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TAXONOMIC DESCRIPTION

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Pyrobaculum igneiluti sp. nov., a novel anaerobic hyperthermophilic archaeon that reduces thiosulfate and ferric iron

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Abstract

A novel anaerobic, hyperthermophilic archaeon was isolated from a mud volcano in the Salton Sea geothermal system in southern California, USA. The isolate, named strain 521^T, grew optimally at 90 °C, at pH 5.5–7.3 and with 0–2.0 % (w/v) NaCl, with a generation time of 10 h under optimal conditions. Cells were rod-shaped and non-motile, ranging from 2 to 7 µm in length. Strain 521^T grew only in the presence of thiosulfate and/or Fe(III) (ferrihydrite) as terminal electron acceptors under strictly anaerobic conditions, and preferred protein-rich compounds as energy sources, although the isolate was capable of chemolithoautotrophic growth. 16S rRNA gene sequence analysis places this isolate within the crenarchaeal genus *Pyrobaculum*. To our knowledge, this is the first *Pyrobaculum* strain to be isolated from an anaerobic mud volcano and to reduce only either thiosulfate or ferric iron. An *in silico* genome-to-genome distance calculator reported <25 % DNA–DNA hybridization between strain 521^T and eight other *Pyrobaculum* species. Due to its genotypic and phenotypic differences, we conclude that strain 521^T represents a novel species, for which the name *Pyrobaculum igneiluti* sp. nov. is proposed. The type strain is 521^T (=DSM 103086^T=ATCC TSD-56^T).

Anaerobic respiratory processes based on the reduction of sulfur compounds or Fe(III) have been proposed to be among the first energy-conserving pathways to develop in the anoxic, high-temperature environment of the early Earth, with important consequences for the geochemistry of the current biosphere [1–5]. Today, similar environments are found in anaerobic sediments and subsurface environments such as deep petroleum reservoirs and submarine hydrothermal vents [6, 7]. The anaerobic hyperthermophiles inhabiting such geothermally heated locales on the sea floor, in oil wells/reservoirs and in other subsurface environments are frequently found to utilize thiosulfate, elemental sulfur and Fe(III) as terminal electron acceptors [3, 7–9].

The Salton Sea geothermal system supports a variety of subsurface hydrothermal features. Located in a rift valley of the San Andreas fault system in southern California, USA, the Salton Sea geothermal system contains the endorheic Salton Sea, hypersaline springs and mud volcanoes. Two main mud volcano systems are expressed at the surface near the southern end of the sea. One field is composed of 1–4 m tall volcanoes that eject a hydrocarbon-rich, anaerobic clay-based liquid at 60–70 °C with significant CO_2 bubbling from a reservoir 120 m below the surface. A second nearby field, recently revealed by the receding of the Salton Sea, ejects fluid of a similar composition at 90–95 °C. Water, mud, gas and petroleum-bearing seeps pervade the geothermal site and have significant variations in temperature, pH, density and solute content [10]. The seeps are driven by CO_2 released from decarbonation reactions occurring at 150–200 °C at a depth between 0.5 and 1.5 km [10]. The petroleum's composition indicated a hydrothermal origin and evidence of biodegradation [10]. Although the geological and thermodynamic characteristics of the Salton Sea geothermal system have been extensively studied, to our knowledge there have been no prior studies analysing the microbial communities living in and below these hot mud volcanoes.

Here, we report the discovery of an isolate named strain 521^T found in the hotter of the two fields. 16S rRNA gene sequence analysis places this isolate in the genus *Pyrobaculum*. The genus *Pyrobaculum* is within the family *Thermoproteaceae*, order *Thermoproteales*, class *Thermoprotei* and phylum *Crenarchaeota* that currently comprises nine established species, with *Pyrobaculum ferrireducens* being the most recently discovered [11, 12]. Members of the genus *Pyrobaculum* are exclusively hyperthermophiles and adopt chemoautotrophic or heterotrophic lifestyles, with

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Abbreviation: DDH, DNA–DNA hybridization.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 521^T is KU720559.
One supplementary figure is available with the online Supplementary Material.

