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Sulfuriflexus mobilis gen. nov., sp. nov., a sulfur-oxidizing bacterium isolated from a brackish lake sediment

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Running head: *Sulfuriflexus mobilis* gen. nov., sp. nov.

Subject category: New taxa: *Proteobacteria*

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain is LC131141.

Summary

A chemolithotrophic sulfur-oxidizing bacterium, strain aks1^T was isolated from sediment of a brackish lake in Japan. The cells were curved rod-shaped and Gram-stain-negative. The G+C content of genomic DNA was 53 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 for chemolithoautotrophic growth, strain aks1^T oxidized thiosulfate, sulfide, and elemental sulfur. The strain could utilize oxygen and nitrate as an electron acceptor for thiosulfate oxidation. Growth was observed at a temperature range of 5–34°C, with optimum growth at 30–32°C. Growth of the strain was observed at a pH range of 6.4–8.7. Phylogenetic analysis based on 16S rRNA gene indicated that the strain is related to members of the family *Granulosicoccaceae* within the order *Chromatiales*, with sequence similarities around 92%. On the basis of its phylogenetic and phenotypic properties, the strain aks1^T (= DSM 102939^T = NBRC 111889^T) is proposed as type strain of a new species of a novel genus, *Sulfuriflexus mobilis* gen. nov., sp. nov.

The original description of the order *Chromatiales* contains 3 families, *Chromatiaceae, Ectothiorhodospiraceae,* and *Halothiobacillaceae* (Imhoff, 2005), and 4 families, *Granulosicoccaceae* (Lee *et al.*, 2007), *Thioalkalispiraceae* (Mori *et al.*, 2011), *Wenzhouxiangellaceae* (Wang *et al.*, 2015), and *Woeseiaceae* (Du *et al.*, 2016) have been added in the order. In the present study, a novel chemolithoautotrophic sulfur-oxidizing bacterium, strain aks1^{T}, was isolated and characterized to be proposed as type species of a new genus is in this order.

The strain aks1^{T} was isolated from sediment of a brackish lake in Japan, Lake Akkeshi. Throughout this study, a bicarbonate-buffered defined medium was used as basal medium. To prepare the medium, following constituents (l^{-1}) were dissolved in distilled water and then autoclaved: 20 g NaCl, 3 g $MgCl_2 \cdot 6H_2O$, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g NH_4Cl, 0.1 g KH_2PO_4, 0.1 g KCl. After cooling down to room temperature, 1 ml trace element solution, 1 ml selenite-tungstate solution, 30 ml $NaHCO_3$ solution, and 1 ml vitamin mixture solution (DSM 141) were aseptically added to the main body of medium. The solutions of trace element, selenite-tungstate and $NaHCO_3$ were prepared as described previously (Widdel & Bak, 1992). Before dispensing into culture containers, the pH of the medium was adjusted to 7.0–7.2. The

52 enrichment culture was established with the basal medium supplemented with elemental
53 sulfur (ca. 0.5 g l^{-1}). After 11 times transfer to fresh medium of the same composition
54 (1–2%), the sole electron donor was changed to 20 mM $Na_2S_2O_3$. Finally, strain was
55 isolated in pure culture by repeated serial dilution in the medium supplemented with
56 $Na_2S_2O_3$. Purity of the isolate was checked by microscopy and sequencing of the 16S
57 rRNA gene fragments amplified with using some PCR primer pairs, as described
58 previously (Higashioka *et al.*, 2012).
59
60 For the characterization of the strain, the basal medium supplemented with 20 mM
61 $Na_2S_2O_3$ was used unless otherwise specified. Culturing experiments were performed in
62 bottles closed with rubber stoppers, and the bottles were incubated without shaking at
63 30°C unless otherwise specified.
64 The Gram-stain test was conducted with a kit (Fluka), and oxidase activity was tested
65 by using an oxidase test reagent (bioMérieux). Catalase activity was assessed by
66 pouring 3% H_2O_2 solution onto a pellet of cells. The genomic G+C content of the DNA
67 was determined with the HPLC methods (Katayama-Fujimura *et al.*, 1984), using a kit
68 (Yamasa Shoyu). Fatty acid profile of the strain was analyzed at the Techno Suruga Co.
69 Ltd (Shizuoka, Japan), by using the Sherlock Microbial Identification System Version

6.0 (MIDI) with database TSBA6.

