Sulfuricystis multivorans gen. nov., sp. nov. and Sulfuricystis thermophila sp. nov., facultatively autotropic sulfur-oxidizing bacteria isolated from a hot spring, and emended description of the genus Rugosibacter

Hisaya Kojima (kojimah@lowtem.hokudai.ac.jp)
Hokkaido University https://orcid.org/0000-0003-3750-8353
Miho Watanabe
Akita Prefectural University
Naoyuki Miyata
Akita prefectural University
Manabu Fukui
Hokkaido University: Hokkaido Daigaku

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Abstract

Strains J5B^T and M52^T are facultatively autotrophic sulfur-oxidizing bacterium isolated from a microbial mat from a hot spring. They were isolated and partially characterized in previous studies, as facultative anaerobes which use nitrate as electron acceptor. In this study, additional characterizations were made to determine their taxonomic status. In both strains, major cellular fatty acids were $C_{16:1}$ ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) and $C_{16:0}$. Their chemotihoautorophic growth was supported by thiosulfate and elemental sulfur. They used some organic acids as growth substrates. Their 16S rRNA gene sequences indicated the highest sequence identities to species in the family *Sterolibacteriaceae*, but the identities were 95% or lower. Values of average nucleotide identity and digital DNA–DNA hybridization between strains J5B^T and M52^T were 87.93% and 34.3%, respectively. Phylogenetic analysis of the 16S rRNA gene sequences indicated that these strains belong to the family *Sterolibacteriaceae* but not to any existing genera. On the basis of phenotypic and genomic characteristics, *Sulfuricystis multivorans* gen. nov. sp. nov., and *Sulfuricystis thermophila* sp. nov. are proposed, with type strains of J5B^T (= BCRC 81386^T = DSM 104688^T = NBRC 112605^T) and M52^T (= BCRC 81387^T = NBRC 114016^T), respectively. An emended description of the genus *Rugosibacter* is also proposed, for its reclassification to the family *Sterolibacteriaceae*.

Introduction

The family *Sterolibacteriaceae* was proposed to encompass six genera, as a part of major reconstruction of the order *Nitrosomonadales* (Boden et al., 2017). According to the List of Prokaryotic Names with Standing in Nomenclature (as of 29 March 2022), the family still comprises the original six genera, *Sterolibacterium*, *Denitratisoma*, *Methyloversatilis*, *Georgfuchsia*, *Sulfuritalea* and *Sulfurisoma*. Before the reclassification, some of these genera had been regarded as members of the family *Rhodocyclaceae* in the order *Rhodocyclales*. Immediately before establishment of the family *Sterolibacteriaceae*, a genus was proposed in the family *Rhodocyclaceae*, with the name of *Rugosibacter* (Corteselli et al., 2017). In that study, phylogenetic analysis of the 16S rRNA gene indicated that *Rugosibacter* belongs to a clade consisting of the six genera mentioned above. The genus *Rugosibacter* was not taken into consideration in the proposal of *Sterolibacteriaceae*, and it is still placed in the family *Rhodocyclaceae* as noted in the original description. The close relatedness between *Rugosibacter* and genera of *Sterolibacteriaceae* has also been indicated by a phylogenetic analysis of concatenated ribosomal proteins (Okubo & Takami, 2021).

The original description of family *Sterolibacteriaceae* states that the family is circumscribed on the basis of 16S rRNA gene sequences, and includes physiologically diverse organisms (Boden et al., 2017). It refers to methylotrophy, autotrophy and anaerobic respiration (with nitrate, ferric iron or manganic manganese), as varied metabolism observed in the family. As other notable functions, anaerobic degradation of aromatic compounds (Weelink et al., 2009; Sperfeld et al., 2019) and arsenate respiration (Watanabe et al., 2017) have also been demonstrated in species belonging to this family.

