Viridibacillus Soli sp.nov., Isolated from Forest Soil in Ailaoshan National Nature Reserve

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Abstract

A Gram-staining positive, rod-shaped, and subterminal endospore-forming bacterium, designated strain YIM B01967^T, was isolated from a forest soil sample collected in Ailaoshan National Nature Reserve, Yuxi City, Xinpin county, Yunnan province, China. Strain YIM B01967^T showed the highest 16S rRNA gene sequence similarity with *Viridibacillus arvi* (99.05%) and *Viridibacillus arenosi* (98.92%). Based on the phylogenetic and 16S rRNA gene sequence results, strain YIM B01967^T was affiliated to the genus *Viridibacillus*. The growth of YIM B01967^T was observed at 15-35 °C (optimum, 28 °C), pH 7.0-9.0 (optimum, pH 7.5) and in the presence of 0-2% (w/v) NaCl (optimum in 2% NaCl). The cell-wall sugars of YIM B01967^T were ribose, glucose, arabinose, galactose, and mannose. The quinone system consisted of the major compound MK-8 and moderate amounts of MK-7. The major fatty acids (> 10%) were iso-$C_{15:0}$, anteiso-$C_{15:0}$, $C_{16:1}$ *ω10c*. The major polar lipids profile included DPG, PME. The cell wall peptidoglycan was the type A4*a* with an L-Lys–D-Asp interpeptide bridge. The genomic DNA G+C content of strain YIM B01967^T was 36.3 mol%. The ANI value and dDDH values between strain YIM B01967^T and *Viridibacillus arvi* DSM 16317^T were 61.03% and 32.1% based on the draft genome sequence. The results support the conclusion that strain YIM B01967^T represents a novel species of the genus *Viridibacillus*, for which proposed the name *Viridibacillus soli* sp. nov. The type strain is YIM B01967^T (= KCTC 43249^T = CGMCC 1.18436^T).

Introduction

The genus *Viridibacillus* belongs to the family *Planococcaceae*, phylum *Bacillales* which was first proposed by Albert et al. (2007) to reclassify 3 species in the genus *Bacillus*. To date, this genus also consists of 3 species, including *Viridibacillus arenosi* (Heyrman et al.2005; Albert et al.2007), *Viridibacillus arvi* (Heyrman et al. 2005; Albert et al.2007), and *Viridibacillus neidei* (Nakamura et al. 2002; Albert et al.2007), they were all separated from the soil. Thakur et al. (2017) identified a strain of *Viridibacillus arenosi* from tea tree rhizosphere entries and named it IHBB7171. They found that stress-tolerance and plant growth-promoting activities by the strain under stressed growth conditions with potential as a broad-spectrum plant growth-promoting rhizobacterium. In July 2019, we isolated the strain YIM B01967^T from a forest soil sample in Ailaoshan National Nature Reserve, Yuxi City, Xinpin county, Yunnan province, China. Through the study of polyphasic taxonomy, strain YIM B01967^T is considered to be a new member of the genus *Viridibacillus*, named *Viridibacillus soli* sp.nov.

Materials And Methods

Strain isolation and culture conditions

The sample of forest soil from Ailaoshan National Nature Reserve, Yuxi, China (24°17'N, 101°53'E. Altitude 2100 m) was used as the source for the isolation of bacterial strains. Isolation was performed by the standard dilution plate method on plate count agar (PCA; Difco) at 28 °C for 4-7days. The isolation

procedure was performed as described by Liu et al. (2017). Pure culture of strain YIM B01967^T was preserved on PCA slants at 4 °C and as glycerol suspensions (20%, w/v) at -80 °C. Besides, it was preserved in lyophilized form in skimmed milk at 4 °C temperature. The reference strain,*Viridibacillus arvi* DSM 16317^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

Phylogenetic and genotypic analysis

The preliminary identification of strain YIM B01967^T was performed based on the 16S rRNA gene sequence and phylogenetic analysis. The genomic DNA was extracted and 16S rRNA gene amplification and sequencing were performed according to the method previously described by Feng et al. (2020). The 16S rRNA gene sequence was compared at the EZBioCloud server (https: //www. ezbio cloud. net; Yoon et al. 2017). Multiple sequence alignments were performed with CLUSTAL_X (Thompson et al.1997). Phylogenetic analysis was performed using the MEGA software package version 7.0 (Kumar et al. 2016). The phylogenetic trees were reconstructed using neighbor-joining (NJ) (Saitou and Nei 1987), maximum-likelihood (ML) (Felsenstein 1981), and maximum-parsimony (MP) (Fitch 1971) with MEGA 7 software (Kumar et al. 2016). The method used to compute evolutionary distances was Kimura's two-parameter (Kimura 1980). The stability of the topology and the phylogenetic tree was evaluated by using bootstrap analysis (Felsenstein 1985), with 1000 replications. The 16S rRNA gene sequence of *Bhargavaea cecembensis* DSM 22132^T was used as an outgroup.

