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***Desulfoferula mesophilus* gen. nov. sp. nov., a mesophilic sulfate-reducing bacterium isolated from a brackish lake sediment**

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Abstract

A novel sulfate-reducing bacterium (strain 12FAK^T) was isolated from sediment sampled from a brackish lake in Japan. Respiratory growth was observed with formate and pyruvate as an electron donor. Sulfate, thiosulfate, elemental sulfur and dimethyl sulfoxide were utilized as an electron acceptor. The isolate grew over a temperature range of 18–42°C (optimum 35–37°C), a NaCl concentration range of 50–450 mM (optimum 150–300 mM) and a pH range of 6.6–7.5. The 12FAK^T genome consists of a circular chromosome with a length of 4.5 Mbp and G+C content of 63.6%. Based on 16S rRNA gene sequence similarity, the closest cultured relative was *Desulfarculus baarsii* 2st14^T (92.2%). Genome-based phylogenetic analysis placed strain 12FAK^T within the family *Desulfarculaceae* but did not affiliate the strain with any existing genus. Taken together, we propose a novel species of a novel genus, *Desulfoferula mesophilus* gen. nov. sp. nov. with the type strain 12FAK^T (=DSM 115219^T = JCM 39399^T).

Introduction

The family *Desulfarculaceae* in the order *Desulfarculales* was originally described to accommodate the genus *Desulfarculus* (Kuever et al. 2005c; Kuever et al. 2005a; Kuever et al. 2005b; Validation list no 107, 2006). The type species of the genus is *Desulfarculus baarsii* (Widdel 1980; Validation list no 107, 2006), which is represented by the strain 2st14^T isolated from a ditch sediment near the University of Konstanz, Germany (Validation list no 7, 1981; Jansen et al. 1984). It oxidized organic substrates (formate, acetate, propionate, butyrate, isobutyrate, 2-methylbutyrate, valerate, isovalerate and higher fatty acids up to 18 carbon atoms) to CO₂ by reducing sulfate to sulfide for growth (Kuever et al. 2005b). Sulfite and thiosulfate were reported as an alternative electron acceptor of the strain 2st14^T. The 2st14^T cells grown with butyrate and sulfate showed activities of carbon monoxide dehydrogenase and formate dehydrogenase (Schauder et al. 1986). The complete genome sequence of the strain 2st14^T was reported (Sun et al. 2010). The genus *Desulfocarbo* was described as the second genus of the order *Desulfarculales* (An and Picardal 2014). *Desulfocarbo indianensis* is a sole species of the genus, and its type strain SCBM^T was isolated from water extracted from a coal bed in Indiana, USA. The strain SCBM^T grew with oxidation of H₂, formate, acetate, pyruvate, butyrate, fumarate, succinate, palmitate, and benzoate coupled with reduction of sulfate. Thiosulfate was also used as an electron acceptor. The draft genome sequence of the strain SCBM^T was reported (An and Picardal 2015). The genus *Dethiosulfatarculus* was reported as a new genus of the family *Desulfarculaceae*. A sole species *Dethiosulfatarculus sandiegensis* is represented by the type strain SPR^T isolated from a methanogenic consortium degrading long-chain paraffins in a sulfate-free,

bicarbonate-buffered seawater mineral medium (Davidova et al. 2016). The strain SPR^T grew fermentatively using yeast extract, pyruvate and oxaloacetate. Alanine weakly supported its fermentative growth. It was reported that acetate and traces of propionate were detected after growth of the strain SPR^T on pyruvate. Respiratory growth was reported with thiosulfate as an electron acceptor in the presence of pyruvate or H₂ plus CO₂. The genome analysis of the strain SPR^T identified Wood-Ljungdahl pathway, methylmalonyl cycle, and dissimilatory sulfate reduction pathway. The draft genome of SPR^T suggested a partial TCA cycle consisting of *re*-citrate synthase (2.3.3.3), aconitase hydratase (4.2.1.3), isocitrate dehydrogenase (1.1.1.42), succinyl-CoA synthetase alpha subunit (6.2.1.5) and malate dehydrogenase (1.1.1.37). Taken together, emended description of the family *Desulfarculaceae* was made to accommodate the genera *Desulfarculus*, *Desulfocarbo* and *Dethiosulfatarculus* (Davidova et al. 2016).