Besides the organisms mentioned above, some strains have been isolated and characterized as members of this family. *Sterolibacteriaceae* bacterium strain J5B^T is a facultatively autotrophic sulfur-oxidizing bacterium, isolated from a microbial mat collected in a hot spring in Japan (Watanabe et al, 2019). As for strain J5B^T, some physiological characteristics have been reported, along with genomic characteristics related to sulfur oxidation (Watanabe et al, 2019). The closest relative of strain J5B^T is also sulfur oxidizer, isolated from the same microbial mat. The sulfur-oxidizing bacterium, strain M52^T, can oxidize arsenite anaerobically (Ospino et al., 2019). The previous studies reported complete genome sequences of these two strains. The genome sequences have been publicized and incorporated in the genome taxonomy database (GTDB) (Parks et al., 2018). In the GTDB release 06-RS202, they are both classified in a genus-level taxon (UBA2250) which has no species with validly published name and regarded as representatives of independent species, respectively.

In this study, additional characterizations of strains J5B^T and M52^T were made to determine their taxonomic status in the family *Sterolibacteriaceae*.

Materials And Methods

Isolation and maintenance of strains

The strains J5B^T and M52^T were isolated from a microbial mat obtained from a hot spring in Japan (42° 57' 53" N 141° 09' 47" E). They were isolated into pure cultures under nitrate-reducing conditions, by using bicarbonate and thiosulfate as sole carbon source and electron donor (Watanabe et al, 2019; Ospino et al., 2019). The strains were maintained in the laboratory with a bicarbonate-buffered medium referred to as “S5 medium” (Kojima et al., 2017), supplemented with 10 mM nitrate. Purity and identity of the cultures were periodically checked by microscopic observation and direct sequencing of the 16S rRNA gene.

Cellular fatty acid analysis

For fatty acid analysis, cells of strains J5B^T and M52^T were grown at 45°C, in a medium consisting of the following constituents (l^{-1}): 1 g $NaNO_3$, 0.5 g yeast extract, 0.5 g Casamino acids, 0.5 g disodium fumarate, 0.5 g disodium succinate, and 50 mg cyanocobalamin. Headspace of the culturing bottles was filled with N_2 gas, and pH of the medium was adjusted to 7.0. Their cellular fatty acid profiles were obtained with the Sherlock Microbial Identification System (MIDI) version 6.0 (database; TSBA6). As for J5B^T, cells grown in the S5 medium under oxic conditions were also subjected to the same analysis.

Physiological characterizations

Effects of temperature on growth of strain M52^T were examined by culturing at various temperatures (15, 18, 22, 25, 28, 30, 32, 35, 37, 40, 42, 45, 48, 50, 53, 55, 57 and 60°C), in the medium used for maintenance. The other tests for phenotypic characterization were all conducted at 45°C.

Chemolithoautotrophic growth under nitrate-reducing conditions was tested in the medium used for the maintenance, by replacing thiosulfate with one of electron donors listed below; elemental sulfur (0.5 g l^{-1}), sulfide (2 mM), tetrathionate (10 mM) and hydrogen gas ($H_2/N_2/CO_2$ 50:40:10 v/v/v; 200 kPa in total pressure). Utilization of organic substrates for heterotrophic growth was tested in 10 mM MOPS-NaOH buffer (pH 7.0), supplemented with $NaNO_3$ (1 g l^{-1}), yeast extract (0.1 g l^{-1}), Casamino acids (0.1 g l^{-1}) and cyanocobalamin (50 mg l^{-1}). The medium was dispensed in closed culture bottles and the headspace was filled with N_2 gas. One of the following organic substrates were added to the medium (mM); pyruvate (5), lactate (5), acetate (5), propionate (2.5), succinate (2.5), fumarate (2.5), malate (2.5), butyrate (2.5), benzoate (2.5), isobutyrate (2.5), methanol (5), ethanol (2.5), formate (5), citrate (5), glucose (2.5), xylose (2.5), phenol (2), *o*-cresol (1), *m*-cresol (1).

