



Bacillus pinisoli sp. nov., Isolated from Soil of a Decayed Pine Tree

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Abstract

A Gram-stain-positive, rod-shaped, facultatively anaerobic, motile and spore-forming bacterium with multiple flagella designated GXH0341^T was isolated from the soil associated with decayed pine tree samples collected from Weizhou Island, Beihai, Guangxi, China. Growth occurred at 4–37 °C (optimum 30 °C), at pH 5.0–11.0 (optimum 8.0) and in the presence of 0–7% (w/v) NaCl (optimum 2%). Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain GXH0341^T was most closely related to *Bacillus mesophilus* DSM 101000^T (98.9%), followed by *Bacillus salitolerans* KC1^T (96.95%) and *Margalitia shackletonii* DSM 18435^T (96.67%). Phylogenetic analysis revealed that strain GXH0341^T represented a separate lineage within the genus *Bacillus*. Peroxidase is positive. The predominant quinone was MK-7 and the cell-wall diagnostic diamino acid was *meso*-diaminopimelic acid. The predominant polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, and two unidentified phospholipids. The major fatty acids are *iso*-C_{14:0}, *iso*-C_{15:0}, *anteiso*-C_{15:0} and *iso*-C_{16:0}. The genome of GXH0341^T comprises the biosynthetic gene cluster for T3PKS, terpene, lassopeptide and RRE-containing element as secondary metabolites. The average nucleotide identity values and the digital DNA-DNA hybridization values between GXH0341^T and *B. mesophilus* DSM 101000^T were 78.22% and 21.00%, respectively, which were in the range of the recommended level for interspecies identity. The results of phenotypic, chemotaxonomic and genotypic analyses clearly indicated strain GXH0341^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus pinisoli* sp. nov. is proposed. The type strain is GXH0341^T (=MCCC 1K07157^T =JCM 35212^T).

Introduction

The genus *Bacillus*, first described by Cohn in 1872 [1], are composed of Gram-positive, rod shaped, aerobic or facultatively anaerobic and endospore-forming bacteria of the family *Bacillaceae* within the phylum *Firmicutes*. As the time went by, more and more bacteria were found and classified into this family/genus. Castellani and Chalmers

assigned the tribe *Bacteroideae*, *Haemophileae*, *Pasteurelleae* and *Proteeae* to this family [2]. Alves et al. assigned the genus *Nosocomiicoccus* [3]. Krishnamurthi et al. assigned the genus *Psychrobacillus* [4]. Zhao et al. assigned the genus *Lysinibacillus* [5] and so on. Among the many criteria of classification, one of the standards for the description of new taxa in this group were published in 2009 according to Logan et al. [6] which proposed minimal standards for describing this genus based on their ability to form spores in the presence of oxygen. As a result, the member of the genus *Bacillus* became large and chaotic, and had been recognized taxonomically inoperable and in need for reclassification. Therefore, in order to clarify the evolutionary relationships and classification of *Bacillus* species, comprehensive phylogenomic and comparative analyses were performed on *Bacillus/Bacillaceae* genomes according to Gupta et al. [7], and they arranged most of the *Bacillus* clades, in addition to the *Subtilis* and *Cereus* clades, in 17 novel distinct clades. As a consequence, the genus *Bacillus* was restricted to only the members of the *Subtilis* and *Cereus* clades. However, despite the detailed classification, a number of strains are

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and the genome sequences of strain GXH0341^T are OL336472 and JAJQKI000000000, respectively. Strain GXH0341^T has been deposited in Marine Culture Collection of China (MCCC) and Japan Collection of Microorganisms (JCM), the preservation number are MCCC 1K07157 and JCM 35212, respectively.

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still classified as ambiguous genera, and are not recommended as new genera. In the course of investigating the bacteria diversity of Weizhou Island, Beihai, Guangxi, China, a novel strain GXH0341^T, which was isolated from the soil associated with decayed pine tree and assessed by the analysis of phenotype and genotype.