The sequencing of the whole genome was performed on the HiSeq X-Ten platform (Illumina). The draft whole-genome sequencing of the strain YIM B01967^T was performed by Majorbio (Shanghai, China). ANIb values were calculated using JSpeciesWS (http://jspecies. ribohost.com/jspeciesws/#analyse) (Richter et al. 2016). The Genome-to-Genome Distance Calculator, version 2.1 was used to calculate the digital DNA-DNA hybridization (dDDH) value (Meier-Kolthoff et al.2013). The dDDH results of recommended formula 2 (identities/HSP length) were used. A phylogenomic tree was constructed based on genomic data using the supermatrix method (Zhi et al.2017).

Morphological, physiological, and biochemical analyses

To determine the differential phenotypic properties, strain YIM B01967^T was subjected to morphological, physiological, and biochemical analyses. Phenotypic characteristics of strain YIM B01967^T were observed using cells grown on PCA medium for 4 days at 28 °C. Cell morphology was examined using a transmission electron microscope (JEM 2100; JEOL). For transmission electron microscopy, cells were negatively stained with 1% phosphotungstic acid before observation. Colony morphology and pigmentation were observed on PCA medium incubated at 28 °C for 4 days. Growth at different temperatures (4, 10, 15, 20, 25, 28, 30, 35, 37, 40, and 45 °C) was examined after incubation on PCA medium for 4 days. Tolerance to NaCl between 0 and 10% (w/v, at intervals of 2%) in plate count broth (PCB; Difco) medium at 28 °C was recorded after 4 days. The ability of the strain to grow at different pH values (4.0-10.0, at 0.5 intervals by using the buffer system described by Tang et al. 2010). Catalase

activity was determined by the production of bubbles after adding 3% H_2O_2 to the tested bacteria (Tarrand and Gröschel 1982). Enzyme activities, production of acid, utilization of different compounds, and the other physiological functions were tested with API ZYM, API 20NE kits (bioMé-rieux), API 50CHB kits, and the Biolog GEN III MicroPlates kits according to the manufacturers' instructions. All tests were completed in duplicate.

Chemotaxonomic characterization

The isolation of the peptidoglycan and analysis of the peptidoglycan structure were done according to published protocols (Schumann 2011; Schleifer and Kandler 1972). Analyses of diaminopimelic acid in the cell wall and sugars of whole-cell hydrolysates were performed according to the procedures described by Lechevalier and Lechevalier (1970) and Tang et al. (2009). Cellular fatty acids were extracted, methylated, and analyzed by using the Sherlock Microbial Identification System (MIDI) according to the manufacturer's instructions. Fatty acid methyl esters were analyzed by using the Microbial Identification Software Package (Sherlock Version 6.1; MIDI databaseTSBA6) (Sasser 1990). The respiratory quinones of YIM B01967^T were extracted from lyophilized cells (Collins et al. 1977), purified by TLC, and then analyzed by HPLC according to the methods of Xie and Yokota (2003). Polar lipids were extracted, examined by two-dimensional TLC, and identified using the procedures described by Collins and Jones (1980) and Minnikin et al. (1979).

Results And Discussion

Molecular phylogenetic analysis

The almost-complete 16S rRNA gene sequence of YIM B01967^T was 1540 bp (GenBank accession number MW386301). Strain YIM B01967^T showed the highest 16S rRNA gene sequence similarity with *Viridibacillus arvi* (99.05%) and *Viridibacillus arenosi* (98.92%). The NJ tree, MP tree, and ML tree for the 16S rRNA shared the same topology and were presented in Fig 1, Fig S1, and Fig S2, respectively.