In this study we report the isolation and characterization of a sulfate-reducing bacterium, designated strain 12FAK^T, whose 16S rRNA gene sequence forms a monophyletic cluster with the members of the family *Desulfarculaceae*.

Materials and Methods

Enrichment and isolation

Strain 12FAK^T was isolated from the sediment of Lake Akkeshi, a brackish lake in Japan. Most of the lake has been covered by eelgrasses (Nakagawa et al. 2018). The sediment sample was taken from a non-eelgrass zone (43°03'40"N, 144°51'35"E) in August 2020. The surface water of the sampling site was characterized using a CastAway-

CTD (SonTek, USA) to have a temperature of 17°C, a specific conductivity of 5.06 mS cm⁻¹, and a practical salinity scale of 32.9. Sediment was sampled using an Eckman-Burge sampler and transferred to a 50 ml polypropylene tube. The sediment sample was stored in cold storage and was inoculated into a bicarbonate-buffered saltwater medium. The medium was composed as follows (l⁻¹): 21 g NaCl, 0.2 g KH₂PO₄, 0.25 g NH₄Cl, 3.4 g MgCl₂·6H₂O, 0.5 g KCl, 0.15 g CaCl₂·2H₂O, 4 g Na₂SO₄, 1 ml trace element solution, 1 ml selenite-tungstate solution, 1 ml vitamin mixture, 1 ml thiamin solution, 1 ml vitamin B₁₂ solution, 30 ml NaHCO₃ solution and 1.5 ml of 1 M Na₂S solution. The trace element solution consisted of as follows (l⁻¹): 12.5 ml 25%-HCl, 2100 mg FeSO₄·7H₂O, 30 mg H₃BO₃, 100 mg MnCl₂·4H₂O, 190 mg CoCl₂·6H₂O, 24 mg NiCl₂·6H₂O, 2 mg CuCl₂·2H₂O, 144 mg ZnSO₄·7H₂O, and 36 mg Na₂MoO₄·2H₂O. The vitamin mixture dissolved 4 mg 4-aminobenzoic acid, 1 mg D(+)-biotin, 10 mg nicotinic acid, 5 mg calcium D(+)-pantothenate and 15 mg pyridoxine dihydrochloride in 100 ml of 10 mM sodium phosphate buffer at pH 7.1. The pH of the medium was adjusted to approximately 7 using 2 M HCl solution. The medium was dispensed into glass bottles, and the gas phase was exchanged with an N₂:CO₂ (80:20) mixture. Before the inoculation, formate and acetate were added into the medium at final concentrations 5 mM and 2 mM, respectively. Preparation of the medium and the aforementioned stock solutions was performed as described previously (Widdel and Bak 1992). Cultivation was performed at 37°C in the dark. After seven subcultures, the culture was serially diluted into 1% agar-solidified medium (agar shake tubes). A reddish-brown colony was transferred into liquid medium, and a second serial dilution in agar shake tubes was performed. Purity was routinely assessed by microscopy and sequencing of 16S rRNA gene fragments amplified by PCR.

Phenotypic and chemotaxonomic characterizations

Strain 12FAK^T was characterized in the medium with 10 mM formate and 2 mM acetate at 37°C unless otherwise specified. The active culture was maintained in the medium with decreased amount of NaCl (13.2 g l⁻¹). Motility and morphology were observed by phase-contrast microscopy (Axioplan 2, Zeiss, Germany). A Gram-stain test was performed using a kit (Fluka, Japan). The cellular fatty acid profile of 12FAK^T grown without acetate was identified according to the Sherlock Microbial Identification System version 6.0 (MIDI, USA) with the Calculation Method MOORE6 (library version 6). Activity of catalase was assessed by pouring 10% H₂O₂ solution onto a fresh cell pellet. Activity of oxidase was tested using oxidase reagent (bioMérieux, France). Growth of the isolate was monitored at 0, 5, 15, 18, 22, 25, 28, 30, 35, 37, 42, 45 and 50°C. Growth was tested in media with varying concentrations of NaCl, 0–500 mM at 50 mM intervals and 500–1000 mM at 100 mM intervals. Growth was tested in bicarbonate-free medium in which pH was adjusted to 5.1, 5.4, 5.6, 6.0, 6.1 with 20 mM MES; 6.3, 6.5, 6.8, 7.0, 7.2 with 20 mM MOPS; 6.6, 7.0, 7.2, 7.5 with 20 mM HEPES; or 6.9, 7.5, 7.9, 8.4 with 20 mM TAPS. Utilization of electron donors was tested by replacing formate and acetate with either 10 mM of formate, acetate, pyruvate, butyrate, valerate, fumarate, succinate, propionate, DL-lactate, malate, citrate, D-glucose, L-arabinose, methanol, ethanol, glycerol, 2.5 mM of benzoate or 1 g l⁻¹ of yeast extract. Growth on H₂ as an electron donor was tested by pressurizing the headspace with 1 atm of H₂ in the presence or absence of 2 mM acetate. Utilization of electron acceptors was assessed in the sulfate-free medium containing either 14 mM thiosulfate, 3 mM sulfite, 2.5 g l⁻¹ elemental sulfur, 15 mM