Effects of temperature on growth were examined by culturing at various temperatures (0, 5, 8, 10, 13, 15, 18, 22, 25, 28, 30, 32, 34, 36, and 37°C). To examine effects of salt concentration, strain aks1 was cultured in modified media with varying concentrations of NaCl (0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 4.5 and 5.0% w/v) and lowered concentration of $MgCl_2 \cdot 6H_2O$ (0.2 gl^{-1}). Effect of pH on the growth was tested with a method previously described (Kojima *et al.*, 2015), with a modification for the NaCl-requiring strain. The test was performed with media containing 2.0% (w/v) NaCl, and tested pH and buffering reagents were as follows; pH 5.9, 6.3, 6.4, 6.5, 6.8 and 7.2 with MES; pH 6.9, 7.0, 7.2, 7.3, 7.4 and 7.6 with PIPES; pH 7.7, 7.8, 7.9 and 8.2 with MOPS; pH 8.2, 8.4, 8.6, and 8.7 with Tricine; pH 8.9, 9.4, 9.6, and 10.0 with CHES.

Utilization of electron donors was tested in the basal medium supplemented with one of the substances listed later. The utilization of electron acceptor was tested with $Na_2S_2O_3$ (10 mM) as an electron donor, in the same medium under anoxic conditions (headspace of the bottles was filled with 4:1 mixture of N_2/CO_2). Heterotrophic growth in complex liquid media was tested under oxic conditions, for Marine Broth 2216 (MB; Difco) and following media all supplemented with 2% NaCl; R2A (Daigo), diluted (1/10) R2A, NB (Difco), and TSB (OXOID).

For phylogenetic analysis, 16S rRNA gene was amplified by PCR using the primer pair 27F and 1492R (Lane, 1991) and then sequenced with a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). For the resulting sequence, phylogenetic analysis was conducted using the program MEGA version 5.22 (Tamura *et al*., 2011).

Cells of aks1^{T} were motile curved rods, 0.9–6.0 µm long and 0.3–0.5 µm wide (Fig. 1). Strain aks1^{T} was Gram-stain-negative, and tests of catalase and oxidase resulted in negative and positive respectively. The G+C content of the genomic DNA assessed by the HPLC-based method was 53 mol%. In the cellular fatty acid profile, major components were summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; 57.3 %) and $C_{16:0}$ (32.2%). The other fatty acids detected were summed feature 9 (iso$C_{17:1}\omega7c$ and/or $C_{16:0}$ 10-methyl; 5.2 %), summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$; 1.7 %), $C_{10:0}$ 3-OH (1.3%), $C_{18:0}$ (0.8%), $C_{14:0}$ (0.6%), $C_{12:0}$ 3-OH (0.4%), $C_{17:0}$ (0.3%) and $C_{10:0}$ (0.2%).

Growth of strain aks1^{T} was observed over a temperature range between 5°C and 34°C, with optimal growth at 30–32°C. Growth of the strain was observed at a pH range of 6.4–8.7, and optimum growth was observed at pH range of 6.8–8.3. Growth was observed in medium with 1–4% NaCl with an optimum of 3%.

