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ORIGINAL PAPER

Desulfofustis limnaeus sp. nov., a freshwater sulfate-reducing bacterium isolated from marsh soil

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Abstract

A novel sulfate-reducing bacterium, strain PPLL^T, was isolated from marsh soil. Cells of strain PPLL^T were rod-shaped with length of 1.5 µm and width of 0.7 µm. Growth was observed at 22–37 °C (optimum 35 °C) and pH 6.8–8.4 (optimum 7.3). Lactate, succinate, fumarate, formate and malate were utilized as electron donors for sulfate reduction. Fermentative growth was not observed on tested organic acids. Besides sulfate, sulfite, thiosulfate and elemental sulfur were utilized as electron acceptors. Hydrogen is used only in the presence acetate or yeast extract. The major fatty acid was $C_{16:0}$. The complete genome of strain PPLL^T was composed of a circular chromosome with length of 4.2 Mbp and G + C content of 57.7 mol%. Sequence analysis of the 16S rRNA gene showed that strain PPLL^T was affiliated with the genus *Desulfofustis* in the family *Desulfocapsaceae*. On the basis of differences in the phylogenetic and phenotypic properties between the strain and the type strain of the genus *Desulfofustis*, strain PPLL^T (DSM 110475^T = JCM 39161^T) is proposed as the type strain of a new species, with name of *Desulfofustis limnaeus* sp. nov.

Keywords Sulfate-reducing bacterium · *Desulfocapsaceae* · *Desulfofustis*

Introduction

The genus *Desulfofustis* contains the sole species *Desulfofustis glycolicus*, which can degrade glycolate and glyoxylate with sulfate respiration (Friedrich et al. 1996). The glycolate is mainly produced as an intermediate of photosynthesis, and, therefore, *Desulfofustis* species may occur in cyanobacterial mats in marine brackish ecosystem (Galushko and Kuever 2019). The strain PerGlyS^T, the type strain of *D. glycolicus*, is a mesophilic and neutrophilic sulfate-reducing bacterium isolated with glycolate from anoxic marine mud in Italy. Based on the genome-based phylogeny (Parks et al. 2018), the genus *Desulfofustis* was proposed to be reclassified in the family *Desulfocapsaceae* within the order *Desulfobulbales* in the class *Desulfobulbia* (Waite et al. 2020). In this study, a novel freshwater sulfate-reducing bacterium designated as the strain PPLL^T was isolated and characterized, as a representative of a new species within the genus *Desulfofustis*.

Materials and methods

Enrichment and isolation

Strain PPLL^T was isolated from soil of a marsh in Hokkaido, Japan (43° 04′ 08″ N 141° 31′ 11″ E). From the same site, a spore-forming sulfate-reducing bacterium was isolated in a previous study (Watanabe et al. 2017). Approximately, 1 ml of the soil slurry was inoculated into 40 ml bicarbonate-buffered sulfide-reduced defined basal medium containing sulfate (Widdel and Bak 1992) to establish the first enrichment culture. One milliliter of 2% (v/v) *p*-xylene solution [in 2, 2, 4, 4, 6, 8-heptamethylnonane, which served as carrier phase (Rabus et al. 1993)] was added to 40 ml of the medium

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as a sole carbon and energy source. The headspace of the bottle was filled with N_2/CO_2 (80:20, v/v), and incubation was carried out in the dark at 28 °C. The grown enrichment culture was subjected to agar shake dilution with 2% (v/v) *p*-xylene solution as described in a previous study (Higashioka et al. 2009). After several months, a beige colony was picked and inoculated into the fresh medium same as for the first enrichment. Subsequently, the grown culture was transferred and diluted to extinction in the bottle of fresh medium filled with $H_2 + CO_2$ gasses ($H_2/N_2/CO_2$, 50:40:10; 2 atm total pressure). After the growth of enrichment, the substrate was changed to lactate (5 mM). After colony isolation from the agar tube dilution using 5 mM lactate, a single colony of strain PPLL^T was obtained. The purity of culture was ascertained routinely by microscopy and verified by denaturing gradient gel electrophoresis of the 16S rRNA gene (Muyzer et al. 1993) for cultures used to perform physiological tests.

