Genomic Molecular Signatures Determined Characterization of *Mycolicibacterium gossypii* sp. nov., a Fast-Growing Mycobacterial Species Isolated From Cotton Field Soil

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Genomic molecular signatures determined characterization of *Mycolicibacterium gossypii* sp. nov., a fast-growing mycobacterial species isolated from cotton field soil

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Abstract

A Gram-positive, acid-fast and rapidly growing rod, designated S2-37^T, that could form yellowish colonies was isolated from soil samples collected from cotton cropping field located in the Xinjiang region of China. The draft genome of strain S2-37^T was 5.1 Mb in length, with a DNA G+C content of 68.4 mol%. 16S rRNA-directed phylogenetic analysis referred that strain S2-37^T was closely related to bacterial species belonging to the genus *Mycolicibacterium* and *Mycobacterium*. Multilocus sequence analysis of three genes (16S rRNA, *hsp65* and *rpoB*) revealed that strain S2-37^T shared high sequence similarities with *Mycolicibacterium litorale* CGMCC 4.5724^T (96.5%) and *Mycobacterium neglectum* CECT 8778^T (95.7%). Digital DNA-DNA hybridization (dDDH) and the average nucleotide identity (ANI) presented that strain S2-37^T displayed the highest values of 39.1% (35.7-42.6%) and 81.28% with *M. litorale* CGMCC 4.5724^T, respectively. And characterization of conserved molecular signatures further confirmed that strain S2-37^T could be well classified into the genus *Mycolicibacterium*. The main fatty acids were identified as C_{16:0}, C_{18:0}, C_{20:3ω3} and C_{22:6ω3}. In addition, polar lipids profile was mainly composed of diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol. Results indicated that strain S2-37^T represented genetically and phenotypically distinct from its closest phylogenetic neighbour, *M. litorale* CGMCC 4.5724^T. Here, we propose a novel species of the genus *Mycolicibacterium*: *Mycolicibacterium gossypii* sp. nov. with the type strain S2-37^T (=JCM 34327^T=CGMCC 1.18817^T).

Keywords: *Mycolicibacterium*, fast-growing, cotton field soil, molecular signatures

Declarations

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Conflicts of interest: The authors declare no conflicts of interest.

Availability of data and material: The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the strain S2-37^T is MW295419. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JAFEV000000000. The version described in this paper is version JAFEV010000000.

Authors' contributions: RRH performed the experiments and finished the draft of the manuscript. RRH, SRY, CZ, XFG and XKC performed strain isolation and phenotypic analyses. RRH and SRY performed genomic analyses. ZQW and YNL assisted to improve the manuscript. WZL designed all the experiments and supervised the manuscript.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: All authors agree to publish this work.

Introduction

The genus *Mycolicibacterium* presents the second largest population of Gram-positive, acid-fast and rod-shaped microbes in the family *Mycobacteriaceae* (Parte et al. 2020). At the time of writing, the genus *Mycolicibacterium* is composed of 92 recognized species with published names (<https://lpsn.dsmz.de>) (Parte et al. 2020), which primarily harbors rapid-growing species isolated from diverse range of environments, including river water, marine sediment and soil (Butler et al. 1993; Brown-Elliott et al. 2010; Zhang et al. 2013). There are also relatively high proportion of members in the genus isolated from clinical specimens, showing pathogenic to humans and animals (Shojaei et al. 2000; Brown-Elliott and Wallace 2002).

It is well documented that mycobacterial species displayed relatively high diversities in their genomic features at species level (Fedrizzi et al. 2017), which has already remarkably enhanced the difficulty accurately characterizing the taxonomic position of microbial species belonging to this population. Much effort has been devoted to primarily delineate different groups that exist within this meaningfully evolved bacterial branch using well established approaches based on analyses of the 16S rRNA gene, 16S-23S spacer, and housekeeping gene concatenated multilocus sequences (Roth et al. 1998; Mignard and Flandrois, 2008; Magee and Ward, 2012). However, the reliability of these methods used to well distinguish sub-groups (e.g., slow- and fast-growing species) of mycobacterial species remains of concern. Recently, Gupta et al. (2018) have developed a robust method consistently supporting the existence of five distinct monophyletic sub-groups of mycobacterial species, which are designated as the *Mycobacterium*, *Mycolicibacterium*, *Mycolicibacter*, *Mycolicibacillus* and *Mycobacteroides* genera. They have identified representative molecular markers in the form of conserved signature indels and proteins, which are uniquely shared by members of the five identified clades.

