Effusibacillus dendaii sp. nov. isolated from farm soil

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Abstract

Gram-positive, rod-shaped, spore-forming, thermophilic, acidophilic bacterium, designated strain skT53^T, was isolated from farm soil in Tokyo, Japan. The strain grew aerobically at 37–55°C (optimum 50°C) and pH 4.0–6.0 (optimum 5.0). Phylogenetic analysis of the 16S rRNA gene sequence showed that the isolate was most closely related to the type strain of *Effusibacillus consociatus* (94.3% similarity). The G + C content of the genomic DNA was 48.22 mol%. MK-7 was the predominant respiratory quinone. The major fatty acids were anteiso-C_{15:0}, iso-C_{15:0}, iso-C_{16:0} and C_{18:3}ω6c. The results of phenotypic and chemotaxonomic, 16S rRNA gene sequence similarity, and whole genome analyses support strain skT53^T as representing a novel species of *Effusibacillus dendaii* sp. nov. is proposed. The type strain is strain skT53^T (= NBRC 114101^T = TBRC 11241^T).

Introduction

When the family *Alicyclobacillaceae* was established (da Costa & Rainey, 2009), it included only the genus *Alicyclobacillus*. Most members of the genus *Alicyclobacillus* are spore-forming, acidophilic and thermophilic bacteria possessing cyclic fatty acids in their cellular fatty acid profiles (Wisotzkey et al., 1992), but ω-alicyclic fatty acids were not detected in several species described recently (Glaeser et al., 2013). Since establishment of the family, the genera *Tumebacillus* (Steven et al., 2008) and *Kyrpidia* (Klenk et al., 2011) have been added to this taxonomic group. Species of the genus *Tumebacillus* are also spore-forming, but strictly aerobic and mesophilic bacteria. The genus *Kyrpidia* contains two species isolated from pond water and sediments in geothermal areas (Bonjour & Arago, 1984; Reiner et al., 2018), which are spore-forming, thermophilic and acidophilic. In addition to these genera, two species of the genus *Alicyclobacillus*, *A. consociatus* and *A. pohliae*, which are phylogenetically distinct from previously known species, have been proposed (Glaeser et al., 2013; Imperio & Marri, 2008). These two species form a distinct phylogenetic cluster between the genera *Alicyclobacillus* and *Tumebacillus* (Glaeser et al., 2013). These two species were proposed as members of the genus *Effusibacillus* along with *Effusibacillus lacus* (Watanabe et al., 2014). In this study, a novel strain, skT53^T isolated from farm soil was taxonomically studied using polyphasic approaches. Thus, we proposed to be a new species of the genus *Effusibacillus*.

Material And Methods

Sampling and isolation

Strain skT53^T 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. This soil was diluted with physiological saline and inoculated into a low nutrient acid medium (I⁻¹) (Difco Nutrient Broth 0.08 mg, gellan gum 6 g, CaCl₂ 0.16 g). The inoculated medium was incubated at 37°C for 4 weeks. In order to isolate the low auxotrophic strain, the

obtained strain was transferred to a nutrient rich LB medium and its growth was confirmed. The strain that did not grow in LB medium was designated as strain skT53^T.

Phenotypic and microscopic analysis and growth conditions

Analysis of cell morphology, Gram stain, catalase and oxidase activities, and examination of optimal medium conditions were performed by the identification service of Techno Suruga Lab Co., Ltd., Shizuoka, Japan. The cell morphology was observed using a stereomicroscope (SMZ800N; Nikon, Japan). Gram staining was observed with a light microscope (BX50F4; Olympus, Japan) using the Gram staining kit (Nissui Pharmaceutical, Japan). Catalase and oxidase activities were determined according to Barrow & Feltham (1993).

