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ORIGINAL PAPER

Bacillus tepidiphilus sp. nov., isolated from tepid spring

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Abstract

A novel *Bacillus* strain, designated SYSU G01002^T, was isolated from a sediment sample collected from tepid spring in Tengchong, Yunnan province, south-west PR China. The 16S rRNA gene sequence analysis showed that the strain SYSU G01002^T shared the highest sequence identity with the type strain of *Bacillus alkalitolerans* (97.7%). Strain SYSU G01002^T grew at pH 6.0–8.0 (optimum, pH 7.0), at 28–55 °C (optimum, 45 °C) and in the presence of 0–2.5% (w/v) NaCl (optimum in the absence of NaCl). It contained *meso*-2,6-diaminopimelic acid as the cell-wall diamino acid and MK-7 as isoprenoid quinone. The major cellular fatty acids were iso-$C_{15:0,}$ iso-$C_{17:0}$ and $C_{16:0}$. The polar were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, and unidentified phospholipid. The genomic DNA G + C content was 38.0 mol %. The digital DNA–DNA hybridization and average nucleotide identity values between SYSU G01002^T and closely related members of the genus *Bacillus* were below the cut-off level recommended for interspecies identity. Based on the above results, strain SYSU G01002^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus tepidiphilus* sp. nov. is proposed. The type strain, SYSU G01002^T (= KCTC 43131^T = CGMCC 1.17491^T).

Keywords Hot spring · *Bacillus* · Polyphasic taxonomy

Introduction

Hot springs once perceived to be sterile but the discovery of *Thermus aquaticus* (Brock 1997) and the valuable enzyme (Taq DNA polymerase) (Chien et al. 1976) suggest this environment not only holds hidden microorganisms but also valuable products. Tengchong (Yunnan Province of China) is the hotspot for the hot springs in China which harbour many unique hot springs with wide microbial diversity (Wang et al. 2014). The temperature of these spring varies from lukewarm (Narsing Rao et al. 2020) to boiling (Wang et al. 2014).

Earlier culture-dependent studies showed that Tengchong hot springs harbour many novel bacterial species (Dong et al. 2015; Narsing Rao et al. 2020). Understanding the importance of the hot spring environment, the present study was conducted to understand a microbial diversity of lukewarm spring located at Tengchong (Yunnan Province of China).

The genus *Bacillus* was first proposed by Cohn (1872) and at the time of writing, the genus comprised more than 280 species (https://lpsn.dsmz.de/genus/bacillus). Members of this genus are Gram-stain-positive, rod-shaped and endospore-former (You et al. 2013; Liu et al. 2018; Rao

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et al. 2019). They have been reported their role in various biogeochemical processes such as sulphur, hydrogen, (Beffa et al. 1996), manganese (Dick et al. 2006) and thiosulfate oxidation (Pérez-Ibarra et al. 2007). They also used as plant growth-promoter and bio-control agents (Cao et al. 2018).

Since the 16S rRNA gene sequence identity of strain SYSU G01002^T with the members of the genus *Bacillus* was low, the present study was conducted to determine its taxonomic position using phenotypic, phylogenetic, chemotaxonomic and comparative genome analysis.

Materials and methods

Strain SYSU G01002^T was isolated from hot spring sediment located in Tengchong, Yunnan province, south-west China (24.95006° N 98.43830° E). Isolation was performed by the serial dilution plate method on trypticase soy agar (TSA) medium (Difco). The purified strain was maintained on TSA slant at 4 °C and as glycerol suspension (20%, w/v) at −80 °C. In addition, it was preserved in lyophilized form in skimmed milk at room temperature. *B. alkalitolerans* KCTC 33631^T was used as a reference strain obtained from the Korean Collection for Type Cultures.

Colony characters of SYSU G01002^T were observed on TSA medium (pH 7.0) incubated for three days at 45 °C. Cell morphology was observed by light microscopy (BH-2 Olympus) and transmission electron microscopy (JEOL JEM-100 CX II). Gram staining was performed by Solarbio's Gram staining kit (China) as per the manufacturer's instructions.

Anaerobic growth was checked using TSA medium supplemented with 5% cysteine and 5% $Na_2S.9H_2O$. The medium was prepared under anaerobic conditions in an anaerobic workstation (A45, Don Whitley Scientific) containing a gas phase of $N_2/H_2/CO_2$ (80:10:10%, by volume).

