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EXPERIMENTAL ARTICLES

Desulfobotulus pelophilus sp. nov., an Alkaliphilic Sulfate-Reducing Bacterium from a Terrestrial Mud Volcano

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Abstract—An alkaliphilic, sulfate-reducing, anaerobic bacterium (strain H1^T) was isolated from a terrestrial mud volcano at the Taman Peninsula, Russia. The cells of the isolate were gram-negative motile vibrios, 1 μm in diameter and 2.0–2.5 μm in length. Strain H1^T grew at 14–42°C (optimum at 37°C), pH 8.5–10.5 (optimum at pH 9.5), and NaCl concentrations of 0.5–6% (wt/vol) (optimum at 0.5–1.5%); pyruvate, lactate, butyrate, caproate, or pelargonate were used as electron donors, and elemental sulfur, sulfite, or sulfate were used as electron acceptors. Pyruvate and lactate were fermented. No growth occurred in the presence of oxygen. Thiosulfate, DMSO, fumarate, nitrate, nitrite, arsenate, selenite, and Fe(III) were not used as electron acceptors. Elemental sulfur, thiosulfate, and sulfite were not disproportionated. Glucose, fructose, sucrose, trehalose, galactose, xylose, fumarate, citrate, yeast extract, and peptone were not fermented. Predominant fatty acids were $C_{20:0}$ (54.2%), $C_{22:0}$ (24.6%), and $C_{18:0}$ (11.1%). The genome of strain H1^T was 3.66 Mb in size and had G + C DNA content of 51.1%. The genome contained the genes encoding the enzymes of dissimilatory sulfate reduction and β-oxidation of fatty acids. According to the results of analysis of the 16S rRNA gene sequence, *Desulfobotulus mexicanus* was the organism most closely related to strain H1^T (98.3% similarity). Based on its phenotypic characteristics and the data of phylogenetic analysis, affiliation of the isolate as member of a novel *Desulfobotulus* species, *Desulfobotulus pelophilus* sp. nov., is proposed, with the type strain H1^T (=DSM 112796^T = VKM B-3697^T =UQM 41590^T).

Keywords: alkaliphile, sulfate reduction, anaerobic bacteria, mud volcano, *Desulfobacterales*

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Mud volcanism is a widespread geological phenomenon, which plays a significant part in the balance of atmospheric methane (Mazzini and Etiope, 2017). Terrestrial mud volcanoes (TMV) result from discharge of the particles of clay and silt, breccia, fluids, and gases from deep sediment layers. The Taman Peninsula is one of the regions with the most intensive mud volcanism. Over 100 active TMV are known in the Kerch-Taman mud volcano province (Gnatenko et al., 1986; Kholodov et al., 2012). Alkaline pH (over 8.5) of the fluids of these environments favor development of alkaliphilic microorganisms (Khomyakova et al., 2020, 2022; Frolova et al., 2021a, 2021b). In TMVs, anaerobic prokaryotes are involved in the biogeochemical cycles of carbon, sulfur, and other elements. Sulfate-reducing bacteria may participate in anaerobic methane oxidation as syntrophic partners of methanotrophic archaea (Knittel and Boetius, 2009). While the data on cultivation of sulfate-reducing bacteria from TMV are not abundant (Alain et al., 2006; Frolova et al., 2021a, 2021b), molecular analysis revealed in these communities the presence of microorganisms phylogenetically related to the known sulfate reducers (Tu et al., 2017; Ren et al., 2018; Merkel et al., 2021). The order *Desulfobacterales*, one of the largest and oldest taxonomic groups of sulfate reducers, is characterized by utilization of fatty acids as carbon and energy sources (Kuever et al., 2015). Members of *Desulfobacterales* inhabit anoxic sediments of freshwater, marine, and soda basins and may belong to various physiological groups (alkaliphiles, psychrophiles, halophilic, magnetotactic, and hydrocarbon-degrading). According to the List of Prokaryotic Names with Standing in Nomenclature, this order comprises 37 genera with validly published names (Parte et al., 2020).

