Thermodesulfobium acidiphilum sp. nov., a thermoacidophilic, sulfate-reducing, chemosynthetic bacterium from a thermal site

Evgenii N. Frolov,1* Ilya V. Kublanov,1 Stepan V. Toshchakov,2 Nazar I. Samarov,2 Andrei A. Novikov,3 Alexander V. Lebedinsky,1 Elizaveta A. Bonch-Osmolovskaya1 and Nikolai A. Chernyh1

Abstract

An obligately anaerobic, sulfate-reducing micro-organism, strain 3127-1T, was isolated from geothermally heated soil (Oil Site, Uzon Caldera, Kamchatka, Russia). The new isolate was a moderately thermoacidophilic anaerobe able to grow with H2 or formate by respiration of sulfate or thiosulfate. The pH range for growth was 3.7–6.5, with an optimum at 4.8–5.0. The temperature range for growth was 37–65°C, with an optimum at 55°C. The G+C content of the genomic DNA was 33.7 mol%. The genome of strain 3127-1T contained two almost identical 16S rRNA genes, differing by a single nucleotide substitution. The closest 16S rRNA gene sequence of a validly published species belonged to Thermodesulfobium narugense Na82T (99.5% similarity). However, the average nucleotide identity of the genomes of strain 3127-1T and T. narugense Na82T and the predicted DNA–DNA hybridization value (GGDC 2.1 BLAST+, formula 2) were as low as 86 and 32.5±2.5 %, respectively. This, together with phenotypic data, showed the new isolate to belong to a novel species, for which the name Thermodesulfobium acidiphilum sp. nov. is proposed. The type strain is 3127-1T (=DSM 102892T=VKM B-3043).

Sulfate-reducing prokaryotes (SRPs) play an important role in both the sulfur and the carbon cycles on Earth using low-molecular-weight organic compounds or H2 as the electron donors for hydrogen sulfide production. The SRP metabolic group includes representatives of five phylogenetic lineages of Bacteria (Deltaproteobacteria, Clostridia, Nitrospirae, Thermodesulfobacteria and Thermodesulfobiaceae) and a few representatives of the archaeal phyla Euryarchaeota and Crenarchaeota [1]. The family Thermodesulfobiaceae was proposed in 2003 to accommodate the genus Thermodesulfobium with a single species, Thermodesulfobium narugense [2]. According to the current taxonomy [3], the family Thermodesulfobiaceae is included in the class Clostridia, although phylogenetic studies show that it may deserve a higher taxonomic status [4, 5]. In this paper, we report the isolation and characterization of a novel anaerobic, chemolithoautotrophic species of the genus Thermodesulfobium isolated from an acidic thermal site on the Kamchatka peninsula and belonging to a physiological group of acido-philic SRP that are not very common [6].

Strain 3127-1T was isolated from geothermally heated soil sampled at the Oil Site, Uzon Caldera, Kronotsky Nature Reserve, Kamchatka, Russia (54° 30.023’ N 160° 00.088’ E, elevation 654 m) in August 2014. A description of the sampling site and its main biogeochemical features were provided elsewhere [6, 7]. Conditions at the sampling site were 60°C, pH 4.8 and Eh +70 mV. Samples were taken anaerobically in tightly stoppered bottles and transported to the laboratory. An enrichment culture was initiated by inoculating the sample [10 % (w/v)] into anaerobically prepared sterile (121°C, 1 h) liquid medium of the following composition (g l–1): NH4Cl, 0.33; KCl, 0.33; MgCl2·6H2O, 0.33; CaCl2·6H2O, 0.33; KH2PO4, 0.33; Na2SO4, 2.8; Na2S·9H2O, 0.5; trace element solution [8], 1 ml; vitamin solution [9], 1 ml; resazurin, 1.0 mg l–1. Sodium sulfide was used as a reducing agent. Resazurin was added as a redox indicator. To adjust the pH of the medium to 4.8, 3 M HCl was added. The medium was dispensed in 5 ml portions into 17 ml Hungate tubes; the head space was filled with H2/CO2 (4 : 1, 151.9 kPa). After 4 days of incubation at 60°C, a pronounced production of sulfide accompanied by microbial growth was observed. The enrichment culture was transferred several times to a fresh medium of the same composition, and then to the same medium solidified with 2 % agar.
(Difco 0140-01). Single red-brown colonies about 2 mm in diameter formed after 2 weeks of incubation. After a second transfer on the solidified medium, a uniformly shaped axenic culture designated strain 3127-1T was obtained. The purity of the culture was confirmed by 16S rRNA gene sequencing and complete genome sequencing.