dimethyl sulfoxide (DMSO), 5 mM nitrate or 3 mM nitrite. Fermentative growth of the strain was assessed by replacing formate and acetate in the sulfate-free media with one of the organic substances for the electron donor test other than H₂.

Genomic characterization

Genomic DNA was extracted with a Wizard Genomic DNA Purification Kit (Promega, USA). Near full-length 16S rRNA gene fragments were amplified with the primer set 27F and 1492R (Lane 1991) and the amplicons were sequenced. Whole genome sequencing was performed using two R9.4.1 Flongle flow cells via a MinION sequencer (Oxford Nanopore Technologies, UK). Library preparation was performed using the Ligation Sequencing Kit (SQK-LSK109), and basecalling was performed using Guppy version 4.3.4 with the High Accuracy model. In addition to Flongle (long read) sequencing, 2x200 bp short read whole genome sequencing data were generated using a DNBSEQ-G400 system (MGI Tech, China). Genomic DNA was sheared using a Focused-ultrasonicator (Covaris, USA), and library preparation was performed using the MGIEasy PCR-Free DNA Library Prep Set (MGI Tech) and MGIEasy Circularization Kit (MGI Tech). Shearing, library preparation, and sequencing were performed by the Bioengineering Lab Co. (Sagamihara, Japan).

Following sequencing, long read data shorter than 1 kb in length were discarded using *reformat.sh* from BBMap version 38.18 (Bushnell B. – sourceforge.net/projects/bbmap). For short read data, the ‘qc’ module of ATLAS version 2.8.1 (Kieser et al., 2020) was used to perform quality control, including adapter removal and quality trimming steps. Sequencing adapters used during library preparation for DNBSEQ were added to the

adapters.fa file, included with ATLAS, to guide adapter removal (R1 adapter: ‘AAGTCGGAGGCCAAGCGGTCTTAGGAAGACAA’; R2 adapter: ‘AAGTCGGATCGTAGCCATGTCGTTCTGTGAGCCAAGGAGTTG’). The long and short read data were then subjected to hybrid assembly using Unicycler version 0.4.8 (Wick et al. 2017), which relied on SPAdes version 3.13.0 (Prjibelski et al. 2020). The resulting complete genome was annotated using DFAST version 1.2.15 (Tanizawa et al. 2018).

The taxonomic position of the complete genome in the Genome Taxonomy Database (GTDB) release 06-RS202 was determined using GTDB-Tk version 1.5.1 (Chaumeil et al. 2020). Average nucleotide identity (ANI) was calculated by ANI Calculator (Yoon et al. 2017). Digital DNA–DNA hybridization (dDDH) values were calculated using Type Strain Genome Server (TYGS) with the recommended settings of the Genome-to-Genome Distance Calculator (GGDC) based on formula 2 (Meier-Kolthoff et al. 2013; Meier-Kolthoff et al. 2022).

Phylogenetic analysis of the 16S rRNA gene was performed with MEGA version 11 (Tamura et al. 2021). To construct the phylogenetic tree, reference sequences (112 in total) were obtained from type strains of species with validly published names under the order *Desulfuromonadales* and families *Desulfobulbaceae*, *Desulfarculaceae* and *Syntrophobacteraceae*, based on the List of Prokaryotic Names with Standing in Nomenclature (LPSN), in March 2023. Sequences from type species of each genus (42 in total) were subjected to further analysis. Two environmental sequences with accession numbers HE797781 and FJ189563 were retrieved from National Center for Biotechnology Information (NCBI) and included for further analysis, because they were

the most closely related environmental sequences to strain 12FAK^T (99.4% identity) and *D. baarsii* 2st14^T (95.1% identity). The resulting set of 44 sequences was aligned using CLUSTALW version 2.1 (Larkin et al. 2007), and evolutionary history was inferred, based on this alignment, using the Neighbor-Joining method with 1000 bootstrap resampling (Felsenstein 1985; Saitou and Nei 1987) and Maximum Likelihood method (Tamura and Nei 1993) with 1000 bootstrap resamplings. Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are expressed as the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1200 comparable positions in the final dataset.