Pyrobaculum neutrophilum (previously classified as *Thermoproteus neutrophilus*) being the only strain that is obligately autotrophic [13, 14]. While most are obligately anaerobic, others are capable of growing under aerobic and microaerophilic conditions [15–17]. Most members were isolated from terrestrial hot springs and solfataric waters, the exception being *Pyrobaculum aerophilum*, which was isolated from a boiling marine water hole [17]. In the absence of oxygen, *Pyrobaculum* members utilize several alternative electron acceptors, including Fe(III), nitrate, sulfur, thiosulfate, sulfite, selenite, selenate and arsenate [11, 12]. No *Pyrobaculum* species has been observed to grow by fermentation [12]. Notably, although prokaryotic genomes do not generally contain introns [18], species in the order *Thermoproteales*, including the genus *Pyrobaculum*, commonly contain regions of introns within rRNA genes that may bias environmental sampling methods that use universal 16S rRNA primers [19, 20].

Sampling was performed at a 95 °C mud volcano at the Salton Sea geothermal system (33.2188° N 115.6007° W). Samples were collected by injecting about 20 ml of hot, anaerobic mud from the outflow of the volcano into a sealed, anaerobic 120 ml serum bottle containing 60 ml of anaerobic minimal medium (see below), reduced by 0.1 ml of 3 % sodium sulfide. Clearing of the included resazurin dye indicated anaerobiosis. Enrichment cultures were started by direct injection of portions of this sample into the medium described below, using thiosulfate (10 mM) as an electron acceptor.

A minimal medium ('AB medium') was used in the initial isolation procedure and in subsequent growth experiments. AB medium contained the following (all concentrations final): PIPES (9 mM), K_2HPO_4 (2 mM), NH_4Cl (18 mM), NaCl (0.36 % w/v) and trace elements solution [21], adjusted to pH 7.0 at 25 °C using 10 M NaOH. Resazurin (0.0001 %, w/v) was added as a redox indicator. The medium was transferred to 30 ml Balch tubes, sealed with rubber stoppers, crimped, and made anaerobic and pressurized by bubbling with 100 % N_2 gas, and then autoclaved [22]. After autoclaving, sterile tryptone/yeast/beef (TYB) extract (0.05 %, w/v, for each ingredient) and sodium thiosulfate (10 mM) were added by syringe as the carbon source and the final electron acceptor, respectively. $Na_2S{\cdot}9H_2O$ (0.03 %, w/v) was added immediately before inoculation to remove any remaining oxygen, with clearing of the resazurin indicating complete anaerobiosis. The anaerobic medium was inoculated with a stock culture (5 %, v/v) and grown at 90 °C in a New Brunswick Innova 3000 water bath shaker at 160 r.p.m. for 5–7 days. Growth was evaluated by direct cell counting under a phase contrast light microscope (Zeiss) at 1000× magnification using oil immersion. Cells were individually counted using a Petroff Hauser Counting Chamber.

To determine the conditions that supported growth, cultures were grown using the procedure described above, across a range of pH values, NaCl concentrations and temperatures. To test for growth dependence on pH, cultures were incubated at the following pH values for 7 days with the indicated buffers (9 mM) substituted for PIPES (pH measured at 25 °C): 5.0, 5.5, 6.3 6.8 (MES), 7.7, 8.5 (PIPES) and 9.0 (EPPS). When corrected for the expected ΔpK_a at 90 °C, the corresponding pH values were 4.0, 4.5, 5.5, 6.1, 7, 7.3 and 8, respectively. To determine the range of salt concentrations that supported growth, cultures were grown in 0, 0.5, 1.0, 2.0, 4.0 and 6.0 % (w/v) NaCl for 7 days. To determine the temperature range for growth, cultures were incubated at 70, 75, 80, 85, 90 and 95 °C for 5 days. Doubling times were calculated by fitting a linear trendline for the logarithmic transformations of each growth curve for each condition and compared. Each condition was repeated in at least duplicate and the average was calculated for each value.

