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***Thiomicrothrix immobilis* sp. nov., a mesophilic sulfur-oxidizing bacterium isolated from sediment of a brackish lake**

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Abstract

A novel sulfur-oxidizing bacterium, strain Am19^T, was isolated from sediment of a brackish lake. Strain Am19^T grew chemolithotrophically on inorganic sulfur compounds, and heterotrophic growth was not observed. Cells were rod-shaped with length of 1.1–3.0 µm and width of 0.5–0.8 µm. Growth was observed at 5–37°C with an optimum growth temperature of 30°C. The pH range for growth was 5.6–8.5 with an optimum pH of 6.6–7.0. Major fatty acids were summed feature 3 (C_{16:1}ω7c and/or C_{16:1}ω6c), summed feature 8 (C_{18:1}ω7c and/or C_{18:1}ω6c) and C_{16:0}. The sole respiratory quinone was ubiquinone-8. The complete genome of strain Am19^T is composed of a circular chromosome with length of 2.5 Mbp and G + C content of 42.7 mol%. Phylogenetic analysis based on genomic data indicated that strain Am19^T belongs to the genus *Thiomicrohabdus* but is distinct from any existing species. Analysis of the 16S rRNA gene supported creation of a new species to accommodate strain Am19^T. On the basis of genomic and phenotypic characteristics, strain Am19^T (= NBRC 114602^T = BCRC 81336^T) is proposed as the type strain of a new species, with name of *Thiomicrohabdus immobilis* sp. nov.

Introduction

The genus *Thiomicrothrix* was established in 2017, within the family *Piscirickettsiaceae* (Boden et al. 2017a). Afterward, the genus description was emended in 2017 and 2021 (Boden et al., 2017b; Liu et al., 2021). In this genus, there are nine species with validly published names at present, including five species which were originally classified in another genus. The reclassified five species are *T. frisia* (Brinkhoff et al., 1999a), *T. chilensis* (Brinkhoff et al., 1999b), *T. arctica*, *T. psychrophila* (Knittel et al., 2005) and *T. hydrogeniphila* (Watsuji et al., 2016). The other four species described in this genus are *T. aquaedulcis* (Kojima & Fukui 2019), *T. indica* (Liu et al., 2020), *T. sediminis* and *T. xiamenensis* (Liu et al., 2021). They are all obligate chemolithoautotrophs which oxidize sulfur compounds. They require oxygen as electron acceptor for growth, and rely on sulfur compounds as electron donor. As exceptional cases, anaerobic growth with nitrite was reported in *T. sediminis*, and hydrogen-dependent growth was observed in *T. hydrogeniphila*. As common chemotaxonomic feature, all species examined share three major fatty acids, C_{16:1}, C_{18:1} and C_{16:0}.

In this study, a novel aerobic and mesophilic autotroph was isolated and characterized, as a representative of a new species in the genus *Thiomicrothrix*.

Materials and methods

Enrichment and isolation

Strains Am19^T was isolated from a sediment collected at a site (43.05N, 144.89E), in Lake Akkeshi, a brackish lake in Japan. The sediment was inoculated into a bicarbonate-buffered medium, which comprised (l⁻¹): 2.5 g Na₂S₂O₃ • 5H₂O, 20 g NaCl, 0.2 g MgCl₂ • 6H₂O, 0.1 g CaCl₂ • 2H₂O, 0.1 g NH₄Cl, 0.1 g KH₂PO₄, 0.1 g KCl, 1 ml trace element solution, 1 ml selenite-tungstate solution, 1 ml vitamin mixture solution, 30 ml NaHCO₃ solution. The medium and respective stock solutions were prepared as described previously (Kojima et al. 2016). After some transfers to medium of the same composition, the composition of the medium was changed to contain 5 g Na₂S₂O₃ • 5H₂O, 20 g NaCl, 3 g MgCl₂ • 6H₂O, and 0.3 g MgSO₄ • 7H₂O. The other components were same as the medium used to establish the first enrichment. This medium containing MgSO₄ is hereafter referred to as the basal medium. Strain Am19^T was isolated by repeated serial dilution with the basal medium. The enrichment and isolation were performed at 22°C. Purity of culture was routinely checked by microscopy and sequencing of the 16S rRNA gene fragments.

