1}). The following substrates did not support

aerobic growth: pyruvate (5 mM), lactate (5 mM), acetate (5 mM), methanol (5 mM), succinate (2.5 mM), fumarate (2.5 mM), butyrate (2.5 mM), isobutyrate (2.5 mM), ethanol (2.5 mM), formate (5 mM), lactose (2.5 mM), glucose (2.5 mM), xylose (2.5 mM). The strain exhibited no growth on R2A, NB, LB, or TSB. Although strain skN76^T was obtained from a nitrate-reducing enrichment culture, anaerobic growth was not observed under the tested conditions. Nitrite (5 mM), nitrate (20 mM), or poorly crystalline Fe(III) oxide (10 mM) did not support growth of the strain as sole electron acceptor for thiosulfate oxidation. The strain might have been growing with trace amounts of oxygen in the enrichment culture, but this possibility needs to be experimentally tested.

The 16S rRNA gene sequence analysis revealed that the closest cultivated relative of strain skN76^T was *A. thiooxydans* m-1^T, with sequence similarity of 93%. These two strains and related environmental clones formed a distinct cluster outside of the order *Chromatiales* encompassing the family *Ectothiorhodospiraceae*, in phylogenetic trees constructed with different methods (Fig. 2, Fig. S1). The PCR products of *aprA* and *cbbL* genes were also sequenced. Phylogenetic analysis revealed that protein coded by *aprA* gene of skN76^T belonged to a phylogenetic lineage referred to as "Apr lineage I" (Meyer & Kuever, 2007b), which is one of two major lineages of sulfur oxidizers (Fig.

148 3). The nucleotide sequence of *cbbL* gene has been deposited in the public database
149 (accession number, LC062959).
150
151 The novel strain skN76^T exhibited low similarity (93%) of the 16S rRNA gene to the
152 closest relative, *A. thiooxydans* m-1^T. The strain is known as extremely acidophilic
153 bacterium (optimum pH for growth is around 2) which requires an external osmotic
154 potential for growth (Hallberg *et al.*, 2011). On the other hand, strain skN76^T grew
155 under neutral to moderately alkaline conditions and optimum growth was observed in
156 the medium of lowest salt concentration. On the basis of these phylogenetic and
157 phenotypic properties, strain skN76^T is proposed to be assigned to a new species of a
158 novel genus, with the name *Sulfurifustis variabilis* gen. nov., sp. nov. As shown in
159 phylogenetic trees previously constructed (Oren, 2014, Rua & Thompson 2014), *A.*
160 *thiooxydans* is phylogenetically isolated from the other cultivated gammaproteobacteria
161 belonging to existing orders. In the phylogenetic analysis including the novel bacterium
162 obtained in this study, the genera *Acidiferrobacter* and *Sulfurifustis* formed a distinct
163 cluster apart from the clade of the class *Chromatiales*, irrespective of tree construction
164 methods (Fig. 2, Fig. S1). Therefore, a novel family and a novel order are proposed to
165 accommodate these genera, with the names *Acidiferrobacteraceae* fam. nov. and

166 *Acidiferrobacterales* ord. nov., respectively.

167

168 **Description of *Sulfurifustis* gen. nov.**

169 *Sulfurifustis* (Sul.fu.ri.fus'tis. L. neut. n. *sulfur* sulfur; L. masc. n. *fustis*, stick; N.L.

170 masc. n. *Sulfurifustis* sulfur-oxidizing stick).

171 Grow chemolithoautotrophically by the oxidation of inorganic sulfur compounds. Based

172 on 16S rRNA gene sequence analysis, affiliated to the family *Acidiferrobacteraceae* in

173 the class *Gammaproteobacteria*. The type species is *Sulfurifustis variabilis*.

174

175 **Description of *Sulfurifustis variabilis* sp. nov.**

176 *Sulfurifustis variabilis* (va.ri.a'bi.lis. L. masc. adj. *variabilis*, changeable, referring to

177 variation of the morphology depending on growth temperatures).

178 Cells are Gram-stain-negative, rod-shaped or filamentous, 0.4–0.6 μm in width. Major

179 components in the fatty acid profile are $C_{16:0}$, summed feature 3, and summed feature 9.

180 Autotrophic growth occurs with oxidation of thiosulfate, tetrathionate, and elemental

181 sulfur. Catalase-positive and oxidase-positive. The temperature range for growth is

182 28–46°C, with an optimum of 42–45°C. The pH range for growth is 6.3–8.8, and

183 optimum growth occurs at pH 6.8–8.2. The G+C content of genomic DNA is 69 mol%.

The type strain skN76^{T} (= DSM 100313^{T} = NBRC 110942^{T}) was isolated from sediment of a freshwater lake in Japan (Lake Mizugaki).

Description of *Acidiferrobacteraceae* fam. nov.

Acidiferrobacteraceae (A.ci.di.fer.ro.bac.te.ra.ce'ae. N.L. n. *Acidiferrobacter* type genus of the family; *-aceae* ending to denote family; N.L. fem. pl. n. *Acidiferrobacteraceae* the family of the genus *Acidiferrobacter*).

Encompasses Gram-stain-negative chemolithoautotrophic bacteria. Based on the 16S rRNA gene sequence analysis, phylogenetically affiliated to the order *Acidiferrobacterales* ord. nov. The type genus is *Acidiferrobacter*.

Description of *Acidiferrobacterales* ord. nov.

Acidiferrobacterales (A.ci.di.fer.ro.bac.te.ra.les. N.L. n. *Acidiferrobacter* type genus of the order; *-ales* ending to denote order; N.L. fem. pl. n. *Acidiferrobacterales* the order of the genus *Acidiferrobacter*).