Effect of pH on growth of strain M52^T was tested with pH-buffered media modified from the medium used for the fatty acid analysis. The media were buffered with 10 mM of MES, MOPS or Tricine, and adjusted to varying pH with NaOH. The tested pH were as follows: 5.4, 5.5, 5.6, 5.8, 5.9, 6.0, 6.1, 6.3, 6.5, 6.7, 6.9, 6.9 and 7.3 with MES; 6.4, 6.6, 6.8, 6.9, 7.1, 7.2, 7.3, 7.4, 7.6, 7.8 and 8.0 with MOPS; 7.0, 7.8, 8.0, 8.2, 8.4, 8.6, 8.8 and 9.0 with Tricine.

Genomic characterization

The complete genome sequences of strains J5B^T and M52^T were obtained in the previous studies (Watanabe et al, 2019; Ospino et al., 2019). As overall genome relatedness indices between them, values of average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) were calculated by using online tools as follows. The ANI was computed by ANI calculator available in EzBioCloud, based on the OrthoANIu algorithm (Yoon et al., 2017). The dDDH value was calculated using Genome-to-Genome Distance Calculator (GGDC) provided by DSMZ (Meier-Kolthoff et al., 2013), applying the formula 2.

The genome sequences were annotated with DFAST (Tanizawa et al., 2018), to identify protein coding sequences and RNA genes. Genes for sulfur oxidation were identified as described previously (Watanabe et al, 2019).

The full sequences of the 16S rRNA gene identified in the genomes were subjected to the blastn search at NCBI against the nucleotide collection (nr/nt) database. Phylogenetic analyses were conducted using the program MEGA version 11 (Tamura et al., 2021). The 16S rRNA gene sequences were aligned with reference sequences, using the MUSCLE algorithm. The references sequences were those of type strains of species with validly published names in the order *Nitrosomonadales* and the genus *Rugosibacter*. The best model for calculation of genetic distances was selected by using the model selection tool in MEGA, as the model with the lowest score of the Bayesian Information Criterion (BIC).

Results And Discussion

Physiological and chemotaxonomic characteristics

The fundamental characteristics of strains J5B^T and M52^T are presented in the species descriptions, and some of them are summarized in Table 1. Their cells grown in the maintenance medium were both motile rods, with width of 0.4–0.5 μm. The cell lengths of strain J5B^T and M52^T were 0.8–2.0 μm and 1.8–3.2 μm, respectively. The strain M52^T grew at 18–55°C, with optimum growth at 50°C. Its pH range for growth was 5.5–8.6, and optimum pH was 6.7–6.9. These characteristics of strain J5B^T have been reported in the previous study (Table 1).

Table 1

Differential characteristics of strains J5B^T, M52^T and type strains of species in the family *Sterolibacteriaceae*. Strains: 1, J5B^T (Watanabe et al., 2019); 2, M52^T (Ospino et al., 2019; this study); 3, *Sterolibacterium denitrificans* Chol-1S^T (Tarlera & Denner, 2003); 4, *Denitratisoma oestradiolicum* AcBE2-1^T (Fahrbach et al., 2006); 5, *Methyloversatilis universalis* FAM5^T (Kalyuzhnaya et al., 2006); 6, *M. thermotolerans* 3t^T (Doronina et al., 2014); 7, *M. discipulorum* FAM1^T (Smalley et al., 2015); 8, *Georgfuchsia toluolica* G5G6^T (Weelink et al., 2009); 9, *Sulfuritalea hydrogenivorans* sk43H^T (Kojima & Fukui, 2011); 10, *Sulfurisoma sediminicola* BSN1^T (Kojima & Fukui, 2014); 11, *Rugosibacter aromaticivorans* Ca6^T (Corteselli et al., 2017). Data were retrieved from respective references.

