Streptomyces Blattellae, A Novel Actinomycete Isolated From the in Vivo of an Blattella Germanica.

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Abstract

During a screening for novel and useful actinobacteria in desert animal, a new actinomycete was isolated and designated strain TRM63209^T. The strain was isolated from *in vivo* of a *Blattella germanica* in Tarim University in Alar City, Xinjiang, north-west China. The strain was found to exhibit an inhibitory effect on biofilm formation by *Candida albicans* American type culture collection (ATCC) 18804. That it belongs to the genus *Streptomyces*. The strain was observed to form abundant aerial mycelium, occasionally twisted and which differentiated into spiral spore chains. Spores of TRM63209^T were observed to be oval-shaped, with a smooth surface. Strain TRM63209^T was found to grow optimally at 28°C, pH 8 and in the presence of 1% (w/v) NaCl. The whole-cell sugars of strain TRM63209^T were rhamnose ribose, xylose, mannose, galactose and glucose, and the principal polarlipids were found to be diphosphatidylglycerol(DPG), Phos-phatidylethanolamine(PE), phosphatidylcholine(PC), phosphatidylinositol mannoside(PIM), phosphatidylinositol(PI) and an unknown phospholipid(L). The diagnostic cell wall amino acid was identified as LL-diaminopimelic acid. The predominant menaquinone was found to be MK-9(H6), MK-9(H2), MK-9(H8), MK-10(H2). The major cellular fatty acids were identified as iso-C16:0, 16:0, anteiso-C15:0, anteiso-C17:0, iso-C15:0 and Sum In Feature 3. Analysis of the 16S rRNA sequence showed that strain TRM63209^T exhibits high sequence similarity to *Streptomyces bungoensis* strain DSM 41781^T 98.20%. A multi-locus sequence analysis of five house-keeping genes (atpD, gyrB, rpoB, recA and trpB) also illustrated that strain TRM63209^T should be assigned to the genus *Streptomyces*. The DNA G + C content of the strain was determined to be 70.2 mol%. The average nucleotide identity relatedness between strain TRM63209^T and the phylogeny vertically related *Streptomyces bungoensis* DSM 41781^T, *Streptomyces phyllanthi* PA1-07^T, *Streptomyces longwoodensis* DSM 41677^T and *Streptomyces caeruleatus* NRRL B-24802^T was respectively found to be 82.76 %, 82.54%, 82.65%, 84.02%. And the in sico DNA-DNA hybridization value to respectively be 26.30%, 25.10%, 26.20%, 29.50%. Therefore, it is concluded that strain TRM63209^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces blattellae* is proposed. The type strain is TRM63209^T (CCTCC AA 2018093^T = LMG 31403= TRM63209^T).

Introduction

The genus *Streptomyces* was initially described in 1943 (Waksman and Henrici 1943) and more than 850 species with valid names of *Streptomyces* have been published (https://www.bacte rio.net/streptomyces.html) (Zhang et al. 2017). The members of the genus are Gram-positive with high G + C content, and species are widely distributed in aquatic and terrestrial ecosystems. *Streptomyces* are aerobic Gram-positive bacteria with well-developed mycelia, which can produce a large number of conidia for reproduction. Many *Streptomyces* undergo morphological differentiation and also have a mycelial phase. *Streptomyces* are highly versatile and produce an abundant array of bioactive secondary metabolites (Genilloud 2017) that have been used as antibiotic, anti-carcinogenic, anti-helminthic and antifungal compounds. Consequently, they are very important for biotechnology, medicine and agriculture (Barka et al. 2015).

Strain TRM63209^T isolated from *in vivo* of an *Blattella germanica* in Tarim University in Alar City, Xinjiang, north-west China(40°55′N,81°29′E). This strain inhibits the formation biofilm of *Candida albicans* ATCC 18804. On the basis of phylogenetic, phenotypic and genetic data, TRM63209^T considered to a novel species of the genus *Streptomyces*, for which the name *Streptomyces blattellae* is proposed.