Cell growth and cell morphology were studied using an Olympus CX-41 phase-contrast microscope. Cells of strain 3127-1T were rod-shaped, 0.5 µm in diameter and 1–5 µm in length, growing singly or in pairs. Stationary-phase cells were non-motile, but motile cells were observed by phase-contrast microscopy in the early exponential phase of growth, and a subpolar flagellum was detected by transmission electron microscopy of whole cells (Fig. 1a). Spore formation was not observed. The fine structure of the cells was studied using a JEM-100 electron microscope. Negative staining of whole cells was performed with 2% phosphotungstic acid. Ultrathin sections of cells of strain 3127-1T revealed a Gram-stain-negative cell wall type with an outer membrane (Fig. 1b). Neither storage compounds nor extensive internal membranes were observed.

Growth experiments were performed in duplicate using Hungate tubes with medium of the same composition as used for the pure culture isolation procedure. To determine optimal growth conditions, strain 3127-1T was cultivated anaerobically at various temperatures (30–70 °C) and pH values [3.5–6.83, adjusted with solutions of HCl (3 M) or NaOH (5%, w/v); both initial and final pH values were measured, and the changes resulting from the culture growth were insignificant]. Strain 3127-1T was a strictly anaerobic bacterium: no growth occurred in sodium sulfide-free medium. The temperature range for growth was 37–65 °C, with an optimum at 54 °C. No growth was detected at 70 °C or above, as well as at 35 °C or below after incubation for 2 weeks. The pH range for growth was 3.7–6.5, with optimal growth at pH 4.8–5.0. Growth was not observed at and below pH 3.5 and at and above pH 6.8. To test the reaction of the new isolate to NaCl concentration, it was anaerobically cultivated at NaCl concentrations from 0 to 30 g l−1 with intervals of 5 g l−1. The isolate grew at salt concentrations of up to 1.0% (w/v) NaCl, but optimal growth was observed if no NaCl was added to the basal medium, where concentrations of Na+ and Cl− were 0.1% and 0.06%, respectively. No growth was observed at and above 1.5% (w/v) NaCl. The doubling time under optimal growth conditions (60 °C, pH 5.0, no addition of NaCl) was 16 h. The strain was sensitive to benzylpenicillin, ampicillin, novobiocin, chloramphenicol, kanamycin, streptomycin, oxacillin, neomycin and polymyxin (all at 50 µg ml−1), but resistant to vancomycin (at 50 and 100 µg ml−1).

The utilization of substrates was tested with a CO2-filled headspace and sulfate as the electron acceptor, at 60 °C. The utilization of electron acceptors was tested in medium without sulfate, under H2/CO2 at 60 °C. Soluble substrates and electron acceptors were added from sterile anaerobic stock solutions before inoculation. Insoluble substrates and electron acceptors were added into tubes with liquid medium prior to sterilization. Utilization of substrates or electron acceptors was recognized by the increase in optical density and decrease in electron donor or acceptor content. Sulfide was measured colorimetrically with N,N-dimethyl-p-phenylenediamine [10]. Sulfate was analysed with a Stayer ion chromatograph (Aquilon) equipped with an IonPack AS4-ASC column (Dionex) and conductivity detector; the eluent was bicarbonate (1.36 mM)/carbonate (1.44 mM) at a flow rate of 1.5 ml min−1. H2 and CO2 were analysed with a Crystal 5000.2 chromatograph (Chromatec) equipped with an NaX zeolite 60/80 mesh 3 m × 2 mm column (Chromatec) for H2 and an Haysep Q 60/100 mesh 3 m × 2 mm column (Chromatec) for CO2. Strain 3127-1T grew optimally chemolithoautotrophically with H2 as the energy source, HCO3-/CO2 as the carbon source and sulfate as the electron acceptor. Sulfide was the sole product of sulfate reduction. In the presence of sulfate, the isolate also used formate (20 mM) as the electron donor, but the growth yield was significantly lower than that with H2. We failed to detect any alcohols or volatile fatty acids on medium with formate or hydrogen as electron donors. Strain 3127-1T was unable to utilize yeast extract, peptone (both 1 g l−1), glucose, lactose, maltose, sucrose (5 mM each), acetate, lactate, pyruvate, malate, propionate, butyrate, fumarate, succinate, citrate, ethanol, propanol, glycerol, methanol (20 mM each) or CO (N2/CO=1:1, 4:1 or 20:1, v/v). With H2/CO2 (80:20), strain 3127-1T utilized thiosulfate (10 mM), but not nitrate (10 mM), nitrite (5 mM), sulfate (5 mM), elemental sulfur (10 g l−1), Fe(III) citrate (10 mM), fumarate (10 mM) or O2 (2 or 5%) as electron acceptors.