Growth at various temperatures (4, 10, 15, 20, 28, 30, 37, 40, 45, 50, 55 and 60 °C) and pH range (pH 4.0–12.0, at intervals of 1.0 pH unit) was determined (performed in triplicates) using TSA as the basal medium. The pH of the basal medium was adjusted using the buffer system as described by Xu et al. (2005). The NaCl tolerance was analysed in modified TSA medium (all the ingredients were the same, expect the NaCl was not added) at concentrations 0–12.0% (w/v, at intervals of 0.5%).

Catalase activity was determined by assessing the production of bubbles on the addition of a drop of 3% (v/v) H_2O_2 on the bacterial culture. Oxidase activity was determined based on the oxidation of tetramethyl-*p*-phenylenediamine (Kovacs 1956). Milk coagulation and peptization, and hydrolysis of starch, and Tweens (20, 40, 60 and 80) were determined as described by Gonzalez et al. (1978). Other tests were performed using API ZYM, API 20NE (bioMérieux) and GEN III Micro Plate (Biolog) assays according to the manufacturer's instructions.

The genomic DNA extraction and PCR amplification of the 16S rRNA gene sequence was performed as described by Li et al. (2007). The obtained 16S rRNA gene sequence was compared with available sequences of cultured species at the EzTaxon database (Yoon et al. 2017). Neighbor-joining (Saitou and Nei 1987) and maximum-likelihood (Felsenstein 1981) trees were reconstructed using the MEGA version 7.0 (Kumar et al. 2016) after multiple alignments of sequences using CLUSTAL_X program (Thompson et al. 1997). Evolutionary distance matrices of phylogenetic trees were calculated according to Kimura's two-parameter model (Kimura 1980). Bootstrap analysis was performed with 1000 replications (Felsenstein 1985).

Chemotaxonomic characteristics were observed using several standard methods under identical conditions. Analysis of the isomer of diaminopimelic acid was performed by TLC method following the procedures described by Hasegawa et al. (1983) and Lechevalier and Lechevalier (1970). For the analysis, a loop of cell mass and 0.2 ml of 0.5 N HCl was taken in an ampule, sealed and hydrolysed in the sand bath for 16 h at 121 °C. The plate was developed using methanol, water and 6 N HCl–pyridine (80:26:4:10, v/v). The plate was visualized using 0.4% of ninhydrin heated at 100 °C for 2 min.

Quinones was extracted and purified as described by Collins et al. (1977) and analysed by HPLC (Kroppenstedt 1982). Polar lipids were extracted as described by Minnikin et al. (1979) and identified by two-dimensional TLC (Collins and Jones 1980). Biomass for cellular fatty acids analysis was harvested from cultures grown on TSA for three days. Cellular fatty acids methyl esters were prepared and analysed according to the standard protocol of the Microbial Identification System (Sherlock version 6.1; MIDI database: TSBA6) Sasser (1990).

Genome sequencing of SYSU G01002^T and *B. alkalitolerans* KCTC 33631^T was performed using a paired-end sequencing method on the Hiseq X platform (Illumina, San Diego, CA, USA). Reads of each data set were filtered, and high-quality paired-end reads were assembled using Velvet (version 1.2.10) (Zerbino and Birney 2008). The rRNAs and tRNAs were predicted using RNAmmer (Lagesen et al. 2007) and tRNAscan-SE (Lowe and Eddy 1997), respectively. Circular representation of SYSU G01002^T genome was performed using the CGView Server (Grant and Stothard, 2008). Pan-genome analysis was carried out via the Anvi'o tool (Eren et al. 2015) using NCBI blast and MCL flag (Buchfink et al. 2015; van Dongen and Abreu-Goodger 2012). The average nucleotide identity (ANI) values were determined using JSpecies (Richter et al. 2016). Digital DNA–DNA hybridization (dDDH) was estimated using the GGDC (Genome-to-Genome Distance Calculator; https://

ggdc.dsmz.de/ggdc.php) version 2.1 with BLAST + and formula 2 (Meier-Kolthoff et al. 2013).