Materials and Methods

Strain Isolation and Culture Conditions

Strain GXH0341^T was isolated from the soil associated with decayed pine tree samples which were collected on Weizhou Island, Beihai, Guangxi, China (21°05′ 31.71″ N, 109°14′05.33″ E), and the samples were placed in a sterile sampling bag, stored at 4 °C and transported back to the laboratory. 2 g of soil samples were added to 20 mL of 0.85% NaCl (w/v) solution and incubated in 30 °C shaking for 1 h, and used it as a reserve solution and diluted to 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵ with sterile distilled water. Various dilutions were coated on 2216E agar medium (MA) and incubated at 30 °C for 72 h. The GXH0341^T was isolated by the method described above and stored in a glycerol suspension (35%, v/v) and stored in -20 °C. *Bacillus mesophilus* (= CCTCC AB 2015209^T) was obtained from China General Microbiological Culture Collection Center (CGMCC), and the strain was incubated on MA medium and used as reference strain for all experiments.

16S rRNA Gene Sequencing and Phylogenetic Analysis

DNA was extracted using the chelex-100 method [8] and PCR amplification of the 16S rRNA gene was performed as described by Chun et al. [9]. The 16S rRNA gene sequence was determined by Sangon Biotech (Guangzhou, PR China). The 16S rRNA gene sequence of GXH0341^T was identified through the EzBioCloud database (<https://www.ezbiocloud.net>) [10]. The Clustal W algorithm was used in MEGA 11 to perform multiple alignments of sequences. The neighbor-joining [11], maximum-likelihood [12] and maximum-parsimony [13] methods were used to reconstruct phylogenetic trees by MEGA software [14]. The evolutionary distances were computed by the Kimura two-parameter method [15]. The stability of the phylogenetic tree topology was evaluated using the bootstrap method, with a total of 1000 replicates [16].

Genomic Analysis

In order to further genotypic analysis, the whole genomic DNA of strain GXH0341^T was sequenced using the Illumina

Hiseq 4000 (Wekemo Tech Co., Ltd. Shenzhen China). The quality control of the raw data was carried out by Fastp v.0.20.1 software [14], and finally the clean data was assembled into genome sequence by Unicycler v.0.4.8 [15]. The estimated digital DNA-DNA hybridization (dDDH) values were calculated using the genome-to-genome distance calculator GGDC 3.0 (<http://ggdc.dsmz.de/>) [17]. The average nucleotide identity (ANI) was analyzed by JSpeciesWS online service (<http://jspecies.ribohost.com/jspeciesws/>) [18]. The genome sequence was annotated by the NCBI Prokaryote Genome Annotation Pipeline (PGAP). The draft genome of GXH0341^T was examined using the antiSMASH 6.0 (<https://antismash.secondarymetabolites.org/>) server to detect putative biosynthetic gene clusters [19]. The phylogenomic tree was constructed through the PATRIC website (<https://www.patricbrc.org/>) [20].

