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Paenibacillus maysiensis sp. nov., a Nitrogen-Fixing Species Isolated from the Rhizosphere Soil of Maize

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Abstract

A novel bacterium SX-49^T with nitrogen-fixing capability was isolated from the rhizosphere soil of maize. Phylogenetic analysis of *nifH* gene fragment and 16S rRNA gene sequence revealed that the strain SX-49^T is a member of the genus *Paenibacillus*. Values of 16S rRNA gene sequence similarity were highest between SX-49^T and *P. jamilae* DSM 13815^T (97.0%), *P. brasiliensis* DSM 14914^T (97.8%), *P. polymyxa* DSM 36^T (97.5%), and *P. terrae* DSM 15891^T (98.8%). The similarity between SX-49^T and other *Paenibacillus* species was < 97.0%. DNA–DNA hybridization values between strain SX-49^T and the four type strains were *P. jamilae* DSM 13815^T: 40.6%, *P. brasiliensis* DSM 14914^T: 27.9%, *P. polymyxa* DSM 36^T: 29.2%, and *P. terrae* DSM 15891^T: 66.4%. The DNA G+C content of SX-49^T was 46.4 mol%. The predominant fatty acids were anteiso-$C_{15:0}$, $C_{16:0}$ and iso-$C_{16:0}$. The predominant isoprenoid quinone was MK-7. The genome contains 5628 putative protein-coding sequences (CDS), 6 rRNAs and 56 tRNAs. The phenotypic and genotypic characteristics, DNA–DNA relatedness, and genome features suggest that SX-49^T represents a novel species of the genus *Paenibacillus*, and the name *Paenibacillus maysiensis* sp. nov. is proposed.

Introduction

The genus *Paenibacillus* was established by Ash et al. [1] based on the analysis of the 16S rRNA gene sequences of group 3 bacilli. Some species of the genus *Paenibacillus* were transferred from the genus *Bacillus*, and further descriptions of novel members increased the number of species of the genus *Paenibacillus* considerably. So far, there are > 110 now (http://www.bacterio.cict.fr/p/paenibacillus.html).

Paenibacillus species have diverse physiological characteristics. They have shown great advantage in agriculture, due to the various enzymes and antimicrobial substances they produced, such as polymyxins and bacitracins [18]. Some strains of the *Paenibacillus* have the ability of nitrogen fixation [3, 10, 11, 19, 20]. So far, > 20 species of *Paenibacillus* have been found to be nitrogen-fixers [33]. A nitrogen-fixing strain SX-49^T is described in this study, representing a novel species of the genus *Paenibacillus*.

Materials and Methods

Isolation

A sample of maize rhizosphere soil was collected in Shaanxi province of China (34°48′N, 109°53′E). 1-g sample was suspended in 9-ml sterile water, stirred for 30 min and heated at 80 °C for 15 min. After that, 100-µl suspension was spread on nitrogen-free medium agar plates in triplicate. The nitrogen-free medium consisted 20 g sucrose, 0.1 g K_2HPO_4, 0.4 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g NaCl, 0.01 g $FeCl_3$, and 0.002 g Na_2MoO_4 per liter water. After incubation at 30 °C for 3 days, single colonies were isolated by streaking plating. Strains were routinely cultured in LD medium (per liter contains 2.5 g NaCl, 5 g yeast, and 10 g tryptone) at 30 °C for further identification and study.

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Nitrogen-Fixing Capability

Conserved amino acid sequences within the *nifH* gene have been exploited to design PCR primers to detect the genetic potential for nitrogen fixation in any environment [2, 21, 26]. A 325 bp fragment of the *nifH* gene was amplified using two degenerate primers for the nitrogenase Fe protein gene, [forward 5′-GGCTGCGATCC(CGA)AAG GCCGA(CT)TC(CGA)ACCCG-3′, reverse 5′-CTG(GCA) GCCTTGTT(CT)TCGCGGAT(CG)GGCATGGC-3′] and sequenced as described by Ding et al. [10].

