Effusibacillus dendaii sp. nov. isolated from farm soil

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Abstract
A Gram-positive, rod-shaped, spore-forming, thermophilic, and acidophilic bacterium, designated as strain skT53T, was isolated from farm soil in Tokyo, Japan. Under aerobic conditions, the strain grew at 35–55 °C (optimum temperature 44–55 °C) and pH 4.0–6.0 (optimum pH 5.0). Phylogenetic analysis of the 16S rRNA gene sequence showed that the isolate was moderately related to the type strain of Effusibacillus consociatus (94.3% similarity). The G+C content of the genomic DNA was 48.2 mol%, and MK-7 was the predominant respiratory quinone in the strain. The major fatty acids were anteiso-C15:0, iso-C15:0, and iso-C16:0. Based on the phenotypic and chemotaxonomic characteristics, as well as 16S rRNA gene sequence similarity and whole genome analyses, strain skT53T represents a novel species in the genus Effusibacillus, for which the name Effusibacillus dendaii sp. nov. has been proposed. The type strain is skT53T (= NBRC 114101T = TBRC 11241T).

Keywords Effusibacillus · Soil · 16S rRNA · Alicyclobacillaceae · Novel species · Firmicutes

Introduction
The family Alicyclobacillaceae was established by da Costa and Rainey (2009) to accommodate only the genus Alicyclobacillus, which includes spore-forming, acidophilic, and thermophilic bacteria with cellular fatty acids comprising ω-alicyclic fatty acids (Wisotzkey et al. 1992). The genera Kyrpidia (Klenk et al. 2011) and Tumebacillus (Steven et al. 2008) have been added to this family since it was first established. Species belonging to the genus Kyrpidia, which contains two species (Bonjour and Arago 1984; Reiner et al. 2018), are spore-forming, thermophilic, and acidophilic; whereas, species belonging to the genus Tumebacillus are spore-forming and mesophilic. Although typical bacteria belonging to the genus Alicyclobacillus possess ω-alicyclic fatty acids, a few members with no detectable ω-alicyclic fatty acids have also been identified; among them, two species named A. consociatus and A. pohliae, which are phylogenetically distinct from previously known species, have been established (Glaeser et al. 2013; Imperio et al. 2008). Based on 16S rRNA gene sequencing analysis, these two species were subsequently reclassified into the new genus Effusibacillus along with E. lacus in 2014 (Watanabe et al. 2014). In the present study, a novel strain, termed skT53T, isolated from farm soil was taxonomically studied using polyphasic approaches. Herein, we propose a new species of the genus Effusibacillus.
Materials and methods

Sampling and isolation

Strain skT53T was isolated from the soil of a farm in Adachi-ku Tokyo (35°47′01.3″ N, 139°47′58.0″ E), where crop failure occurred in 2014.

Briefly, 1 g of soil sample was suspended in 4.5 mL of sterile saline, and the suspension was allowed to stand for 10 min prior to the recovery of the supernatant. The supernatant was serially diluted and plated on SKT medium containing (per liter) 0.8 mg Difco nutrient broth, 6 g gelatin gum, and 0.16 g CaCl2. The inoculated plates were incubated at 37 °C for 4 weeks. To select microorganisms that exhibited growth only in oligotrophic media, colonies formed were transferred to both SKT and LB media plates. Microorganisms that showed growth on SKT medium without demonstrating growth on LB medium were selected. Selected microorganisms were suspended in sterile saline and incubated at 80 °C for 30 min to enable the formation of spores, and each suspension was then plated onto SKT medium. After incubation at 37 °C for 4 weeks, growth of nine microorganisms was observed; these were then collected and stored for further analysis. The nine microorganisms were suspended individually in sterile saline and incubated at 80 °C for 30 min to facilitate spore formation. After incubation, sporulation of all nine microorganisms was confirmed using a Witz spore staining kit (Muto Pure Chemicals, Japan). The 16S rRNA genes of the nine microorganisms were amplified through PCR with the primers 27f (= 8f) and 1492r (Turner et al. 1999; Loy et al. 2002), and their sequences were analyzed. Among the nine microorganisms, we selected the one with the lowest similarity in 16S rRNA gene sequences compared to those with known sequences and designated it as strain skT53T.