	1	2	3	4	5	6	7	8	9	10	11
Optimum temp.	45–50	50	30–32	28–30	37	30–37	25–37	25–30	25	30–32	30–34
Temp. range	28–55	18–55	15–35	4–38	10–42	10–45	7–37	20–37	8–32	8–34	20–35
Optimum pH	6.7–7.4	6.6–6.9	7.0	7.0-7.2	8.0	7.0-7.5	6.6-8.0	7.3	6.7–6.9	7.8–8.1	6.5
pH range	5.8–8.7	5.5–8.6	5.8-8.0	6.4–8.5	6.5-9.0	6.5–8.5	8.8-8.0	6.6-9.0	6.4–7.6	6.8–8.8	6.5–7.5
Electron acceptor											
oxygen	+	+	+	+	+	+	+	-	+	+	+
nitrate	+	+	+	+	-	+	+	+	+	+	-
Electron donor											
methanol	-	-	ND	-	+	+	+	-	-	-	ND
ethanol	-	-	-	-	+	+	+	-	-	-	ND
thiosulfate	+	+	ND	-	ND	ND	ND		+	+	ND
hydrogen	-	-	ND	ND	ND	ND	ND	-	+	+	ND
benzoate	-	-	-	-	ND	ND	ND	-	+	-	ND
phenol	-	-	-	-	ND	ND	+	+	-	-	ND
m-cresol	-	-	ND	-	ND	ND	ND	+	ND	-	ND
isobutyrate	-	+	-	+	ND	ND	ND	ND	+	-	ND
pyruvata	+	+	-	+	-	ND	+	-	+	+	ND
lactate	+	+	-	+	ND	ND	ND	-	+	+	-
acetate	+	+	+	+	-	+	+	-	+	+	-

	1	2	3	4	5	6	7	8	9	10	11
propionate	-	+	+	+	ND	ND	ND	-	+	+	-
succinate	+	+	-	+	+	+	+	-	+	+	-
fumarate	+	+	-	+	ND	+	ND	-	+	+	ND
malate	+	+	-	ND	+	ND	+	-	+	+	ND
butyrate	+	+	+	+	ND	ND	ND	-	+	-	ND
glocose	-	-	-	-	+	+	+	-	-	-	ND
xylose	-	-	-	-	ND	ND	ND	-	-	-	ND
citrate	-	-	-	-	+	-	ND	-	-	-	-
formate	-	-	-	-	+	+	+	ND	-	-	+

The strain M52^T was isolated and maintained under thiosulfate-oxidizing conditions, without organic carbon source. Chemolithoautotrophic growth of the strain was also supported by electron donors of tetrathionate and elemental sulfur. On the other hand, hydrogen gas and sulfide did not support growth strain M52^T. As reported previous, strain M52^T can grow on acetate and lactate. The culturing experiments in this study revealed that the following organic acids are also used as growth substrates; pyruvate, propionate, succinate, fumarate, malate, and butyrate and isobutyrate. None of tested sugars, alcohols or cresols supported growth of strain M52^T.

The cellular fatty acid profiles of strains J5B^T and M52^T are shown in Table 2. For strain J5B^T, effects of growth conditions on fatty acid composition was investigated by analyzing cells grown autotrophically and heterotrophically. Under both conditions, $C_{16:0}$ was the most abundant fatty acid, followed by summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$). These two major components accounted for 78–80% of total, irrespective of the culture conditions. The third most abundant fatty acid differed depending on the growth conditions. That was cyclo-$C_{17:0}$ under the heterotrophic condition, whereas that under the autotrophic condition was summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$). The fatty acid profile of strain M52^T was similar to that of J5B^T grown under the same conditions, sharing the major fatty acids (Table 2).

Table 2

Cellular fatty acid profiles of strains J5B^T and M52^T, grown on at 45°C. Minor fatty acids (< 1% in all samples) are not shown. Summed features consist of fatty acids that could not be separated, as follows: summed feature 3, $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; summed feature 8, $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$.

Fatty acid	J5B^T / autotrophic	J5B^T / heterotrophic	M52^T / heterotrophic
$C_{14:0}$	0.98	1.54	3.13
$C_{16:0}$	44.93	51.47	43.97
$C_{18:0}$	1.24	0.90	0.58
$C_{10:0}$ 3OH	2.86	2.70	2.37
cyclo-$C_{18:0}$	5.77	10.87	13.33
Summed feature 3	35.52	26.98	32.61
Summed feature 8	8.40	4.13	3.44

Genomic characteristics

Complete genome sequences of strains J5B^T and M52^T were independently obtained in the previous studies. The values of ANI and dDDH between them were calculated to be 87.93% and 34.3%, respectively. These values are lower than thresholds for species delineation (Richter & Rosselló-Móra, 2009; Meier-Kolthoff et al., 2013), suggesting that strains J5B^T and M52^T are representatives of different species.