In this study, a putative novel species (strain S2-37^T) belonging to mycobacterial species was isolated from soil samples of cotton cropping field of Xinjiang in PR China. Multilocus sequence analysis of three genes (16S rRNA, *hsp65* and *rpoB*) presented that strain S2-37^T shared high sequence similarities with microbial species belonging to both the genus *Mycolicibacterium* and *Mycobacterium*. In the previous work, conserved molecular signatures (indels and proteins) have been clearly identified as specific markers that could be used to distinguish most members belonging to these two genera confidently (Gupta et al. 2018). Therefore, genome sequence of strain S2-37^T was mapped to these documented molecular features specific for each genus, ending up with the conclusion that this strain was taxonomically classified into the genus *Mycolicibacterium* in combination with following polyphasic approaches.

Materials and methods

Isolation of the novel strain and cultivation

Strain S2-37^T was isolated from a soil sample collected from a cotton cropping field (86°20' N 44°62' E, Xinjiang, China). The isolation procedures were described in the previous report with some modifications (Hopkins et al. 1991). Soil samples (5 g) were firstly naturally dried, then mixed with 100 ml of sterilized water in 250 ml flasks. In order to efficiently elute microbes firmly attached to soil particles, Sample were placed on a shaker with vibration frequency of 160 r/min at 30 °C for 30 minutes, followed by homogenization treatment for 1 min using a sonicator (XO-3200DT, Nanjing Xianou laboratory equipment Co., Ltd) with a frequency of 40 kHz. Then, 100 µl serially diluted samples were plated on Gause's synthetic No.1 agar (G1, 20 g soluble starch; 1 g KNO₃; 0.5 g K₂HPO₄; 0.5 g MgSO₄·H₂O; 0.5 g NaCl; 0.01 g FeSO₄; 20 g Agar; 1000 ml distilled water; pH 7.2-7.4) for cultivation at 30 °C in the following 14 days. Bacterial colonies were sub-cultured on G1 agar until pure isolates were achieved. Strain S2-37^T was maintained aerobically on G1 agar at 30 °C, and stored at -80 °C in G1 broth

supplemented with equal volume of 50% (v/v) sterilized glycerol for preservation (Prakash et al. 2013).

Phylogenetic analysis based on 16S rRNA gene and multilocus sequences

Genomic DNA of strain S2-37^T was extracted and its 16S rRNA gene was achieved by PCR amplification using universal primers 27F and 1492R as described by Fan et al. (2008), followed by sequence alignment in order to preliminarily locate the taxonomic position of the strain S2-37^T using the BLAST function embedded in NCBI database (Federhen 2012). GGDC web server (available at <http://ggdc.dsmz.de/>) was employed for gene phylogenetic and similarity analysis between 16S rRNA gene sequences of S2-37^T and all published *Mycolicibacterium* type strains collected from the LPSN database (Parte et al. 2020). Respectively, the phylogenetic trees were reconstructed based on 16S rRNA gene sequences of strains S2-37^T and its 22 closely phylogenetic relatives using the neighbor-joining (NJ) (Saitou and Nei 1987), maximum-parsimony (MP) (Sourdis and Nei 1988) and maximum-likelihood (ML) (Steel and Rodrigo 2008) algorithms supported by the software package MEGA 7.0 (Kumar et al. 2016), with *Bacillus subtilis* NTCC 6051^T as the outgroup. The topology of phylogenetic trees was evaluated by performing a bootstrap analysis based on 1000 replications (Felsenstein 1985).