The present work describes a strain of anaerobic, salt-tolerant, obligately alkaliphilic sulfate-reducing bacterium isolated from a mud volcano at the Taman Peninsula and identified as a new *Desulfobotulus* species.

MATERIALS AND METHODS

Source of isolation. The sample of the mud volcano fluid containing liquid and solid fractions was collected in May 2017 from an active gryphon of the Gnilaya Gora terrestrial mud volcano, Taman Peninsula, Krasnodar krai, Russia, GPS coordinates: 45.251° N, 37.436° E. The temperature at the sampling site was 21°C, pH 8.5, Cl^- concentration, 15.7 mM, and SO_4^{2-} concentration, 5.3 mM. The sample was collected anoxically into a plastic tube with a tight screw cap and was transported to the laboratory at ambient temperature for further investigation.

Media and cultivation. For the isolation and routine cultivation of strain H1^T, an anoxic, reduced brackish medium was used containing the following (g/L distilled water): KH_2PO_4, 0.33; NH_4Cl, 0.33; KCl, 0.33; $CaCl_2 \cdot 6H_2O$, 0.33; $NaHCO_3$, 2.00; $MgCl_2 \cdot 6H_2O$, 0.33; NaCl, 20.0; resazurin, 0.001; vitamin solution (Wolin et al., 1963), 1 mL; and trace element solution (Slobodkin et al., 2012), 1 mL. The medium was prepared with boiling and cooling under a continuous N_2 flow, then the reducing agent ($Na_2S \cdot 9H_2O$) was added. The medium was dispensed (10 mL) into 17-mL Hungate tubes and autoclaved for 60 min at 121°C. After sterilization, pH was 9.0. Sodium pyruvate (10 mM) and sodium sulfate (14 mM) were added from sterile stock solutions prior to inoculation.

Phenotypic characteristics. Cell morphology and motility were examined in 48-h liquid cultures under a Zeiss Primo Star phase contrast microscope. Growth experiments were carried out in triplicate. For morphological, physiological, and metabolic characterization, strain H1^T was grown in the medium used for its isolation, if not stated otherwise. The ranges of temperature, pH, and salinity were determined in reduced medium with pyruvate and sulfate. The salinity range studied was 0–10% NaCl (wt/vol). Different pH values were adjusted using the following buffers (Good's buffers, Sigma-Aldrich, 30 g/L): MES (pH 6 and 6.5), HEPES (pH 7 and 7.5), Tricine (pH 8.0 and 8.5), CAPSO (pH 9.0 and 9.5), and CAPS (pH 10 and 11). The experiments with sulfur compounds and oxygen were carried out in unreduced medium.

Analytical procedures. Gaseous products of metabolism were analyzed by gas chromatography on a HayeSep N 80/100 mesh column at 40°C and flow rate of 20 mL/min. The carrier gas was argon. Sulfide was determined colorimetrically with dimethyl-*p*-phenylenediamine (Trüper and Schlegel, 1964).

Fatty acid composition. The composition of cellular fatty acids was determined as described previously (Slobodkina et al., 2020), using direct methylation of lyophilized biomass and chromato-mass spectral analysis; the concentrations were determined by internal normalization using the peak areas of the full ionic current of the fatty acid methyl esters.

DNA isolation, sequencing, and full genome analysis. DNA isolation for determination of the nucleotide sequence of the 16S rRNA gene and full-genome sequencing was carried out using the FastDNA Spin Kit (MP Bio), according to the manufacturer's protocol. The 16S rRNA gene was amplified using the universal bacterial primers 27F, 357F, 530F, 1114F, 342R, 519R, and 1492R (Weisburg et al., 1991). PCR products were sequenced by the Sanger method. Preliminary phylogenetic screening of the 16S rRNA gene sequences was carried out using the GenBank database (Benson et al., 1999) with the BLAST software package (Altschul et al., 1990). For more accurate determination of the phylogenetic position of the isolate, the 16S rRNA gene sequence was aligned with those of the reference strains with Clustal W (Thompson, 1997). Phylogenetic analysis was carried out using MEGA 7.0 (Kumar et al., 2016). Statistical significance of the branching order was determined by bootstrap analysis of 1000 alternative trees (Felsenstein, 1985) constructed by the methods implemented in the MEGA 7 software package.