Phenotypic and microscopic analysis and growth conditions

Analysis of cell morphology, assessment using Gram staining, determination of catalase and oxidase activities, and examination of optimal medium conditions were performed by the Identification Service of Techno Suruga Lab Co., Ltd., Japan. Cell morphology was observed using a stereomicroscope (SMZ800N; Nikon, Japan). Gram staining results were observed under a light microscope (BX50F4; Olympus, Japan) using a Gram staining kit (Nissui Pharmaceutical, Japan). Catalase and oxidase activities were determined according to the method described by Barrow and Feltham (1993). To assess anaerobic growth, the growth of the strain skT53 on a yeast extract mineral medium plate (DSM Medium 259) was analyzed as described previously (Hamada et al. 2012). The

Chemotaxonomic analysis

Menaquinone extraction was performed according to the methods described by Collins et al. (1977), and analysis was performed using HPLC (Kroppenstedt 1982). Polar lipids were extracted from 100 mg of freeze-dried cells, purified using the methods described by Minnikin et al. (1979), and analyzed via thin-layer chromatography using chloroform/methanol/water (65:25:4, by volume) in the first direction and chloroform/acetic acid/methanol/water (80:18:12:5, by volume) in the second. Cellular fatty acid methyl esters were identified and quantified by gas chromatography (6890 N; Agilent Technologies, USA) according to the standard protocol of the Sherlock Microbial Identification System (Sasser 1990) with the Sherlock Midi software (version 6.2) and the TSBA6 database. Amino acids of peptidoglycans were identified using the Sherlock Microbial Identification System (Sasser 1990). To assess anaerobic growth, the growth of the strain skT53 on a yeast extract mineral medium plate (DSM Medium 259) was analyzed as described previously (Hamada et al. 2012). The
isomer of diaminopimelic acid (DAP) in the cell wall peptidoglycan was determined as described by Hasegawa (1983).

The 16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene fragment was amplified using the universal primers 9F and 1510R. The nucleotide sequence of the amplified fragment was determined by Fasmac Co., Ltd. (Japan), using the primers 9F, 515F, 1099F, 536R, 926R, and 1510R (Lane et al. 1985; Turner et al. 1999; Lane 1991; Nakagawa et al. 2001). The almost-complete 16S rRNA gene sequence (1471 nt) of strain skT53T was compared with that of the type strains of species with valid published names using EzBioCloud (Yoon et al. 2017a). The CLUSTAL X program (Thompson et al. 1997) was used to align the 16S rRNA gene sequence of strain skT53T with the corresponding sequences of the family Alicyclobacillaceae. Phylogenetic trees were reconstructed using the neighbor-joining (NJ) (Saitou et al. 1987), maximum-likelihood (ML) (Felsenstein 1981), and maximum-parsimony (MP) (Fitch 1971) algorithms using the MEGA X program (Kumar et al. 2018). The resultant tree topologies were evaluated by performing bootstrap analysis (Felsenstein 1985) based on 1000 replicates.

Genome sequencing and analysis

Genomic DNA extraction of the strain skT53T was conducted using cultured cells and the Wizard® Genomic DNA Purification Kit (Promega, USA). Genome sequencing was performed by Macrogen Japan Co., Ltd. (Japan) using the PacBio RSII. The reads of each strain were assembled using the FALCON-integrated version 2.14. The DNA G+C content of strain skT53T was 48.2 mol%. The consensus phylogenetic tree was constructed based on the data of a multi-locus alignment of core genes in the strain skT53T with related species in the NCBI Assembly database using the automated multi-locus species tree (autoMLST) (https://autolst.ziemertlab.com) (Alanjary et al. 2019).

The average nucleotide identity (ANI) and the digital DNA–DNA hybridization (dDDH) values were used to calculate genomic similarities between strain skT53T and the type strains of other Effusibacillus species. The ANI based on BLAST was determined using the ANI Calculator (https://www.ezbiocloud.net/tools/ani) (Yoon et al. 2017b; Goris et al. 2007), and the dDDH values were calculated using the Genome-to-Genome Distance Calculator 2.1 (GGDC; http://ggdc.dsmz.de/distcalc2.php) (Auch et al. 2010; Meier-Kolthoff et al. 2013a, b). Formula 2 was applied to dDDH analysis.

Results and discussion

The cells of strain skT53T were rod shaped, and Gram staining results indicated that the cells were Gram positive. The diameter of the cells grown on yeast extract mineral medium ranged from 0.6 to 0.8 µm, and their length ranged from 2 to 10 µm. Spores were observed at high temperatures. The phenotypic characteristics of the strain skT53T are shown in Table 1.