Genomic DNA was extracted from strain S2-37^T using Bacterial Genomic DNA Rapid Extraction kit (Cat. No. B518225) supported by Sangon Biotech (Shanghai, China). Library construction, quality control and analysis were performed following the methods described by Zhu et al. (2001). The draft genome of S2-37^T was sequenced using Illumina HiSeq 4000 PE150 (Patnaik et al. 2016) at Beijing TSINGKE Bioinformatics Technology Co., Ltd, and assembled using SOAPdenovo (Luo et al. 2012), SPAdes (Bankevich et al. 2012) and Abyss software (Simpson et al. 2009), respectively. The final genome assembly was achieved by integrating three assemblies in CISA software (Lin and Liao 2013).

It has been reported that 16S rRNA-directed phylogenetic analysis could not precisely locate the taxonomic position of all known rapid-growing mycobacteria (Wallace et al. 1993; Brown et al. 1999; Brown-Elliott and Wallace 2002; Wallace et al. 2002), whose members are more closely related to each other. Therefore, phylogenetic analyses were further performed in this study using concatenated sequences composed of the 16S rRNA, *hsp65* and *rpoB* genes, which were previously proposed as suitable phylogenetic markers for the classification of mycobacteria (Ringuet et al. 1999; Adékambi et al. 2003). The *hsp65* and *rpoB* gene sequences of species were achieved by BLAST against corresponding genomes using the *hsp65* (DQ841180) and *rpoB* (DQ534005) genes of *Mycolicibacterium rutilum* JS14^T as references. The phylogenetic trees were reconstructed based on the three-gene (16S rRNA, *hsp65* and *rpoB*) concatenated sequences following the same approaches as 16S rRNA-directed phylogenetic trees were built.

Genomic analysis

Functional categories of the strain S2-37^T genome were predicted using the Cluster of Orthologous Group of Proteins (COG) (Tatusov et al. 2003). AntiSMASH was employed to predict biosynthetic gene clusters of strain S2-37^T (Blin et al. 2019). Functional gene annotation and metabolic pathway prediction were performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al. 2016). The virulence factor database (VFDB) was employed to predict virulence factors of strain S2-37^T (Liu et al. 2019). Digital DNA-DNA hybridization (dDDH) analysis was performed using an automated high-throughput platform for genome-based taxonomy called Type (Strain) Genome Server (TYGS; <http://tygs.dsmz.de/>) (Meier-Kolthoff et al. 2013), with the genome of strain S2-37^T as the only query sequence. Additionally, average nucleotide identity (ANI) was calculated with the OrthoANIu algorithm using the server-based software (available at <http://www.ezbiocloud.net/tools/ani>) (Yoon et al. 2017).

Divergence analysis of conserved molecular signatures

Conserved molecular signatures, including indels (CSIs) and unique proteins (CSPs), specific for the genus *Mycolicibacterium* and *Mycobacterium* were directly collected from the work described by Gupta et al. (2018). Conserved protein sequences of different species with CSIs were achieved by BLASTp searches against the NCBI non-redundant (nr) database (Altschul et al. 2005), and the identified orthologous proteins with a minimum of 50% in sequence identity were used for divergence analyses. Phylogenetic analyses were performed using the software package MEGA 7.0 after multiple alignments of the sequence data with CLUSTAL W (Kumar et al. 2016). Phylogenetic trees were reconstructed using the neighbor-joining (NJ) algorithms (Saitou and Nei 1987). Partial sequence alignments of a conserved region of orthologous proteins from different species were displayed in same patterns as the previous work did (Gupta et al., 2018). Phylogenetically closely related species to strain S2-37^T (*Mycolicibacterium litorale* CGMCC 4.5724^T, *Mycolicibacterium monacense* DSM 44395^T, *Mycobacterium neglectum* CECT 8778^T and *Mycobacterium lehmannii* SN 1900^T) and randomly chosen species (*Mycolicibacterium austroafricanum* DSM 44191^T, *Mycolicibacterium smegmatis* NCTC 8159^T, *Mycobacterium asiaticum* DSM 44297^T, *Mycobacterium avium* 104^T and *Mycobacterium bohemicum* DSM 44277^T) were employed for the divergence analyses. The CSPs profile of strain S2-37^T was identified using BLASTp searches against the NCBI non-redundant (nr) database, in order to evaluate its phylogenetic relationship with genus *Mycolicibacterium* and *Mycobacterium*. Full names will always be used throughout the context for species belonging to the genus *Mycobacterium*, in order to avoid confusion when referring to species from the genus *Mycolicibacterium*.