Each experiment characterizing the isolate was carried out 3 times at 50 °C. (excluding growth temperature test) using Yeast Extract Mineral medium (DSM Medium 259). The composition of the medium is 3.5 g/l Na₂HPO₄ · 12H₂O, 1.0 g/l K₂HPO₄, 0.03 g/l MgSO₄ · 7H₂O, 0.5 g/l NH₄Cl, 4.0 g/l Yeast Extract to pH 5.0 (excluding pH test). It was adjusted. Cultures were incubated at 10 different temperatures ranging from 37°C to 80°C. The utilization of organic substrates was tested in basal medium buffered with 20 mM MES-NaOH (pH 5.0) under oxygen conditions of (l⁻¹): 1.0 g MgSO₄ · 7H₂O, 0.1 g CaCl₂ · 2H₂O, 0.1 g NH₄Cl, 0.1 g KH₂PO₄, 0.1 g KCl, 1 ml trace element solution, 1 ml selenite tungstate solution, 1 ml vitamin mixed solution, 1 ml vitamin B₁₂ solution, 1 ml thiamine solution. All stock solutions were prepared as described by Widdel & Bak (1992). Each substrate was added to defined media at a final concentration of 10 mM. Substrates tested included carbohydrates (D-glucose, D-galactose, D-arabinose, sucrose, cellobiose, D-fructose, maltose, mannose, melibiose, D-sorbitol, trehalose, D-xylose, *N*-acetylglucosamine) and organic acids (acetate, disodium succinate hexahydrate). Yeast extract was also tested at a final concentration of 2 g/l. The sugar oxidation test was carried out by API50CH (BioMérieux, Japan). The enzyme activity test was carried out by APIZYM (BioMérieux, Japan).

Chemotaxonomic analysis

Menaquinones were extracted according to Collins et al. (1977) and were analysed by HPLC (Kroppenstedt, 1982). Polar lipids were extracted from 100 mg freeze-dried cells, purified using the method described by Minnikin et al. (1979) and analysed by thin-layer chromatography using chloroform/methanol/water (65:25:4, by vol.) in the first direction and chloroform/acetic acid/methanol/water (80:18:12:5, by vol.) in the second. Cellular fatty acid methyl esters were analysed by gas chromatography (6890N; Agilent Technologies) 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.

16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene fragment was amplified with universal primers 27f and 1492r. The nucleotide sequence of the amplified fragment was determined by the service of Fasmac Co., Ltd. in Kanagawa,

Japan. The 16S rRNA gene sequence of strain skT53^T was compared with those of type strains of species with validly published names using EzBioCloud (Yoon et al., 2017). The clustal_x program (Thompson et al., 1997) was used to align the almost-complete 16S rRNA gene sequences (both 1471 nt) of strain skT53^T with corresponding sequences of the family *Alicyclobacillaceae*. Phylogenetic trees were reconstructed by the neighbour-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 bootstrap analysis (Felsenstein, 1985) based on 1000 replicates.

Genome sequencing and analysis

Genomic DNAs of strain skT53^T were prepared from cultured cells according to the procedure Wizard® Genomic DNA Purification Kit (Promega). Genome sequencing was carried out by MacroGen Japan Co., Ltd. in Kyoto, Japan using the PacBio RSII. The reads of each strain were assembled using the FALCON-integrate version2.14 program. The DNA G+C contents of strain skT53^T was 48.22 mol%. The consensus phylogenetic tree was built from a multi-locus alignment of core gene in strain skT53^T with related species in the NCBI Assembly database by using The Automated Multi-Locus Species Tree (autoMLST) (<https://automlst.ziemertlab.com>) (Alanjary et al., 2019).

Results And Discussion

Cells of strain skT53^T were rod-shaped. The diameter of 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 higher temperatures. As a result of Gram staining, the cells were suggested to be of Gram-positive type. The phenotypic characteristics of strain skT53^T strain are shown in Table 1.

Strain skT53^T did not grow with the sugars tested. It was grown aerobically on yeast extract, acetate and disodium succinate hexahydrate. No sugar was oxidized in the sugar oxidation test using API50CH. In the enzyme activity test using APIZYM, the activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine allyl amidase, acid phosphatase, and naphthol-AS-BI-phosphohydrazase were observed.

