Sulfuritortus calidifontis gen. nov., sp. nov., a novel sulfur oxidizer isolated from a hot spring microbial mat

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Subject category: New taxa: *Proteobacteria*

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain is LC193823.

Summary

A novel sulfur-oxidizing autotrophic bacterium, strain J1A^T was isolated from a hot spring microbial mat. The cells were Gram-stain-negative, catalase-negative and oxidase-positive. As sole electron donor for chemolithoautotrophic growth, strain J1A^T utilized sulfide, thiosulfate, elemental sulfur, and tetrathionate. The G+C content of genomic DNA was 66 mol%. Major cellular fatty acids (>40% of total) were $C_{16:0}$ and summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$). The predominant quinone was Q-8. Phylogenetic analysis of the 16S rRNA gene indicated that strain J1A^T is a relative of *Thiobacillus* species, but share with them only 93% or lower sequence similarities. On the basis of its properties, strain J1A^T (= DSM 103923^T = NBRC 112474^T) is proposed as type strain of a new species of a novel genus, *Sulfuritortus calidifontis* gen. nov., sp.

The genus *Thiobacillus* formerly included phylogenetically diverse bacteria capable of chemolithoautotrophic growth. Over 20 species have been proposed in this genus (Kelly *et al*., 2005), but it currently includes only 4 species with validly published name (Orlygsson & Kristjansson, 2014). At present the genus *Thiobacillus* is classified in the family *Hydrogenophilaceae* within the order *Hydrogenophilales*, whose type genus is the genus *Hydrogenophilus* (Garrity *et al*., 2005a; 2005b). The polyphyly of the family has already been indicated (Orlygsson & Kristjansson, 2014), and the genus *Thiobacillus* is phylogenetically separated from the genus *Hydrogenophilus* suggesting that reclassification of the genus is desirable. In the present study, a novel chemolithoautotrophic sulfur-oxidizing bacterium related to the *Thiobacillus* species was isolated and characterized.

A sulfur-oxidizing enrichment culture was established with an inoculum of microbial mat, obtained from Jyozankei hot spring in Japan (42° 57' 53" N 141° 09' 47" E). The microbial mat was developed on a concrete wall on which the spring water was running down. At the sampling time, temperature of the running water was approximately 43°C and its pH was 7.8. For enrichment and isolation, a bicarbonate-buffered low-salt defined medium previously described (Kojima & Fukui, 2011) was used with some

modification. The composition of the medium was as follows (l^{-1}): 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 trace element solution, 1 ml selenite-tungstate solution, 1 ml vitamin mixture solution, 30 ml $NaHCO_3$ solution. The vitamin mixture solution contained following ingredients (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 other stock solutions were prepared as described previously (Widdel & Bak, 1992). The enrichment and isolation were performed at 45°C under anoxic conditions created by filling the headspace of the culturing containers with N_2/CO_2 (80:20 [vol · vol^{-1}]). A piece of microbial mat was inoculated into the medium, and elemental sulfur (ca. 0.5 g l^{-1}) and nitrate (10 mM) were added to the medium as electron donor and acceptor, rescectively. The enrichment culture was repeatedly inoculated to the fresh medium. After the 6 times of transfer, sulfur as the sole electron donor was replaced with thiosulfate (10 mM). The second thiosulfate-oxidizing culture was subjected to agar shake dilution (Widdel and Bak 1992). A colony was picked up in the same medium, and the resulting culture was subjected to agar shake dilution again. After verification of purity by microscopy and repeated sequencing of the 16S rRNA gene, the obtained culture was designated as

68 strain J1A^T.