Acetylene reduction assays were carried out to test nitrogenase activity of strain SX-49^T and reference *Paenibacillus* strains, including *P. jamilae* DSM 13815^T, *P. brasiliensis* DSM 14914^T, *P. polymyxa* DSM 36^T, *P. peoriae* DSM 8320^T, *P. terrae* DSM 15891T, and *P. durus* ATCC 35681^T. Strains were cultured in nitrogen-deficient medium (per liter contains: 10.4 g Na_2HPO_4, 3.4 g KH_2PO_4, 26 mg $CaCl_2{\cdot}2H_2O$, 30 mg $MgSO_4$, 0.3 mg $MnSO_4$, 36 mg ferric citrate, 7.6 mg $Na_2MoO_4{\cdot}2H_2O$, 10 μg *p*-aminobenzoic acid, 5 μg biotin, 4 g glucose, and 2 mM glutamate). After 24 h at 30 °C, strains were incubated under acetylene for 3 days before acetylene reduction assays were performed on GC-2010 Plus gas chromatograph (SHIMADZU, Japan) as described previously [3, 32] to measure nitrogenase activity. All treatments were in three replicates and experiments were repeated more than three times.

Genome Sequencing, Assembly, and Annotation

The draft genome sequence was produced by using Illumina paired-end sequencing technology at the BGI-Shenzhen. Assembly was conducted using SOAP de-novo v. 1.04 assembler [16]. Gene prediction was made using Glimmer v. 3.0 [9]. Annotation of protein-coding sequence was performed by using the Basic Local Alignment Search Tool (BLAST) against the COG, Kyoto Encyclopedia of Genes and Genomes (KEGG) databases and NCBI nr protein database.

Molecular Characterization

The 16S rRNA gene sequence (1518 bp) of SX-49^T was acquired from a PCR product using highly specific forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and universal reverse primer 1492R (5′-GGTTACCTTGTTACGACT T-3′). A preliminary phylogenetic analysis was accomplished using EzTaxon database [4]. The phylogenetic tree calculating evolutionary distance matrices was constructed by the neighbor-joining method [27] using MEGA (version 7.0) [15]. Bootstrap analysis was conducted on 1000 replications [12].

Genomic DNA was extracted and purified as described by Yoon et al. [36]. Estimation of the G+C content was accomplished with *Escherichia coli* K12 as standard using the thermal melting protocol [8]. DNA–DNA relatedness was determined by Ziemke's method [37].

Phenotypic Characterization

Cell morphology was obtained by scanning electrical microscopy (SEM), after incubated on endospore-forming medium agar plate [yeast extract 0.07%, tryptone 0.1%, glucose 0.1%, $(NH_4)_2SO_4$, 0.02%, $MgSO_4{\cdot}7H_2O$, 0.02%, K_2HPO_4, 0.1% (w/v), pH 7.2] for 72 h. Physiological and biochemical characteristics were determined in comparison with *P. jamilae* DSM 13815^T, *P. brasiliensis* DSM 14914^T, *P. terrae* DSM 15891^T, and *P. polymyxa* DSM 36^T. A series of experiments were performed according to Gordon et al. [13], Priest et al. [23], and Rhodes-Roberts [24], including Gram staining, nitrate reduction, production of dextrin, optimal temperature and pH for growth, activities of catalase and oxidase, Voges–Proskauer reaction, and growth inhibition by NaCl and lysozyme. Hydrolysis of casein and starch was tested by Cowan and Steel's method [7]. Acid production was tested using medium containing 1 g $(NH)_2HPO_4$, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.2 g KCl, 0.2 g yeast extract, 10 g sugars or alcohols dissolved in 1 l water [31]. To confirm aerotactic ability, bacterial cells were incubated by mixing with semi-solid medium in test tubes at 40–50 °C, followed by incubation at 30 °C for 3 days.

Chemotaxonomy

Strains were incubated in LD medium at 30 °C for 2 days. The compositions of cellular fatty acid were analyzed according to the method described by Komagata and Suzuki [14] using Sherlock Identification System (MIDI) [28].

Cellular menaquinones and respiratory quinones were extracted, purified, and analyzed by HPLC according to the method described by Collins [5]. Polar lipid was extracted by Minnikin et al.'s method [22], and was identified by two-dimensional TLC by Collins et al.'s method [6]. The type of diamino acid of the cell wall peptidoglycan was determined as described by Schleifer and Kandler [29] and Komagata and Suzuki [14].