As reported previously, strains J5B^T and M52^T are chemolithoautotrophs growing on thiosulfate. According to these physiological properties, genes for sulfur oxidation and carbon fixation were identified in their genomes. As those for sulfur oxidation, genes encoding proteins involved in the Sox-Dsr-Soe pathway were identified in the genome of strain M52^T. The Sox-Dsr-Soe pathway is one of the three core pathways of sulfur oxidation, and shared by sulfur oxidizers in the family *Sterolibacteriaceae*, i.e., *Sulfuritalea hydrogenivorans* sk43H^T, *Sulfurisoma sediminicola* BSN1^T and strain J5B^T (Watanabe et al. 2019). These sulfur oxidizers seem to fix inorganic carbon via the Calvin–Benson–Bassham cycle. They have genes encoding key enzyme of the cycle, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). They all have the *cbbM* gene which encodes form II RuBisCO, whereas the *cbbLS* genes encoding form I RuBisCO were identified only in genomes of strain M52^T and *Sulfurisoma sediminicola* BSN1^T. *Sulfuritalea hydrogenivorans* sk43H^T is known to have key genes for anaerobic aromatics degradation and arsenate respiration (Sperfeld et al., 2019; Watanabe et al. 2017). These genes were not identified in the genomes of strains J5B^T and M52^T.

Taxonomic assignment

The strains J5B^T and M52^T have two copies of the 16S rRNA gene in their genomes, respectively. The copies of strain J5B^T are both 1544 nt in length, and there are five mismatches between their sequences. Those of strain M52^T are different in length, 1544 nt and 1534 nt respectively. These sequences indicated the highest sequence identities to species belonging to family *Sterolibacteriaceae*, but the identities were 95% or lower. The sequence identities between strains J5B^T and M52^T ranged 98.3–98.8%. The sequence variations between the copies of each strain did not affect the phylogenetic analysis described below, because they were located at positions with gaps and thus excluded from the calculation.

Phylogenetic positions of strains J5B^T and M52^T within the order *Nitrosomonadales* are shown in Fig. 1, as the maximum likelihood tree of the 16S rRNA gene. The tree indicated that the strains belong to the family *Sterolibacteriaceae* but not to any existing genera. This means that a novel genus should be created to accommodate strains J5B^T and M52^T. The tree also indicate that the genus *Rugosibacter* should be reclassified to the family *Sterolibacteriaceae*, at least at present. The creation of a novel genus for strains J5B^T and M52^T is supported by the genome-based taxonomy, available in GTDB (release 06-RS202).

Conclusion

On the basis of the results of the previous and present studies, strain J5B^T is proposed as the type strain of a novel species of a new genus, with the name *Sulfuricystis multivorans* gen. nov., sp. nov. In addition, strain M52^T is proposed as the type strain of another species of the novel genus, with the name *Sulfuricystis thermophila* sp. nov. Further, emended description of the genus *Rugosibacter* is also proposed to indicate its affiliation to the family *Sterolibacteriaceae*.

Description of Sulfuricystis **gen. nov.**

Sulfuricystis (Sul.fu.ri.cys'tis. L. neut. n. *sulfur* sulfur; Gr. fem. n. *kystis*, a bag; N.L. fem. n. *Sulfuricystis* sulfur-oxidizing bag).

Grows by oxidation of sulfur compounds. Facultatively anaerobic and neutrophilic. Gram-stain-negative. Major cellular fatty acids are $C_{16:0}$ and $C_{16:1}$ ($C_{16:1}$ω7c and/or $C_{16:1}$ω6c). The type species is *Sulfuricystis multivorans*.