The genome of strain H1^T was sequenced using MiSeq (Illumina, San Diego, California, United States). Gene search and annotation were carried out with the RAST server (Brettin et al., 2015). SEED was used to distribute the hypothetic genes according to the subsystem categories (Overbeek et al., 2014). The taxonomic position of strain H1^T was verified using two methods: average nucleotide identity (ANI), as determined using the EzBioCloud ANI calculator (https://www.ezbiocloud.net/tools/ani) (Yoon et al., 2017) and *in silico* DNA-DNA hybridization, as determined using the Genome-to-Genome Distance Calculator (http://ggdc.dsmz.de) (Meier-Kolthoff et al., 2013).

The 16S rRNA gene sequence of strain H1^T was deposited to GenBank/EMBL under accession no. MW872673.

The full genome sequence of strain H1^T was deposited to GenBank/EMBL under accession no. JAPFPW010000000.

RESULTS

Isolation of the pure culture. Enrichment cultures were obtained by inoculation of the environmental sample ~10% vol/vol) into the sterile anoxic medium with pyruvate and sulfate. Abundant microbial growth was observed after 48-h incubation of the enrichments at 30°C. After three sequential transfers (5% vol/vol), tenfold serial dilutions of the culture were used to inoculate the same liquid medium. Only one morphological type of the cells was found in the highest dilution showing growth (10^{-8}). Tenfold dilutions were repeated twice more, and the culture grown in the last dilution was designated strain H1^T. Results of the 16S rRNA gene sequencing confirmed the purity of this

culture. No colonies could be obtained under anoxic conditions either by the roll-tube method or in 1% Gelrite gellan gum or 1% agar.

The cells of strain H1^T were vibrios, 1 μm in diameter and 2.0–2.5 μm in length, growing singly and motile due to the single polar flagellum. No endospores were observed during 30 days of cultivation.

Growth physiology. Strain H1^T grew at the temperatures from 14 to 42°C, with the optimum at 37°C. After 20-day incubation, no growth occurred at 50°C or higher and at 10°C or lower. The pH range for growth was 8.5 to 10.5, with the optimum at pH 9.5; no growth occurred at pH 8.0 and lower or at pH 11.0 and higher. Strain H1^T grew at NaCl concentrations in the medium from 0.5 to 6.0% (wt/vol) with the optimum at 0.5–1.5%; no growth occurred at NaCl concentrations 7% NaCl or above.

Electron donors and acceptors. Strain H1^T could use organic acids as electron donors and sulfur compounds as electron acceptors for growth. Addition of yeast extract (0.2 g/L) stimulated growth slightly, but was not necessary. The highest cell concentration (~5×10^7 cells/mL) was observed on pyruvate, both with sulfate or without electron acceptors. Pyruvate, lactate (10 mM), butyrate (10 mM), caproate (5 mM), caprylate (1 mM), and pelargonate (1 mM) could be used as electron donors in the presence of sulfate as an electron acceptor. In this case, sulfide and acetate were the reaction products. The compounds not used as electron donors (10 mM, if another concentration is not indicated) with sulfate as an electron acceptor were: acetate, formate, formate + acetate (2 mM) as a carbon source, propionate succinate, fumarate, malate, methanol, ethanol, butanol, isobutanol, glycerol, ribose, glucose, fructose, tributyrate, valerate, palmitate (1 mM), stearate (1 mM), oleate (1 mM), trioleate (1 mM), yeast extract (2 g/L), and molecular hydrogen ($H_2 + CO_2$, 80 : 20% in the gas phase). Elemental sulfur (5 g/L), sulfite (10 mM), and sulfate (14 mM) were used as electron acceptors in the presence of butyrate as an electron donor. Thiosulfate, dimethyl sulfoxide (DMSO), fumarate, nitrate, nitrite, arsenate, and selenite (all at 10 mM), as well as ferrihydrite (poorly crystalline Fe(III) oxide, 90 mM), were not used by strain H1^T as electron acceptors with butyrate as an electron donor. The strain was incapable of growth under aerobic or microaerobic conditions (up to 3% oxygen).