69 For the characterization of the obtained strain, “medium S4” (Kojima *et al*, 2016)
70 supplemented with 10 mM $Na_2S_2O_3$ was used unless otherwise specified (headspace of
71 culture bottles was filled with the air). Gram-stain test was conducted with a kit (Fluka).
72 Catalase activity was assessed by pouring 3% H_2O_2 solution onto the cells. Oxidase
73 activity was tested by using an oxidase test reagent (bioMérieux). The G+C content of
74 the genomic DNA was determined by HPLC method (Katayama-Fujimura *et al.*, 1984),
75 using a kit (Yamasa Shoyu). The analyses of fatty acids and quinone were carried out at
76 the Techno Suruga Co. Ltd (Shizuoka, Japan), with cells grown at 45°C for 2 days. The
77 cellular fatty acid profile was analyzed by using the Sherlock Microbial Identification
78 System version 6.0 (MIDI), according to the standard protocol with database TSBA6.
79 The isoprenoid quinones were extracted by chloroform/methanol extraction (Bligh &
80 Dyer, 1959) and analyzed by HPLC with a multiwavelength detector.

81 Utilization of electron donors was tested in the medium used for the isolation, under
82 aerobic conditions. Heterotrophic growth with complex liquid media was tested for R2A
83 (Daigo), diluted (1/10) R2A, nutrient broth (NB, Difco), Luria-Bertani broth (LB,
84 Merck) and tryptone soya broth (TSB, OXOID) at 45°C under aerobic conditions.

85 Effects of the temperature on growth were examined under thiosulfate-oxidizing and

86 nitrate-reducing conditions in the medium used for the isolation. To test the effect of
87 temperatures, the strain was cultured at 10, 13, 15, 18, 22, 25, 28, 30, 37, 45, 48, and
88 50°C. The effect of pH on the growth was tested at 37°C, with a method previously
89 described (Kojima *et al*., 2015). The tested pH and buffering reagents used were as
90 follows; pH 6.1, 6.2, 6.3, 6.6, 6.7 and 6.8 with MES; pH 7.0, 7.2, 7.3, 7.5 and 7.7 with
91 MOPS; pH 8.0 and 8.4 with Tricine; pH 8.7, 8.9, 9.2, 9.3, and 9.6 with CHES. The
92 effect of salt concentration was tested by culturing strain J1A^T in the presence of
93 varying concentrations of NaCl (0, 1, 2, 3, 4 and 5% w/v), at 45°C under the oxic
94 conditions.
95 For phylogenetic analysis, almost entire 16S rRNA gene was amplified with the primer
96 pair 27F and 1492R (Lane, 1991). The resulting PCR product was directly sequenced.
97 The obtained sequence of strain J1A^T was aligned with reference sequences retrieved
98 from the public database (GenBank/EMBL/DDBJ) by using the program CLUSTAL X
99 version 2.1 (Larkin *et al*., 2007). The reference sequences included 35 species
100 representing all betaproteobacterial orders and 6 environmental clone sequences. The
101 environmental sequences were selected on the basis of their high sequence similarity
102 with strain J1A^T (97% or more) as revealed by a BLAST search. All positions with gaps
103 were excluded from the calculation, and 1295 positions were used to construct

104 phylogenetic trees. Phylogenetic trees were reconstructed by using the methods of
105 maximum likelihood, neighbor-joining and minimum evolution with the program
106 MEGA version 7.0.20 (Kumar *et al*., 2016).
107
108 The isolated bacterium, strain J1A^T was facultative anaerobe which grows under oxic
109 or nitrate-reducing conditions. Cells of strain J1A^T were motile and curved to spiral rods,
110 1.3–4.5 µm long and 0.4–0.5 µm wide (Fig. S1). The growth of the strain J1A^T was
111 observed over a temperature range between 15°C and 48°C, with an optimum growth at
112 45°C. The range of pH for growth was 6.2–8.7, and the optimum pH was 6.8–7.3.
113 Negative effect of NaCl on the growth was observed at concentration of 1% and strain
114 J1A^T exhibited no growth in the presence of 2% or more NaCl.
115 The cells were Gram-stain-negative, catalase-negative and oxidase-positive. The G+C
116 content of the genomic DNA of was 66 mol% (HPLC). The major cellular fatty acids
117 were $C_{16:0}$ (48.6%) and summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; 40.3 %). The
118 other fatty acids detected were summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$; 3.9 %),
119 $C_{10:0}$ 3-OH (2.4%), $C_{17:0}$ (1.2%), cyclo-$C_{17:0}$ (1.2%), $C_{18:1}\omega9c$ (1.1%), $C_{18:0}$ (0.6%),
120 $C_{14:0}$ (0.5%), $C_{17:1}\omega8c$ (0.2%), $C_{16:1}\omega5c$ (0.1%), $C_{15:1}\omega6c$ (0.1%). The predominant
121 quinone was identified as Q-8 (95%). As minor quinones, Q-7 and Q-9 were also