Description of Sulfuricystis multivorans **sp. nov.**

Sulfuricystis multivorans (mul.ti.vo'rans. L. adj. multus many; L. part. adj. vorans devouring, eating; N.L. part. adj.multivorans devouring various substrates).

In addition to properties listed in the genus description, cells are rod-shaped, 0.8–2.0 μm long and 0.4–0.5 μm wide. Uses oxygen and nitrate as electron acceptor. Under nitrate-reducing conditions, grows

chemolithoautotrophically on thiosulfate and elemental sulfur, but not on sulfide, tetrathionate, or hydrogen gas. Grows heterotrophically on pyruvate, lactate, acetate, propionate, succinate, fumarate, malate, and butyrate. Does not grow on benzoate, isobutyrate, methanol, ethanol, formate, citrate, glucose, xylose, phenol, *o*-cresol, and *m*-cresol. Temperature range for growth is 28–55°C, with an optimum of 45–50°C. Growth occurs at pH 5.8–8.7, with an optimum of pH 6.7–7.4. G + C content of genomic DNA of the type strain is 62.4 mol%.

The type strain J5B^T (= BCRC 81386^T = DSM 104688^T = NBRC 112605^T) was isolated from a microbial mat of a hot spring in Japan.

The GenBank/EMBL/DDBJ accession numbers for the chromosome and two plasmids of type strain are AP018718 and AP018719-AP018720, respectively.

Description of Sulfuricystis thermophila **sp. nov.**

Sulfuricystis thermophila (ther.mo.phi'la. Gr. adj. *thermos* hot; Gr. adj. *philos* loving; N.L. fem. adj. *thermophila* heat-loving).

In addition to properties listed in the genus description, cells are rod-shaped, 1.8–3.2 μm long and 0.4–0.5 μm wide. Uses oxygen and nitrate as electron acceptor. Under nitrate-reducing conditions, grows chemolithoautotrophically on thiosulfate, tetrathionate and elemental sulfur, but not on sulfide or hydrogen gas. Grows heterotrophically on pyruvate, lactate, acetate, propionate, succinate, fumarate, malate, and butyrate and isobutyrate. Does not grow on benzoate, methanol, ethanol, formate, citrate, glucose, xylose, phenol, *o*-cresol, and *m*-cresol. Temperature range for growth is 18–55°C, with an optimum of 50°C. Growth occurs at pH 5.5–8.6, with an optimum of pH 6.6–6.9. G + C content of genomic DNA of the type strain is 63.6 mol%.

Type strain M52^T (= BCRC 81387^T = NBRC 114016^T) was isolated from a microbial mat of a hot spring in Japan.

The GenBank/EMBL/DDBJ accession number for the complete genome of the type strain is AP019373.

Emended description of Rugosibacter **(**Corteselli et al., 2017)

Rugosibacter (Ru.go.si.bac'ter. L. adj. *rugosus* wrinkled; N. L. masc. n. *bacter* a rod; N. L. masc. n. *Rugosibacter* a wrinkled rod) Cells are Gram-strain-negative and non-motile. Aerobic. Catalase-negative and oxidase-positive. Heterotrophic growth occurs on organic acids. Predominant fatty acids are summed feature 3 ($C_{16:1}$w7c and/or $C_{16:1}$w7c) and $C_{16:0}$. The major polar lipids are phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and phospholipid. The major respiratory quinone is ubiquinone-8. Phylogenetically, belongs the family *Sterolibacteriaceae*. The type species is *Rugosibacter aromaticivorans*.