Encompasses the family *Acidiferrobacteraceae* fam. nov. Based on the 16S rRNA gene sequence analysis, phylogenetically affiliated to the class *Gammaproteobacteria*. The type genus is *Acidiferrobacter*.

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REFERENCES

Boschker, H.T., Vasquez-Cardenas, D., Bolhuis, H., Moerdijk-Poortvliet, T.W., Moodley, L. (2014). Chemoautotrophic carbon fixation rates and active bacterial communities in intertidal marine sediments. *PLoS One* **9,** e101443.

Hallberg, K.B., Hedrich, S., Johnson, D.B. (2011). *Acidiferrobacter thiooxydans*, gen. nov. sp. nov.; an acidophilic, thermo-tolerant, facultatively anaerobic iron and sulfur-oxidizer of the family *Ectothiorhodospiraceae*. *Extremophiles* **15,** 271–279.

Katayama-Fujimura, Y., Komatsu, Y., Kuraishi, H. & **Kaneko, T. (1984).** Estimation of DNA base composition by high-performance liquid chromatography of its Nuclease PI hydrolysate. *Agric Bio. Chem* **48**, 3169–3172.

Kelly, D.P. & Wood, A.P. (2000). Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov., *Halothiobacillus* gen. nov. and *Thermithiobacillus* gen. nov. *Int J Syst Evol Microbiol* **50**, 511–516.

Kelly, D.P. & Wood, A.P. (2013). Proposal for a new class within the phylum *Proteobacteria*, *Acidithiobacillia* classis nov., with the type order *Acidithiobacillales*, and emended description of the class *Gammaproteobacteria*. *Int J Syst Evol Microbiol* **63,** 2901–2906.

Kojima, H. & Fukui, M. (2011). *Sulfuritalea hydrogenivorans* gen. nov., sp. nov., a facultative autotroph isolated from a freshwater lake. *Int J Syst Evol Microbiol* **61,** 1651–1655.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, chapter 6, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.

Meyer, B. & Kuever, J. (2007a). Molecular analysis of the diversity of sulfate-reducing and sulfur-oxidizing prokaryotes in the environment, using *aprA* as functional marker gene. *Appl Environ Microbiol* **73**, 7664–7679.

Meyer, B. & Kuever, J. (2007b). Molecular analysis of the distribution and phylogeny of dissimilatory adenosine-5'-phosphosulfate reductase-encoding (*aprBA*) among sulfur oxidizing prokaryotes. *Microbiology* **153**, 3478–3498.

Oren, A. (2014). The family *Ectothiorhodospiraceae*. In The Prokaryotes, 4th edn, pp. 199–223. Edited by Rosenberg E., DeLong E. F., Lory S., Stackebrandt E., Thompson F. Berlin: Springer.

Rua, C.P.J. & Thompson, F. (2014). The Unclassified Genera of Gammaproteobacteria: *Alkalimonas* , *Arenicella* , *Chromatocurvus* , *Congregibacter* , *Gallaecimonas* , *Halioglobus* , *Marinicella* , *Methylohalomonas* , *Methylonatrum* , *Orbus* , *Plasticicumulans* , *Porticoccus* , *Sedimenticola* , *Simiduia* , *Solimonas*. In The Prokaryotes, 4th edn, pp. 749–768. Edited by Rosenberg E., DeLong E. F., Lory S., Stackebrandt E., Thompson F. Berlin: Springer.

Selesi, D., Schmid, M. & Hartmann, A. (2005). Diversity of green-like and red-like ribulose-1,5-bisphosphate carboxylase/oxygenase largesubunit genes (*cbbL*) in differently managed agricultural soils. *Appl Environ Microbiol* **71,** 175–184.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731–2739.

Watanabe, T., Kojima, H. & Fukui, M. (2015). *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. *Int J Syst Evol Microbiol* **65**, 1504–1508.

Watanabe, M., Kojima, H. & Fukui, M. (2014). Proposal of *Effusibacillus lacus* gen. nov. sp. nov., and reclassification of *Alicyclobacillus pohliae* as *Effusibacillus pohliae* comb. nov. and *Alicyclobacillus consociatus* as *Effusibacillus consociatus* comb. nov.

Int J Syst Evol Microbiol **64,** 2770–2774.

Widdel, F. & Bak, F. (1992). Gram-negative mesotrophic sulfate-reducing bacteria. In *The Prokaryotes* 2nd edn, vol. 4, pp. 3352–3378. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer-Verlag.

Figure legends

Fig. 1 Phase-contrast micrographs of strain skN76^T, grown at 45°C (upper panel) and 28°C (upper panel). Bars, 5 μm.

Fig. 2 Minimum-evolution tree showing the phylogenetic position of skN76^T within the class *Gammaprotepbacteria* based on the 16S rRNA gene sequence analysis. This tree was constructed using 1415 sites, and identical tree was obtained with the neighbor-joining method. A tree constructed with the maximum-likelihood method is shown in Fig. S1. *Desulfatitalea tepidiphila* is included as an outgroup. Numbers on nodes represent percentage values of 1000 bootstrap resampling (values larger than 50 are shown).

Fig. 3 Phylogenetic position of AprA amino acid sequence of strain skN76^T within "Apr lineage I". Tree was constructed with the neighbor-joining method (117 amino acid positions were used) with 1000 bootstrap resamplings (bootstrap values are shown at the nodes). Bar, 0.01 substitutions per amino acid position.