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Sulfuricaulis limicola gen. nov., sp. nov., a novel sulfur oxidizer isolated from a lake

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Running head: *Sulfuricaulis limicola* gen. nov., sp. nov.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain is LC030205. The accession numbers for *aprA* genes are LC030206–LC030207, and *cbbL* gene is LC030208.

Summary

A novel sulfur-oxidizing bacterium, strain HA5^{T} was isolated from sediment of a lake in Japan. The cells were rod-shaped (0.3–0.5 × 1.2–6.0 μm) and Gram- stain-negative. The G+C content of genomic DNA was 63 mol%. The major components in the cellular fatty acid profile were $C_{16:0}$ and summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$). As electron donor to support autotrophic growth, the strain oxidized thiosulfate, tetrathionate, and elemental sulfur. Growth was observed at temperature range of 8–37°C, and optimum growth was observed at 28–32°C. Growth of the strain was observed at pH range of 6.1–9.2. Optimum growth of the isolate was observed in medium without NaCl. Phylogenetic analysis based on 16S rRNA gene indicated that the strain belongs to the family *Acidiferrobacteraceae* in the class *Gammaproteobacteria*. The closest relative was *Sulfurifustis variabilis* skN76^{T}, with the highest sequence similarity of 93%. On the basis of its phylogenetic and phenotypic properties, the strain HA5^{T} (= DSM 100373^{T} = NBRC 110752^{T}) is proposed as type strain of a new species of a novel genus, *Sulfuricaulis limicola* gen. nov., sp. nov.

The family *Acidiferrobacteraceae* in the order *Acidiferrobacterales* was recently established to accommodate two monospecies genera of chemolithoautotrophs, *Acidiferrobacter* and *Sulfurifustis* (Kojima *et al*., 2015). *Acidiferrobacter thiooxydans* is an acidophilic iron oxidizer which requires an osmotic potential for growth (Hallberg *et al.*, 2011). *Sulfurifustis variabilis* is a neutrophilic sulfur oxidizer characterized by variable morphology (Kojima *et al*., 2015). In the present study, a novel autotrophic sulfur oxidizer related to these bacteria was isolated and characterized.

The strain HA5^{T} was isolated from sediment of a lake in Japan, Lake Harutori. Lake Harutori is a meromictic lake located in a residential area (Kubo *et al*., 2014). The sediment sample was manually obtained from a shoreside site with 0.2 m water depth. During the process of enrichment and isolation, media modified from a bicarbonate-buffered medium (Kojima & Fukui, 2011) were used. The compositions of media are summarized in Table 1. All media were prepared with the methods previously described. Briefly, basal constituents were dissolved in distilled water, autoclaved, and then cooled down to room temperature under anoxic conditions. The other constituents were separately prepared as sterile solutions, and aseptically added to the main body of media. The following solutions in Table 1 were prepared as described previously

(Widdel & Bak, 1992); trace element solution, selenite-tungstate solution, vitamin mixture solution (show as "vitamin mixture A" in Table 1), vitamin B_{12} solution, thiamine solution, 1M $NaHCO_3$ solution, and 1 M $Na_2S_2O_3$ solution. Composition of the other solution of vitamin mixture, "vitamin mixture B", was as follows (l^{-1}); 2 mg biotin, 2 mg folic acid, 10 mg pyridoxine-HCl, 5 mg thiamine-HCl·$2H_2O$, 5 mg riboflavin, 5 mg nicotinic acid, 5 mg calcium D(+) pantothenate, 5 mg 4-aminobenzoic acid, 5 mg lipoic acid, and 0.1 mg cyanocobalamine. The final pH of the all media was adjusted to 7.0–7.2. The enrichment culture was established with the medium S1 supplemented with elemental sulfur (ca. 0.5 g l^{-1}), and successively transferred to the media S2 and S3. Finally, a colony was picked up from agar-solidified medium. More detailed procedures for enrichment and isolation are described in supplementary material. Purity of the isolate was checked by microscopy and sequencing of the 16S rRNA gene fragments amplified with universal PCR primer pairs.