106 The isolate grew chemolithotrophically on thiosulfate (20 mM), sulfide (2 mM) and
107 elemental sulfur (0.5 g l^{-1}). Hydrogen gas (Air/H_2; 4:1, v/v; 125 kPa total pressure),
108 tetrathionate (20 mM) and sulfite (5 mM) did not support autotrophic growth of the
109 strain. The following organic substrates did not support growth of strain aks1^T: pyruvate
110 (5 mM), lactate (5 mM), acetate (5 mM), methanol (5 mM), succinate (2.5 mM),
111 fumarate (2.5 mM), butyrate (2.5 mM), isobutyrate (2.5 mM), ethanol (2.5 mM),
112 formate (5 mM), lactose (2.5 mM), glucose (2.5 mM), xylose (2.5 mM). The strain
113 exhibited no growth on MB or other complex media tested. Nitrate (20 mM) supported
114 anaerobic growth of strain as sole electron acceptor for thiosulfate oxidation.
115 Among characterized strains of species with validly published names, *Thioprofundum*
116 *hispidum* gsp61^T showed the highest sequence similarity to strain aks1^T (93%), followed
117 by the type strains of *Granulosicoccus* species (92%) and some other bacteria in the
118 order *Chromatiales*. By constructing phylogenetic trees, it was revealed that strain aks1^T
119 forms a monophyletic cluster with *Granulosicoccus* species (Fig. 2, Fig S1). The
120 methods of neighbor-joining and minimum-evolution generated trees of identical
121 topology (Fig. 2), but a different tree was obtained with the maximum-likelihood
122 method (Fig. S1). In all trees, aks1^T represents a sister group of the genus
123 *Granulosicoccus*, and they form a cluster with the genera *Thioalkalispira* and

124 *Thiohalophilus*. The genus *Granulosicoccus* is the sole genus in the family
125 *Granulosicoccaceae*, whereas *Thioalkalispira* and *Thiohalophilus* belong to the family
126 *Thioalkalispiraceae*. The other genus of the family *Thioalkalispiraceae*, genus
127 *Thioprofundum* was positioned apart from these genera (Fig. 2, Fig S1). This
128 phylogenetic isolation of *Thioprofundum* from the other genera was also shown in
129 phylogenetic trees previously constructed (Mori & Suzuki 2014; Mori *et al.*, 2015),
130 suggesting that the family *Thioalkalispiraceae* is not monophyletic. As pointed out
131 previously, it is difficult to clarify phylogenetic relationships among the families in the
132 order *Chromatiales* (Mori *et al.*, 2015), and reclassification of some taxa may be
133 required in the future. However, it seems reasonable to place the strain aks1^T in the
134 family *Granulosicoccaceae* at this point.
135 Differential properties of strain aks1^T and related genera are summarized in Table 1. In
136 contrast to heterotrophic *Granulosicoccus* species, growth of strains aks1^T was not
137 observed in complex media including the MB, or synthetic medium supplemented with
138 organic substrates. Differences between strains aks1^T and *Granulosicoccus* species are
139 apparent in cell morphology, oxygen requirement, catalase activity (Table 1). On the
140 basis of its distinct phenotypic properties and isolated phylogenetic position, strain
141 aks1^T is proposed to be assigned to a new species of a novel genus in the family

Granulosicoccaceae, with the name *Sulfuriflexus mobilis* gen. nov., sp. nov.

Description of *Sulfuriflexus* gen. nov.

Sulfuriflexus (Sul.fu.ri.fle'xus. L. neut. n. *sulfur* sulfur; L. masc. n. *flexus*, a bending; N.L. masc. n. *Sulfuriflexus* sulfur-oxidizing bending).

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 family *Granulosicoccaceae*. The type species is *Sulfuriflexus mobilis*.

Description of *Sulfuriflexus mobilis* sp. nov.

Sulfuriflexus mobilis (mo'bi.lis. L. masc. adj. *mobilis*, movable, motile).

Cells are curved rod-shaped, 0.9–6.0 μm in length and 0.3–0.5 μm in width. Autotrophic growth occurs with oxidation of thiosulfate, sulfide and elemental sulfur. Oxidase-positive and catalase-negative. Growth occurs at temperatures 5–34°C, with optimum growth at 30–32°C. The pH range for growth is 6.4–8.7. The G+C content of genomic DNA is 53 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 aks1^T (= DSM 102939^T = NBRC 111889^T)

was isolated from sediment of a brackish lake in Japan (Lake Akkeshi).

ACKNOWLEDGMENTS

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Table 1. Differential properties of strain aks1^{T} and related genera. Genera: 1, *Granulosicoccus*; 2, *Thioalkalispira*; 3, *Thiohalophilus*. The properties of *Granulosicoccus* were compiled from the description of type strains representing 4 species in this genus, *G. antarcticus* IMCC3135^{T} (Lee *et al*., 2007), *G. coccoides* Z 271^{T} (Kurilenko *et al*., 2010), *G. marinus* IMCC3490^{T} (Baek *et al*., 2014) and *G. undariae* W-BA3^{T} (Park *et al*., 2014). Those of *Thioalkalispira* and *Thiohalophilus* are from Sorokin *et al*., 2002 and Sorokin *et al*., 2007, respectively.