Phenotypic characterization

In experiments for phenotypic characterizations, strain Am19^T was cultured at 30°C in the basal medium unless otherwise specified. Cell morphology was observed with phase-contrast light microscopy. The Gram staining test was conducted with a Gram-color kit (Merck). Activities of catalase and oxidase were assessed as described previously (Kojima & Fukui 2016)

Effect of temperature on growth was examined by culturing at 0, 5, 8, 10, 13, 15, 18, 22, 25, 28, 30, 32, 37 and 45°C in the basal medium. Effect of salt concentration on growth was examined with modified versions of the basal medium, which contained no MgCl₂ and varying concentrations of NaCl, ranging from 0 to 1400 mM at 100 mM intervals.

For test of pH effect on growth, media of various pH prepared as below. From the basal medium, MgCl₂ was excluded and bicarbonate concentration was reduced to 1 g l⁻¹. Depending on the pH to be tested, the modified media were buffered with 20 mM of MES, MOPS or TAPS. All ingredients were mixed and then sterilized by filtration after pH adjustment with NaOH or HCl. The pH tested and buffering reagents were as follows; pH 3.3, 3.7, 4.2, 5.0, 5.1, 5.6, 6.1, 6.4, 6.6, 6.8, 7.0 and 7.2 with MES; pH 6.4, 7.0, 7.2, 7.4, 7.6, 7.8, 7.9, 8.5 and 8.8 with MOPS; pH 8.1, 8.2, 8.5, 8.7 and 9.1 with TAPS.

Utilization of growth substrates was tested with the basal medium, by replacing

thiosulfate with one of the followings (mM; unless otherwise specified); tetrathionate (20), elemental sulfur (0.5 g/l), sulfite (5), sulfide (2), acetate (5), formate (5), succinate (2.5), fumarate (5), isobutyrate (2.5), methanol (5), ethanol (2.5), glucose (2.5), xylose (2.5), maltose (5), arabinose (5), cellobiose (1). Heterotrophic growth was also tested in complex liquid media listed below; Reasoner's 2A broth (R2A) broth, one-tenth-strength R2A, nutrient broth, LB broth Miller and tryptone soya broth. Utilization of electron acceptor was tested under anoxic condition, with the basal medium supplemented with nitrate (20 mM) or nitrite (5 mM).

Chemotaxonomic characterization

For analyses of cellular fatty acids and respiratory quinone, the basal medium was simplified by omitting MgCl₂. Strain Am19^T was grown in the simplified basal medium at 30°C. The fatty acid profile was obtained with the Sherlock Microbial Identification System (MIDI) version 6.0 (database; TSBA6). Respiratory quinones were extracted as described previously (Bligh & Dyer, 1959), and then analyzed with HPLC.

Genomic characterization

Whole genome sequencing was performed using the platforms of Illumina NextSeq and Nanopore GridION, to obtain short and long reads respectively. The resulting reads

from the platforms were assembled by using Unicycler (Ver 0.4.7). The reconstructed genome sequence was annotated with DFAST (Tanizawa et al., 2018).

A genome-based taxonomic classification was made with reference to the Genome Taxonomy Database (GTDB) (Parks et al., 2018). Taxonomic position in GTDB release 95 was determined by analyzing the genome of strain Am19^T with GTDB-Tk (Chaumeil et al., 2020).

To estimate genome relatedness between Am19^T and its close relatives, values of average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) were calculated, by using tools provided by Kostas lab (<http://enve-omics.ce.gatech.edu/>) and DSMZ, respectively. In the calculation of dDDH with Genome-to-Genome Distance Calculator (GGDC), the formula 2 was applied (Meier-Kolthoff et al., 2013).

Phylogenetic analysis based on the 16S rRNA gene

Phylogenetic analysis of the 16S rRNA gene was conducted using MEGA version 11 (Tamura et al., 2021). The gene sequence of strain Am19^T was aligned with those of type strains in the genus *Thiomicrobacter*, using the MUSCLE algorithm. *Sulfuriflexus mobilis* aks1^T was included in the analysis as an outgroup. With the resulting alignment, genetic distances were calculated by excluding positions with gaps using the best model

selected by the model selection tool in MEGA.

Results

Physiological and chemotaxonomic characteristics

Cells of strain Am19^T were Gram-stain-negative, rod-shaped, non-motile, 0.5–0.8 µm in width and 1.1–3.0 µm in length. They were catalase-negative and oxidase-positive. Strain Am19^T grew at 5–37°C with optimum growth at 30°C. Its growth was observed at pH range of 5.6–8.5, with optimum growth at pH 6.6–7.0. In the test of salt concentration, growth was observed in the presence of 1300 mM or lower concentrations of NaCl. Strain Am19^T did not require NaCl for growth.

