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

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A novel autotrophic bacterium, strain skN76T, 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 growth temperature. Cells grown at the optimum temperature were rod-shaped (0.9–3.0 μm long and 0.3–0.5 μm wide), whereas a filamentous form was observed when the strain was cultured at the lowest permissive growth temperatures. The G+C content of genomic DNA was 69 mol%. The major components in the fatty acid profile were C₁₆ : ₀, summed feature 3 (C₁₆ : ₁υ7c and/or C₁₆ : ₁υ6c) and summed feature 9 (iso-C₁₇ : ₁υ9c and/or 10-methyl C₁₆ : ₀). Phylogenetic analysis based on 16S rRNA gene sequences indicated that the closest cultivated relative of strain skN76T was Acidiferrobacter thiooxydans m-1T, with sequence similarity of 93%. On the basis of its phylogenetic and phenotypic properties, strain skN76T (DSM 100313T = NBRC 110942T) is proposed as the type strain of a novel 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 among four classes in the phylum Proteobacteria (Kelly & Wood, 2000; Williams & Kelly, 2013; Watanabe et al., 2015). One of them, Acidiferrobacter thiooxydans m-1T, 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 freshwater sediment as described previously (Watanabe et al., 2014). The basal medium used for enrichment and isolation was bicarbonate-buffered low-salt defined medium, described previously (Kojima & Fukui, 2011). As electron donor and acceptor, elemental sulfur (approx. 0.5 g l⁻¹) and nitrate (20 mM), respectively, were added to the medium just before inoculation. From the enrichment culture, an isolate was obtained by agar shake dilution (Widdel & Bak, 1992), using the basal medium supplemented with 20 mM thiosulfate and 20 mM nitrate. The headspace of the agar tubes was filled with a gas mixture of N₂/CO₂ (80 : 20, v/v), but no reductant was added to the medium and thus dissolved oxygen was not eliminated. Well-separated colonies were picked up and placed into a slightly modified medium supplemented with 20 mM sodium thiosulfate and 20 mM nitrate. The composition of the modified medium was almost identical to that of the original medium, but vitamin solutions were replaced with a single vitamin mixture (1 ml l⁻¹) which contained the following constituents (l⁻¹): 2 mg biotin, 2 mg folic acid, 10 mg pyridoxine hydrochloride, 5 mg...
thiamine hydrochloride dihydrate, 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 cyano-
cobalamine. The picked-up colonies were cultivated
under oxic conditions in closed bottles, and one of the
resulting pure cultures was designated strain skN76T. All
cultivation for enrichment and isolation was carried out
at 45 °C. Purity of the isolate was checked by microscopy
and sequencing of the 16S rRNA gene fragments amplified
with several universal PCR primer pairs.

For the characterization of the strain, the modified medium
(altered vitamin composition as described above) sup-
plemented 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 without shaking.

The Gram-stain test was conducted with a kit (Fluka). Cat-
alase activity was assessed by pouring 3 % H2O2 solution
onto a pellet obtained by centrifugation of culture. Oxidase
activity was tested with a pellet of cells, by using an oxidase
test reagent (bioMérieux). The genomic G+C content of
the DNA was determined by 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 analysed at Techno Suruga (Shizuoka,
Japan), by using the Sherlock Microbial Identification
System (version 6.0, database TSBA6; MIDI).

The utilization of electron acceptors was tested in
medium amended with 20 mM sodium thiosulfate under
anoxic conditions (headspace of the bottles was filled with
N2/CO2). Utilization of growth substrate was tested in medium with lowered thiosulfate concentration (0.4 mM),
supplemented with one of the substrates listed below. Aerobic
growth in ordinary complex liquid media was tested for R2A
(Daigo), NB (Difco), LB (Merck) and TSB (OXOID) 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 concen-
tration 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
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 Na2S2O3, 5H2O, 1 g NaHCO3,
0.2 g MgCl2·6H2O, 0.1 g CaCl2, 0.1 g NH4Cl, 0.1 g
KH2PO4, 0.1 g KCl, 1 ml trace element solution, 1 ml sele-
nite-tungstate solution and 1 ml vitamin mixture described
above. Depending on the final pH, one of the buffering
reagents listed below was added to each medium at a
final concentration of 20 mM. All ingredients were mixed
and then sterilized by filtration after pH adjustment.
To adjust pH, NaOH solution was used except for media
of the lowest pH (5.4–5.8), which were prepared with
HCl. The pH tested and buffering reagents were as follows;
pH 5.4, 5.7, 5.8, 6.1, 6.2, 6.3, 6.4 and 6.7 with MES; pH 6.8
and 7.0 with 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 Tricine; pH 8.7,
8.9, 9.1, 9.4 and 9.6 with CHES.

A fragment of the 16S rRNA gene was amplified with the
primer pair 27F and 1492R (Lane, 1991), and the resulting
PCR product was directly sequenced. Phylogenetic analysis
was performed with the program MEGA version 5.05
(Tamura et al., 2011). Fragments of the aprA gene
(encoding adenosine-5′-phosphosulfate reductase) were
amplified and sequenced with the primers Apr-1-FW and
Apr-5-RV (Meyer & Kuever, 2007a). The cbbL gene encod-
ing form I ribulose-1,5-bisphosphate carboxylase/oxyge-
nase was amplified with primers cbbLGF1 (Selesi et al.,
2005) and 898E (Boschker et al., 2014), and then directly
sequenced.