Pyruvate and lactate (10 mM each) were fermented with formation of acetate and hydrogen as the end products. Strain H1^T did not ferment formate, fumarate, citrate, succinate, fructose, sucrose, trehalose, galactose, xylose, peptone, or yeast extract after three weeks of incubation. The strain was incapable of disproportionation of elemental sulfur, thiosulfate, and sulfite both in the presence of ferrihydrite (acting as as a sulfide-scavenging agent.) and without it, in the bottles with large volumes of the gas phase.

Fatty acid composition. Cellular fatty acids of strain H1^T were represented by a mixture of unbranched saturated and unsaturated acids: $C_{20:0}$ (54.2%), $C_{22:0}$ (24.6%) and $C_{18:0}$ (11.1%). $C_{16:0}$ (3.8%), $C_{24:0}$ (2.4%), $C_{18:1}$ ω7c (2.2%), and $C_{18:1}$ ω9c (1.8%) were also present.

Phylogeny. The 16S rRNA gene sequences of strain H1^T obtained by amplification with the universal bacterial primers and by full genome sequencing were identical. Comparison of 1539 nucleotides of the 16S rRNA gene sequence of strain H1^T with the GenBank sequences (Benson et al., 1999) revealed that the isolate belonged to the genus *Desulfobotulus*, class *Deltaproteobacteria* with 98.31% similarity to the sequence of *Desulfobotulus mexicanus* (Pérez-Bernal et al., 2020). Reconstruction of the 16S rRNA-based phylogenetic tree showed that strain H1^T represented a monophyletic lineage clearly separated from the most closely related species (Fig. 1).

The result of pairwise comparison of the average nucleotide identity of the genomes of strain H1^T and its closest relative, *D. mexicanus* (DSM 105758^T), was 88.7%. The value of *in silico* DNA-DNA hybridization between H1^T and *D. mexicanus* (DSM 105758^T) according to the recommended formula 2 was 26.10%. Both values were considerably lower than the thresholds recommended for delineation between prokaryotes at the species level, i.e., 95–96% (for ANI) and 70% (for DNA-DNA hybridization) (Meier-Kolthoff et al., 2013; Rodriguez and Konstantinidis, 2016).

General characteristics of the genome. The genome of strain H1^T assembled out of 86 contigs had the overall length of 3656775 nt and N50 of 160366 nt. The G + C content of genomic DNA was 51.1%. The genome contained 3783 nucleotide sequences encoding proteins and 55 RNA genes. Most of the annotated genes were responsible for the synthesis of amino acids and their derivatives (151), protein metabolism (147), carbohydrate metabolism (101), respiration (83), cofactors, vitamins, prosthetic groups, and pigment formation (55).

The genome of strain H1^T contained the genes of the Embden–Meyerhof–Parnas pathway, including the NAD-dependent glyceraldhyde-3-phosphate dehydrogenase (WP_265423680), triose phosphate isomerase (WP_265423679), glucose-6-phosphate isomerase (WP_265426043), phosphoglycerate kinase (WP_265423283), enolase (WP_265423752), pyruvate kinase (WP_265423750), 6-phosphofructokinase (WP_265423438), fructose-bisphosphate aldolase (WP_265423362), and phosphoglycerate mutase (WP_265426113). The gene encoding hexokinase, which catalyzes glucose phosphorylation at the first stage of glycolysis, was, however, absent. The tricarboxylic acids cycle was encoded incompletely and did