In addition to 16S rRNA gene-based phylogenetic analysis, a phylogeny based on conserved protein sequences was constructed using GToTree version 1.6.12 (Lee 2019). Alongside the three existing genomes from all species within the family *Desulfarculaceae*, we selected genomes from the type species of the type genera of nine other families. Additionally, three genomes were selected from three genera within the family *Syntrophobacteraceae*, which lacks a genome from its type genus's type species. Predicted proteins from these genomes, together with the genomes of strain 12FAK^T and an outgroup species, were searched for 74 single-copy conserved proteins within the “Bacteria.hmm” set defined in GToTree. Alignment of protein hits, masking of sequence alignments, and concatenation of sequence alignments was performed using default settings in GToTree. IQ-TREE version 2.2.0-beta was used to construct a phylogeny (Minh et al. 2020), using the concatenated sequence alignment, via the LG+F+R4 evolutionary model.

Results

Physiological and chemotaxonomic characteristics

Cells of strain 12FAK^T were motile, Gram-stain-negative, oval shaped (Fig. S1), 1.5–2.0 µm in length, 0.6–0.8 µm in width. Catalase and oxidase tests were both negative. The major cellular fatty acids were *anteiso*-C_{15:0} (50.9%), summed feature 10 (C_{18:1ω7c} and unknown, 18.9%), C_{18:0} (8.4%), C_{16:0} (7.7%) and C_{15:0} (5.6%) (Table S1). Growth of the isolate was observed over a temperature range of 18–42°C, a NaCl concentration range of 50–450 mM, and a pH range of 6.6–7.5. Its optimum growth was observed at 35–37°C and pH 6.9–7.2 in the presence of 150–300 mM NaCl.

Respiratory growth of the isolate was observed with formate and pyruvate as an electron donor. During growth with formate, sulfide was detected in the culture supernatant by the colorimetric method (Cline 1969). Respiratory growth of the strain 12FAK^T was not observed with the other organic substrates tested or with H₂. Strain 12FAK^T used sulfate, thiosulfate, elemental sulfur and DMSO as electron acceptors for growth. Fermentative growth was not observed with organic substrates tested. Table 1 summarizes differences in energy metabolism of strains in the family *Desulfarculaceae*. Within the family, fermentative growth is currently only known to be performed by *Dethiosulfatarculus sandiegensis* SPR^T, which is the sole bacterium incapable of dissimilatory sulfate reduction in the family.

Genomic characteristics and phylogenetic position

The complete genome of the strain 12FAK^T is 4,479,191 bp long and consists of a single circular chromosome with a G+C content of 63.6%. The genome of strain 12FAK^T includes all required genes for the Wood-Ljungdahl pathway. Genes for the citric acid cycle were all detected except the citrate synthase gene. We identified a putative gene encoding *Re*-citrate synthase (FAK_10550), which is related to isopropylmalate synthase rather than the common *Si*-citrate synthase (Li et al. 2007). Some prokaryotes encode *Re*-citrate synthase rather than *Si*-citrate synthase and synthesize citrate from acetyl-CoA and oxaloacetate, such as *Ignicoccus hospitalis* (Jahn et al. 2007).

The 16S rRNA gene sequence of the strain 12FAK^T showed the highest identity (92.2%) against that of *Desulfarculus baarsii* 2st14^T and formed a robust cluster with an environmental sequence (HE797781) derived from anoxic brackish photosynthetic biofilms, as indicated by the GenBank record. This cluster was distinct from the others in phylogenetic trees constructed using the Maximum Likelihood method (Fig. 1) and Neighbor-Joining method (not shown). Phylogenetic analysis based on conserved protein sequences affiliated strain 12FAK^T with the family *Desulfarculaceae* but not with any existing genus (Fig. S2). At least 95% of the 74 conserved proteins were identified for each genome used in the phylogeny. Values of ANI and dDDH were 73.3% and 18.6% (12FAK^T–*D. indianensis* SCBM^T), 72.1% and 17.7% (12FAK^T–*D. baarsii* 2st14^T), and 69.2% and 18.2% (12FAK^T–*D. sandiegensis* SPR^T). The GTDB-Tk assigned strain 12FAK^T to the family *Desulfarculaceae* but not to any existing genus.