Results and discussion

Strain SYSU G01002^T was aerobic, Gram-stain-positive and rod-shaped (0.5–0.7 × 1.2–5.9 µm). Endospores were located at the terminal and show the presence of peritrichous flagella. Strain SYSU G01002^T was positive for catalase which was similar to *B. alkalitolerans* KCTC 33631^T. In API ZYM tests, strain SYSU G01002^T was negative for alkaline phosphatase, esterase lipase (C8), cystine arylamidase, α-chymotrypsin, β galactosidase and *N*-acetyl-β-glucosaminidase while *B. alkalitolerans* KCTC 33631^T was positive. In API 20NE tests, strain SYSU G01002^T was negative for nitrate reduction and aesculin hydrolysis whereas *B. alkalitolerans* KCTC 33631^T and *B. subterraneus* COOI3B^T were positive (Kanso et al. 2002). In Biolog GEN III MicroPlate, strain SYSU G01002^T was positive for dextrin, L-alanine, D-gluconic acid, methyl pyruvate, D-lactic acid methyl ester and β-hydroxy-DL-butyric acid while *B. alkalitolerans* KCTC 33631^T was negative. Detailed differentiating features between strain SYSU G01002^T and its closet members are mentioned in (Table 1).

Table 1 Differential characteristic features of strain SYSU G01002^T and closely related members

Characteristic	1	2	3
Temperature range for growth (°C)	28–55	10–42	37–40
pH range for growth	6–8	7–9	7–9
NaCl concentration growth range (%)	2.5	6.0	9.0
Oxidase	+	+	−
Hydrolysis of starch	−	+	+
Aesculin hydrolysis	−	+	+
Nitrate reduction	−	+	+

Strains: 1, SYSU G01002^T (data from this study); 2, *B. alkalitolerans* KCTC 33631 ^{T}(data from this study); 3, *B. subterraneus* COOI3B^T (Kanso et al. 2002)

+Positive; − negative

The 16S rRNA gene sequence analysis showed that the strain SYSU G01002^T shared the highest sequence identity with the type species of *B. alkalitolerans* (97.7%). The neighbor-joining tree (Fig. 1) showed that strain SYSU G01002^T clustered with the members of the genus *Bacillus*. A similar topology was found when the tree reconstructed using the maximum-likelihood tree (Fig. S1).

Strain SYSU G01002^T contained *meso*-2,6-diaminopimelic acid as the cell-wall diamino acid, which was the same as for other members of the genus *Bacillus* (You et al. 2013; Rao et al. 2019; Liu et al. 2018). The isoprenoid quinone present in strain SYSU G01002^T was MK-7. The major cellular fatty acids of strain SYSU G01002^T were iso-$C_{15:0,}$ iso-$C_{17:0}$ and $C_{16:0}$. The cellular fatty acid composition of strain SYSU G01002^T varied when compared with *B. alkalitolerans* KCTC 33631^T (Table S1). The polar lipids of strain SYSU G01002^T were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, and an unidentified phospholipid (Fig. S2). The polar lipid profile of *B. alkalitolerans* T3-209^T reported to consist phosphatidylglycerol, unidentified phospholipids and unidentified aminolipid (Liu et al. 2018) while *B. subtilis* DSM 10^T consist diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and unknown aminophospholipids (Kämpfer et al. 2006).

The genome of SYSU G01002^T contained 63 contigs with a total size of 3,370,610 bp with an N_{50} length of 92,587 bp. The genomic DNA G + C content was 38.0 mol%. A total of 12 rRNAs and 84 tRNAs were predicted. A