To identify the electron donors that strain 521^T was capable of oxidizing, the following reductants were added at 3.0 mM and the cultures were incubated for 5 days, unless otherwise specified: cellobiose, trehalose, maltose, glucose, mannitol, pyruvate, lactose, lactate, acetate, sorbitol, sucrose, galactose, starch (0.05 %, w/v), gelatin (0.05 %, w/v), beef extract, yeast extract, peptone, casamino acids, tryptone/yeast extract/casamino acids (TYC, 0.07 %, w/v, each), or TYB (0.05 %, w/v, each). To test for fermentative ability, the isolate was incubated anaerobically with 0.05 % TYB without the addition of an external electron acceptor. Electron acceptor utilization was determined using the following oxidants at 10 mM: sodium thiosulfate, nitrate, sulfate, sulfite, L-cystine and oxidized glutathione. Elemental sulfur and Fe(III) (ferrihydrite) were tested as electron acceptors at 10 g l^{-1}. Fe (III) (ferrihydrite) was prepared as described by Lovley [23].

To determine if strain 521^T was capable of chemolithoautotrophic growth via H_2, cultures were bubbled with 50:50 % CO_2/H_2 instead of the N_2 gas prior to autoclaving in the standard AB medium described above with 10 mM thiosulfate and with and without TYB (0.001 %, w/v). The culture was incubated at 90 °C under vigorous shaking for 7 days.

Genomic DNA was isolated from 1 litre of harvested cells grown in standard media (as described above) using the Qiagen DNeasy Blood and Tissue Kit. The genome sequence was determined at Molecular Research, LP. Fifty nanograms of DNA was used to prepare the genomic library using the Nextera DNA Sample Preparation Kit (Illumina). The library insert size was determined by Experion Automated Electrophoresis Station (Bio-Rad) and ranged from 300 to 850 bp (average 500 bp). The pooled library (12 pM) was loaded into a 600 Cycles v3 Reagent cartridge (Illumina) and the sequencing was performed on a Miseq system (Illumina).

The isolate's 16S rRNA gene was initially isolated via PCR amplification of genomic DNA using primers Arch-8-F (5′-TCCGGTTGATCCTGCC-3′) and Arch-1492-R (5′-GGC TACCTTGTTACGACTT-3′). From the annotated genome, the isolate's complete 16S rRNA gene sequence was compared with those of several other archeal strains. All 16S rRNA gene sequences were complete with the exception of

Pyrobaculum organotrophum. Introns were removed from sequences prior to alignment using CLUSTAL W [24]. Evolutionary history was inferred using the maximum-likelihood method in MEGA version 6 [25, 26]. Evolutionary distances were computed using the maximum composite likelihood method [27].

To determine whether the isolate represented a novel species in the genus *Pyrobaculum*, its genome was compared with the complete genomes of eight other established *Pyrobaculum* species: *P. calidifontis* JCM 11548^T (CP000561.1), *P. islandicum* DSM 4184^T (CP000504.1), *P. aerophilum* IM2^T (AE009441.1), *P. oguniense* TE7^T (CP003316.1), *P. arsenaticum* DSM 13514^T (NR102989.2), *P. neutrophilum* V24Sta^T (NR102765.2) *P. ferrireducens* 1860^T (CP003098) and '*P. yellowstonensis*' WP30 (CP012158.1). As indicated by quotation marks, '*Pyrobaculum yellowstonensis*' is an effectively published species but not validly published [28]. *P. neutrophilum* was previously classified as *Thermoproteus neutrophilum* [13] and *P. organotrophum* JCM 9190^T was not compared because its complete genome was unavailable. Genomes were compared *in silico* by an online genome-to-genome distance calculator (http://ggdc.dsmz.ge) using GGDC 2.0 BLAST Plus [29–31]. For each comparison, the calculator returned three DNA–DNA hybridization (DDH) values, based on different criteria, expressed in confidence intervals. The DDH values calculated by the recommended Formula 2 were compared.

Cells of strain 521^T were isolated by multiple rounds of dilution to extinction. Isolation was confirmed by 16S rRNA gene amplification and sequencing, which resulted in a clean sequence indicating the presence of only one archaeal species, and by phase contrast microscopy, which confirmed that only rod-shaped cells were present in cultures of the isolate.