Materials And Methods

Isolation of *Streptomyces* strain and culture conditions

As part of a program to unravel the diversity of symbiotic actinomycetes in insect–microbe and to discover novel actinomycetes and novel natural products, strain TRM 63209^T was isolated from the *in vivo* of an *Blattella germanica*, the Tarim University, Alar, Xinjiang Province, north-west China. *Blattella germanica* is washed with sterile distilled water to remove surface impurities. The surface was sterilized in 70% ethanol for 60 seconds and then washed three times in sterile distilled water. Grind it to a powder and suspend in sterile distilled water incubated on a rotary shaker at 180 rpm 37℃ for 30 min (Liu et al., 2017), ultrasound 3min, and the suspension was appropriately diluted before being spread onto Czapek's agar (Wiese et al., 2008) supplemented with Nystatin (100 mg/ml) and nalidixic acid (50 mg/ml) (Arocha-Garza et al., 2017). After 21 days of incubation at 28 ℃,the isolate was transferred and purified on International Streptomyces Project (ISP) 4 medium (Shirling□Gottlieb, 1966) and the spore and mycelia maintained as glycerol suspensions (20 %, v/v) at -80℃.

Phenotypic characterisation

Cultural characteristics were determined by methods used in the ISP(1–7) (Shirling & Gottlieb, 1966), nutrient agar (NA) (Waksman, 1961), Gause's synthetic agar no.1 and Czapek's agar (Wiese et al., 2008) for 28℃ at 14 days. For colour determination, the colour of colony of strain TRM63209^T were compared with the Inter-Society Colour Council–National Bureau of Standards (ISCC-NBS) Colour Charts standard sample no. 2106 (Kelly, 1964). Microscopic observation of spores and mycelia were observed by light microscopy (Axioskop 20; Zeiss) and scanning electron microscopy SEM (Quanta; FEI) after Incubation for 7 days at 28℃ on the optimal medium ISP3. The growth temperature range 5–55°C (4, 10, 15, 18, 28, 35, 36, 37, 38, 39, 40, 45, 50 and 55°C) and tolerance to NaCl concentrations (0, 1, 3, 5, 8, 10, 13, 15, 18 and 20%, w/v) was tested on ISP3 agar medium after culturing for 28℃ at 2 weeks. The pH range was adjusted separately with buffer (pH 4.0–5.0, 0.1 M citric acid/0.1 M sodium citrate; pH 6.0–8.0, 0.1 M KH_2PO_4 0.1 M NaOH; pH9.0–10.0, 0.1 M $NaHCO_3$ 0.1 M Na_2CO_3; pH 11.0–12.0, 0.05 M Na_2HPO_4 0.1 M NaOH) and incubated at 37℃ for 2 weeks in ISP3 medium. The pH range was controlled separately with buffer and incubated at 37℃ for 2 weeks in ISP3 medium.

Chemotaxonomy

Isomers of diaminopimelic acid were analysed following the method of Hasegawa et al (1983). The whole cell sugar composition was analysed following the method of Staneck and Roberts (1974). Polar lipids in cells of strain TRM63209^T were extracted and examined by two-dimensional TLC and identified following the methods of Minnikin et al (1984). Menaquinones were extracted using the method of Collins (1985) and subjected to HPLC analysis (Groth et al. 1997). The cellular fatty acid composition was determined using the Microbial Identification System (MIDI Sherlock version 6.0) (Sasser 1990).