Results and Discussion

Nitrogen-Fixing Capability

Since bacteria in the soil sample were cultured in nitrogen-free medium on the purpose of isolating nitrogen-fixing

strain, strain SX-49^T is possible to have nitrogen-fixing capability. To confirm genes encoding nitrogenase are contained in its genome, a 325 bp segment of the *nifH* gene was amplified and sequenced. Phylogenetic analysis showed that the *nifH* gene of strain SX-49^T was clustered with species of genus *Paenibacillus* (Fig. 1), revealing that strain SX-49^T probably is a nitrogen-fixing *Paenibacillus* strain. Acetylene reduction assays were performed to verify the nitrogenase activity of SX-49^T. As shown in Table 1, strain SX-49^T exhibited nitrogenase activity, which was relatively high among nitrogen-fixing *Paenibacillus* species.

Table 1 Nitrogenase activity of strain SX-49^T compared with some nitrogen-fixing type strains of *Paenibacillus* species

Strain	Nitrogenase activity [nmol C_2H_4 (mg protein h)$^{-1}$]
P. jamilae DSM 13815^T	123.9 ± 12.7
P. brasiliensis DSM 14914^T	523.4 ± 10.3
P. polymyxa DSM 36^T	416.5 ± 17.1
P. peoriae DSM 8320^T	38.7 ± 4.8
P. terrae DSM 15891^T	110.4 ± 11.2
P. durus ATCC 35681^T	2327.7 ± 24.2
Strain SX-49^T	1124.6 ± 11.9

Results are mean ± SD of three determinations

Molecular Characterization

A phylogenetic tree based on 16S rRNA gene sequences was constructed using the neighbor-joining method (Fig. 2). Comparison of 16S rRNA gene sequences showed that SX-49^T is clustered with the species of the genus *Paenibacillus*. While similarities between strain SX-49^T and other species of the genus *Paenibacillus* were below 97.0%, strain SX-49^T showed high similarities of 16S rRNA gene sequence with *P. jamilae* DSM 13815^T (97.0%), *P. brasiliensis* DSM 14914^T (97.8%), *P. polymyxa* DSM 36^T (97.5%), and *P. terrae* DSM 15891^T (98.8%).

Given the high similarity between SX-49^T and its closest phylogenetic relatives, DNA–DNA hybridization analysis was conducted. DNA–DNA relatedness between strain SX-49^T and *P. jamilae* DSM 13815^T, *P. brasiliensis* DSM 14914^T, *P. polymyxa* DSM 36^T, *P. terrae* DSM 15891^T were 40.6, 27.9, 29.2, and 66.4%, respectively, values below 70%, indicating that strain SX-49^T is a novel species [34].

The average nucleotide identity (ANI) between two genomes is the most promising method for prokaryotic species definition [25]. Since 66.4% DNA–DNA hybridization between strain SX-49^T and *P. terrae* DSM 15891^T was close to the boundary at 70%, ANI value was calculated. The ANI value between SX-49^T and *P. terrae* HPL-003 is 85.2%; the value below 95% supporting that SX-49^T is a novel species [35].

The DNA G+C content of strain SX-49^T was 46.4 mol%, ranging between 39 and 54% of validly named *Paenibacillus* species [30].

Phenotypic Characteristics

Strain SX-49^T is Gram-positive and facultatively anaerobic. Colonies on LD agar medium were round, convex, cream white, glossy, typically 1.0–2.0 mm in diameter, and uneven in margins after 72 h of incubation at 30 °C. Ellipsoidal spores were visible, positioned centrally or paracentrally in swollen sporangia (Fig. 3).

In order to determine physiological and biochemical characteristics of SX-49^T in comparison with *P. jamilae* DSM 13815^T, *P. brasiliensis* DSM 14914^T, *P. polymyxa* DSM 36^T, and *P. terrae* DSM 15891^T, a series of tests were carried