Cells were exclusively rod-shaped and non-motile ranging from 2 to 7 µm in length. Doubling times were slightly responsive to changes in pH, with the doubling time being lowest between pH 5.5 and 7.3 and higher when the medium was more acidic or basic (Fig. S1a, available in the online Supplementary Material). pH 5.5 at 90 °C yielded the optimal doubling time of 14.3 h. No growth was observed at pH 3 and below or pH 9 and above. Doubling times were also mildly responsive to variations in NaCl concentrations, with 0–2.0 % (w/v) NaCl conferring the lowest doubling times, while 4.0 and 6.0 % (w/v) conferred higher values (Fig. S1b). NaCl at 0.5 % (w/v) conferred the optimal doubling time of 10.5 h. No growth was observed at NaCl concentrations of 7.0 % (w/v) and greater. Growth was most sensitive to temperature, as doubling times trended downward as temperature increased, with incubation at 90 °C conferring the lowest doubling time (Fig. S1c). Growth at 90 °C yielded the optimal doubling time of 10.0 h. No growth was observed at 65 °C and lower.

Strain 521^T was unable to grow under aerobic or microaerophilic conditions. For energy sources, it grew optimally in TYB and other proteinaceous sources including peptone, casamino acids and gelatin. Strain 521^T was unable to grow with starch, cellobiose, galactose, sucrose, acetate, trehalose, sorbitol, maltose hydrate, glucose, mannitol, sucrose, pyruvate, lactose or lactate as substrates. Of the electron

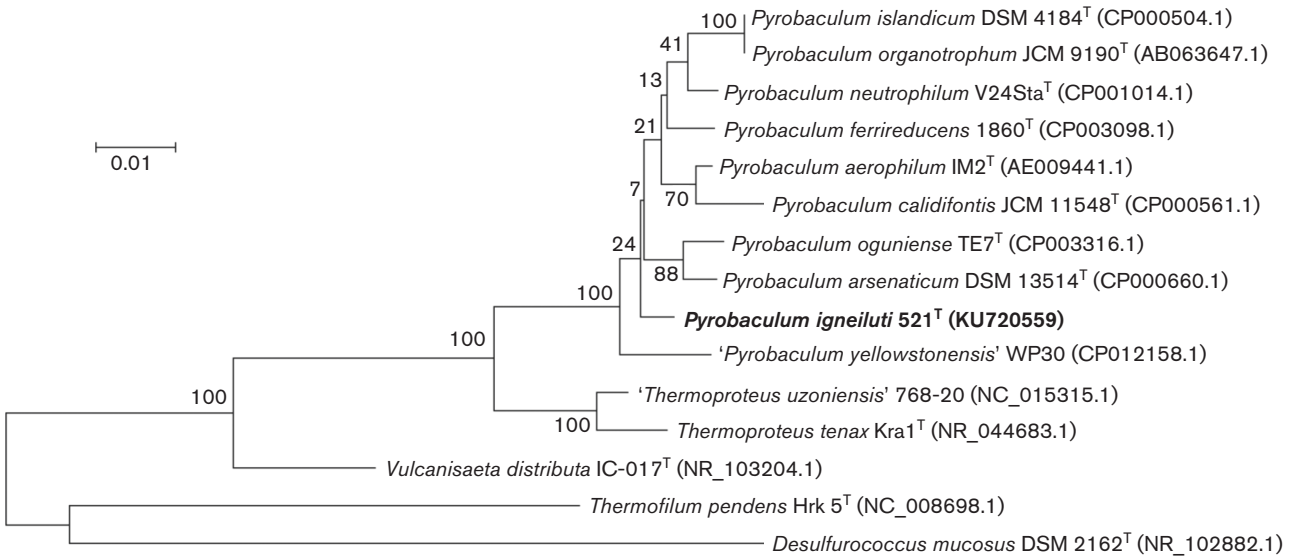

Fig. 1. Phylogenetic tree comparing the 16S rRNA gene sequence of strain 521^T to those of other species in the class *Thermoprotei*. Only validly published or effectively published species (marked by quotation marks) were included. Gaps, missing data and introns were removed from each sequence prior to alignment. Each number indicates the bootstrap value from 500 trials. Bar, 1 substitution per 100 nt. GenBank accession numbers are shown in parentheses.