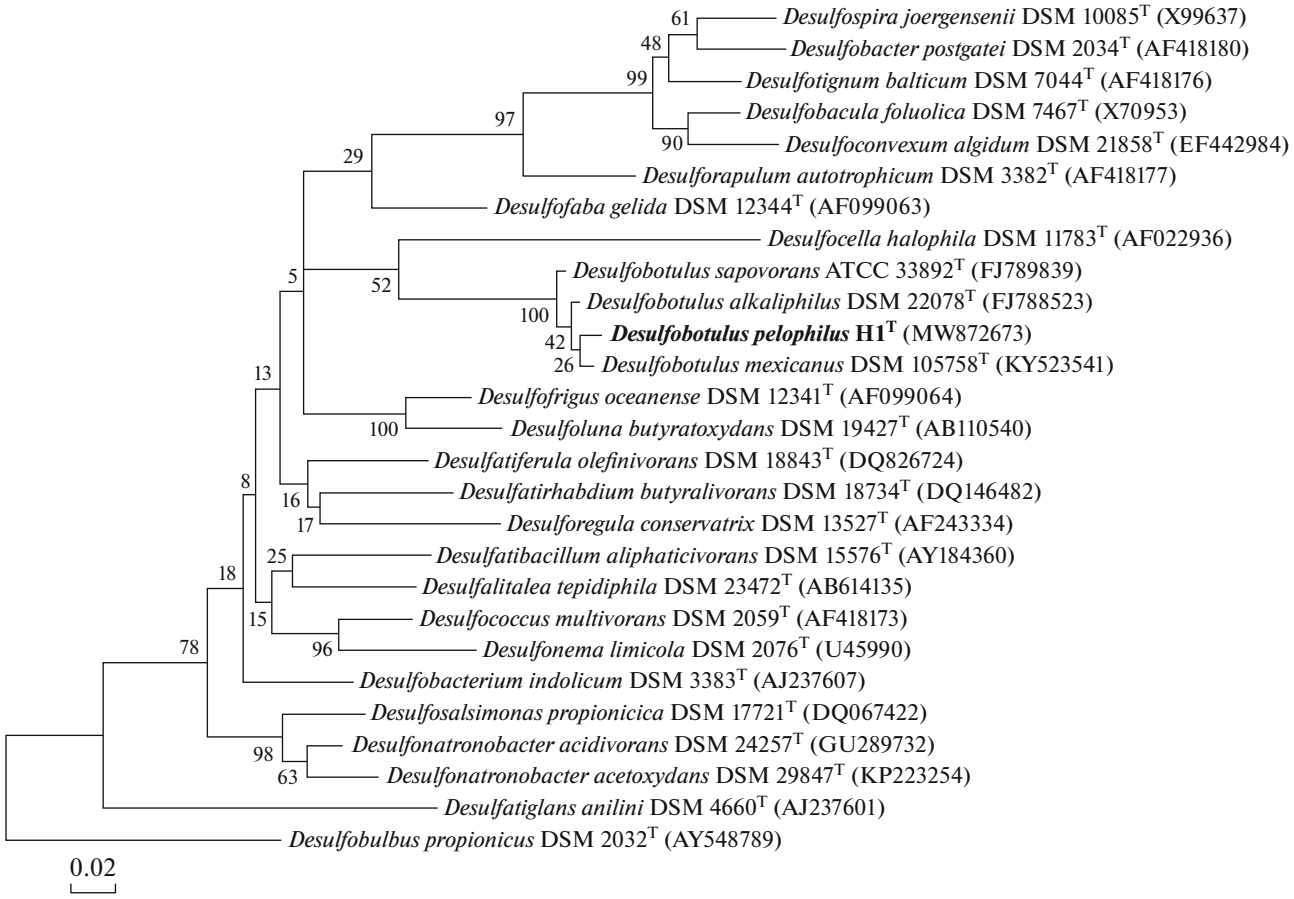

Fig. 1. Phylogenetic tree constructed using the 16S rRNA gene sequences and showing the position of strain H1^T and related microorganisms. The tree was reconstructed using the maximum-likelihood method. The trees constructed using the neighbor-joining and minimum-evolution algorithms showed the same topology. Each numeral indicates the value of initial load of 1000 replicates. Scale bar, 0.020 replacements per nucleotide position. The GenBank accessionnumbers are indicated in parentheses.

not contain the genes for malate dehydrogenase, succinate dehydrogenase, and succinyl-CoA synthetase.