Phenotypic characterization

In experiments for phenotypic characterizations, strain PPLL^T was cultured at 35 °C in the basal medium supplemented with 5 mM lactate and 0.05% (w/v) yeast extract, unless otherwise specified. Cell morphology was confirmed by phase-contrast microscopy (Axioplan 2; Zeiss). The fatty acid profile was carried out by the identification services of Techno Suruga Laboratory with the Sherlock Microbial Identification System (MIDI) version 6.0 (database; MOORE6). For cellular fatty acid analysis, strain PPLL^T was grown in the basal medium supplemented with 10 mM lactate and 0.05% (w/v) yeast extract.

Effect of temperature on growth was examined by culturing at 18, 22, 25, 28, 32, 35, 37, 42, 45 and 50 °C. In the test of pH effect on growth, bicarbonate in the basal medium was replaced with MES, MOPS, or TAPS (20 mM). The pH of the modified media was adjusted with NaOH, to pH 5.8, 6.2, 6.4, 6.5 and 6.8 (MES), pH 6.5, 7.0, 7.3, 7.5 and 7.8 (MOPS), pH 7.8, 8.0, 8.2, 8.4, 8.6, 8.8 and 9.0 (TAPS). Effect of salt concentration on growth was examined by culturing with various concentration of NaCl, ranging from 0 to 3.0% (w/v) at 0.5% intervals.

Utilization of growth substrates was tested with the basal medium, supplemented with one of the following substrates (mM; unless otherwise specified); formate (5), acetate (5), propionate (5), lactate (5), glycolate (5), butyrate (2), isobutyrate (2), malate (5), succinate (5), fumarate (5), benzoate (2), citrate (5), methanol (5), ethanol (5), glucose (5) and yeast extract (0.05% w/v). Hydrogen-dependent growth was tested under a gas mixture of H_2, N_2 and CO_2 (50:40:10 v/v/v, 200 kPa total pressure), with or without supplement of acetate (1 mM) or yeast extract (0.05%). Fermentative growth was tested in sulfate-free medium supplemented with lactate (5), succinate (5), malate (5), pyruvate (5) or fumarate (5). As electron accepters, thiosulfate (10), elemental sulfur (0.5% w/v), sulfite (1 and 5), nitrate (10) and poorly crystalline Fe (III) oxide (20) were tested. The production of acetate during sulfate reduction was assessed using acetate colorimetric assay kit (Sigma-Aldrich) and supernatant of well-grown culture supplemented with 5 mM lactate.

Genomic characterization and phylogenetic analysis

Whole-genome sequencing was performed using the platforms of Illumina NextSeq and Nanopore GridION. Short and long reads from the platforms were subjected to hybrid assembly using Unicycler (Ver 0.4.7). The assembled genome sequence was annotated with DFAST web pipeline (Tanizawa et al. 2018). In order to evaluate genomic distance between strain PPLL^T and related organisms, values of average amino acid identity (AAI) (Rodriguez R and Konstantinidis 2014) and percentage of conserved proteins (POCP) (Qin et al. 2014) were calculated, using EzAAI version 1.0 (Kim et al. 2021) and modified scripts based on data_file_4.sh (Rodriguez-R and Konstantinidis 2016), respectively. The Genome-to-Genome Distance Calculator (GGDC) calculation was carried out using GGDC 3.0 server (Meier-Kolthoff et al. 2022). For reconstruction of metabolic pathway, eggNOG-mapper version 2.1.6 annotation server (Cantalapiedra et al. 2021), NCBI BLASTP analysis was utilized for amino acid sequences of strain PPLL^T genome. The phylogenetic affiliation based on genome-based taxonomy of strain PPLL^T was investigated using GTDB-Tk version 1.5.0 (Chaumeil et al. 2020) with the GTDB database release 202. The 16S rRNA gene sequence of strain PPLL^T was retrieved from the complete genome and was aligned with reference sequences using the program CLUSTAL X version 2.1 (Larkin et al. 2007). Selection of the best nucleotide substitution models and construction of phylogenetic trees were performed using MEGA version 10.0.5 (Kumar et al. 2018).

Results

Physiological and chemotaxonomic characteristics

Cells of strain PPLL^T were non-motile, rod-shaped, 0.7 µm in width, 1.5 µm in length. In the cellular fatty acid profile, $C_{16:0}$ accounting for 32.7% was predominant. Other major components accounting > 10% of total were $C_{18:1}\omega7c$ summarized with unknown acids (17.0%), $C_{16:1}\omega7c$ (14.4%) and $C_{16:1}\omega5c$ (13.3%).