Table 1. Physiological characteristics of strain 521^T compared to other species of the genus *Pyrobaculum*

Strains: 1, 521^T (data from this study); 2, *P. neutrophilum* V24Sta^T [14]; 3, *P. islandicum* DSM 4184^T [11]; 4, *P. organotrophum* JCM 9190^T [11]; 5, *P. aerophilum* IM2^T [17]; 6, *P. arsenaticum* DSM 13514^T [36]; 7, *P. oguniense* TE7^T [16]; 8, *P. calidifontis* JCM 11548^T [15]; 9, *P. ferrireducens* 1860^T [12]; 10, '*P. yellowstonensis*' WP30 [37]. +, Positive; −, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10
Source of isolation	Anaerobic mud volcano, Salton Sea, USA	Terrestrial hot spring, Kerlingarfjoll, Iceland	Solfataric and geothermal waters, Iceland	Solfartic water, Iceland	Boiling marine water hole, Ischia, Italy	Terrestrial hot spring, Pisciarelli Solfatara, Naples, Italy	Terrestrial hot spring, Ogunicho, Japan	Terrestrial hot spring, Calamba Laguna, Philippines	Terrestrial hot spring, Kamchatka, Russia	Sulfidic geothermal spring, Yellowstone National Park, USA
Morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Temperature (°C)										
Optimum	90	85	100	102	100	ND	90–94	90–95	90–95	75
Range	70–95	ND	74–102	78–102	75–104	68–100	70–97	75–100	75–98	60–94
pH										
Optimum	5.5–7.3	6.5	6.0	6.0	7.0	ND	6.3–7.0	7.0	6.0–7.0	4.6–6.6
Range	4.0–8.0	ND	5.0–7.0	5.0–7.0	5.8–9.0	ND	5.4–7.4	5.5–8.0	5.5–7.5	3.6–9.0
Oxygen requirement	Strictly anaerobic	Strictly anaerobic	Strictly anaerobic	Strictly anaerobic	Facultatively microaerobic	Strictly anaerobic	Facultatively aerobic	Facultatively aerobic	Strictly anaerobic	Strictly anaerobic
Autotrophic growth	+	+	+	−	+	+	−	−	−	−*
Organic carbon sources supporting growth	TYB, casamino acids, peptone, gelatin	None (obligate autotroph)	Yeast extract, peptone, meat extract, cell homogenate	Yeast extract, peptone, meat extract, cell homogenate	Yeast extract, tryptone, peptone, casamino acids, proprionate, acetate	Yeast extract, meat extract, peptone, brain heart infusion	Yeast extract, trypticase, peptone	Yeast extract, tryptone, peptone, casamino acids	Yeast extract, peptone, tryptone	Yeast extract, casmino acids, tryptone, peptone, soy broth*
Electron acceptors supporting growth										
Fe(III)	+	ND	+†	ND	+†	+†	ND	+†	+	−
Nitrate	−	ND	ND	ND	+	−	−	+	+	−
Sulfur	−	+	+	+	−	+	+	−	−	+
Thiosulfate	+	ND	+	−	+‡	+	+	−	+	−*
Sulfite	−	ND	+	−	ND	ND	ND	−	−	ND
Sulfate	−	ND	−	−	ND	ND	ND	−	−	−‡
L-Cystine	−	ND	+	+	ND	ND	+	ND	ND	ND
Glutathione (oxd.)	−	ND	+	+	ND	ND	+	ND	ND	ND

*Data from Jay and Inskeep [20].

†Data from Feinberg *et al.* [38].

‡Data from Huber *et al.* [36].

acceptors tested, strain 521^T was only able to grow in the presence of thiosulfate and Fe(III) (ferrihydrite). The presence of sulfide as the final product of thiosulfate reduction was detected by the methylene blue assay and through the use of a solid state Hg/Au microelectrode system [32, 33] Growth in ferrihydrite initially resulted in a cloudy black solution that cleared after the first day of growth. Soluble Fe^{2+} as the final product was detected by the ferrozine assay [34]. The isolate was unable to grow in the presence of elemental sulfur, sulfite, nitrate, sulfate, L-cystine or oxidized glutathione. No fermentation was observed. Growth was observed under autotrophic conditions and was enhanced by the addition of a trace amount of TYB (0.001 %, w/v).

The *in silico* genome-to-genome calculator estimated the following DDH percentages between strain 521^{T}and the type strains of the following species (mean±SD): *P. calidifontis* (17.60±2.23), *P. islandicum* (16.60±2.20), *P. aerophilum* (16.60±2.20), *P. oguniense* (17.70±2.24), *P. arsenaticum* (18.50±2.26), *P. neutrophilum* (17.30±2.22), *P. ferrireducens* (17.30±2.22), '*P. yellowstonensis*' (19.80±2.30). When each genome was compared to itself, all strains returned a DDH percentage of 100.