The genome of strain H1^T contained a complete set of genes required for dissimilatory sulfate reduction (Pereira et al., 2011), including sulfate adenylyl transferase Sat (WP_26542348), inorganic pyrophosphatase (WP_265426333), the AprA (WP_265426087) and AprB (WP_265426088) subunits of the APS reductase, the components of the dissimilatory sulfite reductase (WP_26542589–WP_265425892), and the electron-transporting complexes DsrMKJOP and QmoABC (WP_265425905–WP_265425909).

The genome of strain H1^T contained two genes encoding the molybdopterin oxidoreductase (WP_265423973 and WP_265426274). The biochemical function of these enzymes is not clear. They are most probably not the catalytic subunit of the *psrA* polysulfide reductase, since the characteristic adjacent genes of such molybdopterin oxidoreductases were not revealed in the genome of strain H1^T. The genome encoded the enzymes rhodanese (WP_265423671) and the hdr-like complex with the *hdrA* (WP_265423457), *hdrB* (WP_265425454), and *hdrC* (WP_265425453) subunits, which are involved in the redox reactions of sulfur compounds, although their biochemical mechanism is presently insufficiently studied (Zhang et al., 2021).

The genome of strain H1^T contained all genes required for β-oxidation of fatty acids, including acyl-CoA dehydrogenase (WP_265423377, WP_265423535, WP_265424553, WP_265424646, WP_265425157, WP_265425230, WP_265425661, WP_265425744, WP_265426163), enoyl-CoA hydratase (WP_265423968, WP_265425159, WP_265425648, WP_265425692), 3-hydroxyacyl-CoA dehydrogenase (WP_265423389, WP_265425692), and 3-ketoacyl-CoA thiolase (WP_265423278, WP_265424057, WP_265423533, WP_265425693).

The genome of strain H1^T contained all the genes encoding the components of the nitrogenase complex required for dinitrogen fixation, including the Mo-Fe- and Fe- proteins of nitrogenase *nifHDK,*

Table 1. Differentiating characteristics of strain H1^T and other *Desulfobotulus* species. All strains use pyruvate as an electron donor and sulfate or sulfite as electron acceptors. ND indicates no data in the literature sources

Characteristics	H1^T (this work)	*D. mexicanus* (Pérez-Bernal et al., 2020)	*D. alkaliphilus* (Sorokin et al., 2010)	*D. sapovorans* (Kuever et al., 2005)
T_{opt}, °C	37	32	32	34
pHopt	9.5	9.0–9.6	9.9–10.1	7.7
NaCl, % (wt/vol)	0.5–6	0.4–6.75	ND	<1.875
Electron donors				
Fatty acids	C_4, C_6, C_8, C_9	C_4, C_6, C_8, C_{10}, C_{14}, C_{16}, C_{18}	C_4, C_6, C_8, C_9	C_4–$C_{16(18)}$
2-Methylbutyrate	–	+	–	+
Lactate	+	–	–	+
Electron acceptors				
Thiosulfate	+	–	+	–
Elemental sulfur	+	+	+	–
Fermentation				
Lactate	+	ND	ND	–
Pyruvate	+	–	+	+
G + C, mol %	51.1	49.0	51.3	53
Source of isolation	Terrestrial mud volcano	Soda lake	Soda lake	Freshwater basin

(WP_265425310–WP_265425314), as well as the proteins required for its asembly and regulation: *nifENB* (WP_265425306–WP_265425309), *nifU* (WP_26542406), *nifS* (WP_265424064), *NifA* (WP_265424773), and *NtrXY* (WP_265425703–WP_265425704).

The genome of strain H1^T contained the genes encoding catalase (WP_265425612), superoxide dismutase (WP_265425250), quinol oxidase *CydAB* (WP_265424027–WP_265424028), and several copies of the genes of the proteins involved in protection against oxidative stress, including the rubredoxin *Rbo* (WP_265424029, WP_265425910) and the rubrerythrin *Rbr* (WP_265425583), which probably carry out this function in *Desulfovibrio vulgaris* (Lumppio et al., 2001).