Physiology and Chemotaxonomy

After culturing on MA for 72 h at 30 °C, the cell morphology of the strain GXH0341^T was observed using a scanning electron microscopy (SUPRA 55 Sapphire, carl AEISS) and transmission electron microscope (HT-7700, Hitachi). Spores were observed with carbol-fuchsin solution. Oxidase activity was measured by 1% (w/v) N, N, N', N'-Tetramethyl-p-phenylenediamine dihydrochloride and oxidized by 3% (v/v) hydrogen peroxide solution [21]. Based on MA medium, growth was observed at different temperatures (0, 4, 15, 25, 30, 37, 45 °C) and NaCl (0–10% w/v, at intervals of 1% NaCl). To determine the range of growth pH (4.0–12.0, at intervals of 1.0 pH unit), we adjusted the pH value using HCl or NaOH and then determined the optimal pH growth value by measuring the value of optical density in 600 nm (OD₆₀₀) such as Xu et al. [22]. According to Gonzalez et al. [23], we hydrolysed gelatin, starch, and Tweens (20, 60, and 80). GXH0341^T was inoculated to MB and cultured on a shaker at 30 °C for 12 h, 75 µL of the bacterial solution was spread on MA, placed in an anaerobic generating bag produced by Merck (Anaerocult A mini) and cultured at 30 °C for 7 days for anaerobic growth test. The motility of the strain was determined using a motility medium (4 g/L agar). Gram staining was carried out according to the method of Allen [24]. Biolog's Gen III ecological board was used to test the carbon utilization of strains. The test panels were performed strictly according to their respective instructions. Susceptibility test discs were used to determine the sensitivity of the strains to antibiotics containing the following amounts (µg per tablet): ampicillin (10), kanamycin (30), amoxicillin (10), cefoxitin (30), carbenicillin (100), bacitracin (0.04), gentamicin (10), tetracycline (30), chloramphenicol (30), streptomycin (10), erythromycin (15), rifampin (5), neomycin (30), streptomycin (300). Other tests were performed using API ZYM, API 50 CH, and API 20NE

test strips (bioMérieux), according to the manufacturer's instructions.

Chemotaxonomic Characteristics

For determination of chemotaxonomic characteristics, strain GXH0341^T and reference strain *Bacillus mesophilus* DSM 101000^T were grown in MB medium for 3 days at 30 °C, harvested by centrifugation, washed with distilled water and freeze-dried. The quinone component was analyzed by menaquinone system using high performance liquid chromatography (HPLC) as described by Collins et al. [25]. The cell-wall amino acids compositions were analyzed as described by Schleifer et al. [26]. The polar lipids were detected by solvent systems using thin-layer chromatography (TLC) [27]. The fatty acid composition was measured according to the instructions of the Sherlock Microbial Identification System (MIDI, version 6.0) [28].

Results and Discussion

Morphological, Physiological and Biochemical Characteristics

The colonies of strain GXH0341^T were pink, rounded, with a convex and smooth surface. The cell of strain GXH0341^T was Gram-stain-positive, rod-shaped approximately 0.5–0.7 µm wide and 2.2–2.6 µm long (Fig. S1) with multiple flagella (Fig. S2) and could produce oval spores outside the cells (Fig. S3). The strain was motile, facultatively anaerobic and its peroxidase was positive, but the oxidase activity was negative. The strain GXH0341^T could grow at 4–37 °C (optimum 30 °C), at pH 5.0–11.0 (optimum 8.0) and in the presence of 0–7% (w/v) NaCl (optimum 2%). The Voges-Proskauer test was negative.

The experimental results indicated that strain GXH0341^T could hydrolyse Tween20, Tween60, and Tween 80. In API 50CH strips, inositol, fructose, potassium 5-ketoglucuronate were positive. In API ZYM strips, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase were positive. Biochemical assays with an API 20NE kits revealed that D-glucose, esculin, 4-nitrophenyl-β-D-galactopyranoside were utilized. GEN III stripes indicated that L-alanine, glucuronamide, acetoacetic acid, L-malic acid, 1% sodium lactate, fusidic acid, D-serine, troleandomycin, rifamycin SV, minocycline, lincomycin, guanidine HCl, niaproof 4, vancomycin, nalidixic acid, lithium chloride, potassium tellurite, aztreonam, sodium butyrate, sodium bromate could be used as carbon sources. And susceptibility

test discs experiments indicated GXH0341^T were sensitive to antibiotics containing the following (µg per tablet): gentamicin (10), tetracycline (30), chloramphenicol (30), streptomycin (10), erythromycin (15), rifampin (5), neomycin (30), streptomycin (300), ampicillin (10), kanamycin (30), amoxicillin (10), carbenicillin (100), bacitracin (0.04) but not sensitive to cefoxitin (30). The comparison of the physiological characteristics of its closely members strains in different experiments are shown in Table 1, besides, all the negative results of test strips are shown in supplementary material Table S5.