Morphology, physiology, and biochemical analysis

Gram staining was performed using the methods described by Smibert and Krieg (1994). Acid fastness was conducted using methods described by Berd (1973). Cell morphology was

determined when grown on Bennett's agar (10 g glucose; 2 g N-Z amine; 1 g beef extract; 1 g yeast extract; 15 g agar; 1000 ml distilled water; pH 7.1-7.5) at 30 °C for 7 days using a light microscope (OLYMPUS BX43F; Olympus Corporation, Tokyo, Japan) and a cold field emission scanning electron microscope (SEM; Hitachi SU8010, Tokyo, Japan). SEM analysis was performed as described by Koon et al. (2019).

Growth with various NaCl concentrations (0-3% at 0.5% intervals and 3-7% at 1% intervals, w/v) and at different temperatures (10, 18, 25, 30, 37 and 40, 45 °C), was examined by growing the strains on Bennett's medium as the basal medium. Growth at different pH values (4.0-10.0, at intervals of 1.0 pH unit) was examined on Bennett's medium using the buffer system described by Xu et al. (2005). Catalase activity was determined using 3% H₂O₂, and gas production was identified as a positive reaction. Urease activity, nitrate reduction and tween 80 hydrolysis were determined using methods described by Kent and Kubica (1985). Resistance to antibiotics was determined using impregnated filter-paper discs (Goodfellow and Orchard 1974) containing cefalotin, cefoxitin, amikacin, ciprofloxacin, clarithromycin, doxycycline, tobramycin and sulfamethylisoxazole. Other biochemical properties of strain S2-37^T were further tested using the API 20 NE and API ZYM systems (bioMérieux) according to the manufacturer's instructions, and results were summarized in the species description.

Chemotaxonomic characterization

The polar lipids profile of the strain S2-37^T was determined using standard thin-layer chromatographic procedures (Minnikin et al. 1984). In addition, cellular fatty acids were extracted from freeze dried biomass of the strain and were saponified and methylated to produce fatty acid methyl esters (FAMES) following the procedure described by Kuykendall et al. (1988). The FAMES were analyzed by gas chromatography (Agilent 6890 instrument) and the resultant peaks were automatically integrated (Lisec et al. 2006). The fatty acids profile was determined using the standard Microbial Identification (MIDI) System, version 4.5, and

the Myco 6 database (Sasser 2001).

Results and discussion

Phylogenetic and genomic analyses

Phylogenetic analyses displayed that strain S2-37^T showed the highest 16S rRNA gene sequence similarity with *Mycolicibacterium pyrenivorans* DSM 44605^T (98.5%), *Mycobacterium neglectum* CECT 8778^T (98.5%), followed by *Mycolicibacterium austroafricanum* DSM 44191^T (98.3%) (Table S1). NJ algorithm-directed phylogenetic analysis exhibited that strain S2-37^T was located in a clade adjacent to *M. pyrenivorans* DSM 44605^T, *Mycolicibacterium aurum* NCTC 10437^T and *M. austroafricanum* DSM 44191^T, with a low bootstrap support (< 50%) (Fig. 1a). The phylogenetic distribution pattern could also be recovered by the MP and ML trees (Fig. S1, available in the online version of this article).