122 detected.

123 Chemolithoautotrophic growth of strain J1A^T was supported by thiosulfate (10, 20
124 mM), sulfide (2 mM), tetrathionate (10 mM), and elemental sulfur (0.5 g l^{-1}). The
125 following electron donors did not support growth of strain J1A^T: acetate, formate,
126 lactate, fumarate, malate, succinate, glucose, mannose and ethanol (all 5 mM). Strain
127 J1A^T exhibited no growth on R2A, diluted R2A, NB, LB, or TSB.

128 The analysis of 16S rRNA gene revealed that strain J1A^T is closely related to members
129 of the genus *Thiobacillus* (Fig. 1, Fig. S2). The sequence similarity with the type strains
130 of the validaded *Thiobacillus* species ranged 92–93%. Environmental clones most
131 closely related to strain J1A^T have been reported from a freshwater aquifer (Gray &
132 Engel, 2013). In the phylogenetic tree constructed with the maximum likelihood method,
133 strain J1A^T and closely related environmental sequences formed a distinct cluster which
134 represents a sister group of the genus *Thiobacillus* (Fig. 1). This branching pattern was
135 consistently observed in phylogenetic trees constructed with the methods of
136 neighbor-joining and minimum evolution, which generated trees of identical topology
137 (Fig. S2). In all trees constructed, the clade including strain J1A^T and *Thiobacillus*
138 species was phylogenetically distant from the genus *Hydrogenophilus* (Fig. 1, Fig. S2).

139

The independent phylogenetic position and low sequence similarities (93% or lower) to the related species suggest that strain J1A^T represents a novel species of a new genus. In addition, its twisted cell morphology is distinct from that of rod-shaped *Thiobacillus* species (Kelly *et al*., 2005). On the basis of these findings, *Sulfuritortus calidifontis* gen. nov., sp. nov. is proposed to accommodate strain J1A^T as the type strain. The phylogenetic analysis also showed that the genera *Thiobacillus* and *Sulfuritortus* should not be placed in the family *Hydrogenophilaceae* or other existing family in the class *Betaproteobacteria* (Fig. 1, Fig. S2). A major reclassification of higher taxa in this class may be required to define taxonomy of the genera *Thiobacillus* and *Sulfuritortus* adequately.

Description of *Sulfuritortus* gen. nov.

Sulfuritortus (Sul.fu.ri.tor'tus. L. neut. n. *sulfur* sulfur; L. masc. n. *tortus* twist, torsion; N.L. masc. n. *Sulfuritortus* sulfur-oxidizing twisted organism)

Grow chemolithoautotrophically by the oxidation of inorganic sulfur compounds. Major cellular fatty acids are $C_{16:0}$ and $C_{16:1}$. The predominant quinone is Q-8. Based on 16S rRNA gene sequence analysis, affiliated to the class *Betaproteobacteria*. The type species is *Sulfuritorttus calidifontis*.

158

159 **Description of *Sulfuritortus calidifontis* sp. nov.**

160 *Sulfuritortus calidifontis* (ca.li.di.fon'tis. L. adj. *calidus* hot; L. n. *fons, fontis* spring,
161 fountain; N.L. gen. n. *calidifontis* of a hot spring).