For the characterization of the strain, the medium S4 supplemented with 20 mM sodium thiosulfate was used unless otherwise specified. All culturing experiments were performed in bottles closed with rubber stoppers, and the bottles were incubated at 28°C without shaking.

The Gram-stain test was conducted with a kit (Fluka). Catalase activity was assessed

71 by pouring 3% H_2O_2 solution onto a pellet obtained by centrifugation of a culture.
72 Oxidase activity was tested by using an oxidase test reagent (bioMérieux). The genomic
73 G+C content of the DNA was determined with the HPLC methods (Katayama-Fujimura
74 *et al.*, 1984), using a Yamasa GC kit (Yamasa Shoyu). Fatty acid profile of the strain
75 was analyzed at the Techno Suruga Co. Ltd (Shizuoka, Japan), by using the Sherlock
76 Microbial Identification System (Version 6.0; database, TSBA6; MIDI). Analysis of
77 polar lipids was carried out by the DSMZ Identification Service, along with cells of
78 *Sulfurifustis variabilis* skN76^T grown at 42°C with the same medium for comparison.
79 Effects of the temperature and salt concentration on growth were examined by
80 culturing the strain at various temperatures (5, 8, 10, 13, 15, 18, 22, 25, 28, 30, 32, 34,
81 37, and 40°C), or in the medium supplemented with varying concentrations of NaCl (0,
82 50, 100, 150, 200, and 300 mM).
83 Utilization of substrate was tested in the medium S4 supplemented with one of the
84 substrates listed later. The utilization of electron acceptor was tested in the medium S4
85 amended with 10 mM sodium thiosulfate under anoxic conditions (headspace of the
86 bottles was filled with N_2/CO_2). Heterotrophic growth in complex liquid media was
87 tested for R2A, NB, LB, and TSB at 28°C under oxic conditions.
88 The effect of pH on the growth was tested as described previously (Kojima *et al.*,

89 2015). The tested pH and buffering reagents were as follows; pH 5.4, 5.7, 5.8, 6.1, 6.2,
90 6.3, 6.4, 6.5, 6.8 with MES; pH 6.8, 7.0 and 7.3 with PIPES; pH 6.9, 7.2, 7.4, and 7.5
91 with MOPS; pH 7.8, 8.1, 8.2, 8.6 and 8.7 with Tricine; pH 8.8, 8.9, 9.2, 9.5, 9.6, and 9.7
92 with CHES.
93 The nearly full-length 16S rRNA gene was amplified with the primer pair 27F and
94 1492R (Lane, 1991), and the resulting PCR product was sequenced. The *cbbL* gene
95 encoding form I ribulose-1,5-bisphosphate carboxylase/oxygenase was amplified with
96 the primer pair cbbLG1F/cbbLG1R (Selesi *et al.*, 2005) and then directly sequenced by
97 using internal primers (Boschker *et al*, 2014). Partial fragments of *aprA* gene encoding
98 adenosine-5'-phosphosulfate reductase were amplified by PCR with the primer pair
99 Apr-1-FW/Apr-5-RV-GC (Meyer & Kuever, 2007). The PCR products were subjected
100 to sequence-dependent separation by denaturing gradient gel electrophoresis (DGGE).
101 DGGE was performed on 6% (wt vol^{-1}) polyacrylamide gel with the thickness of 1.5
102 mm and denaturant gradient ranging from 20 to 50% (100% was defined as 7 M urea
103 and 40% [vol vol^{-1}] formamide). Electrophoresis was run in 0.5 × TAE buffer (20 mM
104 Tris, 10 mM acetic acid, 0.5 mM EDTA; pH 8.3) at a voltage of 200 V and temperature
105 of 60°C. DNA fragments in the resulting DGGE bands were amplified with the same
106 primer pair again, and then sequenced.