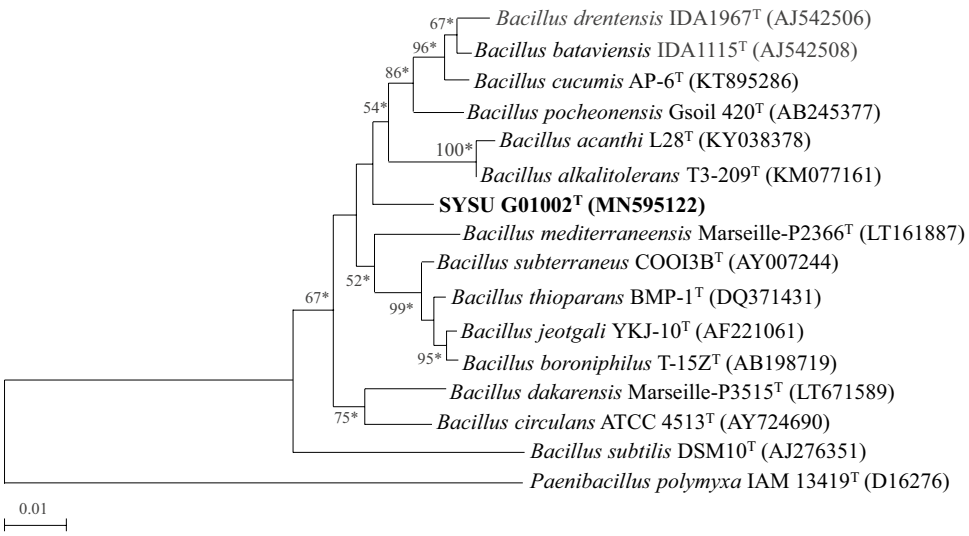

Fig. 1 Neighbor-joining phylogenetic tree showing the relationships between strain SYSU G01002^T and related species. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at branch points. Bar, 0.01 substitutions per nucleotide position. An asterisk indicates conserved clades recovered when the phylogenetic tree was reconstructed using the maximum-likelihood method

comparative genomic feature between SYSU G01002^T and its closet members of the genus *Bacillus* are mentioned in Table S2. Fig. S3 shows the circular genomic feature view of strain SYSU G01002^T. A comparison of SYSU G01002^T with closely related *Bacillus* members regarding coding sequences (CDSs) shows a dissimilar distribution. The pan-genome analysis suggests that (Fig. S4), the number of singleton gene clusters between SYSU G01002^T and its closet members of the genus *Bacillus* varied. The ANI and dDDH values between SYSU G01002^T and other closely related *Bacillus* members (Table S3) were below the cut-off level (ANI 95–96% and dDDH 70%) for bacterial species delineation (Richter and Rosselló-Móra 2009; Goris et al. 2007; Meier-Kolthoff et al. 2013).

Taxonomic conclusion

Based on the above results, strain SYSU G01002^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus tepidiphilus* sp. nov. is proposed.

Description of *Bacillus tepidiphilus* sp. nov

Bacillus tepidiphilus (te.pi.di'phi.lus. L. adj. masc. *tepidus* lukewarm; Gr. masc. adj. *philös* friendly to; N.L. masc. n. *tepidiphilus* liker of lukewarm conditions).

Cells are Gram-stain-positive, rod-shaped (0.5–0.7 × 1.2–5.9 μm), motile and endospore-forming. Colonies are cream-coloured and round. Aerobic growth on TSA medium occurs at pH 6.0–8.0 (optimum, pH 7.0), at 28–55 °C (optimum, 45 °C) and in the presence of 0–2.5% (w/v) NaCl (optimum in the absence of NaCl). Positive for catalase and oxidase. Hydrolysis of starch and Tweens 20, 40 and 80 are negative but positive for Tween 60. In API ZYM tests, positive for esterase (C4), acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-glucosidase.

In API 20NE tests, positive for gelatin hydrolysis and assimilation of D-maltose, D-Mannitol and D-glucose. With the Biolog GEN III MicroPlate, positive for, dextrin, sucrose, α-D-glucose, D-fructose, D-mannitol, *myo*-inositol, glycyl-L-proline, L-alanine, L-arginine, L-glutamic acid, D-gluconic acid, methyl pyruvate, D-lactic acid methyl ester, citric acid, β-hydroxy-DL-butyric acid, acetoacetic acid, propionic acid and acetic acid. The polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and an unidentified phospholipid. The diagnostic diamino acid of peptidoglycan is meso-2,6-diaminopimelic acid and MK-7 as the only respiratory quinone. The major cellular fatty acids are iso-$C_{15:0,}$ iso-$C_{17:0}$ and $C_{16:0}$. The genomic DNA G + C content is 38.0 mol%. The type strain, SYSU G01002^T (= KCTC 43131^T = CGMCC 1.17491^T), was isolated from a sediment sample collected from tepid spring in Tengchong, Yunnan province, south-west PR China. The GenBank accession numbers for the 16S rRNA gene and the genome sequence are MN595122 and WIAQ00000000, respectively.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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