Multilocus sequence directed phylogenetic analyses displayed that strain S2-37^T showed higher sequence similarities with *Mycolicibacterium litorale* CGMCC 4.5724^T (96.5%) and *M. austroafricanum* DSM 44191^T (96.4%) as compared to 16S rRNA-directed close relatives *M. pyrenivorans* DSM 44605^T (82.5%) and *Mycobacterium neglectum* CECT 8778^T (95.7%) (Table S1), both of which were located in a clade completely separated from where strain S2-37^T was as displayed in NJ algorithm-directed phylogenetic analysis (Fig. 1b). The topology structure was also recovered by the MP and ML trees (Fig. S2, available in the online version of this article).

The assembled genome of S2-37^T was 5.9 Mbp with 12 contigs (all >500 bp, with an N50 length of 495170 bp) and the sequencing coverage was approximately ×100. The total length was 5843440 bp and the G+C content was 68.43 mol%. The results of functional annotation based on the COG database showed that genes involved in lipid transport and metabolism accounted for the largest proportion, except for genes with general function prediction only (Fig. S3). The antiSMASH biosynthetic gene clusters of S2-37^T were shown in Supplementary

table 2, which could provide ideas for guiding the screening of active secondary metabolites. According to the KEGG analysis, the majority of genes were involved in cell metabolism (Fig. S4), and there were some genes classified into the category associated with human diseases (Table S3). This suggested that S2-37^T was a potential pathogen of human being, the feature of which was shared by many species belonging to the genus *Mycolicibacterium* (Gupta et al. 2018). According to the VFDB analysis, strain S2-37^T was predicted to be able to produce mycolic acid (see details in Table S4), which was considered as a virulence factor shared by large number of mycobacteria (Tortoli 2003).

Respectively, we then determined that strain S2-37^T presented dDDH values (average and confidence intervals in parentheses) of 39.1% (35.7-42.6%) and 34.9% (35.0-38.4%) with *M. litorale* CGMCC 4.5724^T and *M. monacense* DSM 44395^T, showing much lower dDDH values of 23.0% (19.7-26.6%) and 20.5% (27.3-24.1%) with *M. austroafricanum* DSM 44191^T and *Mycobacterium neglectum* CECT 8778^T. This provided a sound evidence supporting the result achieved from the phylogenetic tree based on multilocus sequences of 16S rRNA, *hsp65* and *rpoB* genes, suggesting closely phylogenetic relationships of strain S2-37^T with *M. litorale* CGMCC 4.5724^T and *M. monacense* DSM 44395^T. The ANI values between strain S2-37^T and *M. litorale* CGMCC 4.5724^T and *M. monacense* DSM 44395^T were 81.28% and 81.09%, respectively. Overall, all calculated values were below the suggested threshold for the delineation of a novel species (Chun et al. 2018).

Divergence analysis of conserved molecular signatures

Respectively, four (LacI family transcriptional regulator, cyclase, CDP-alcohol phosphatidyltransferase and phosphatidylserine synthase) and two (UPF0182 family protein and 23S rRNA (guanosine(2251)-2'-O)-methyltransferase RlmB) conserved proteins with CSIs specific for most members of the genus *Mycolicibacterium* and *Mycobacterium* were employed for the divergence analysis. Phylogenetic analyses based on whole sequences of four

244 conserved proteins (LacI family transcriptional regulator, CDP-alcohol
245 phosphatidyltransferase, UPF0182 family protein and RlmB) indicated that strain S2-37^T was
246 more closely related to members from the genus *Mycolicibacterium* as compared to those from
247 the genus *Mycobacterium*. And in the partial sequence alignments of these four proteins, the
248 amino acid insertion patterns of strain S2-37^T were consistent with the molecular signatures
249 (Fig. 2; Fig. S5b, d, e), which were specific for most members within the genus
250 *Mycolicibacterium* identified by Gupta et al. (2018). However, conserved regions of protein
251 sequences (cyclase and phosphatidylserine synthase) did not give a clear clue where strain S2-
252 37^T could be taxonomically positioned (Fig. S5a, c). The CSPs profile of strain S2-37^T was
253 summarized in table 1. It was clearly shown that strain S2-37^T possessed some identified CSPs
254 specific for members of the genus *Mycocilibacterium*, but none for members of the genus
255 *Mycobacterium* and other slow growers of mycobacterial species. Thus, we concluded that
256 strain S2-37^T represented a novel bacterial species in the genus *Mycolicibacterium*.