162 Cells are motile, Gram-stain-negative, 1.3–4.5 μm long, 0.4–0.5 μm wide and curved to
163 spiral rods in shape. Facultatively anaerobic. Autotrophic growth occurs with oxidation
164 of sulfide, thiosulfate, tetrathionate and elemental sulfur. Catalase-negative and
165 oxidase-positive. The temperature range for growth is 15–48°C, with an optimum of
166 45°C. The pH range for growth is 6.2–8.7, and optimum growth occurs at pH 6.8–7.3.
167 The G+C content of genomic DNA is 66 mol%. The type strain J1A^{T} (= DSM 103923^{T}
168 = NBRC 112474^{T}) was isolated from microbial mat of a hot spring in Japan.

169

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174 **Conflicts of interest statement**

175 The authors declare that there is no conflict of interest.

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228

229 Figure legend

230

231 Fig. 1 Maximum-likelihood tree based on the 16S rRNA gene sequence of strain J1A^T,

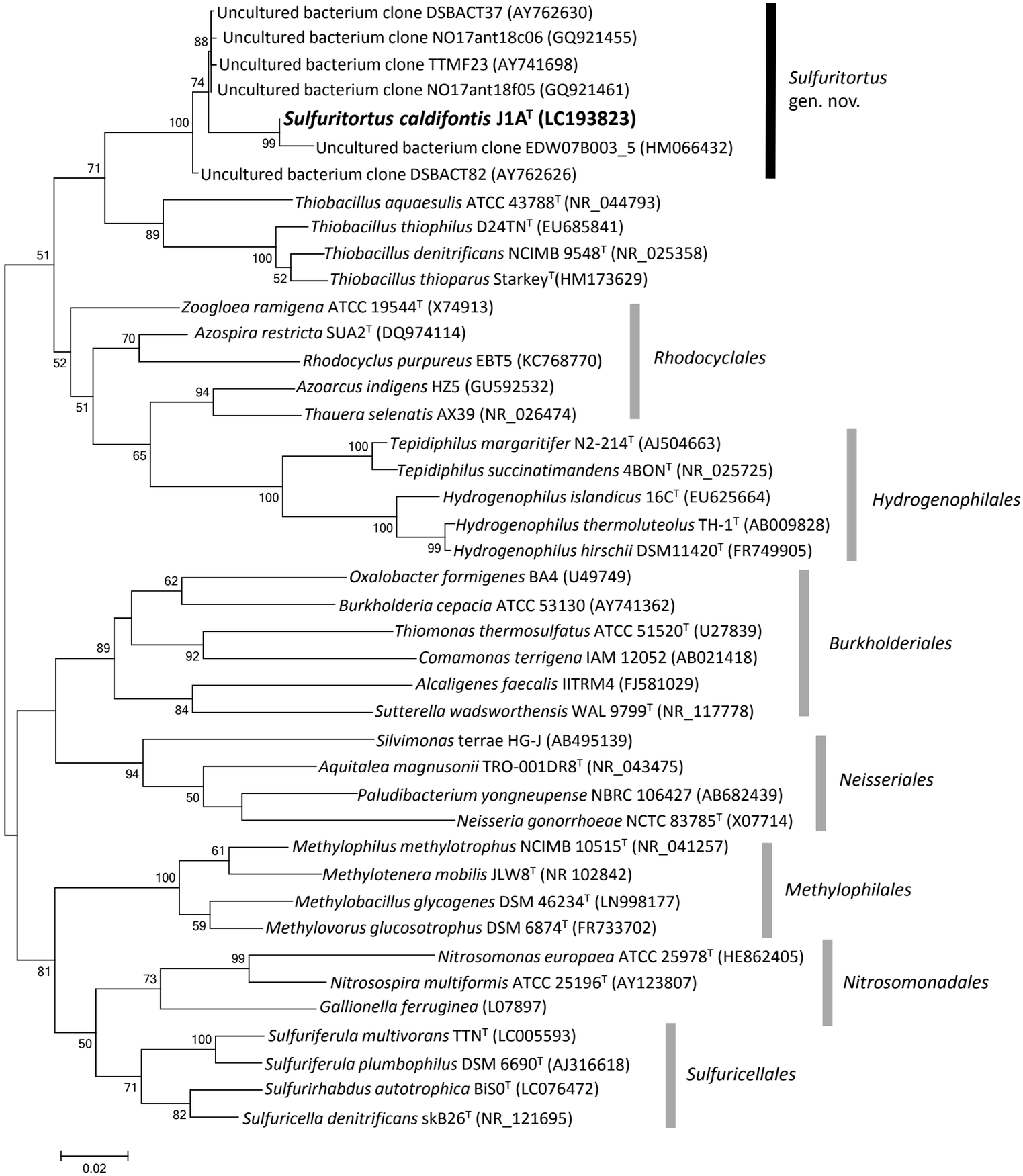
232 related environmental sequences and species from all orders of the class