16S rRNA Gene Sequencing and Phylogenetic Analysis

The complete 16S rRNA gene sequence of GXH0341^T was determined (1506 bp, GenBank/EMBL/DDBJ accession number OL336472). Based on 16S rRNA gene sequence analyzed of GXH0341^T showed the highest sequence similarity with *Bacillus mesophilus* DSM 101000^T (98.90%), followed by *Bacillus salitolerans* KC1^T (96.95%) and *Margalitia shackletonii* DSM 18435^T (96.67%). Phylogenetic analysis using the neighbor-joining algorithm revealed that strain GXH0341^T represented a separate lineage within the genus *Bacillus* (Fig. 1), and the phylogenetic position was also confirmed by the trees generated using the maximum-likelihood (Fig. S4) and maximum-parsimony (Fig. S5) methods. For further comparative observation, the phylogenetic tree with the addition of the core *Bacillus* species is shown in (Fig. S8). As the strain which we report a novel bacterium in this study and the most similar strains *B. mesophilus* DSM 101000^T, *Bacillus salitolerans* KC1^T are all defined as members of ambiguous genera and do not belong to any of the genera as defined by Gupta et al. [5]. Therefore, this novel bacterium GXH0341^T was temporarily classified as a member of *Bacillus*.

Genomic Analysis

The genome sequence of GXH0341^T, which was deposited in the GenBank/EMBL/DDBJ database under accession number JAJQI000000000, was used for further analysis. The genome size of GXH0341^T was about 4,004,187 bp, it contained 38 number of contigs, 302 number of subsystems, 4100 number of coding sequences and 111 number of RNAs. The N50 value was 242,785 bp, N75 value was 141,909 bp, and the average genome coverage was 149.72×. The longest and the shorted contig size of the whole genome were 446,877 bp and 1311 bp, respectively. The results annotated by the antiSMASH 6.0 database showed that GXH0341^T had one Type III Polyketide synthase (T3PKS) which involved in the biosynthesis of alkyl-O-dihydrogeranyl-methoxyhydroquinones. The genome of

Table 1 Comparison of phenotype characteristics of strains GXH0341^T and related strains

| Characteristic | 1 | 2 | 3 |
|-----------------------------------|----------------|----------------|---------------|
| Colony color | Pink | Pink | Cream |
| Group at/with | | | |
| pH(optimum) | 5.0–11.0 (8.0) | 6.0–11.0 (8.0) | 6.5–9.0 (8.0) |
| Temperature (optimum) (°C) | 4–37 (30) | 15–40 (35) | 25–45 (37) |
| NaCl (optimum) (% w/v) | (0–7) 2% | (0–7) 3% | (0–10) 4% |
| Tween80 | + | – | – |
| Casein | – | – | + |
| API ZYM | | | |
| Valine arylamidase | + | – | – |
| β-glucuronidase | – | + | – |
| α-galactosidase | + | + | – |
| β-galactosidase | + | + | – |
| API 50CH | | | |
| Inositol | + | – | + |
| D-Mannose | – | + | + |
| Maltose | – | + | + |
| Glycerol | – | + | – |
| D-Xylose | – | + | + |
| Sucrose | – | + | + |
| Voges-Proskauer test | – | + | – |
| API 20NE | | | |
| L-Tryptophan | – | + | – |
| GEN III | | | |
| γ-Amino-butyric acid | + | – | ND |
| D-Fucose | – | + | ND |
| Sensitivity | | | |
| Ampicillin (10 µg per tablet) | S | R | ND |
| Kanamycin (30 µg per tablet) | S | R | S |
| Amoxicillin (10 µg per tablet) | S | R | S |
| Cefoxitin (30 µg per tablet) | R | S | S |
| Carbenicillin (100 µg per tablet) | S | R | ND |
| Bacitracin (0.04 µg per tablet) | S | R | R |

Strains: 1, GXH0341^T (this study); 2, *Bacillus mesophilus* DSM 101000^T (this study); 3, *Bacillus salitolerans* KC1^T [31]