The draft genome of strain YIM B01967^T contained 112 contigs, with a total length of 4,553,251 bp and an N50 length of 171,059 bp (GenBank accession number JAEOAH000000000), and genome coverage of 14.0´. The DNA G+C content of strain YIM B01967^T was determined from the genome to be 36.3 mol%. Strain YIM B01967^T genome was annotated with 4,444 genes, included 4,200 protein-coding genes, 60 rRNA genes, 50 tRNA genes, 5 ncRNA genes, and 184 pseudogenes. In contrast, the draft genome of the reference strain *Viridibacillus arvi* DSM 16317^T consists of 4,758,570 bp with an N50 contig length of 244,670 bp and a G+C content of 35.0 mol%. The ANI value between strain YIM B01967^T and *Viridibacillus arvi* DSM 16317^T was 61.03% based on the draft genome sequence, which was lower than the 95.0% cut-off for species demarcation (Richter et al. 2016). The DNA-DNA hybridization values between strain YIM B01967^T and *Viridibacillus arvi* DSM 16317^T was 32.1%, which was much lower than the threshold value (70%) recommended for distinguishing novel prokaryotic species (Chun et al. 2018). The phylogenomics tree of YIM B01967^T with the closely related strains was presented in Fig 2.

Morphological, physiological, and biochemical analyses

Strain YIM B01967^T was Gram-positive, spore-forming, motile rods. Endospores were round and located terminally in a swollen or slightly swollen sporangium (Fig S3). Growth of cells occurred at a temperature ranging between 15 and 37 °C (optimum, 28 °C), pH 6.0-9.0 (optimum, pH 7.5) and in the presence of 0-2% (w/v) NaCl (optimum in 2% NaCl). Strain YIM B01967^T was Catalase-positive and oxidase-negative, and this feature was also present in *Viridibacillus arvi* DSM 16317^T and *Viridibacillusarenosi* DSM 16319^T.

Chemotaxonomic characterization

The cell wall amino acids of strain YIM B01967^T contained aspartic acid, glutamic acid, alanine and lysine, this result was same as the reference strain *Viridibacillus arvi* DSM 16317^T , indicating that the peptidoglycan was the type A4a (Schleifer and Kandler 1972) with an L-Lys–D-Asp interpeptide bridge (Fig S4). Ribose, glucose, arabinose, galactose, and mannose were the major whole-cell sugars of strain YIM B01967^T. The quinone system consisted of the major compound MK-8 and moderate amounts of MK-7 in strain YIM B01967^T, which was identical to that found in members of the genus *Viridibacillus* (Albert et al. 2007). The cellular fatty acids profile of strain YIM B01967^T consisted of iso-$C_{15:0}$ (34.64%), anteiso-$C_{15:0}$ (12.72%) and $C_{16:1}$ *ω10c* (11.53%) as major fatty acids (>10%), and $C_{16:1}$*ω7c* alcohol(8.95%), anteiso-$C_{17:0}$(6.15%), iso-$C_{16:0}$(6.02%), Summed Feature 4(iso I-$C_{17:1}$/anteiso B)(5.02%), iso-$C_{14:0}$(3.68%), iso-$C_{17:1}$ *ω10c*(3.06%), iso-$C_{17:0}$(2.94%) and $C_{16:0}$(2.21%) as minor fatty acids(>1%). The polar lipids of the strain YIM B01967^T were diphosphatidylglycerol (DPG), phosphatidylmethylethanolamine (PME), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and two unidentified phospholipids (PL1, PL2). (Fig S5). The polar lipid profile consisted of the major compounds diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylethanolamine and major to moderate amounts of an unknown aminophospholipid (APL1) and moderate to minor amounts of two unknown phospholipids (PL1, PL2) and three unknown polar lipids in *Viridibacillus arvi* DSM 16317^T. Chemotaxonomic analyses including cell-wall peptidoglycan, whole-cell fatty acids, cell-wall sugars, and polar lipids exhibited the close similarity of strain YIM B01967^T to type strains of the closely related species, which confirmed its affiliation to the genus *Viridibacillus*, with sufficient differences to warrant its proposal as representing a novel species of the genus *Viridibacillus*.

The detailed differentiating phenotypic and chemotaxonomic characteristics features between YIM B01967^T and the reference strains were given in Table 1.

Consequently, based on the above findings, we characterized strain YIM B01967^T as a novel species within the genus *Viridibacillus*, for which the name *Viridibacillus soil* sp.nov.is proposed.

Description of *Viridibacillus soli* sp.nov.