Cells of the isolate HA5^T were motile Gram-stain-negative rods, 1.2–6.0 μm long and 0.3–0.5 μm wide (Fig. 1). The tests of catalase and oxidase resulted in negative and positive respectively. The G+C content of the genomic DNA was 63 mol%. The cellular fatty acid profile is shown in Table 2. Major components in the profile were summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; 45.0 %) and $C_{16:0}$ (40.9%). Polar lipid profile of strain HA5^T is shown in Fig. 2. Growth of the strain was observed over a temperature range between 8°C and 37°C, with an optimum at 28–32°C. Growth of the strain was observed at pH range of 6.1–9.2. Within this range, relationship between the growth and pH was apparently inconsistent and thus optimum pH could not be determined. As a possible reason for this, growth might have been affected by reagents for pH buffering and adjustment. Optimum growth was observed in medium without NaCl, and a negative effect of NaCl on the growth was observed at concentrations 50 mM or higher. Slow growth was observed in the medium containing 200 mM NaCl, but no growth was observed in the medium containing 300 mM or more NaCl.

The isolate grew chemolithotrophically on thiosulfate (10–20 mM), tetrathionate (20 mM), and elemental sulfur (0.5 g l^{-1}), but not on sulfide (2 mM) and sulfite (5 mM). The following substrates did not support heterotrophic growth of the strain: pyruvate (5

125 mM), lactate (5 mM), acetate (5 mM), methanol (5 mM), succinate (2.5 mM), fumarate
126 (2.5 mM), butyrate (2.5 mM), isobutyrate (2.5 mM), ethanol (2.5 mM), formate (5 mM),
127 lactose (2.5 mM), glucose (2.5 mM), xylose (2.5 mM). The strain exhibited no growth
128 on R2A, NB, LB, or TSB.

129 Despite the fact that the isolate experienced anoxic culturing conditions during the
130 enrichment, anaerobic growth of the isolated strain HA5^T was not observed under the
131 tested conditions. Nitrite (5 mM), nitrate (20 mM), or poorly crystalline Fe(III) oxide
132 (10 mM) did not support growth of the strain as sole electron acceptor at the tested
133 concentrations.

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135 The 16S rRNA gene sequence analysis revealed that the closest cultivated relatives of
136 strain HA5^T was *Sulfurifustis variabilis* skN76^T and *Acidiferrobacter thiooxydans* m-1^T,
137 with sequence similarity of 93% and 92%, respectively. The novel strain and related
138 environmental clones formed a distinct cluster within the family *Acidiferrobacteraceae*
139 (Fig. 3). Some environmental clones very closely related to strain HA5^T have been
140 reported from slightly alkaline sediment of an eutrophic reservoir (Qu *et al*., 2008),
141 whereas *Acidiferrobacter*-like sequences were reported from acidic environments
142 (Mendez *et al*., 2008; Garcia-Moyano *et al*., 2012).

The PCR product of *cbbL* gene was directly sequenced. Phylogenetic position of the obtained sequence is shown in supplementary Fig. S1. In case of the *aprA* gene, PCR products could not be directly sequenced presumably because the strain possesses two copies of *aprA* genes with different sequences. In the DGGE analysis of the *aprA* gene, one distinct band and four weak and proximate bands were observed (Fig. S2). The latter 4 bands were identical in the sequence of amplified region, suggesting that these bands were formed because of sequence variations in the degenerated primers. As a result, two *aprA* gene sequences belonging to distinct lineages were obtained (Fig. S3).

The novel strain HA5^T exhibited low similarity (93%) of the 16S rRNA gene to the closest relative, *S. variabilis* skN76^T (Kojima *et al.*, 2015). One of the outstanding characteristics of the strain skN76^T, changes in morphology depending on growing temperature, was not observed in HA5^T. Differences between the strains HA5^T and skN76^T are also apparent in the cellular fatty acid profile (Table 2), polar lipid profile (Fig. 2) and other characteristics (Table 3). Therefore, strain HA5^T is proposed to be assigned to a new species of a novel genus, with the name *Sulfuricaulis limicola* gen. nov., sp. nov.