+ Positive, – negative, *R* resistant, *S* sensitive, *ND* not determined

GXH0341^T contains one terpene type of secondary metabolite biosynthesis gene cluster, with a similarity of 33% to synthesize carotenoid. It is also consistent with the phenotypic feature that the colonies color of GXH0341^T is pink. In addition, GXH0341^T also had two secondary metabolite biosynthesis gene clusters which were RRE-element containing cluster (RRE-containing), and Lasso peptide cluster (lassopeptide), which may involve in biosynthesis and regulatory responses, while no biosynthetic gene cluster was found in *Bacillus mesophilus* DSM 101000^T. Some genomic information was compared between GXH0341^T and *B. mesophilus* DSM 101000^T (Table 2). The DNA G+C content was 37.0 mol%, which was slightly higher than those of strain *B. mesophilus* DSM 101000^T which of

DNA G+C content was 36.8 mol% [29]. Based on JSpeciesWS online service, the ANI value between GXH0341^T and *B. mesophilus* DSM 101000^T was 78.22%, which were less than the suggested cut-of 95–96% ANI value for species delineation [30]. The dDDH value between GXH0341^T and *B. mesophilus* DSM 101000^T was 21.00%, which was clearly below the 70% cut-off for species delineation. The ANI and dDDH values between GXH0341^T and the related type strain were seen in Table 3. The phylogenomic tree (Fig. S6) confirmed the relationship between GXH0341^T and *B. mesophilus* DSM 101000^T. Based on above results we proposed the view temporarily that strain GXH0341^T represents a novel species of the genus *Bacillus*.