Viridibacillus soli (so'li. L. neut. gen. n. soli of soil, the source of the type strain)

Cells are straight, round-ended, Gram-positive, motile rods (0.6-0.8×2.5-3.0 mm), occurring singly and in pairs. Growth occurs at temperature range of 15-37 °C (optimum, 28 °C), pH 6.0-9.0 (optimum, pH 7.5) and in the presence of 0-2% (w/v) NaCl (optimum 2% NaCl). Strain YIM B01967^T is catalase-positive and oxidase-negative. In API 20NE, positive for urease, *β*-glucosidase, and malic acid, nitrate is reduced, gelatin is hydrolyzed. In API ZYM, positive for alkaline phosphatase, esterase, lipid esterase, leucine aramidase, chymotrypsin, acid phosphatase, and naphthol-*AS-BI*-phosphohydrolase. In the Biolog GEN III MicroPlate, positive for N-acetyl-d-glucosamine, N-acetyl-*β*-d-mannosamine, D-galactose, 3-methyl-glucose, inosine, D-mannitol, D-arabitol, myoinositol, glycerol, D-glucose-6-PO_4, D-fructose-6-PO_4, gelatin, L-alanine, L-arginine, L-glutamic acid, L-histidine, L-pyroglutamic acid, L-serine, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, quinic acid, D-saccharic acid, D-lactic acid, methyl ester, L-lactic acid, α-keto-glutaric acid, L-malic acid, Tween 40, *α*-hydroxy-butyric acid, *β*-hydroxy-D, L-butyric acid, acetic acid, 1% sodium lactate, nalidixic acid, aztreonam. In the API 50CHB gallery, acid is not produced from any of the carbohydrate substrates. Cell wall peptidoglycan is of the type A4*a* with an L-Lys–D-Asp interpeptide bridge. The cell-wall sugars are ribose, glucose, arabinose, galactose, and mannose. The quinone system consists of the major compound MK-8 and moderate amounts of MK-7. The fatty acids (>5% of total fatty acids) are iso-$C_{15:0,}$ anteiso-$C_{15:0}$, $C_{16:1}\omega10c$, $C_{16:1}\omega7c$ alcohol, anteiso-$C_{17:0}$, iso-$C_{16:0}$. The polar lipids profile include diphosphatidylglycerol, phosphatidylglycerol, phosphatidylmethylethanolamine phosphatidylethanolamine, and moderate to minor amounts of two unknown phospholipids (PL1, PL2). The DNA G+C content of the type strain is 36.3 mol% based on the draft genome sequence.

The type strain, YIM B01967^T (= KCTC 43249 ^{T} = CGMCC 1.18436 ^{T}), was isolated from a soil sample collected from a forest in Ailaoshan National Nature Reserve, Yuxi City, Xinpin county, Yunnan province, China. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence and the draft genome sequence are MW386301 and JAEOAH000000000 respectively.

Abbreviations

PCA Plate count agar

PCB Plate count broth

DPG Diphosphatidylglycerol

PME Phosphatidylmethylethanolamine

PG Phosphatidylglycerol

PE Phosphatidylethanolamine

PL Unidentified phospholipid

Declarations

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Conflict of interest: The authors declare that they have no conflicts of interest.

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Tables

Table 1 Phenotypic and chemotaxonomic characteristics that differentiate strain YIM B01967^T from closely related reference strains

Characteristic	1	2	3
Colony colour	Light yellow	White	White
Swelling of sporangia	No obvious enlargement	Slight	Slight
Growth conditions			
Temperature (°C)	15-37	5-40	5-37
Growth in 5 % NaCl	-	-	+
Anaerobic growth	w	w	-
API 20NE			
Urease	+	+	-
β-Glucosidase	+	-	-
Malic acid	+	-	-
Nitrate reduced	+	-	+
Gelatin hydrolysis	+	-	+
Aesculin hydrolysis	+	-	-
ONPG	-	w	-
API ZYM			
Casein hydrolysis	-	w	-
Acid production from(50CHB)			
N-acetylglucosamine	-	w	-
D-fructose	-	+	-
Myo-inositol	+	-	-
D-arabitol	+	-	-
DNA G + C content (mol%)	36.3	35.0	35.0
Polar lipids	DPG, PME, PG, PE, 2PL	DPG, PG, PE, APL, 2PL	DPG, PG, PE, APL, 2PL
Major fatty acids(>10%)	iso-$C_{15:0}$, anteiso-$C_{15:0}$, $C_{16:1}$ *ω10c*	iso-$C_{15:0}$, anteiso-$C_{15:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$

Strains: (1) YIM B01967^T; (2) *Viridibacillus arvi* DSM 16317^T (data from Heyrman et al. 2005; Albert et al. 2007); (3) *Viridibacillus arenosi* DSM 16319^T (data from Heyrman et al. 2005; Albert et al. 2007).