Strain PPLL^T grew at 22–37 °C with optimum growth at 35 °C, and grew at pH range of 6.8–8.4 with the optimum pH of 7.2. No growth was observed in the presence of 0.5% or higher concentrations of NaCl. In the presence of sulfate, formate, lactate, succinate, fumarate and malate

supported heterotrophic growth of PPLL^T. Growth on H_2 gas was observed only in the presence of acetate or yeast extract. Sulfate, sulfite, thiosulfate and elemental sulfur were used as electron acceptor. Nitrate and Fe (III) oxide did not support growth of strain PPLL^T as electron acceptor. Fermentative growth was not observed with the tested substrates. Acetate production was observed under sulfate-reducing conditions with lactate.

The complete genome of strain PPLL^T was reconstructed as a 4,232,141 bp of circular chromosome with 57.7 mol% of G + C content. It was predicted to contain 3843 protein-coding genes, 50 tRNA genes, and 9 rRNA genes. Based on the metabolic pathway reconstruction, gene sets for anaerobic alkylbenzene degradation were not identified in the genome of strain PPLL^T. The genome possesses all genes for complete Wood–Ljungdahl pathway, although strain PPLL^T did not grow autotrophically. Strain PPLL^T did not use nitrate as electron acceptor, but its genome has *narGHI* and *nrfHA* genes, that are the key components for dissimilatory nitrate reduction to ammonia. In addition, *nirK* and *norB* genes, that are essential genetic components for nitrate reduction to nitrous oxide were also identified. The gene of *nosZ* was not found in the genome. As key enzyme for glycolate oxidation, membrane-associated methylene-blue-dependent glycolate dehydrogenase (E.C. 1.1.99.14) is encoded in the genome of *D. glycolicus* PerGlyS^T, but its gene was not found in the genome of strain PPLL^T.

In the genome-based taxonomy according to the GTDB, strain PPLL^T was classified into the genus *Desulfofustis*, but not into any existing species. The POCP value between strain PPLL^T and *D. glycolicus* PerGlyS^T was 73.1%, within the genus boundary range of 60–80%. The AAI between them was 75.8%, which is clearly higher than proposed threshold for genera delineation, 50%. These genomic distance calculations suggested that the strain PPLL^T should be a new species of the genus *Desulfofustis*. Based on the 16S rRNA gene sequence, the sequence similarity between strain PPLL^T and its closest relative *D. glycolicus* PerGlyS^T was 97.2% (compared length: 1,406 nucleotide positions). This value is lower than 98.7–99% of the threshold value for species delineation between two bacterial strains (Stackebrandt 2006). In addition, a low ANI value between these strains (78.8%) was shown in the analysis with GTDB-tk. An ANI value of 95% is widely utilized for the species demarcation, and therefore the result indicated that these strains should not be classified to same species. The GGDC value between these species was 20.4% and was lower than 70% of species boundary, which is also supported that these strains can be discriminated at a species level. The phylogenetic tree reconstruction based on the 16S rRNA gene sequences revealed that strain PPLL^T could be a representative of a novel species of the genus *Desulfofustis* in the family *Desulfocapsaceae* (Fig. 1).

The differential properties between strain PPLL^T and *D. glycolicus* PerGlyS^T are shown in Table 1. The utilization of electron acceptors and donors indicated that these strains have different metabolic characteristics. In addition, these strains differ in tolerance and requirement