Description of *Sulfuricaulis* gen. nov.

Sulfuricaulis (Sul.fu.ri.cau'lis. L. neut. n. *sulfur* sulfur; L. masc. n. *caulis*, stalk; N.L. masc. n. *Sulfuricaulis* sulfur-oxidizing stalk).

Cells are motile and Gram-stain-negative. Grow chemolithoautotrophically by the oxidation of inorganic sulfur compounds. As determined by 16S rRNA gene sequence analysis, belonging to the family *Acidiferrobacteraceae*. The type species is *Sulfuricaulis limicola*.

Description of *Sulfuricaulis limicola* sp. nov.

Sulfuricaulis limicola (li.mi'co.la. L. n. *limus*, mud; L. suff. *-cola* (from L. n. *incola*), dweller; N.L. n. *limicola*, mud-dweller).

Cells are rod-shaped, 1.2–6.0 μm in length and 0.3–0.5 μm in width. Autotrophic growth occurs with oxidation of thiosulfate, tetrathionate, and elemental sulfur. Catalase-negative and oxidase-positive. Growth occurs at temperatures 8–37°C, with optimum growth at 28–32°C. The pH range for growth is 6.1–9.2. The G+C content of genomic DNA is 63 mol%. Major cellular fatty acids are $C_{16:0}$ and summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$). The type strain HA5^T (= DSM 100373^T = NBRC 110752^T) was isolated from sediment of a lake in Japan (Lake Harutori).

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252 Dworkin, W. Harder & K.-H. Schleifer. New York: Springer-Verlag.

253 Table 1. Compositions of media used for enrichment and isolation, shown as amounts of constituents contained in 1 L of media. See
254 text for details.

	medium S1	medium S2	medium S3	medium S4
Basal constituents	1.0 g $MgSO_4$. $7H_2O$, 0.2 g $CaCl_2$. $2H_2O$, 0.2 g KNO_3, 0.2 g KH_2PO_4	0.2 g $MgCl_2$. $6H_2O$, 0.1 g $CaCl_2$. $2H_2O$, 0.1 g NH_4Cl, 0.1 g KH_2PO_4, 0.1 g KCl, 1.7 g $NaNO_3$	0.2 g $MgCl_2$. $6H_2O$, 0.1 g $CaCl_2$. $2H_2O$, 0.1 g NH_4Cl, 0.1 g KH_2PO_4, 0.1 g KCl, 20 mM MOPS/NaOH (pH 7.0)	0.2 g $MgCl_2$. $6H_2O$, 0.1 g $CaCl_2$. $2H_2O$, 0.1 g NH_4Cl, 0.1 g KH_2PO_4, 0.1 g KCl
Trace elements	1 ml trace element solution, 1 ml selenite-tungstate solution	1 ml trace element solution, 1 ml selenite-tungstate solution	1 ml trace element solution, 1 ml selenite-tungstate solution	1 ml trace element solution, 1 ml selenite-tungstate solution
Vitamin	1 ml vitamin B_{12} solution	1 ml vitamin mixture A, 1 ml vitamin B_{12} solution, 1 ml thiamine solution	1 ml vitamin mixture A, 1 ml vitamin B_{12} solution, 1 ml thiamine solution	1ml vitamin mixture B
Thiosulfate	–	1.5 ml $Na_2S_2O_3$ solution	1.5 ml $Na_2S_2O_3$ solution	0.4 ml $Na_2S_2O_3$ solution
Bicarbonate	30 ml $NaHCO_3$ solution	30 ml $NaHCO_3$ solution	20 ml $NaHCO_3$ solution	30 ml $NaHCO_3$ solution
Headspace	Air/CO_2 (80 : 20, v/v)	N_2/CO_2 (80 : 20, v/v)	Air	Air

255 Table 2. Cellular fatty acid content (as percentages of the total) of strain HA5^T and its

256 closest relative.