Strain Am19^T grew chemolithotrophically on thiosulfate, tetrathionate, sulfide and elemental sulfur. None of the tested organic substrate supported growth of the strain. No heterotrophic growth was observed in the complex media tested. In the presence of thiosulfate, anaerobic growth was not observed in the medium containing nitrate or nitrate as sole electron acceptor.

Major components in the cellular fatty acid profile of strain Am19^T were summed feature 3 (C₁₆:1ω7c and/or C₁₆:1ω6c; 46%), summed feature 8 (C₁₈:1ω7c

and/or C_{18:1}ω6c; 33%) and C_{16:0} (14%). The other fatty acids detected (>0.1% of total) were C_{17:1}ω6c (1.3%), C_{18:0} (1.0%), C_{17:0} (1.0%), C_{10:0} (0.8%), C_{17:1}ω8c (0.7%), C_{14:0} (0.6%), C_{18:1}ω5c (0.5%), C_{10:0} 3-OH (0.8%), C_{16:1}ω5c (0.3%), C_{12:0} (0.2%), summed feature 2 (C_{12:0} aldehyde, unknown 10.928, C_{14:0} 3-OH and/or iso-C_{16:1} I; 0.2%), C_{15:1}ω6c (0.2%), C_{20:1}ω7c (0.1%).

In the analysis of quinones, only ubiquinone-8 was detected.

Genomic characteristics and phylogenetic position

The complete genome of strain Am19^T was reconstructed from reads of DNBSEQ and GridION. The sequencing coverage was 825.8-fold. The genome consists of a single circular chromosome with size of 2,539,444 bp and G+C contents of 42.7%. In the genome, 2251 protein-coding sequences, 9 rRNA genes and 46 tRNA genes were predicted.

The reconstructed genome was analyzed with GTDB-tk, for phylogenetic analysis based on 120 conserved proteins. As a result, strain Am19^T was classified into the genus *Thiomicrothabodus*. On the other hand, the analysis also indicated that strain Am19^T does not belong to any existing species with validly published name.

The values of dDDH and ANI between strain Am19^T and genome-sequenced strains in the genus *Thiomicrobacter* are shown in Table 1. The values are lower than threshold for species delineation, 70% and 95–96% for dDDH and ANI, respectively (Richter & Rosselló-Móra, 2009; Meier-Kolthoff et al., 2013).

Three copies of the 16S rRNA gene were identified in the genome, and they have an identical sequence. The sequence indicated high identities to the 16S rRNA gene sequences of *Thiomicrobacter* species. Among species with validly published names, *T. frisia* indicated the highest identity of 97.7% (Table 2). Phylogenetic tree based on the 16S rRNA gene sequences is shown in Fig. 1. The tree indicated that strain Am19^T belongs to the genus *Thiomicrobacter* but is phylogenetically distinct from existing species.

Conclusions

The genomic characteristics of strain Am19^T, including the 16S rRNA gene sequence, indicated that this novel isolate is close relative of the *Thiomicrobacter* species but should not be classified into any existing species. Between strain Am19^T and type strains of existing species, the 16S rRNA gene sequence identity, dDDH and ANI values were lower than species delineation thresholds (Tables 1 and 2). Within the genus,

strain Am19^T and existing species are differentiated by combinations of phenotypic characteristics (Table 2). On the basis of these genomic and phenotypic characteristics, strain Am19^T is proposed to be assigned to a novel species of the genus *Thiomicrothabodus*, with the name *Thiomicrothabodus immobilis* sp. nov.

Description of *Thiomicrothabodus immobilis* sp. nov.

Thiomicrothabodus immobilis (im.mo'bi.lis. L. masc./fem. adj. *immobilis*, non-motile, pertaining to a feature of the type strain).

Cells are rod-shaped, 1.1–3.0 µm in length and 0.5–0.8 µm in width. Strictly aerobic. Gram-stain-negative. Catalase-negative and oxidase-positive. Chemolithoautotrophic growth occurs with thiosulfate, tetrathionate, elemental sulfur and sulfide. Acetate, formate, succinate, fumarate, isobutyrate, methanol, ethanol, glucose, xylose, maltose, arabinose and cellobiose are not utilized as growth substrates. Growth of the type strain is observed at temperature range from 5°C to 37°C, with an optimum growth at 30°C. The pH range for growth is 5.6–8.5, and the optimum pH was 6.6–7.0. The G+C content of genomic DNA of type strain is 42.7 mol% (genome).