Cellular fatty acid (CFA) profiles were determined by GC-MS (Thermo Scientific Trace GC Ultra DSQ II, HP-5MS column, E70 eV) of methyl ester derivatives prepared from 5 mg of freeze-dried cell material treated by anhydrous HCl/MeOH [2]. CFA contents were determined as percentages of the total ion current peak area. Analysis of the CFAs revealed C16:0 (42.4% of the total fatty acids) as the major component. The following fatty acids were also detected: C18:0 (18.8%), C19:0Δ (13.6%), C21:0Δ (4.9%), C20:0 (4.9%), C12:0 (4.7%), C18:1ω9 (4.2%), C14:0 (3.4%), C22:0 (2.1%), C10:0 (0.5%) and C15:0 (0.5%). Polar lipids of...
strain 3127-1\(^T\) determined by two-dimensional TLC as described earlier [11] included phosphatidylglycerol, an unidentified phosphoglycerolipid and six more unknown lipids detected by phosphomolybdic acid only. Respiratory lipiquiones were extracted with cold acetone from cells disrupted by grinding in liquid N\(_2\) and further separated by TLC. The excised bands were analysed by tandem MS (LCQ ADVANTAGE MAX) and the compounds were identified by their ionized masses. The dominant quinone species was identified as menaquinone MK-7. MK-8 and MK-9 were detected as minor fractions.

The genome of strain 3127-1\(^T\) was sequenced using an Illumina Miseq platform and assembled into a single chromosome using Spades 3.6.0 [12]. The \textit{in silico} determined G+ C content of the genomic DNA of strain 3127-1\(^T\) was 33.7 mol\%. The genome encoded two 16S rRNA genes with a single nucleotide polymorphism. A \textit{BLASTN} search against 16S rRNA gene sequences of validly published micro-organisms using the \textit{EZTaxon-e} server [13] revealed that either of the isolate 3127-1\(^T\) 16S rRNA gene sequences was 99.5\% similar to either of the two 100\% identical genes of \textit{T. narugense} Na82\(^T\). Next, \textit{BLAST} hits were 83–81\% similar to the query sequences and mainly belonged to various \textit{Firmicutes} species, but also to representatives of the phyla \textit{Synergistetes} and \textit{Nitrospirae}. The average nucleotide identity of the genomes of strain 3127-1\(^T\) and \textit{T. narugense} Na82\(^T\), determined with the ANI calculator (http://enve-omics.ce.gatech.edu/ani/), was 86\%, which is far below the 95\% species-delimiting value [14, 15]. The \textit{in vitro} DNA–DNA hybridization value between strain 3127-1\(^T\) and \textit{T. narugense} Na82\(^T\) that was predicted by GGDC 2.1 \textit{BLAST+} ([16], http://ggdc.dsmz.de/distcalc2.php) using the recommended formula 2 was 32.5 ±2.5\% (the currently accepted species-delimiting value being 70\% [17]).

A comparison of the proteins encoded by the genomes of strain 3127-1\(^T\) and \textit{T. narugense} Na82\(^T\) was performed, after their annotation with \textit{RAST} [18], by using the \textit{SEED} [18] Sequence Based Comparison tool and then an ad hoc script written to average the results of the comparison of individual proteins by \textit{SEED}. This analysis produced an average value of 90\% amino acid sequence identity for the proteins shared by strain 3127-1\(^T\) and \textit{T. narugense} Na82\(^T\).

About 5\% of the proteins encoded in the genome of strain 3127-1\(^T\) and about 8\% of the proteins encoded in the genome of \textit{T. narugense} Na82\(^T\) had no homologues in the counterpart genome.