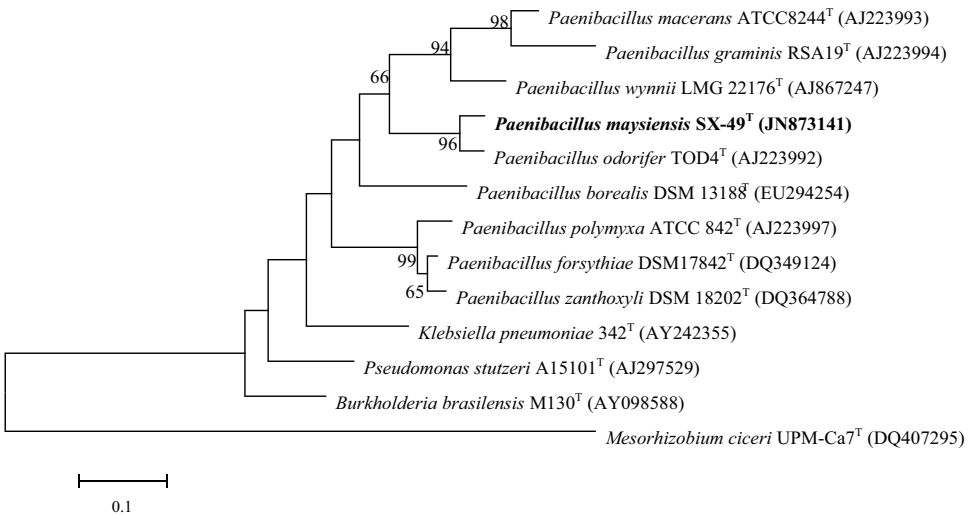

Fig. 1 Phylogenetic tree based on partial *nifH* sequences (292 nt fragment), compared using the neighbor-joining method, showing the position of strain SX-49^T. The numbers at branching points stand for bootstrap values from 1000 replicates. Only values > 50% are shown. Bar 0.1 substitutions per nucleotide position

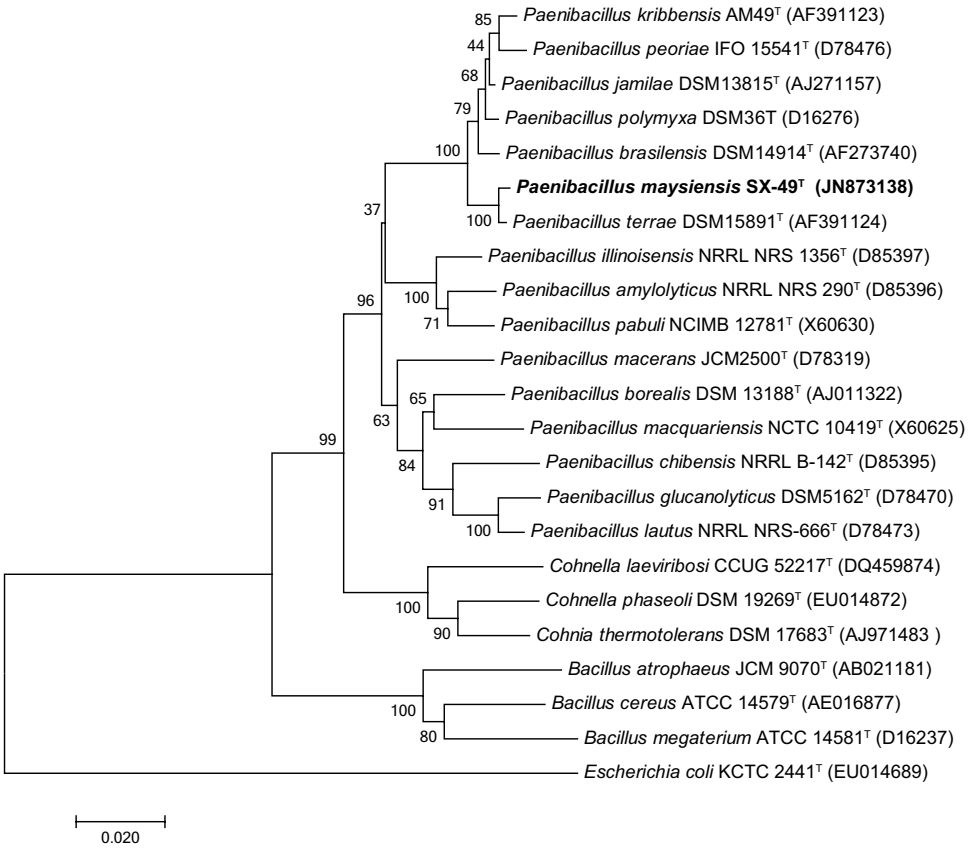

Fig. 2 Neighbor-joining phylogenetic tree based on 16S r RNA gene sequences, positioning strain SX-49^T among species of genus *Paenibacillus*. *E. coli* KCTC 2441^T was used as an out group. Bootstrap analysis was performed with 1000 cycles. Only bootstrap values > 50% are shown at the branch points. Bar 0.02 substitutions per nucleotide positions