Phylogenetic analyses

Genomic DNA extraction and PCR amplification of the 16S rRNA gene from strain TRM63209^T were performed following Chun and Goodfellow (1995). The purified PCR product was cloned into the vector pMD19-T (Takara) and sent to Sangon for gene sequencing. Multiple alignments with sequences from closely related *Streptomyces* species and calculations of sequence similarity were performed using the EzTaxon-e server (Kim et al. 2012). Phylogenetic analyses were performed using MEGA version 7.0 (Kumar et al. 2016) selecting the neighbour-joining (Saitou and Nei 1987), Maximum-Evolution (Rzhetsky and Nei 1993) and maximum-likelihood (Felsenstein 1981) algorithms. Topologies of the resultant trees were evaluated using the Felsenstein's (1985) resampling method with 1,000 replications. AtpD, gyrB, rpoB, recA and trpB genes were obtained using primers and amplification conditions as previously described (Guo et al. 2008; Hatano et al. 2003). Phylogenetic relationships were reconstructed using the Neighbour-Joining algorithm as described above.

The whole genome of TRM63209^T was sequenced by Oxford Nanopore technologies. The G + C content of strain TRM63209^T was obtained by whole genome sequencing. The Average nucleotide identity (ANI) was determined as described by Lee et al. (2015). DNA–DNA relatedness values were determined online according to the method of Meierkolthoff et al. (2013). DNA-DNA hybridization (dDDH) values were calculated at the Genome-to Genome Distance Calculator (GGDC) website using formula 2, as originally described by Auch et al. (2010) and updated by Meier-Kolthoff et al. (2013). Anti-SMASH was used to predict the biosynthetic gene clusters of strain TRM63209^T (Blin et al. 2013).

Antifungal and antibacterial activity

C. albicans ATCC 18,804 was obtained from China Center for Type Culture Collection, and was cultured with Sabouraud dextrose agar/broth (SDA/SDB). Unless specified otherwise, ISP3 was used to culture TRM63209^T strain.

A 4% (v/v) inoculum of well-growing strain TRM63209^T was used to culture strain TRM63209^T in ISP3 liquid culture medium (20 g oatmeal and 1 ml trace salt per L distilled water) and incubated at 28 ℃ with shaking at 180 rpm for 10 days. Cells were removed by centrifugation to leave the supernatant, which was kept at 4 ℃ for further screening of biofilm inhibition. The effect of the strain TRM63209^T growth supernatants on static biofilm formation was measured according to Balasubramanian et al. (2017). Briefly, test organism cells were diluted 1:100 with fresh SDB to bring the test cell suspension to a concentration of 1 × 10^8 cells per mL. 100 µL aliquots of cells were added to the wells of a 96 well plate and 100 µL of supernatants was added, then the plates inoculated with *C. albicans* were incubated at 37 ℃ for 72 h. Wells without the supernatant (100 µL SDB) was used as blank control. After crystal violet staining, the absorbance was measured at 490 nm by an enzyme-linked immunosorbent assay reader (Bio-Rad). Relative activity of biofilm formation was indicated as Relative Biofilm Formation % (RBF %) calculated the following formula: RBF % = Treated OD_{490}/Untreated OD_{490} × 100%.

Results And Discussion

Strain TRM63209^T was observed to grow optimally on ISP 3 and ISP 2, and showed moderate growth on ISP 1, ISP 4, ISP 5, nutrient agar and Gause's synthetic agar no. 1, with slow growth on ISP 6, ISP 1 and Czapek's medium. Light yellow soluble pigment was produced in ISP5 and Greenish White soluble pigment was produced in ISP6, the colour of other the aerial mycelium is white, other no diffusible pigment was produced on the media test, the color of ISP2 substrate mycelium is light yellow(**Table 1**). Morphological characteristics of strain TRM63209^T were observed using SEM (**Fig. 1**). The strain was observed to form an abundant white aerial mycelium, occasionally twisted, which differentiates into spiral spore chains. Each spore was observed to be oval-shaped with a smooth surface (Fig. 1). Strain TRM63209^T was found to grow only at 5–55 ℃, pH 4.0–12.0 and 0–20% (w/v) NaCl, with optimal growth at 28 ℃, pH 8.0 and with 1% (w/v) NaCl. Other physiological characteristics of strain TRM63209^T are listed in the species description and in **Table 1**.