257 Strains: 1, HA5^T; 2, *Sulfurifustis variabilis* skN76^T (Kojima *et al*., 2015).

Fatty acid	1	2
C9:0	0.2	0.1
iso-C10:0		0.4
C10:0	6.2	9.2
C10:0 3OH	1.4	0.2
C12:0 3OH		0.6
C14:0	0.7	0.4
iso-C16:0		0.2
C16:0	40.9	43.6
iso-C17:0		0.8
C17:0	0.4	0.5
C18:0		1.8
C19:0 cycloω9c		0.4
Summed Feature 3	45.0	17.2
Summed Feature 8	2.2	3.8
Summed Feature 9	3.0	21.1

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Table 3. Differential properties of strain HA5^T and *Sulfurifustis variabilis* skN76^T.

Strains: 1, HA5^T; 2, *S. variabilis* skN76^T (Kojima *et al*., 2015).

Characteristics	1	2
DNA G+C content (%)	63	69
Catalase	-	+
Optimal growth temperature	28–32	42–45
Temperature range	8–37	28–46
pH range	6.1–9.2	6.3–8.9
NaCl range (mM)	0–200	0–450

Figure legends

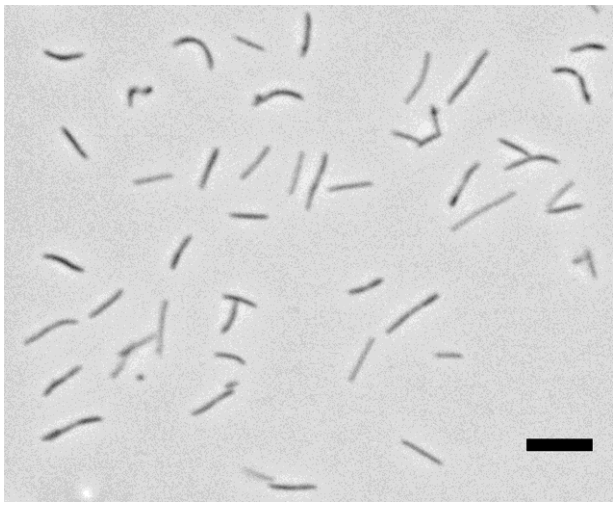

Fig. 1 Phase-contrast micrographs of strain. Bar, 5 μm.