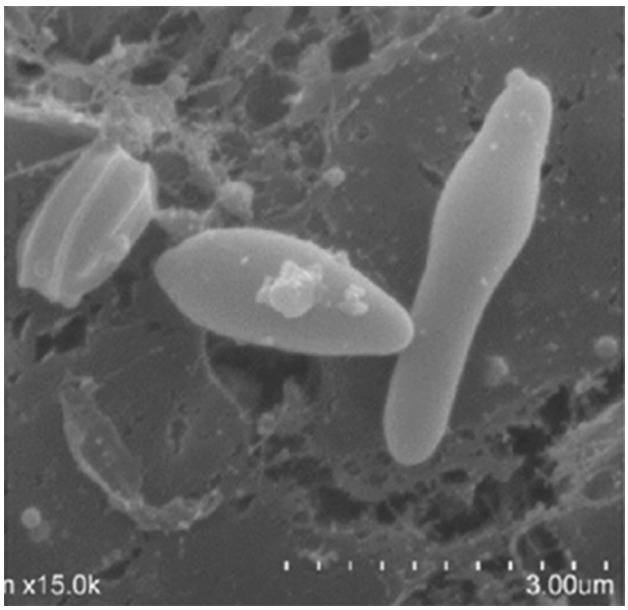

Fig. 3 SEM image of vegetative cells and spores of strain SX-49^T (bar 3 μm)

out following the proposed minimal standards for describing new taxa of aerobic, endospore-forming bacteria [17]. The NaCl concentration range for the growth of strain SX-49^T was 0.1–4% (w/v), with optimal growth at 0.2–0.3%. The pH range for growth was 5.0–9.0, while pH 7.0 was optimal. The temperature range for growth is 10–45 °C, with optimal growth at 30 °C. Strain SX-49^T was determined to be catalase negative and oxidase negative. Strain SX-49^T has the ability to reduce nitrate to nitrite, positive for the Voges–Proskauer reaction, and negative for the methyl red reaction, which differentiated SX-49^T from the most related *P. terrae* DSM 15891^T .

The physiological and biochemical characteristics of SX-49^T are shown in Table 2. Compared with type strains of closely related *Paenibacillus* species, strain SX-49^T exhibited nearly identical phenotypic characteristics. However, characteristics on temperature range, hydrolysis of tween 20, and acid production from mannitol differentiated strain SX-49 from type strains of closely related species.

Chemotaxonomic Characteristics

In order to determine the composition of cellular fatty acid, SX-49^T and type strains *P. jamilae* DSM 13815^T, *P. brasiliensis* DSM 14914^T, *P. polymyxa* DSM 36^T, *P. terrae* DSM 15891^T were incubated in LD medium at 30 °C for 2 days. As shown in Table 3, major fatty acid constitution of SX-49^T was anteiso-$C_{15:0}$ (61.3%). $C_{16:0}$ (6.2%), iso-$C_{15:0}$ (3.9%), iso-$C_{16:0}$ (5.3), and anteiso-$C_{17:0}$ (9.6%) are also important

Table 2 Differential phenotypic characteristics between strain SX-49^T and its closest type strains of the genus *Paenibacillus*

Characteristic	1	2	3	4	5
Temperature range (°C)	10–45	10–40	10–40	10–40	10–40
pH range	5.0–9.0	5.0–9.0	5.0–9.0	5.0–9.0	5.0–9.0
Voges–Proskauer reaction	+	+	+	−	+
Nitrate reduction	+	+	+	+	−
Methyl red reaction	−	−	−	+	−
Hydrolysis of					
Tween 20	−	−	+	+	+
Starch	+	+	+	+	+
Acid production from					
Sucrose	+	+	+	+	+
Sorbierite	−	−	−	−	−
Mannitol	−	+	+	+	+
Glucose	+	+	+	+	+
Raffinose	+	+	+	+	+
L-Arabinose	+	+	−	+	+
DL-Sodium malate	−	−	−	−	−
Sodium citrate	+	+	+	+	+
D-Trehalose	+	+	+	+	+
D-Galactose	+	+	+	+	+
Maltose	+	+	+	+	+
DNA G+C content (mol%)	46.4	43.9	44.5	47.5	45.2

Species: 1. strain SX-49^T; 2. *P. jamilae* DSM 13815^T; 3. *P. brasiliensis* DSM 14914^T; 4. *P. terrae* DSM 15891^T; 5. *P. polymyxa* DSM 36^T

+, Positive reaction; −, negative reaction

Table 3 Fatty acid content (%) of strain SX-49^T and some other type strains of the *Paenibacillus* genus