Conclusions

12FAK^T utilized formate and pyruvate but not H₂, acetate, benzoate, propionate, butyrate and other fatty acids as an electron donor. Unlike the other members in the family *Desulfarculaceae*, the novel strain was oval-shaped and utilized elemental sulfur as an electron acceptor. As determined by 16S rRNA gene- and genome-based phylogenetic analyses, the novel strain belongs to the family *Desulfarculaceae* but not any existing genus. On the basis of phylogenetic and phenotypic properties, we propose a novel species of a novel genus *Desulfoferula mesophilus* gen. nov., sp. nov., for the strain 12FAK^T.

Description of *Desulfoferula* gen. nov.

Desulfoferula (De.sul.fo.fe'ru.la. L. pref. *de* from; L. n. *sulfur* sulfur; L. fem. n. *ferula* a stick, cane; N.L. fem. n. *Desulfoferula* a sulfate-reducing stick).

Cells are motile, Gram-stain-negative and non-spore forming. Respires sulfate. Is a member of the family *Desulfarculaceae*. Within the family *Desulfarculaceae*, utilization of elemental sulfur as an electron acceptor is confirmed. The type species is *Desulfoferula mesophilus* sp. nov.

Description of *Desulfoferula mesophilus* sp. nov.

Desulfoferula mesophilus (me.so'phi.lus. N.L. masc. adj. *mesophilus* mesophilic, referring to the mesophilic temperature optimum of the bacterium).

Cells are oval-shaped, 1.5–2.0 µm in length, 0.6–0.8 µm in width and non-spore-

forming. Strictly anaerobic. Gram-stain-negative. Catalase-negative and oxidase-negative. Growth occurs with formate and pyruvate as electron donors in the presence of sulfate as an electron acceptor. Acetate, butyrate, valerate, fumarate, succinate, propionate, DL-lactate, malate, citrate, D-glucose, L-arabinose, methanol, ethanol, glycerol, benzoate, yeast extract and H₂ are not utilized as growth substrates. Sulfate, thiosulfate, elemental sulfur and DMSO are respired. Not fermentative. Growth is observed at a temperature range of 18°C–42°C with optimum growth at 35–37°C. The pH range for growth is 6.6–7.5, and the optimum pH was 6.9–7.2. Grows within a NaCl concentration range of 50–450 mM, with the optimum range 150–300 mM NaCl. The G+C content of genomic DNA of the type strain is 63.6%.

The type strain 12FAK^T (= DSMZ 115219^T = JCM 39399^T) was isolated from sediment of Lake Akkeshi, a brackish lake in Japan.

The complete genome sequence of strain 12FAK^T, along with raw sequence read data, is available at NCBI/EMBL/DDBJ under BioProject PRJDB16193.

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Competing Interests

Authors disclose no competing financial or non-financial interests that are directly
or indirectly related to the work submitted for publication.

Author Contributions

Tomohiro Watanabe and Manabu Fukui directed the study. Sediment sample was
collected by Tatsuya Yabe and Manabu Fukui. Tatsuya Yabe enriched and isolated the
novel strain, and the strain was characterized by Tomohiro Watanabe and Tatsuya Yabe.
Genome sequencing and analysis were performed by Tomohiro Watanabe and Jackson
Tsuji. The manuscript was written by Tomohiro Watanabe in consultation with all authors.

Table 1. Differences in energy metabolism in the family *Desulfarculaceae*. All members of the family showed no growth on ethanol, lactate and malate as an electron donor. Strains: 12FAK^T (in this study); *Desulfarculus baarsii* 2st14^T (Widdel 1980; Devereux et al. 1989; Kuever et al. 2005b; Kuever 2014; Galushko and Kuever 2019); *Desulfocarbo indianensis* SCBM^T (An and Picardal 2014); *Dethiosulfatarculus sandiegensis* SPR^T (Davidova et al. 2016). nr, not reported.