The major cellular fatty acids (>10% of the total) of strain skT53^T were anteiso-C_{15:0} (18.26%), iso-C_{15:0} (17.40%), iso-C_{16:0} (15.36%). The total cell fatty acid profile of the strain was shown in Table 2. The respiratory quinones of this strain were MK-7 (97.5%) and MK-8 (2.4%). Polar lipids are diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylmethylethanolamine (PME), three unidentified phospholipids (PL) and two unidentified polar lipids (L). All the chemotaxonomic data are consistent with the description of the genus *Effusibacillus*.

The 16S rRNA gene sequence indicated that the closest related species to strain skT53^T were *Effusibacillus consociatus* CCUG53762^T strain (94.3%; Glaeser et al., 2013), *Effusibacillus lacus* skLN1^T (93.4%; Watanabe et al., 2014) and *Effusibacillus pohliae* MP4 (93.5%; Imperio et al., 2008). Meanwhile,

strain skT53^T showed less than 91.3% 16S rRNA gene sequence similarity with the other members of the family *Alicyclobacillaceae*. These features are also in line with the classification of strain skT53^T in the genus *Effusibacillus*. Phylogenetic analysis based on the 16S rRNA gene sequences revealed that strain skT53^T form a clade with the members of genus *Effusibacillus* supported by high bootstrap value (Fig. 1), and ML and MP algorithms (Supplemental Fig. S1 and S2).

The phylogenetic tree based on MLSA (Fig. 2) supported the relationship between strain skT53^T and the members of the *Effusibacillus* in the phylogenetic tree based on 16S rRNA gene sequence. DNA–DNA relatedness values were estimated from genome sequences by digital DNA–DNA hybridization (DDH) using Formula 2 of the Genome-to-Genome Distance Calculator 2.1 (Meier-Koluthoff et al., 2013). Strain skT53^T exhibited levels of DNA–DNA relatedness of 22.5% to *Effusibacillus lacus* DSM 27172^T, and 18.8% to *Effusibacillus pohliae* DSM 22757^T; both of these values are clearly below the 70% threshold for the definition of bacterial species (Wayne et al., 1987). The average nucleotide identity (ANI) was calculated using the web-based program ANI Calculator (<http://www.ezbiocloud.net/tools/ani>) (Yoon et al., 2017). Strain skT53^T exhibited levels of ANI of 71.99% to *Effusibacillus pohliae* DSM 22757^T and 71.76% to *Effusibacillus lacus* DSM 27172^T. These results are significantly below 95~96% (Goris et al., 2007) recommended cut-off points for ANI values.

On the basis of the data presented, we conclude that strain skT53^T represent a novel species of the genus *Effusibacillus*, for which the name *Effusibacillus dendaii* sp. nov. is proposed.

Description of *Effusibacillus dendaii* sp. nov.

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

Aerobic, Gram-positive, spore-forming rods measuring 0.6-0.8× 2-10 µm. Temperature range for growth is 37-55°C; optimum growth temperature is 50°C. pH range for growth is 4.0-6.0; optimum pH is 5.0. Oxidase-test positive, but catalase-test negative. Utilizes yeast extract, acetate and disodium succinate hexahydrate as carbon sources. The major fatty acids are anteiso-C_{15:0}, iso-C_{15:0} and iso-C_{16:0}. The major respiratory quinone is MK-7. The polar lipids contain diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmethylethanolamine, three unidentified phospholipids and two unidentified polar lipids. The in silico genomic DNA G+C content of the type strain is 48.22 mol%.

The type strain, skT53^T (NBRC 114101^T = TBRC 11241^T), was isolated from farm soil in Adachi-ku, Tokyo, Japan.

Declarations

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Tables

Table 1. Differential properties of skT53^T and representatives of other species in the genus *Effusibacillus* and other species in the family *Alicyclobacillaceae*.