Fatty acid	1	2	3	4	5
Saturated straight-chain					
$C_{14:0}$	1.15	2.26	1.50	1.48	2.20
$C_{16:0}$	6.22	7.04	11.52	7.58	9.81
$C_{17:0}$	0.99	0.78	0.99	0.79	1.35
Branched fatty acid					
Iso-$C_{14:0}$	1.34	2.26	2.69	2.27	3.74
Anteiso-$C_{15:0}$	61.27	50.44	49.54	54.46	53.28
Iso-$C_{15:0}$	3.89	6.45	2.58	4.70	4.81
Iso-$C_{16:0}$	5.26	7.75	11.10	7.58	10.12
Anteiso-$C_{17:0}$	9.58	5.59	6.51	6.88	6.44
Iso-$C_{17:0}$	1.94	2.08	0.81	1.76	1.31
Unsaturated fatty acids					
$C_{18:1\ \omega9c}$	2.06	1.49	1.93	2.02	2.63
$C_{16:1\ \omega11c}$	0.69	0.88	2.62	0.99	2.08

Species: 1. strain SX-49^T; 2. *P. brasiliensis* 14914^T; 3. *P. jamilae* 13815^T; 4. *P. terrae* 15891^T; 5. *P. peoriae* DSM 8320^T

fatty acid constitution in strain SX-49^T, in conformity to genus *Paenibacillus* (Table 3). The major menaquinone of SX-49^T is MK-7, in accordance with genus *Paenibacillus* [30]. The polar lipids detected by two-dimensional TLC are diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG) (Supplementary Fig. 1), in agreement with the profile of genus *Paenibacillus*. The isomer type of diamino acid of the cell wall peptidoglycan was identified as *meso*-diaminopimelic acid.

Genomic Features

The draft genome of strain SX-49^T has been uploaded to GenBank with accession number NZ_ASRY00000000. The genome of strain SX-49^T is composed of a single circular molecule of 5.65 Mb with an average G+C content of 46.4%. Totally 5628 putative protein-coding sequences (CDS), 6 rRNAs, and 56 tRNAs were identified. The genome of strain SX-49^T contains a *nif* gene cluster, enables it to fix nitrogen. The *nif* gene cluster is composed of *nifB, nifH, nifD, nifK, nifE, nifN, nifX, hesA*, and *nifV* [35].

On the basis of its molecular, phenotypic, and chemotaxonomic characteristics, strain SX-49^T is considered to represent a novel species of the genus *Paenibacillus*, and the name *Paenibacillus maysiensis* sp. nov. is proposed. The type strain is SX-49^T.

Description of *Paenibacillus maysiensis* sp. nov.

Paenibacillus maysiensis (may. si. en' sis. L. gen. n. maysiensis of maize, where the type strain SX-49^T was isolated).

Cells are motile, Gram-positive. In slightly swollen sporangia, an ellipsoidal spore is formed and located in central or paracentral position of cells. Colonies on LD medium are circular, convex, cream white, with diameter 1.0–2.0 mm. Nitrogen fixation positive. The growth temperature is 10–45 °C, optimal at 30 °C. The growth pH range is 5.0–9.0, optimal at pH 7.0. NaCl concentration of 0.1–4% (w/v) is tolerable for growth, optimal at 0.2–0.3%. Voges–Proskauer reaction is positive and nitrate can be reduced to nitrite. Methyl red reaction is negative. Substrates utilized for growth and acid production are as follows: sucrose, glucose, raffinose, L-arabinose, sodium citrate, D-trehalose, D-galactose, and maltose. Catalase negative and oxidase negative. The major menaquinone is MK-7. The predominant fatty acid is anteiso-$C_{15:0}$. The major polar lipids are DPG, PE, and PG. *Meso*-diaminopimelic is the diagnostic diamino acid in the cell wall. The DNA G+C content of type strain SX-49^T is 46.4 mol%. The genome consists of a single circular molecule of 5.7 Mb with totally 5628 putative protein-coding sequences (CDS), 6 rRNAs, and 56 tRNAs identified.

The type strain, SX-49^T (=CGMCC 1.15332 =JCM 31028), was isolated from rhizosphere soil sample of

maize, acquired in Shaanxi Province, P. R. China (34°48′N, 109°53′E). The GenBank (EMBL) accession number for the 16S rRNA gene sequence is JN873138. The Digital Protologue Taxon Number is TA00403.

Compliance with Ethical Standards

Conflict of interest No conflict of interest is declared.

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