Characteristics	aks1	Genera		
		1	2	3
Cell morphology	Curved rod	Coccoid	Spiral rod	Rod
Motility	+	+/-*	+	-
Heterotrophic growth	-	+	-	-
Anaerobic growth	+	-	-	+
Catalase	-	+	+	ND

*One of the 4 species is non-motile and the others are motile.

Figure legends

Fig. 1 Phase-contrast micrograph of strain aks1^{T}. Bar, 5 μm.

Fig. 2 Minimum-evolution tree showing the phylogenetic position of aks1^{T} within the order *Chromatiales* based on the 16S rRNA gene sequence analysis. This tree was constructed using *ca.* 1200 sites. *Sulfuricaulis limicola* and *Acidiferrobacter thiooxydans* are included as outgroup species. Neighbor-joining method yielded a tree of identical topology. Numbers on nodes represent percentage values of 1000 bootstrap resampling (values less than 50 are not shown).

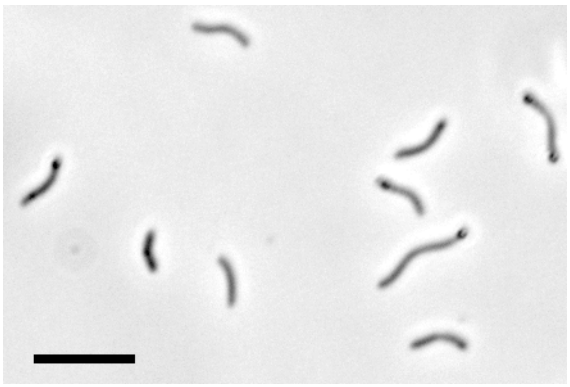

Fig.1

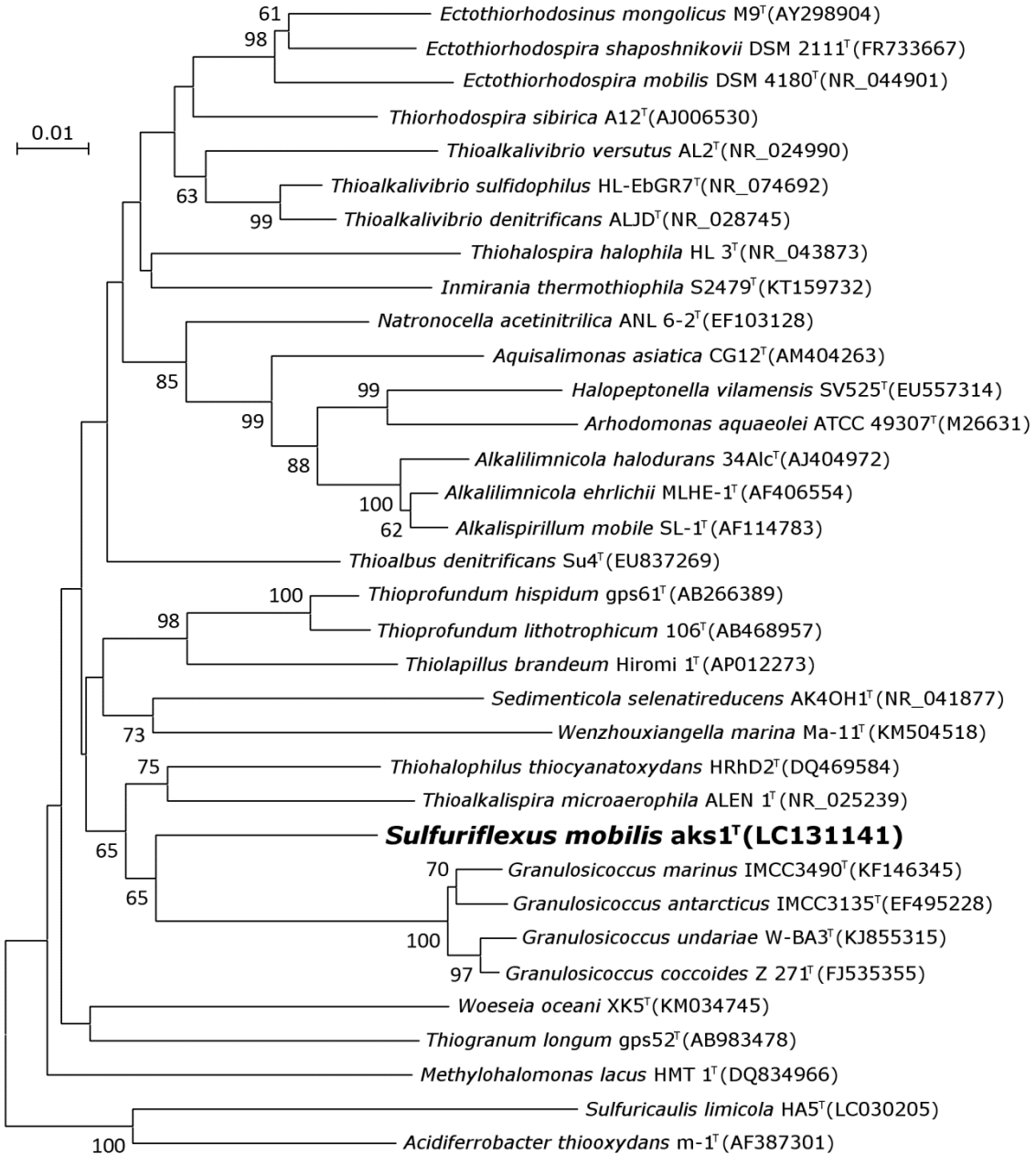
0.01
61
98
Ectothiorhodosinus mongolicus M9T(AY298904)
Ectothiorhodospira shaposhnikovii DSM 2111T(FR733667)
Ectothiorhodospira mobilis DSM 4180T(NR_044901)
Thiorhodospira sibirica A12T(AJ006530)
Thioalkalivibrio versutus AL2T(NR_024990)
63
Thioalkalivibrio sulfidophilus HL-EbGR7T(NR_074692)
99
Thioalkalivibrio denitrificans ALJDT(NR_028745)
Thiohalospira halophila HL 3T(NR_043873)
Inmirania thermothiophila S2479T(KT159732)