233 *Betaproteobacteria*. Bootstrap values larger than 50 (percentages of 1000 replications)

234 are shown at nodes. Bar, 0.02 changes per position.

235

236


Uncultured bacterium clone DSBACT37 (AY762630)
Uncultured bacterium clone NO17ant18c06 (GQ921455)
Uncultured bacterium clone TTMF23 (AY741698)
Uncultured bacterium clone NO17ant18f05 (GQ921461)
Sulfuritortus caldifontis J1A^T (LC193823)
Uncultured bacterium clone EDW07B003_5 (HM066432)
Uncultured bacterium clone DSBACT82 (AY762626)
Sulfuritortus gen. nov.
Thiobacillus aquaesulis ATCC 43788^T (NR_044793)
Thiobacillus thiophilus D24TN^T (EU685841)
Thiobacillus denitrificans NCIMB 9548^T (NR_025358)
Thiobacillus thioparus Starkey^T(HM173629)
Zoogloea ramigena ATCC 19544^T (X74913)
Azospira restricta SUA2^T (DQ974114)
Rhodocyclus purpureus EBT5 (KC768770)
Azoarcus indigens HZ5 (GU592532)
Thauera selenatis AX39 (NR_026474)
Rhodocyclales
Tepidiphilus margaritifer N2-214^T (AJ504663)
Tepidiphilus succinatimandens 4BON^T (NR_025725)
Hydrogenophilus islandicus 16C^T (EU625664)
Hydrogenophilus thermoluteolus TH-1^T (AB009828)
Hydrogenophilus hirschii DSM11420^T (FR749905)
Hydrogenophilales
Oxalobacter formigenes BA4 (U49749)
Burkholderia cepacia ATCC 53130 (AY741362)
Thiomonas thermosulfatus ATCC 51520^T (U27839)
Comamonas terrigena IAM 12052 (AB021418)
Alcaligenes faecalis IITRM4 (FJ581029)
Sutterella wadsworthensis WAL 9799^T (NR_117778)
Burkholderiales
Silvimonas terrae HG-J (AB495139)
Aquitalea magnusonii TRO-001DR8^T (NR_043475)
Paludibacterium yongneupense NBRC 106427 (AB682439)
Neisseria gonorrhoeae NCTC 83785^T (X07714)
Neisseriales
Methylophilus methylotrophus NCIMB 10515^T (NR_041257)
Methylotenera mobilis JLW8^T (NR 102842)
Methylobacillus glycogenes DSM 46234^T (LN998177)
Methylovorus glucosotrophus DSM 6874^T (FR733702)
Methylophilales
Nitrosomonas europaea ATCC 25978^T (HE862405)
Nitrosospira multiformis ATCC 25196^T (AY123807)
Gallionella ferruginea (L07897)
Nitrosomonadales
Sulfuriferula multivorans TTN^T (LC005593)
Sulfuriferula plumbophilus DSM 6690^T (AJ316618)
Sulfurirhabdus autotrophica BiS0^T (LC076472)
Sulfuricella denitrificans skB26^T (NR_121695)
Sulfuricellales
0.02