Declarations

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Figures

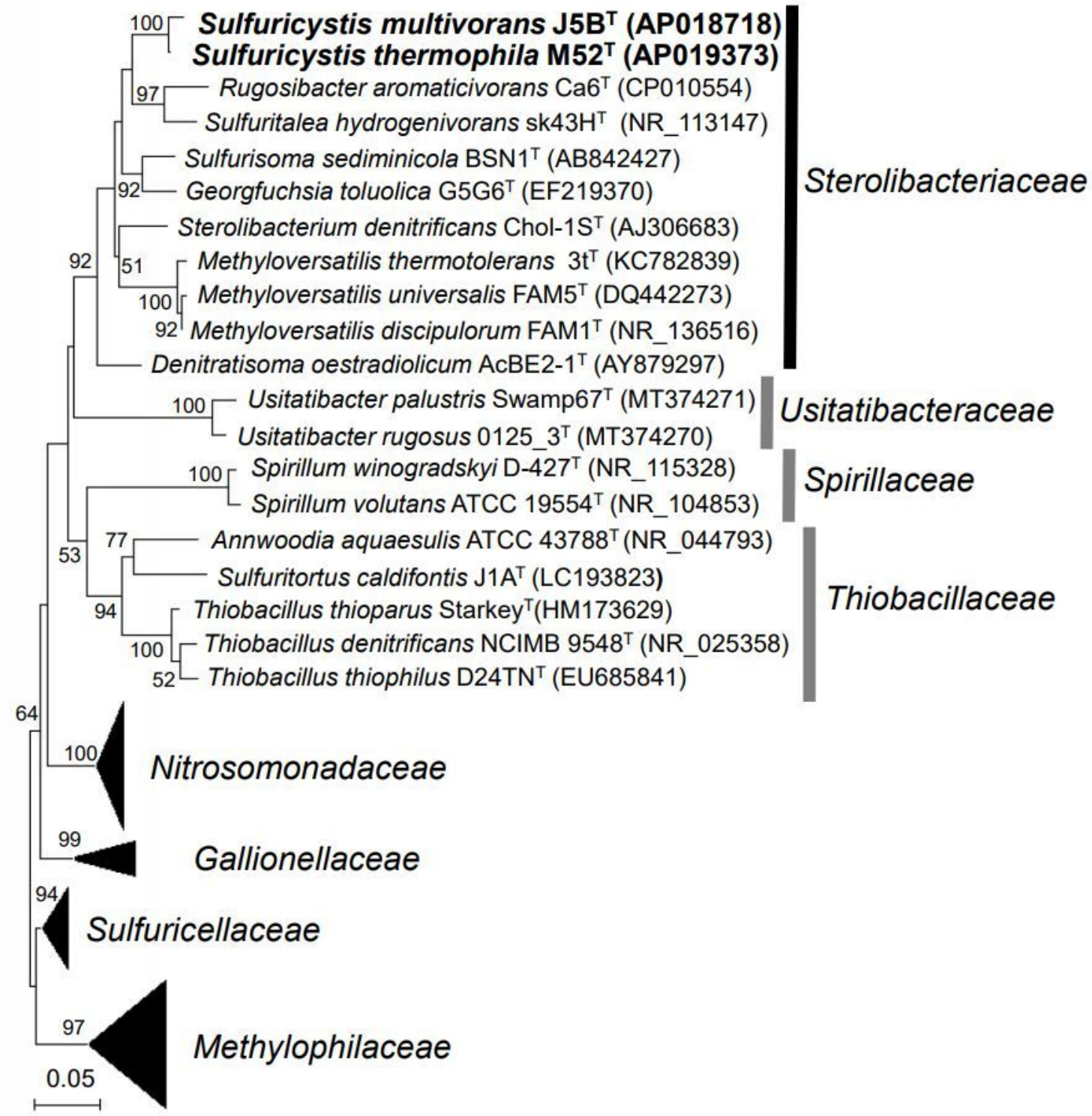

Figure 1

Maximum likelihood tree of the 16S rRNA gene sequences, showing phylogenetic positions of strains J5B^T and M52^T within the order *Nitrosomonadales*. The Kimura 2-parameter model with a discrete gamma distribution and invariable sites was used as the best model with the lowest BIC. All positions containing gaps and missing data were eliminated and there were 1,279 positions in the final dataset. Numbers on nodes represent percentage values of 1,000 bootstrap resampling (values greater than 50 are shown). Names of all strains included in the analysis are shown in full version of the tree provided as Figure S1.

Supplementary Files

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- Fig.S1220330.pdf