257 **Table 1.** Similarity of conserved signature proteins (CSPs) between strain S2-37^T and members
258 of the genus *Mycocilibacterium*, slow growers of mycobacterial species and the genus
259 *Mycobacterium*.

Gene or protein ^a	Accession number of reference protein	Sequence coverage (%)	Sequence identity (%)	Specificity
Hypothetical protein	WP_048630777.1	96	71.59	<i>Mycocilibacterium</i>
Hypothetical protein	WP_048632025.1	-	-	
Hypothetical protein	WP_048632497.1	56	57.78	
Hypothetical protein	WP_048634851.1	-	-	
Hypothetical protein	WP_048633467.1	49	47.47	
Hypothetical protein	WP_048633322.1	96	40.94	
Hypothetical protein	WP_048631132.1	48	29.07	
Hypothetical protein	WP_048634509.1	70	46.03	
Hypothetical protein	WP_048630657.1	41	47.62	
Hypothetical protein	WP_048632441.1	60	48.77	
PPE Family protein	YP_177721.1	6	38.24	Slow-Growers of

PE Family protein PE36	YP_178025.1	-	-	mycobacterial
PE Family protein	WP_011725130.1	-	-	species
MAP_RS07685	WP_003874405.1	-	-	
Histone-like protein HNS	NP_218639.1	-	-	
Rv4010	YP_004837050.1	-	-	<i>Mycobacterium</i>
Membrane protein	NP_217322.1	-	-	

^aPreviously presented by Gupta et al. (2018).

The symbol: -, not detected.

Intriguingly, we observed that *Mycobacterium neglectum* CECT 8778^T and *Mycobacterium lehmannii* SN 1900^T displayed similar CSIs patterns with members of the genus *Mycolicibacterium*. It has been reported that these two species were capable of forming colonies within 7 days (Nouioui et al. 2017, 2018), and their most phylogenetically closely related species (e.g., *Mycobacterium aurum*, *Mycobacterium mageritense* and *Mycobacterium vanbaalenii*) have been reclassified into the genus *Mycolicibacterium* (Gupta et al. 2018). Therefore, we suggested that *Mycobacterium neglectum* and *Mycobacterium lehmannii* should be modified to *Mycolicibacterium neglectum* and *Mycolicibacterium lehmannii*, respectively.

Morphology, physiology, and biochemical analysis

Strain S2-37^T showed more robust growth on Bennett's agar as compared to G1 and R2A agar. It could form round, yellowish colonies stably on Bennett's agar. SEM observation revealed that strain S2-37^T is rod-shaped, approximately 1.0-1.5 µm in length and 0.4 µm in diameter (Fig. 3). Positive for catalase activity and negative for Tween 80 hydrolysis, other results achieved from strains S2-37^T, *M. litorale* CGMCC 4.5724^T and *M. monacense* DSM 44395^T, were summarized in Table 2. All of these tests were carried out in duplicate using the standard inoculum.

Table 2. Differential characteristics between strain S2-37^T and closely related species in the genus *Mycolicibacterium*.

Characteristic	1	2	3
Growth at pH 4.0	+	+	+

Growth with 4% NaCl	-	+	+
Temperature range (°C)	18-40	25-45	25-45
Catalase	+	+	+
Urease	-	+	+
Nitrate reduction	-	+	-
Tween 80 hydrolysis	-	+	+
<i>Antibiotics resistance:</i>			
Cefalotin	+	-	-
Cefoxitin	+	-	-
Amikacin	-	-	-
Ciprofloxacin	-	-	-
Clarithromycin	-	+	+
Doxycycline	-	-	-
Tobramycin	-	-	-
Sulfamethylisoxazole	-	-	-

Strains: 1, S2-37^T; 2, *M. litorale* CGMCC 4.5724^T; 3, *M. monacense* DSM 44395^T. All data was from this study. The symbols: +, Positive; -, negative.