Cells of strain skN76T 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 skN76T exhibited filamentous
morphology when it was grown at 28 °C or 30 °C. The tests for cat-
alase and oxidase activities were both 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 skN76T grown at 45 °C were C16:0 (43.6 %),

![Fig. 1. Phase-contrast micrographs of strain skN76T, grown at
45 °C (a) and 28 °C (b). Bars, 5 μm.](image-url)
summed feature 9 (iso-C₁₇:1ω9c and/or 10-methyl C₁₆:0; 21.1 %) and summed feature 3 (C₁₆:1ω7c and/or C₁₆:1ω6c; 17.2 %). The other fatty acids detected were C₁₀:0 (9.2 %), summed feature 8 (C₁₈:1ω7c and/or C₁₈:1ω6c; 3.8 %), C₁₈:0 (1.8 %), iso-C₁₇:0 (0.8 %), C₁₂:0 3-OH (0.6 %), C₁₇:0 (0.5 %), C₁₄:0 (0.4 %) and iso-C₁₀:0 (0.4 %).

Growth of strain skN76ᵀ was observed over a temperature range between 28 and 46 °C, with an optimum at 42–45 °C. The range of pH for growth was pH 6.3–8.9, and the optimum pH was 6.8–8.2. Optimum growth was obtained from a nitrate-reducing enrichment culture, but this possibility needs to be tested experimentally.

16S rRNA gene sequence analysis revealed that the closest cultivated relative of strain skN76ᵀ was *A. thiooxydans* m-1ᵀ, with sequence similarity of 93 %. These two strains and related environmental clones formed a distinct cluster outside of the order *Chromatiaceae* encompassing the family *Ectothiorhodospiraceae*, in phylogenetic trees reconstructed with different methods (Fig. 2 and Fig. S1 available in the online Supplementary Material). The PCR products of *aprA* and *cbbL* genes were also sequenced. Phylogenetic analysis revealed that the protein coded by the *aprA* gene of skN76ᵀ belonged to a phylogenetic lineage referred to as ‘Apr lineage I’ (Meyer & Kuver, 2007b), which is one of two major lineages of sulfur oxidizers (Fig. 3).

The novel strain, skN76ᵀ, exhibited low similarity (93 %) of the 16S rRNA gene sequence to that of the closest phylogenetic relative, *A. thiooxydans* m-1ᵀ. The latter strain is known as an extremely acidophilic bacterium (optimum pH for growth is around pH 2) which requires an external osmotic potential for growth (Hallberg et al., 2011). By contrast, strain skN76ᵀ grew under neutral to moderately alkaline conditions and optimum growth was observed in the medium of lowest salt concentration (0–50 mM NaCl). On the basis of these phylogenetic and
phenotypic properties, strain skN76^T is proposed to be assigned to a novel species of a novel genus, with the name *Sulfurifustis variabilis* gen. nov., sp. nov. As shown in phylogenetic trees reconstructed previously (Oren, 2014; Rua & Thompson 2014), *A. thiooxydans* is phylogenetically isolated from the other cultivated gammaproteobacteria belonging to existing orders. In the phylogenetic analysis including the novel bacterium obtained in this study, the genera *Acidiferrobacter* and *Sulfurifustis* gen. nov. formed a distinct cluster apart from the clade of the class *Chromatiales*, irrespective of tree construction methods (Figs. 2 and S1). Therefore, a novel family and a novel order are proposed to accommodate these genera, with the names *Acidiferrobacteraceae* fam. nov. and *Acidiferrobacterales* ord. nov., respectively.

**Description of Sulfurifustis gen. nov.**

*Sulfurifustis* (Sul.fu.ri.fus'tis. L. neut. n. sulfur sulfur; L. masc. n. fustis stick; N.L. masc. n. *Sulfurifustis* sulfur-oxidizing stick).

Grow chemolithoautotrophically by the oxidation of inorganic sulfur compounds. Based on 16S rRNA gene sequence analysis, phylogenetically affiliated to the order *Gammaproteobacteria*. The type species is *Sulfurifustis variabilis*.

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

*Sulfurifustis variabilis* (va.ri.a’bi.lis. L. masc. adj. variabilis changeable, referring to variation of the morphology depending on growth temperatures).

Cells are Gram-stain-negative, rod-shaped or filamentous, 0.3–0.5 μm in width. Major components in the fatty acid profile are C16:0 β summed feature 3 (C16:07c and/or C16:06c) and summed feature 9 (iso-C17:1ω9c and/or 10-methyl C16:0). Autotrophic growth occurs with oxidation of thiosulfate, tetrathionate and elemental sulfur. Catalase-positive and oxidase-positive. The temperature range for growth is 28–46 °C, with an optimum of 42–45 °C. The pH range for growth is pH 6.3–8.9, and optimum growth occurs at pH 6.8–8.2.

The type strain, skN76^T (=DSM 100313^T=NBRC 110942^T), was isolated from sediment of a freshwater lake in Japan (Lake Mizugaki). The G+C content of genomic DNA of the type strain is 69 mol%.

**Description of Acidiferrobacteraceae fam. nov.**

*Acidiferrobacteraceae* (A.ci.di.fer.ro.bac.te.ra.ce. 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 16S rRNA gene sequence analysis, phylogenetically affiliated to the order *Acidiferrobacterales*. 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 16S rRNA gene sequence analysis, phylogenetically affiliated to the class *Gammaproteobacteria*. The type genus is *Acidiferrobacter*.

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References