The type strain Am19^T (= NBRC 114602^T = BCRC 81336^T) was isolated from sediment of a brackish lake in Japan (Lake Akkeshi). The GenBank/EMBL/DDBJ

accession numbers for the complete of strain Am19^T is AP024202.

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Statements and Declarations

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Table 1. Values of dDDH and ANI between strain Am19^T and genome-sequenced strains in the genus *Thiomicrothabodus*

Strain (accession number)	dDDH (%)	ANI (%)
<i>Thiomicrothabodus</i> sp. Kp2 (GCA_000478585.1)	21.1	80
<i>Thiomicrothabodus</i> sp. Milos-T2 (GCA_000702325.1)	20.9	80
<i>Thiomicrothabodus</i> sp. HH3 (GCA_013391695.1)	15.8	79
<i>Thiomicrothabodus</i> sp. HH1 (GCA_013391765.1)	16.2	78
<i>T. aquaedulcis</i> HaS4 ^T (GCA_004001325.1)	16.2	78
<i>T. indica</i> 13-15A ^T (GCA_004293625.1)	16.5	78
<i>T. sediminis</i> G1 ^T (GCA_005885815.1)	16.8	79
<i>T. xiamenensis</i> G2 ^T (GCA_013282625)	16.4	79
<i>T. arctica</i> DSM 13458 ^T (GCA_000381085.1)	16.4	78
<i>T. chilensis</i> DSM 12352 ^T (GCA_000483485.1)	16.3	79

Table 2. Differential properties of strain Am19^T and type strains of *Thiomicrothabodus* species.

Strains: 1, Am19^T; 2, *T. frisia* JB-A2^T (Brinkhoff et al., 1999a); 3, *T. chilensis* Ch-1^T (Brinkhoff et al., 1999b); 4, *T. arctica* SVAL-E^T (Knittel et al., 2005); 5, *T. psychrophila* SVAL-D^T (Knittel et al., 2005); 6, *T. hydrogeniphila* MAS2^T (Watsuji et al., 2016); 7, *T. aquadulcis* HaS4^T (Kojima & Fukui, 2019); 8, *T. indica* 13-15A^T (Liu et al., 2020); 9, *T. sediminis* G1^T (Liu et al., 2021); 10, *T. xiamenensis* G2^T (Liu et al., 2021). All strains are obligate chemolithoautotrophs which oxidize thiosulfate, tetrathionate and sulfur. NR, not reported.

	1	2	3	4	5	6	7	8	9	10
Optimum pH for growth	6.6-7.0	6.5	7	7.3-8.0	7.5-8.5	6	6.6-7.4	7	7.5	6.5
pH range for growth	5.6-8.5	4.2-8.5	5.3-8.5	6.5-9.0	6.5-9.0	5.0-8.0	6.2-8.8	5.0-9.0	6.0-9.0	5.5-8.0
Optimum temperature for growth (°C)	30	32-35	32-37	11.5-13.2	14.6-15.4	30	22	28	30	28
Temperature range for growth (°C)	5-37	3.5-39	3.5-42	-2.0-20.8	-2.0-20.8	2-40	0-25	10-45	10-40	4-45
Fourth most abundant fatty acid*	C _{17:1}	NR	C _{18:0}	C _{14:1}	C _{12:1}	C _{12:0}	C _{18:0}	C _{10:0} 3-OH	C _{18:0}	C _{10:0} 3-OH
16S sequence identity to strain Am19 ^T (%)	100	97.7	96.5	97.3	96.7	97.1	95.3	94.9	96.2	94.7

*Following to C_{16:1}, C_{18:1} and C_{16:0}.

Fig. 1 Maximum-likelihood tree showing the phylogenetic position of strain Am19^T within the genus *Thiomicrohabdus*, based on the 16S rRNA gene sequences. A discrete gamma distribution was used to model differences in evolutionary rates among sites. The rate variation model allowed for some sites to be invariable. All positions containing gaps and missing data were eliminated, leaving a total of 1407 positions in the final dataset. Numbers on nodes represent percentage values of 1000 bootstrap resampling.