The isolate's 16S rRNA gene sequence did not contain any introns. Fig. 1 shows the phylogenetic relationship between strain 521^T and the other members of the class *Thermoprotei*. Based on the phylogenetic tree reconstructed, strain 521^T was most closely related to the type strains of *P. arsenaticum* (98.9 % 16S rRNA gene sequence similarity), *P. oguniense* (98.7 %) and '*P. yellowstonensis*' (98.0 %).

Strain 521^T was a rod-shaped, obligately anaerobic, heterotrophic archaeon growing optimally at 90 °C. It was incapable of fermentative growth and required the addition of thiosulfate or Fe(III) (ferrihydrite) as an electron acceptor to support growth. 16S rRNA gene sequencing placed this strain within the genus *Pyrobaculum* and genome-genome distance calculations estimated DDH between the strain and other established *Pyrobaculum* members to be no more than 20 %, far below 70 %, the accepted threshold for prokaryotic species differentiation [35]. Therefore, strain 521^T is classified as representing a novel species, for which the name *Pyrobaculum igneiluti* sp. nov. is proposed, and the tenth established member of the genus *Pyrobaculum*. Like most established *Pyrobaculum* species, strain 521^T is rod-shaped, hyperthermophilic and neutrophilic, although it is able to grow between pH 4 and 8, a significant range (Table 1). Similar to other heterotrophic *Pyrobaculum* species, the preferred carbon sources were protein-rich nutrients (e.g. TYB, peptone, gelatin, casamino acids). Strain 521^T was also capable of chemolithoautotrophic growth with H_2 and CO_2, albeit less robust than under heterotrophic conditions.

Among the *Pyrobaculum* species most closely related to *P. igneiluti* based on 16S rRNA gene sequence similarity, there are several physiological differences. While *P. igneiluti*, *P. arsenaticum* and '*P. yellowstonensis*' are all strictly anaerobic, *P. oguniense* is a facultative aerobe capable of living under aerobic and microaerophilic conditions [16, 36, 37]. Of the four, *P. igneiluti* and *P. arsenaticum* are facultative autotrophs, and '*P. yellowstonensis*' grows optimally at a relatively lower pH than the other three, reflecting the acidic conditions of the geothermal spring from which it was isolated [36, 37]. For the electron acceptors tested here, no two species had an identical combination of electron acceptors utilized. Thus, despite having close phylogenetic relationships, these *Pyrobaculum* species nonetheless display widely varying phenotypes.

To our knowledge, strain 521^T is the first *Pyrobaculum* strain to be isolated from a mud volcano and to reduce only either thiosulfate or ferric iron. Continued studies will further determine the mechanism by which thiosulfate and ferric iron reduction occurs in *P. igneiluti* and provide greater insight into the microbial diversity found in the Salton Sea geothermal system.

DESCRIPTION OF *PYROBACULUM IGNEILUTI* SP. NOV.

Pyrobaculum igneiluti (ig.ne.i.lu'ti. L. adj. *igneus* fiery; L. neut. n. *lutum* mud; N.L. gen. n. *igneiluti* of fiery mud).

Cells are non-motile straight rods 2–7 µm in length. Growth occurs at pH 4–8 at 90 °C (optimum, pH 5.5–7.3), at 75–95 °C (optimum, 90 °C) and with 0–6 % (w/v) NaCl (optimum, 0–2 %). Strictly anaerobic and uses TYB, casamino acids, peptone and gelatin as energy sources. Autotrophic growth in H_2/CO_2 is also observed. Unable to oxidize cellobiose, galactose, sucrose, acetate, trehalose, sorbitol, maltose hydrate, glucose, mannitol, sucrose, pyruvate, lactose or lactate. For terminal electron acceptors, only able to reduce thiosulfate and Fe(III) (ferrihydrite). Unable to reduce sulfate, sulfite, elemental sulfur, nitrate, L-cystine or oxidized glutathione.

The type strain, 521^T (=DSM 103086^T=ATCC TSD-56^T), was isolated from a mud volcano in the Salton Sea geothermal system in southern California, USA.

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

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