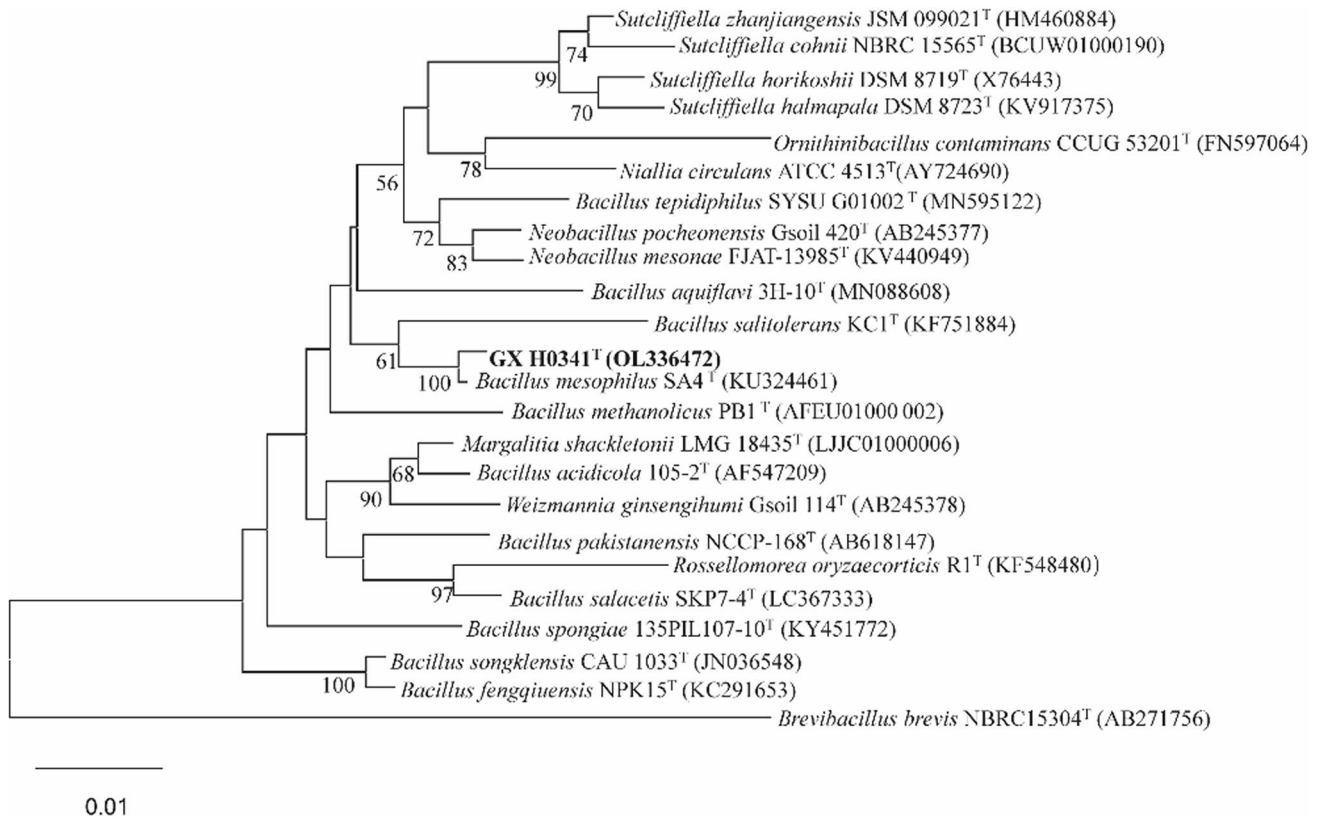


Fig. 1 The neighbor-joining tree based on the 16S rRNA gene sequences showing the phylogenetic relationship of strain GXH0341^T with related taxa. The evolutionary distances were computed using the Kimura 2-parameter method. The sequence of *Brevibacillus bre-*

vis NBRC 15304^T (AB271756) was used as an outgroup. Bootstrap support values were calculated from 1000 replicates and only values above 50% were shown. Bar, 0.01 substitutions per nucleotide position

Chemotaxonomic Characteristics

The predominant quinone of strain GXH0341^T was found to be MK-7 and the peptidoglycan contained *meso*-diaminopimelic acid as the diagnostic diamino acid. The polar lipids of GXH0341^T were found to be included diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC), and two unidentified phospholipids (Fig. S7) while *Bacillus mesophilus* DSM 101000^T contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, two aminophospholipids (APL) and seven unidentified phospholipids [29]. The major fatty acid of strain GXH0341^T were *iso*-C_{14:0} (23.8%), *iso*-C_{15:0} (18.5%), *anteiso*-C_{15:0} (18.2%) and *iso*-C_{16:0} (21.4%), which were similar to the reference strain *B. mesophilus* DSM 101000^T. Besides, the content of *iso*-C_{14:0} in strain GXH0341^T was much higher than in the reference strain *B. mesophilus* DSM 101000^T while the other strains showed small differences (shown in supplement material Table 4).

Taxonomic Conclusion

Based on 16S rRNA gene sequencing, the strain GXH0341^T formed a cluster with *Bacillus mesophilus* DSM 101000^T of the genus *Bacillus*. ANI and DDH values between strain GXH0341^T and its closely relative *Bacillus* species were both much lower than the species definition threshold value (95–96% for ANI and 70% for DDH). The phenotypic, physiological, chemotaxonomic, and genetic comparative analysis of strain GXH0341^T supported that it is a novel species of the genus *Bacillus*. Therefore, strain GXH0341^T is considered to be a novel *Bacillus* species, and proposed as *B. pinisoli* sp. nov.

Description of *Bacillus pinisoli* sp. nov.

Bacillus pinisoli (pi.ni.so'li. L. fem. n. *pinus* pine; L. neut. n. *solum* soil; N.L. gen. n. *pinisoli* of pine soil).