	12FAK ^T	2st14 ^T	SCBM ^T	SPR ^T
Morphology				
Shape	Oval	Vibrio	Vibrio	Vibrio
Length (µm)	1.5–2.0	1.5–4.0	1.5–2.4	nr
Width (µm)	0.6–0.8	0.5–0.7	0.4–0.5	nr
Mobility	+	+	+	+
Flagellation	nr	+	+	+
Genomic characteristics				
Genome size (bp)	4,479,191	3,655,731	5,114,044	5,940,380
Number of scaffolds	1	1	334	83
G+C content (%)	63.6	65.7	63.0	52.0
Growth conditions				
Temperature (optimum) (°C)	18–42 (35–37)	20–39 (35–39)	16–37 (30)	15–45 (30–37)
pH (optimum)	6.6–7.5 (nr)	6.5–8.2 (7.3)	6.5–8.0 (7.0)	5.8–7.2 (6.0–7.2)
NaCl (optimum) (mM)	50–450 (150–300)	0–nr (120–342)	0–300 (nr)	171–1369 (513–1027)
Electron donor				

H ₂	–	–	+	+
Formate	+	+	+	–
Acetate	–	+	+	–
Propionate	–	+	–	–
Butyrate	–	+	+	–
Palmitate	nr	+ ^a	+	–
Pyruvate	+	–	+	+
Fumarate	–	–	+	–
Succinate	–	–	+	–
Benzoate	–	–	+	nr
<i>Electron acceptor</i>				
Sulfate	+	+	+	–
Sulfite	–	+	–	–
Thiosulfate	+	+	+	+
Elemental sulfur	+	–	–	–
DMSO	+	nr	–	–
Fermentative growth	–	–	–	+
<i>Isolation source</i>	Brackish lake sediment, Japan	Freshwater ditch sediment, Germany	Coal-bed water, USA	Paraffin-degrading consortium enriched from marine sediments, USA

^a It was described that the strain 2st14^T utilizes higher fatty acids up to 18 carbon atoms (Kuever et al. 2005b).

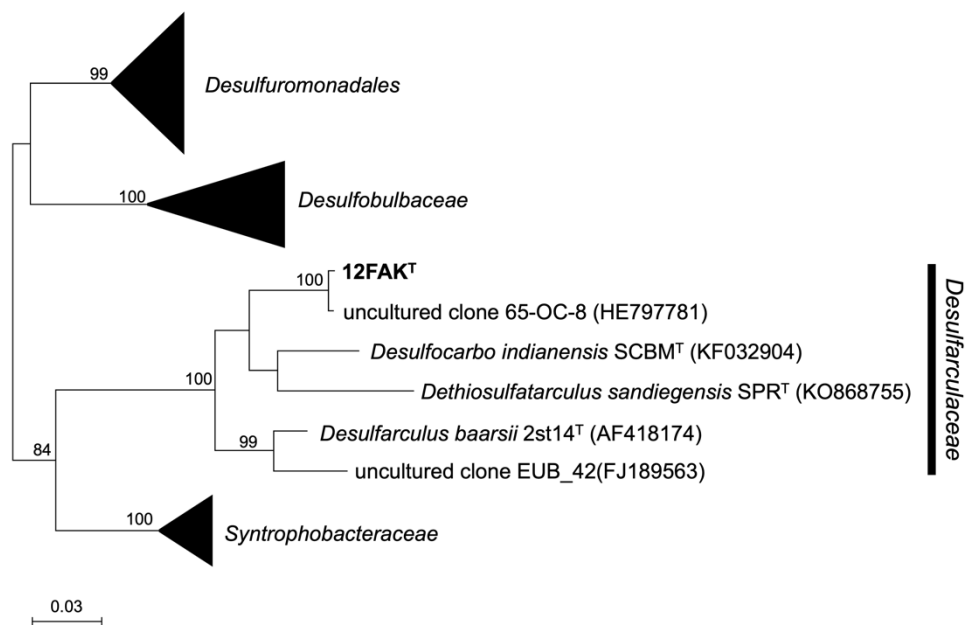


Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences. The tree was constructed using the Maximum Likelihood method with 100 bootstrap resamplings. Bootstrap values higher than 50% are shown. Bar, 0.03 substitutions per nucleotide position.

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