+ Positive, - negative, w weak. All data were obtained from this study except where indicated.

Figures

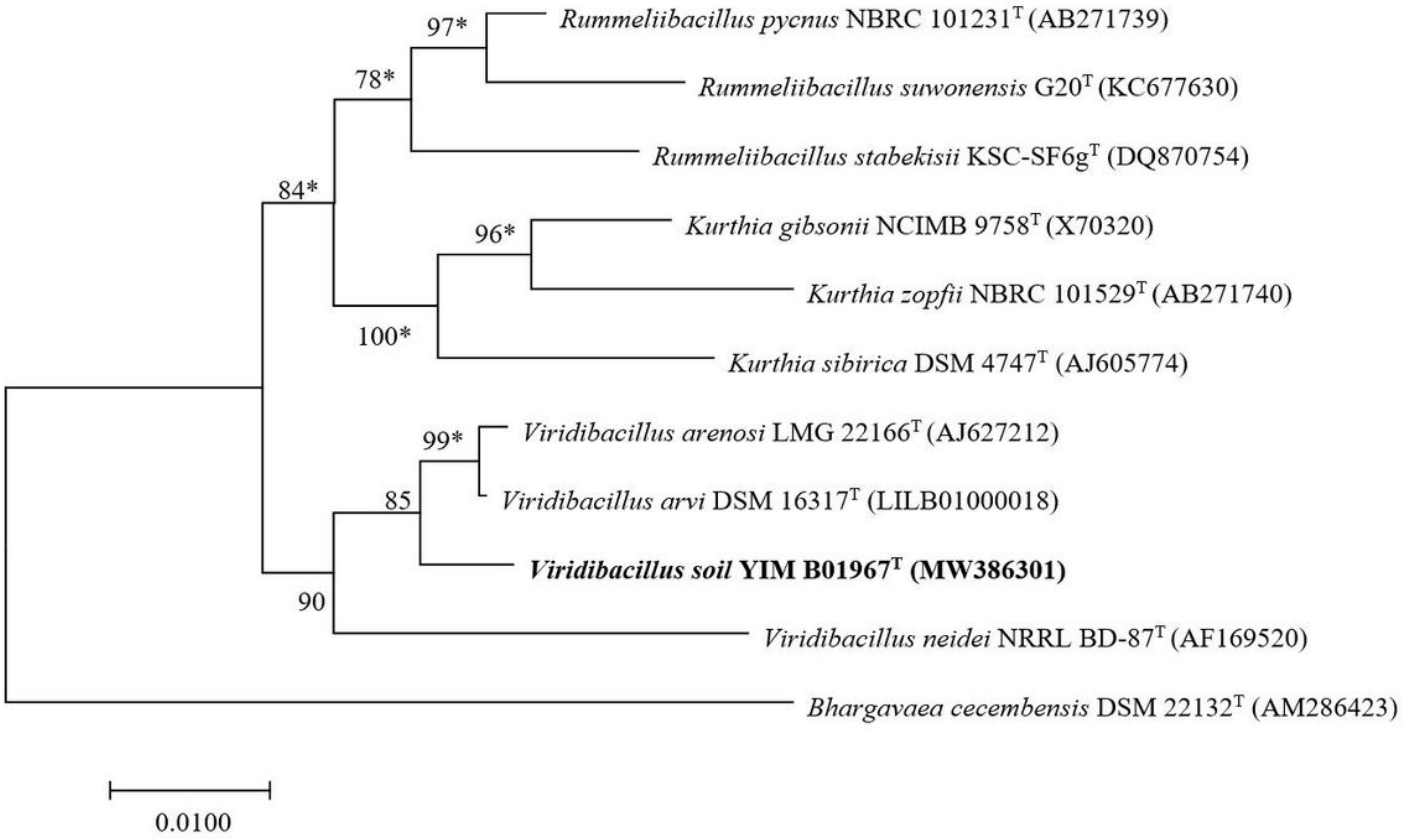

Figure 1

Neighbour-joining tree based on 16S rRNA gene sequences showing the relationships between strain YIM B01967T and some members of the family Planococcaceae. Bootstrap values (>70%) based on 1000 replicates were shown at the branch nodes. Asterisks (*) indicate that the corresponding branches were also recovered in trees generated with the maximum parsimony and maximum likelihood methods. Bhargavaea cecembensis LMG 24411T was used as an out group. Bar, 1% substitutions per nucleotide position.