The new isolate was compared with \textit{T. narugense} Na82\(^T\), the only validly published member of this genus, isolated from Narugo hot spring [2]. Both organisms had rod-shaped cells with a Gram-stain-negative type of cell wall and were thermophilic, obligate anaerobes capable of growing autotrophically by the oxidation of H\(_2\) or formate with sulfate or thiosulfate. The DNA G+C contents of \textit{T. narugense} Na82\(^T\) and strain 3127-1\(^T\) were similar: 35.1 (33.9 mol % according to our \textit{in silico} genome analysis) and 33.7 mol %, respectively. However, comparisons of metabolic and physiological characteristics of the two strains revealed a few significant differences (Table 1). While \textit{T. narugense} Na82\(^T\) could use nitrate and nitrite as the terminal electron acceptor, strain 3127-1\(^T\) could not. \textit{T. narugense} Na82\(^T\) showed no motility under the microscope, while motile cells of strain 3127-1\(^T\) were observed in the early exponential phase of growth, and a subpolar flagellum was detected. Both strain 3127-1\(^T\) and \textit{T. narugense} Na82\(^T\) are thermoacidophilic sulfate reducers, but the new isolate has a lower pH growth optimum.

Thus, isolate 3127-1\(^T\) differed from \textit{T. narugense} in the above-mentioned phenotypic features; the average nucleotide identity of the genomes of strain 3127-1\(^T\) and the \textit{T. narugense} type strain, as well as the \textit{in vitro} DNA–DNA hybridization value between these strains predicted by GGDC 2.1 \textit{BLAST+} using the recommended formula 2, were considerably below the species-delimiting values. Therefore, it is suggested that strain 3127-1\(^T\) should be assigned to a novel species, for which the name \textit{Thermodesulfofibium acidiphilum} sp. nov. is proposed.

**DESCRIPTION OF \textit{THERMODESULFOFIBIUM ACIDIPHILUM} SP. NOV.**

\textit{Thermodesulfofibium acidiphilum} [a.ci.di’phi.lum. N.L. n. acidum (from L. adj. acidus sour) an acid; N.L. neut. adj. philum (from Gr. adj. philos -ê·on) friend, loving; N.L. neut. adj. acidiphilum acid-loving].

Cells are single rods, 0.5 µm in diameter and 1–5 µm in length. Stationary-phase cells are non-motile, while in the early exponential phase of growth motile cells can be observed, and a subpolar flagellum can be detected using electron microscopy. Spore formation is not observed. Moderately thermoacidophilic. Chemolithotrophic and strictly anaerobic. Growth occurs between 37 and 65°C, with an optimum at 54°C. The pH range for growth is 3.7–6.5, with an optimum pH of 4.8–5.0. Growth does not occur above 1 % (w/v) NaCl. The doubling time is 16 h under optimum growth conditions. Sulfate and thiosulfate

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>\textit{Strain 3127-1}(^T) (current study)</th>
<th>\textit{T. narugense} Na82(^T) [2]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimensions (µm)</td>
<td>0.5×1–5</td>
<td>0.5×2–5</td>
</tr>
<tr>
<td>Motility</td>
<td>Variable</td>
<td>–</td>
</tr>
<tr>
<td>pH range</td>
<td>3.7–6.5</td>
<td>4.4–6.5</td>
</tr>
<tr>
<td>pH optimum</td>
<td>4.8–5.0</td>
<td>5.5–6.0</td>
</tr>
<tr>
<td>Electron acceptors:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrate</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Nitrite</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>G+C content of DNA (mol%)</td>
<td>33.7</td>
<td>35.1 (33.9)*</td>
</tr>
</tbody>
</table>

*Our \textit{in silico} genome analysis.
are used as electron acceptors, but not sulfite, elemental sulfur, Fe(III), fumarate, nitrate, nitrite or O2. Electron donors utilized in the presence of sulfate or thiosulfate are H2 and formate. No growth occurs with yeast extract, peptone, glucose, lactose, maltose, sucrose, acetate, lactate, pyruvate, malate, propionate, butyrate, fumarate, succinate, citrate, ethanol, propanol, glycerol, methanol or CO2, either in the absence or in the presence of sulfate or thiosulfate. The major cellular fatty acid is C16:0. Minor components are C18:0, C19:0, C16:0, C12:0, C20:0, C18:1ω9c, C14:0, C22:0, C10:0 and C15:0. The dominant respiratory lipoquinone is MK-7.

The type strain is 3127-1T (=DSM 102892T=VKM B-3043T), isolated from geothermally heated soil sampled at the Oil Site, Uzon Caldera, Kronotsky Nature Reserve, Kamchatka, Russia. The G+C content of the genomic DNA of the type strain is 33.7 mol%.

Funding information
This work was supported by grant 14-24-00165 from the Russian Science Foundation. CFA and polar lipid assays were supported by President of Russia grant MK-4530.2015.4.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The manuscript does not contain description of experimental work with humans or experiments conducted using animals.

References

Five reasons to publish your next article with a Microbiology Society journal
1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as ‘excellent’ or ‘very good’.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.