DISCUSSION

Strain H1^T was isolated from a terrestrial mud volcano at the Taman Peninsula. It is an anaerobic alkaliphilic mesophilic sulfate-reducing bacterium.

Phylogenetic analysis based on the 16S rRNA gene sequences showed that strain H1^T formed a separate lineage within the genus *Desulfobotulus* (Kuever et al., 2005), of the family *Desulfobacteraceae*, order *Desulfobacterales*, phylum *Pseudomonadota*. At the time of publishing, the genus *Desulfobotulus* was represented by three species with validly published names: *D. sapovorans* (Kuever et al., 2005), *D. alkaliphilus* (Sorokin et al., 2010), and *D. mexicanus* (Pérez-Bernal et al., 2020). Identity of the 16S rRNA nucleotide sequences between strain H1^T and *D. mexicanus* (DSM 105758^T) was 98.31%. Pairwise comparisons of the average nucleotide identity (ANI) and *in silico* DNA–DNA hybridization between strain H1^T and *D. mexicanus* (DSM 105758^T) also supported identification of the isolate as a new *Desulfobotulus* species.

Members of the genus *Desulfobotulus* are geographically widespread. They have been detected in diverse ecosystems, including freshwater habitats and alkaline soda or crater lakes (GBIF Secretariat, 2021; GBIF Backbone Taxonomy. Checklist dataset https://doi.org/10.15468/39omei). Strain H1^T is the first member of this genus isolated from a terrestrial mud volcano. The new isolate had the growth ranges of pH, temperature, and salinity, which were close to the parameters of its habitat.

The metabolic potential encoded in the genome of strain H1^T was in agreement with the phenotypic data. All *Desulfobotulus* species are characterized by utilization of aliphatic fatty acids in dissimilatory sulfate reduction. The differentiating characteristics of strain H1^T and the known *Desulfobotulus* species are presented in Table 1. The most noticeable differences were its higher temperature optimum and ability to ferment lactate. Thus, based on our data, we propose to assign strain H1^T to a new *Desulfobotulus* species, *Desulfobotulus pelophilus* sp. nov.

Description of *Desulfobotulus pelophilus* sp. nov.

Desulfobotulus pelophilus (pe.lo'phi.lus. Gr. m. n. *pêlos*, mud, N. Lat. adj. *philus -a -um*, loving; from Gr. adj. *philos -ê -on*, loving; N. Lat. adj. *pelophilus*, loving

mud, since the species was isolated from a mud volcano).

The cells are motile vibrios, 1 μm in diameter and 2.0–2.5 μm in length. Growth occurs at 14–42°C (optimum at 37°C), pH 8.5–10.5 (optimum at 9.5), and NaCl concentrations (wt/vol) 0.5–6% (optimum at 0.5–1.5%). Pyruvate, lactate, butyrate, caproate, caprylate, and pelargonate are used as electron donors; elemental sulfur, sulfite, and sulfate are used as electron acceptors. Pyruvate and lactate are fermented. No growth occurs in the presence of oxygen. Thiosulfate, DMSO, fumarate, nitrate, nitrite, arsenate, selenite, and Fe(III) are not used as electron acceptors. No disproportionation of elemental sulfur, thiosulfate, or sulfite. Glucose, fructose, sucrose, trehalose, galactose, xylose, fumarate, citrate, yeast extract, and peptone are not fermented. Predominant cellular fatty acids are $C_{20:0}$, $C_{22:0}$, and $C_{18:0}$.

The type strain H1^T (=DSM 112796^T = VKM B-3697^T = UQM 41590^T) was isolated from a terrestrial mud volcano at the Taman Peninsula. Genome size is 3.66 Mb. The G + C content of genomic DNA is 51.1% (full genome sequencing).

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest.

This article does not contain any studies involving animals or human participants performed by any of the authors.

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