Natronocella acetinitrilica ANL 6-2T(EF103128)
Aquisalimonas asiatica CG12T(AM404263)
85
99
Halopeptonella vilamensis SV525T(EU557314)
99
Arhodomonas aquaeolei ATCC 49307T(M26631)
88
Alkalilimnicola halodurans 34AlcT(AJ404972)
100
Alkalilimnicola ehrlichii MLHE-1T(AF406554)
62
Alkalispirillum mobile SL-1T(AF114783)
Thioalbus denitrificans Su4T(EU837269)
100
Thioprofundum hispidum gps61T(AB266389)
98
Thioprofundum lithotrophicum 106T(AB468957)
Thiolapillus brandeum Hiromi 1T(AP012273)
Sedimenticola selenatireducens AK4OH1T(NR_041877)
73
Wenzhouxiangella marina Ma-11T(KM504518)
75
Thiohalophilus thiocyanatoxydans HRhD2T(DQ469584)
Thioalkalispira microaerophila ALEN 1T(NR_025239)
Sulfuriflexus mobilis aks1T(LC131141)
65
70
Granulosicoccus marinus IMCC3490T(KF146345)
65
Granulosicoccus antarcticus IMCC3135T(EF495228)
100
Granulosicoccus undariae W-BA3T(KJ855315)
97
Granulosicoccus coccoides Z 271T(FJ535355)
Woeseia oceani XK5T(KM034745)
Thiogranum longum gps52T(AB983478)
Methylohalomonas lacus HMT 1T(DQ834966)
Sulfuricaulis limicola HA5T(LC030205)
100
Acidiferrobacter thiooxydans m-1T(AF387301)

Fig.2

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Supplementary material

Sulfuriflexus mobilis gen. nov., sp. nov., a sulfur-oxidizing bacterium isolated from a brackish lake sediment