Characteristic	1	2	3	4	5	6
Anaerobic growth	+	+	–	+	–	–
Optimal growth temperature	37-55	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%)	48.22	50.8	47	55.1	60.3	53.1
Major fatty acids	anteiso-C _{15:0} , iso-C _{15:0} , iso-C _{16:0}	iso-C _{14:0} , iso-C _{15:0} , iso-C _{16:0}	iso-C _{15:0} , anteiso-C _{15:0}	iso-C _{15:0} , iso-C _{16:0} , iso-C _{17:0}	ω-cyclohexane-C _{17:0} , ω-cyclohexane-C _{19:0}	iso-C _{15:0}
Aerobic growth on:						
D-Glucose	–	+	+	+	+	+
D-Fructose	–	+	–	+	+	+
Cellobiose	–	+	–	+	+	+
Sucrose	–	–	+	+	+	w
Melibiose	–	–	–	+	+	w
<i>N</i> -Acetylglucosamine	–	+	+	+	–	w

Strains: 1, skT53^T; 2, *Effusibacillus lacus* skLN1^T; 3, *Effusibacillus consociatus* CCUG 53762^T; 4, *Effusibacillus pohliae* CIP 109385^T; 5, *Alicyclobacillus acidocaldarius* 104-1A^T; 6, *Tumebacillus permanentifrigoris* Eur1 9.5^T. Data for strains 2, 3, 4, 5 and 6 were taken from Watanabe et al. (2014).

+, Growth; –, no growth; w, slight growth; nd, not detected.

Table 2. Cellular fatty acid contents (percentage of total) of strain skT53^T and the closest relatives

Fatty acid	1	2	3	4
iso-C _{13:0}	0.52	-	-	-
iso-C _{14:0}	7.99	24.93	7.5	3.0
C _{14:0}	0.63	1.98	0.8	-
iso-C _{15:0}	20.14	14.93	12.9	39.4
anteiso-C _{15:0}	25.85	11.3	20.4	10.3
C _{15:0}	-	-	-	2.4
C _{15:1} ω6c	0.38	-	-	-
iso-C _{16:1} H	0.38	1.88	8.4	-
iso-C _{16:0}	17.95	36.31	-	14.0
C _{16:1} ω5c	-	-	1.5	-
C _{16:0}	7.00	5.86	3.9	2.7
iso-C _{17:0}	5.31	2.05	7.5	19.6
iso-C _{17:0} ω9c	-	-	0.9	-
anteiso-C _{17:0}	2.36	0.76	2.7	6.2
C _{17:1} ω6c	0.56	-	-	-
C _{17:0}	8.05	-	-	-
iso-C _{18:1} H	-	-	1.9	-
iso-C _{18:0}	0.46	-	-	-
C _{18:0}	0.63	-	-	-
C _{18:1} 2OH	0.46	-	-	-
Summed Features*				
Sum In Feature 3	0.42	-	-	-
Sum In Feature 4	0.92	-	-	-

Strains: 1, skT53^T; 2, *Effusibacillus lacus* skLN1^T; 3, *Effusibacillus consociatus* CCUG 53762^T; 4, *Effusibacillus pohliae* CIP 109385^T. 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 with the MIDI System. Summed feature 3 contains C_{16:1} ω7c / C_{16:1} ω6c. Summed feature 4 contains C_{17:1} iso I / anteiso B.

Figures

Figure 1

Phylogenetic tree based on 16S rRNA gene sequences created using the neighbour-joining method in MEGA X (Kumar et al., 2018), showing the position of strain skT53T and type strains within the family Alicyclobacillaceae. Numbers at branching points refer to percentages of bootstrap values over 50% derived from 1000 replications. Bar, 0.01 substitutions per nucleotide position.

Figure 2

The consensus phylogenetic tree was built from a multi-locus alignment of core genes in strain skT53T with the family Alicyclobacillaceae species in the NCBI Assembly database by using the Automated Multi-Locus Species Tree (autoMLST) (<https://automlst.ziemertlab.com>).

Supplementary Files

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- [FigureS1.jpg](#)
- [FigureS2.jpg](#)