Chemotaxonomic characterization

The major polar lipids of strain S2-37^T were identified as diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol (Fig. S6). The major fatty acids (>10%) of strain S2-37^T were composed of C_{16:0}, C_{18:0}, C_{20:3}ω3 and C_{22:6}ω3, of which the last two types of fatty acids were not detected or only traced in small amounts (< 1%) in *M. litorale* CGMCC 4.5724^T and *M. monacense* DSM 44395^T (Reischl et al. 2006; Zhang et al. 2012).

In conclusion, the phenotypic, chemotaxonomic and phylogenetic data supports the delineation of strain S2-37^T as a novel species of the genus *Mycolicibacterium*. We propose the name *Mycolicibacterium gossypii* sp. nov. for the species.

Description of *Mycolicibacterium gossypii* sp.nov.

Mycolicibacterium gossypii (gos.sy'pi.i. N.L. gen. n. gossypii of the cotton genus Gossypium).

Cells are aerobic, Gram-stain-positive, acid-fast, non-spore-forming and short rods. It could form smooth, yellowish colonies on Bennett's agar within 7 days at 30 °C. Cells are approximately 0.4 μm in diameter and 1.0-1.5 μm long. Positive for catalase activity and

negative for Tween 80 hydrolysis. In API 20NE tests, arginine dihydrolase activity is positive. Negative for nitrate reduction, indole production, β -galactosidase, β -glucosidase, gelatinase and urease activities, as well as fermentation of D-glucose. In addition, results are negative for assimilation of D-glucose, L-arabinose, D-mannitol, maltose, gluconate, N-acetylglucosamine, adipic acid, malic acid, capric acid and phenylacetic acid, but positive for citrate acid. In API ZYM tests, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -glucosidase activities are positive. Negative for lipase (C14), trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -rucosidase activities. Susceptible to amikacin, ciprofloxacin, clarithromycin, doxycycline, tobramycin and sulfamethylisoxazole, but resistant to cefalotin and cefoxitin. The major fatty acids (>10%) are C_{16:0}, C_{18:0}, C_{20:3} ω 3 and C_{22:6} ω 3. The polar lipids profile is mainly composed of diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol. The genome size of strain S2-37^T is 5.9 Mbp and the DNA G+C content of the type strain is 68.4 mol%. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA sequence is MW295419. This Whole Genome Shotgun project has been deposited at GenBank/EMBL/DDBJ under the accession number JAFEVR000000000. The version described in this paper is version JAFEVR010000000.

The type strain, S2-37^T (=JCM 34327^T=CGMCC 1.18817^T), was isolated from cotton field soil in Xinjiang, PR China.

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Figure captions

Fig. 1. Phylogenetic trees reconstructed by the neighbour-joining method based on 16S rRNA (a) and multilocus gene (16S rRNA, *hsp65* and *rpoB*) (b) sequences, which show the phylogenetic relationships between strain S2-37^T and its closely related species. Bootstrap percentages (based on 1000 replications) above 50% are shown at the nodes. The GenBank accession numbers for the 16S rRNA gene and genomic sequences are shown in parentheses. *Bacillus subtilis* subsp. *subtilis* JCM 1465^T was employed as the outgroup. Filled circles indicate that the corresponding nodes are also recovered in trees reconstructed by the ML and MP algorithms. Bar, 0.02 (a) and 0.05 (b) substitutions per nucleotide position.

Fig. 2. The phylogenetic tree displayed in the left column is reconstructed by the neighbour-joining method based on the whole sequences of LacI family transcriptional regulator from tested members of the genus *Mycocilbacterium* and *Mycobacterium*, conserved region alignment of which is displayed in the right column. Sequence information for additional CSIs is shown in Supplementary Figure 5.