Cells are Gram-stain-positive, rod-shaped approximately 0.5–0.7 μm wide and 2.2–2.6 μm long, with multiple

Table 2 Comparison of genome features and secondary metabolite biosynthesis gene clusters between GXH0341^T and the reference strain

| | GXH0341 ^T | <i>Bacillus mesophilus</i> DSM 101000 ^T |
|--------------------------|----------------------|---|
| GenBank accession number | JAJQI000000000 | NZ_ JAAIWM000000000 |
| Total sequence length | 4,004,187 | 4,268,430 |
| Number of contigs | 38 | 22 |
| Genome Coverage | 149.72× | 118.0× |
| Contig N50 | 242,785 | 450,611 |
| Contig L50 | 6 | 3 |
| Median GC mol% | 37.0 | 36.8 |
| rRNAs | 111 | 113 |
| antiSMASH v.6.0.1 | | |
| T3PKS | 1 | 0 |
| RRE-containing | 1 | 0 |
| Terpene | 1 | 0 |
| Lasso peptide | 1 | 0 |
| Redox-cofactor | 0 | 0 |

T3PKS Type III Polyketide synthase, *RRE-containing* RRE-element containing cluster, Terpene; lasso peptide: Lasso peptide cluster, *Redox-cofactor* Redox-cofactors such as PQQ

Table 3 ANI and dDDH values between GXH0341^T and the reference strain

| Strain 1 | Strain 2 | ANI (%) | dDDH (%) | 16S rRNA gene identity (%) |
|----------------------|--|---------|----------|----------------------------|
| GXH0341 ^T | <i>Bacillus mesophilus</i> DSM 101000 ^T | 78.22 | 21.00 | 98.90 |

flagella, facultatively anaerobic, motile and spore-forming. On MA, colonies are pink, rounded, with a convex and smooth surface. Growth occurs at 4–37 °C (optimum 30 °C), at pH 5.0–11.0 (optimum 8.0) and in the presence of 0–7% (w/v) NaCl (optimum 2%). Peroxidase was positive, but the oxidase activity and the Voges–Proskauer test was negative. Hydrolysis of Tween20, Tween 60, and Tween 80 are all positive. In API 50CH strips, inositol, fructose, potassium 5-ketogluconate are positive. In API ZYM strips, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase are positive. Biochemical assays with API 20NE kits reveal that D-glucose, esculin, 4-nitrophenyl-β-D-galactopyranoside are utilized. GEN III stripes indicate that L-alanine, glucuronamide, acetoacetic acid, L-malic acid, 1% sodium lactate, fusidic acid, D-serine, troleandomycin, rifamycin SV, minocycline, lincomycin, guanidine HCl,

niaproof 4, vancomycin, nalidixic acid, lithium chloride, potassium tellurite, aztreonam, sodium butyrate, sodium bromate can be used as carbon sources. Sensitive to antibiotics containing the following (μg per tablet): gentamicin (10), tetracycline (30), chloramphenicol (30), streptomycin (10), erythromycin (15), rifampin (5), neomycin (30), streptomycin (300), ampicillin (10), kanamycin (30), amoxicillin (10), carbenicillin (100), bacitracin (0.04) but not sensitive to cefoxitin (30). The predominant quinone was MK-7 and the cell-wall diagnostic diamino acid was *meso*-diaminopimelic acid. The predominant polar lipids are diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC), and two unidentified phospholipids. The major fatty acid (> 5%) are *iso*-C_{14:0}, *iso*-C_{15:0}, *anteiso*-C_{15:0} and *iso*-C_{16:0}. The DNA G + C content of the type strain is 37.0 mol%.

The type strain GXH0341^T (=MCCC 1K07157^T=JCM 35212^T), was isolated from the soil of a decayed pine tree samples in Weizhou Island, Beihai, Guangxi, China.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00284-022-03130-x>.

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Author Contributions MJ and YJ designed the study; YH, HC and SQ performed research; YZ, JW, XC and YNI analyzed data. Ying Huang wrote the manuscript. All of the authors contributed to the manuscript revision process and read and approved the submitted version.

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Declarations

Conflict of interest All authors declare that they have no conflicts of interest.

Ethical Approval This article does not contain any studies with human participants performed by any of the authors.

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