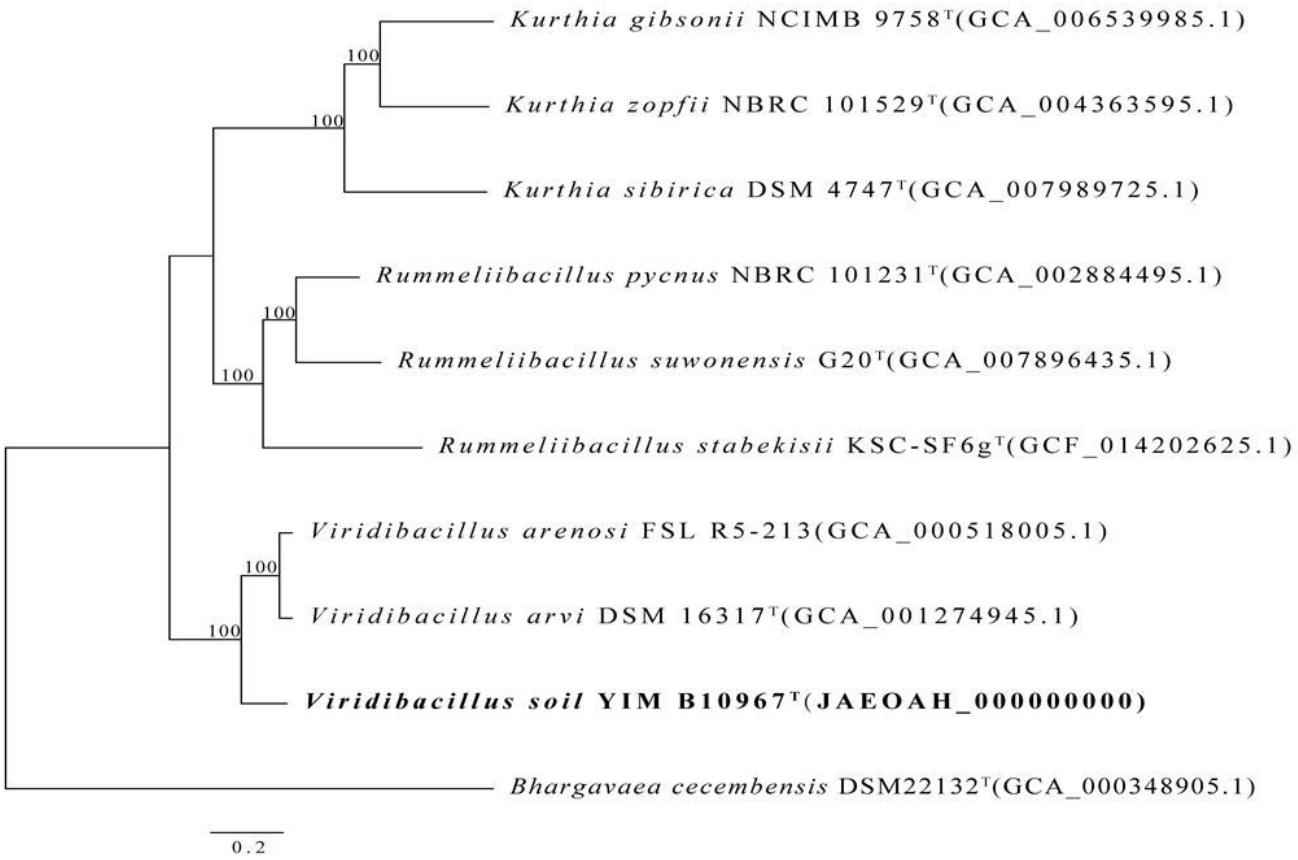

Figure 2

Phylogenomics tree of YIM B01967T with the closely related strains using the supermatrix method (containing 672 single-copy orthogroups, 171,059 genes). Bhargavaea cecembensis LMG 24411T was used as an out group. Bootstrap analysis was carried out using 100 replications. Percentage bootstrap values (>70%) are given at branching points.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterial.doc
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- YIMB01967KCTC43249.pdf