Table 1 Differential properties of skT53T and representatives of other species in the genus Effusibacillus and some other species in the family Alicyclobacillaceae

| Characteristic                                  | 1   | 2   | 3   | 4   | 5   | 6   |
|------------------------------------------------|-----|-----|-----|-----|-----|-----|
| Anaerobic growth                               | w   | +   | −   | +   | −   |     |
| Optimal growth temperature                     | 44–55| 50–52| 30  | 55  |     | 60–65| 25–30|
| pH range for growth                            | 4.0–6.0| 7.0–8.5| 5.5–10.5| 4.5–7.5|     | 2.0–6.0| 5.5–9.0|
| Oxidase/catalase                               | +/− | +/− | Weak/− | −/− | −/Weak | −/− | −/− |
| DNA G+C content (mol%)                         | 48.2| 50.8| 47  | 55.1| 60.3| 53.1|
| Major fatty acids                              | anteiso-C15:0, iso-C14:0, iso-C16:0 | iso-C14:0, iso-C15:0, iso-C16:0 | iso-C15:0, anteiso-C15:0 | iso-C15:0, iso-C16:0, iso-C17:0 | ω-cyclohexane-C17:0, ω-cyclohexane-C19:0 | iso-C15:0 |

Aerobic growth on:

D-Glucose − + + + + +
D-Fructose − + − + + +
Cellubiose − + − + + +
Sucrose − − + + + +
Melibiose − − − + + +
N-Acetylglicosamine − + + + − s

Strains: 1, skT53T; 2, Effusibacillus lacus skLN1T; 3, Effusibacillus consociatus CCGU 53762T; 4, Effusibacillus pohliae CIP 109385T; 5, Alicyclobacillus acidocaldarius 104-1A; 6, Tumebacillus permanentifrigoris Eur1 9.5T. Data for strains 2–6 were obtained from Watanabe et al. (2014)

+, growth; −, no growth; w, weak growth; s, slight growth
The skT53T strain did not grow with the sugars tested but grew aerobically on yeast extract, acetate, fumarate, D-lactate and succinate. No sugar was oxidized in the sugar oxidation test using API50CH. The strain showed weak anaerobic growth under the conditions described in the Materials and methods. Evaluation of enzyme activity using APIZYM revealed that the strain skT53T exhibits alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine allyl amidase, acid phosphatase, and naphthol-AS-BI-phosphohydразide activities.

The major cellular fatty acids (> 10% of the total) of strain skT53T were anteiso-C15:0 (18.26%), iso-C15:0 (17.40%), and iso-C16:0 (15.36%); the total cell fatty acid profile of the strain is shown in Table 2. The respiratory quinones in the strain were MK-7 (97.5%) and MK-8 (2.4%). Polar lipids included diphosphatidylglycerol, phosphatidylglycerol, phosphatidylmethylethanolamine, three unidentified phospholipids, and two unidentified polar lipids. The peptidoglycan sample contained alanine, glutamic acid, and meso-diaminopimelic acid. All chemotaxonomic data are consistent with the description of the genus Effusibacillus.

The 16S rRNA gene sequence indicated that the species most closely related to strain skT53T were *E. consociatus* CCUG53762T strain (94.3%; Glaeser et al. 2013), *E. lacus* skLN1T (93.4%; Watanabe et al. 2014) and *E. pohliae* MP4 (93.5%; Imperio et al. 2008). Strain skT53T showed less than 91.3% 16S rRNA gene sequence similarity with other members of the family Alicyclobacillaceae. These results are also in line with the classification of strain skT53T in the genus Effusibacillus. Phylogenetic analysis based on the 16S rRNA gene sequences revealed that strain skT53T forms a clade with members of the genus *Effusibacillus* supported by a high bootstrap value (Fig. 1) and ML and MP algorithms (Supplemental Fig. S1 and S2). As shown in Fig. 1 (NJ), strain skT53T formed a clade with members of the genus *Effusibacillus*, and the genera *Alicyclobacillus*, *Tumebacillus*, and *Effusibacillus* formed independent clades. In Supplemental Fig. S1 (ML) and S2 (MP), strain skT53T formed a clade with members of the genus *Effusibacillus* and *Tumebacillus*. Members of the genus *Tumebacillus* formed a daughter clade against the genus *Effusibacillus*. These results show that strain skT53T phylogenetically belongs to the genus *Effusibacillus*. In addition, these results based on the 16S rRNA gene sequences were consistent with the phylogenetic analysis based on the multi-locus alignment of core genes (Fig. 2).