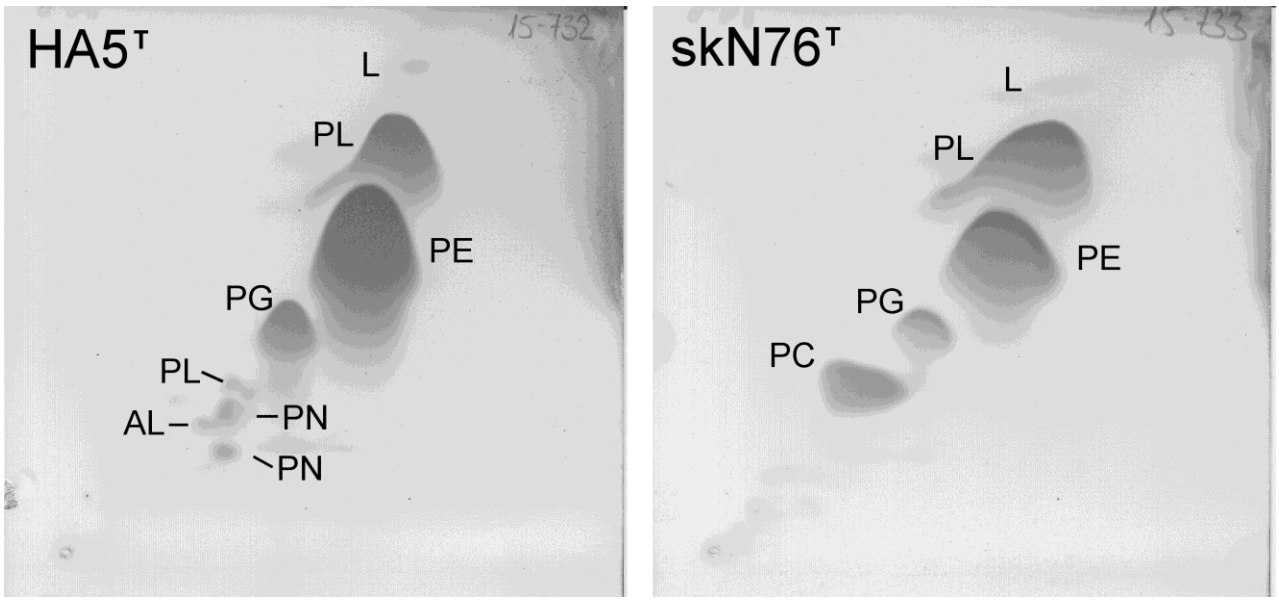

Fig. 2 Polar lipid profiles of strain HA5^{T} and *Sulfurifustis variabilis* skN76^{T}. PG = phosphatidylglycerol, PE = phosphatidylethanolamine, PN = Phosphoaminolipid, PC = Phosphatidylcholine, PL = phospholipids, AL = aminolipids L = lipids.

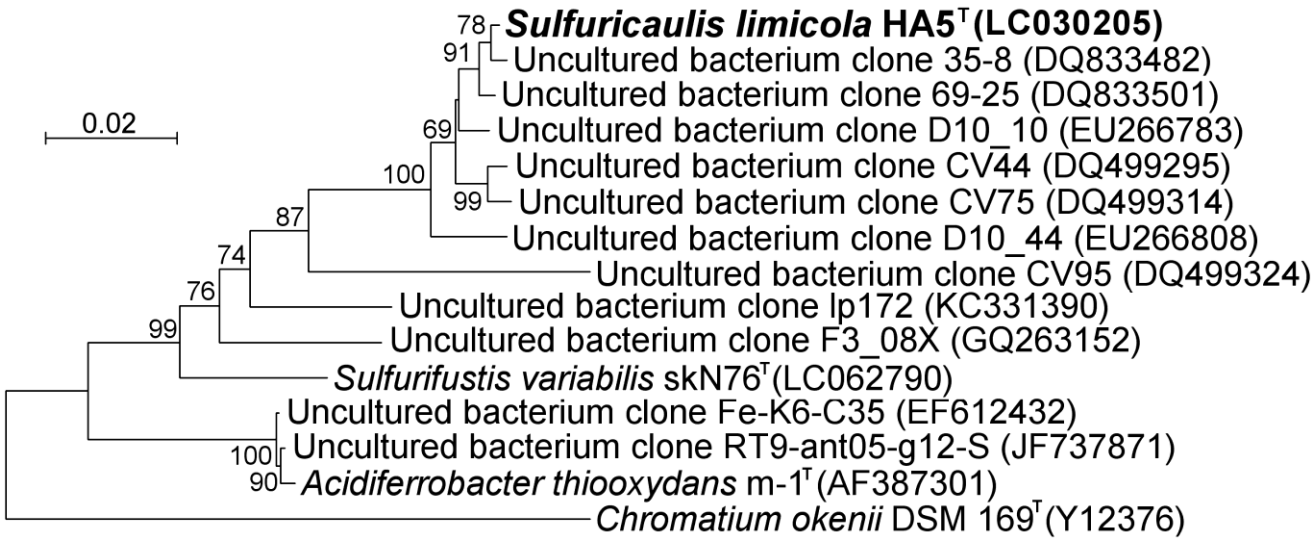

Fig. 3 Minimum-evolution tree showing the phylogenetic position of HA5^T within the family *Acidiferrobacteraceae* based on the 16S rRNA gene sequence analysis. This tree was constructed using 1429 sites, and identical tree was obtained with the neighbor-joining method. *Chromatium okenii* is included as an outgroup. Numbers on nodes represent percentage values of 1000 bootstrap resampling.