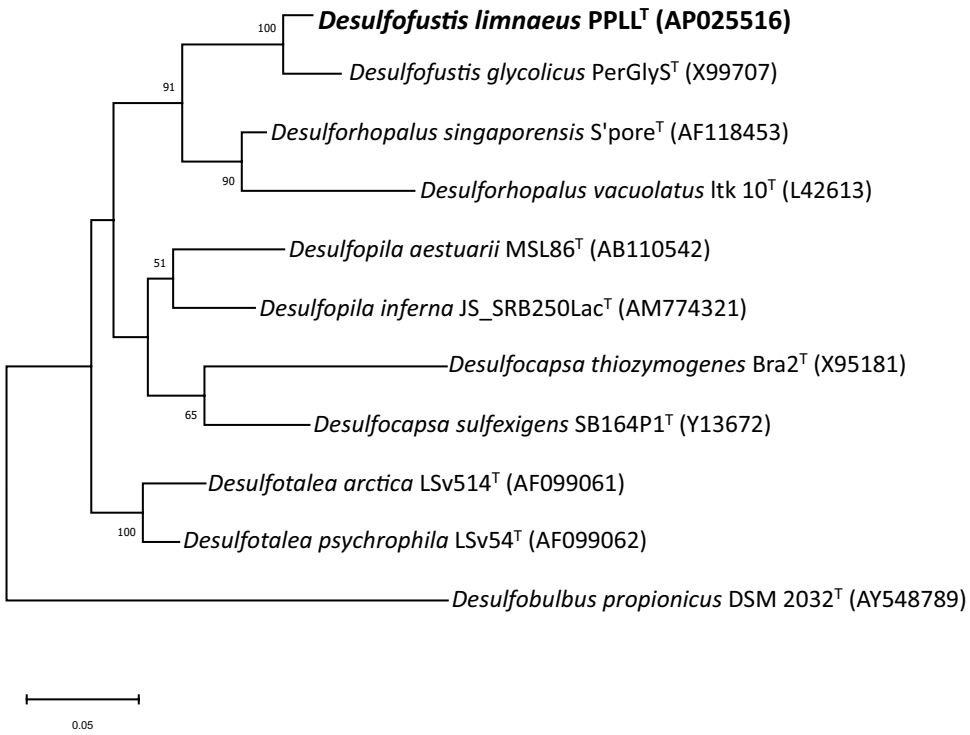

Fig. 1 Maximum-likelihood tree based on 16S rRNA gene sequences of strains PPLL^T and reference strains of the family *Desulfocapsaceae*. A total of 1477 positions were used in the final dataset. *Desulfobulbus propionicus* DSM 2032 ^{T} was used as an outgroup. The phylogenetic tree was inferred using the maximum likelihood method and Kimura 2-parameter model + Gamma distributed with Invariant sites. Bootstrap values (percentages of 1000 replications) only 50% or more are shown at nodes

Table 1 The differential properties between strain PPLL^T and *D. glycolicus* PerGlyS^T

Characteristics	*D. limnaeus* PPLL^T sp. nov	*D. glycolicus* PerGlyS^T
Isolation source	Marsh soil	Marine sediment
Morphology	Straight rod	Straight or slightly curved rod
Cell size (μm)	0.7 × 1.5	0.55 × 2.0–4.5
Optimum temperature for growth (°C)	35	28
Optimum NaCl concentration (%)	0	2.0
Compounds used as electron donors and carbon sources		
Glycolate	−	+
Yeast extract	−	+
H_2/acetate	+	+
Formate	+	−
Electron acceptor used		
Sulfate	+	+
Sulfite	+	+
Thiosulfate	+	-
Sulfur	+	+
Nitrate	−	−
Genome size (Mbp)	4.2	5.0

+ Positive, − negative

of NaCl. The type strain of *D. glycolicus* does not grow in freshwater medium, whereas strain PPLL^T grows only under freshwater conditions. Based on the phenotypic, genomic and phylogenetic analysis, we conclude that the strain PPLL^T represents a novel species of the genus *Desulfofustis* for which the name *D. limnaeus* sp. nov. is proposed.

Description of *Desulfofustis limnaeus* sp. nov.

Desulfofustis limnaeus (*lim'nae.us.* Gr. Masc. adj. *limnaîos*, living in limnic waters, swamps; N.L. masc. adj. *limnaeus*, living in freshwaters and swamps).

In addition to the characteristics given in the description of the genus, the following properties are observed. Growth occurs at 22–37 °C (optimum 35 °C), at pH 6.8–8.4 (optimum pH 7.2), and with 0% NaCl. Grows on lactate, succinate, formate, fumarate and malate; acetate, propionate, glycolate, citrate, butyrate, iso-butyrate, glucose and alcohols are not utilized. Uses sulfate, sulfite, thiosulfate and elemental sulfur as an electron acceptor; nitrate and Fe (III) are not utilized. Growth on H_2 gas is observed only in the presence of yeast extract or acetate. Fermentative growth is not observed. Predominant fatty acid is $C_{16:0}$. The G + C content of genomic DNA of the type strain is 57.7 mol%. The type strain PPLL^T (= DSM 110475 = JCM 39161) was isolated from marsh soil. The GenBank/EMBL/DDBJ accession number for the complete genome and the 16S rRNA gene sequence of strain PPLL^T are AP025516 and LC702440.

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Data availability The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene and genome sequence of strain PPLL^T are LC702440 and AP025516. The genome sequence of the strain PPLL was published in public database.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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