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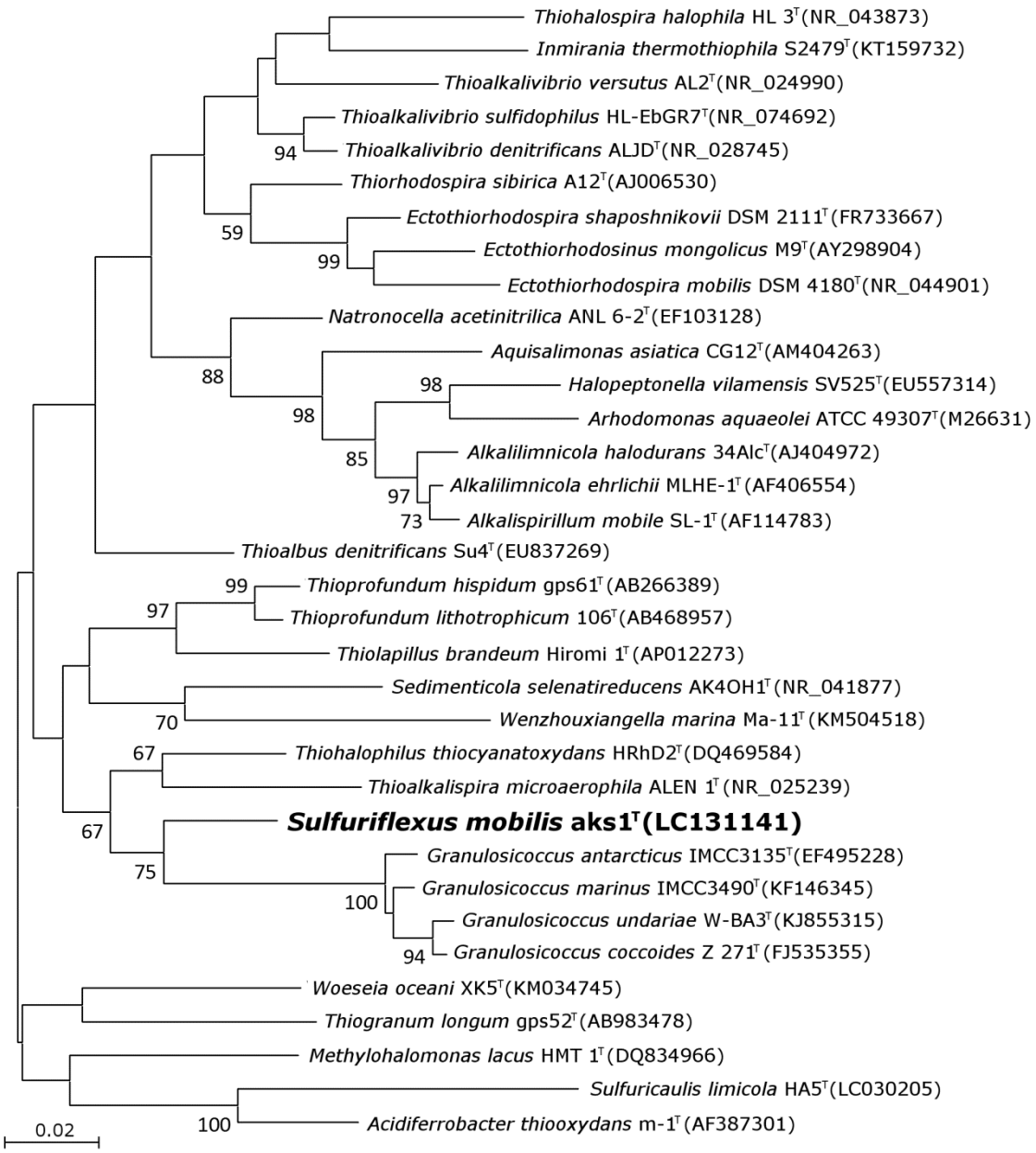

Fig S1. Maximum-likelihood tree showing the phylogenetic position of of aks1^{T} within the order *Chromatiales* based on the 16S rRNA gene sequence analysis.