Strain skT53T exhibited dDDH values of 22.5% for *E. lacus* DSM 27172T and 18.8% for *E. pohliae* DSM 22757T; both values are clearly below the 70% threshold for the definition of bacterial species (Wayne et al. 1987). Strain skT53T exhibited an ANI of 72.0% for *E. pohliae* DSM 22757T and 71.8% for *E. lacus* DSM 27172T. These results are significantly below 95–96% (Goris et al. 2007) recommended cutoff points for ANI values.

Based on the data presented, we conclude that strain skT53T represents a novel species of the genus *Effusibacillus*, for which the name *E. dendaii* sp. nov. is proposed.

**Description of *Effusibacillus dendaii* sp. nov.**

*Effusibacillus dendaii* (den.dai’i. N.L. gen. masc. n., *Dendai’s dendaii* (the Japanese abbreviation name of Tokyo Denki University, where this isolate was initially characterized)).

The isolate is a facultatively anaerobic, gram-positive, spore-forming, and rod-shaped bacterium with a cell size of 0.6–0.8 × 2–10 µm. The temperature range for growth is 35–55 °C, and the optimum growth temperature is 44–55 °C. The pH range for growth is 4.0–6.0, and the

| Table 2 | Cellular fatty acid contents (percentage of total) of strain skT53T and the closest relatives |
|---------|------------------------------------------------------------------------------------------|
| **Fatty acid** | **1** | **2** | **3** | **4** |
| iso-C13:0 | 0.52 | - | - | - |
| iso-C14:0 | 7.99 | 24.93 | 7.5 | 3.0 |
| C15:0 | 0.63 | 1.98 | 0.8 | - |
| iso-C15:0 | 20.14 | 14.93 | 12.9 | 39.4 |
| anteiso-C15:0 | 25.85 | 11.3 | 20.4 | 10.3 |
| C16:0 | - | - | - | - |
| C15:0 9c | 0.38 | - | - | - |
| iso-C16:1 H | 0.38 | 1.88 | 8.4 | - |
| iso-C16:0 | 17.95 | 36.31 | - | 14.0 |
| C16:1 9c | - | - | 1.5 | - |
| C16:0 | 7.00 | 5.86 | 3.9 | 2.7 |
| iso-C17:0 | 5.31 | 2.05 | 7.5 | 19.6 |
| iso-C17:0 9c | - | - | 0.9 | - |
| anteiso-C17:0 | 2.36 | 0.76 | 2.7 | 6.2 |
| C17:0 9c | 0.56 | - | - | - |
| C17:0 | 8.05 | - | - | - |
| iso-C18:1 H | - | - | 1.9 | - |
| iso-C18:0 | 0.46 | - | - | - |
| C18:0 | 0.63 | - | - | - |
| C18:2 9c | 0.46 | - | - | - |
| **Summed Features** | 0.42 | - | - | - |
| **Sum In Feature 3** | 0.92 | - | - | - |

Strains: 1, skT53T; 2, *Effusibacillus lacus* skLN1T; 3, *Effusibacillus consociatus* CCUG 53762T; 4, *Effusibacillus pohliae* CIP 109385T.

Data for strains 2, 3, and 4 were taken from Watanabe et al. (2014) and Glaeser et al. (2013). –, Not detected/not reported.

*-summed features are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system. Summed feature 3 comprises C16:1 9c/C16:1 9c. Summed feature 4 comprises C17:1 iso I/anteiso B.
optimum pH was 5.0. It is oxidase positive but catalase negative. It utilizes yeast extract, acetate, fumarate, D-lactate, and succinate as carbon sources. The major fatty acids are anteiso-C\textsubscript{15:0}, iso-C\textsubscript{15:0}, and iso-C\textsubscript{16:0}. The major respiratory quinone is MK-7. The polar lipids comprise diphosphatidylglycerol, phosphatidylglycerol,
phosphatidylethanolamine, phosphatidylmethyl ethanolamine, three unidentified phospholipids, and two unidentified polar lipids. The diagnostic diamino acid in the cell wall peptidoglycan is meso-DAP. The in silico genomic DNA G + C content of the type strain is 48.2 mol%.

The type strain, skTS5{T (NBRC 114101 {T = TBRC 11241{T)}, was isolated from farm soil in Adachi-ku, Tokyo, Japan.

Supplementary Information The online version contains supplementary material available at [link].

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Declarations

Conflict of interest The authors declare no conflict of interest.

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