Fig. 3. Scanning electron micrograph exhibiting cell morphology of strain S2-37^T. The cells used for investigation were cultured on Bennett's agar at 30 °C for 4 d. Magnification, ×20.0 k. Scale bar, 2.0 μm.

Captions of supplementary figures and tables

Fig. S1. Phylogenetic trees reconstructed by the maximum-parsimony **(a)** and maximum-likelihood **(b)** methods based on 16S rRNA gene sequences. Bootstrap values with more than 50% are shown on the nodes as percentages of 1,000 replicates. The GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. *Bacillus subtilis* subsp. *subtilis* JCM 1465^T was employed as the outgroup. Bar, 20 **(a)** and 0.02 **(b)** substitutions per nucleotide position.

Fig. S2. Phylogenetic trees reconstructed by the maximum-parsimony **(a)** and maximum-likelihood **(b)** methods based on the multilocus gene (16S rRNA, *hsp65* and *rpoB*) sequences. Bootstrap values with more than 50% are shown on the nodes as percentages of 1,000 replicates. The GenBank accession numbers for the 16S rRNA gene and genomic sequences are shown in parentheses. *Bacillus subtilis* subsp. *subtilis* JCM 1465^T was employed as the outgroup. Bar, 100 **(a)** and 0.05 **(b)** substitutions per nucleotide position.

Fig. S3. Predicted functional categories of strain S2-37^T based on COG orthologous groups.

Fig. S4. Predicted metabolic pathway of strain S2-37^T genome using KEGG database.

Fig. S5. Phylogenetic trees displayed in the left column are reconstructed by the neighbour-joining method based on the whole sequences of cyclase **(a)**, CDP-alcohol phosphatidyltransferase **(b)**, phosphatidylserine synthase **(c)**, UPF0182 family protein **(d)** and RlmB **(e)** from tested members of the genus *Mycocilibacterium* and *Mycobacterium*, conserved region alignments of which are displayed in the right column.

Fig. S6. Polar lipids profile of strain S2-37^T following two-dimensional TLC and detection with molybdatophosphoric acid. The solvent system was composed of chloroform-methanol-water (65:25:4, by vol.) for the first development and of chloroform-methanol-glacial acetic acid-water (80:12:15:4, by vol.) for the second development. DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PI, Phosphatidylinositol.

Table S1. Sequence similarities between strain S2-37^T and closely related species in the genus *Mycolicibacterium* and *Mycobacterium*.

Table S2. Biosynthetic gene clusters in the draft genome of strain S2-37^T predicted by antiSMASH.

Table S3. The predicted pathways of strain S2-37^T genome classified into the category of human diseases.

Table S4. The predicted virulence factors of strain S2-37^T using VFDB database.

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Figures

Figure 1

Phylogenetic trees reconstructed by the neighbour-joining method based on 16S rRNA (a) and multilocus gene (16S rRNA, hsp65 and rpoB) (b) sequences, which show the phylogenetic relationships between strain S2-37T and its closely related species. Bootstrap percentages (based on 1000 replications) above 50% are shown at the nodes. The GenBank accession numbers for the 16S rRNA gene and genomic sequences are shown in parentheses. *Bacillus subtilis* subsp. *subtilis* JCM 1465 T was employed as the

outgroup. Filled circles indicate that the corresponding nodes are also recovered in trees reconstructed by the ML and MP algorithms. Bar, 0.02 (a) and 0.05 (b) substitutions per nucleotide position.

Figure 2

The phylogenetic tree displayed in the left column is reconstructed by the neighbour-joining method based on the whole sequences of LacI family transcriptional regulator from tested members of the genus *Mycolicibacterium* and *Mycobacterium*, conserved region alignment of which is displayed in the right column. Sequence information for additional CSIs is shown in Supplementary Figure 5.

Figure 3

Scanning electron micrograph exhibiting cell morphology of strain S2-37T . The cells used for investigation were cultured on Bennett's agar at 30 °C for 4 d. Magnification, ×20.0 k. Scale bar, 2.0 μm

Supplementary Files

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