The whole-cell sugars of strain TRM 63209^T were rhamnose, ribose, xylose, galactose, glucose and mannose, and the principal phospholipids were found to be diphosphatidylglycerol(DPG), Phos-phatidylethanolamine(PE), phosphatidylcholine(PC), phosphatidylinositol mannoside(PIM), phosphatidylinositol(PI) and an unknown phospholipid(L)(**supplementary Fig. S1, Fig. S3**). The diagnostic cell wall amino acid was identified as LL-diaminopimelic acid (**supplementary Fig. S2**). The predominant menaquinone was found to be MK-9(H6), MK-9(H2), MK-9(H8), MK-10(H2) (**Supplementary Fig. S4)**. The major cellular fatty acids (> 5%) were identified as iso-C16:0(26.5%), 16:0 (21.4%), anteiso-C15:0 (9.8%), anteiso-C17:0 (9.7%), iso-C15:0 (6.7%) and Sum In Feature 3 (5.7%). Fatty acids present in smaller amounts (> 1%) were iso-C17:0 (4.3%), iso-C16:1 H (1.9%), iso-C14:0 (1.9%), 18:1 w9c (1.6%), 15:0(1.4%), Sum In Feature 9 (1.4%), anteiso-C17:1 w9c (1.4%), 14:0 (1.2%) and Sum In Feature 5 (1.7%)(**supplementary Table S1**). It utilized D-mannitol, trehalose, sucrose, L-rhamnose, galactose, raffinose, maltose, cellobiose, sorbose as carbon sources but not chitosan, xylan, L-arabinose, starch, melezitose, glucose, xylose, fructose, Inositol or xylitol. Comparison of the physiological properties of strain TRM 63209 ^{T} with other species of *Streptomyces* is shown in **Table 2**.

Phylogenetic analysis based on the 16S rRNA gene sequence revealed that strain TRM 63209 ^{T} belongs to the genus *Streptomyces*, with high sequence similarity to *Streptomyces bungoensis* DSM 41781^T (GenBank accession no. KQ948892; 98.20%), *Streptomyces* PA1-07^T (GenBank accession no. LC125632; 98.14%), *Streptomyces longwoodensis* DSM41677^T (GenBank accession no. KQ948572; 98.00%) and *Streptomyces caeruleatus* NRRL B-24802^T (GenBank accession no. KQ948975; 98.00%). Strain TRM63209^T was found to form an unique clade that was different from other closely related species (**Fig. 2**). The apparent close relationship with *Streptomyces bungoensis* DSM 41781T did not receive high bootstrap support and was not supported by the other two (Maximum Likelihood and Minimum-Evolution) tree building methods (**supplementary Fig. S5, Fig. S6**). According to the guiding principles of Rong and Huang

(2012), in multi-locus sequence analysis (MLSA) it is considered that pairs with evolutionary distances greater than 0.007 belong to different species. A MLSA of five house-keeping genes (atpD, gyrB, recA, rpoB, and trpB) indicated that the MLSA distances between strain TRM63209^T and similar species were greater than the 0.007 threshold (**Fig. S7**). Therefore, it was concluded that TRM63209^T represents a distinct species belonging to the genus *Streptomyces*.

The G + C content in the draft genome sequence of strain TRM63209^T was determined to be 70.2 mol %. The complete genome of strain TRM63209^T has a size of 8.49 Mb, did not distribute among chromosomes and plasmids. In its genome, 7804 genes were annotated, of which 7732 are putative protein-coding genes. The number of hypothetical proteins is 2367, corresponding to 31% of the total number of putatively annotated proteins. 60 tRNAs and seven copies of the 16S rRNA gene were identified. The genomic characteristics of the compared strains are quite heterogeneous (**Table S3**). The ANI relatedness between strain TRM63209^T and the phylogenetically related strain *Streptomyces bungoensis* DSM 41781^T, *Streptomyces phyllanthi* PA1-07^T, *Streptomyces longwoodensis* DSM 41677^T and *Streptomyces caeruleatus* NRRL B-24802^T was respectively determined to be 82.76 %, 82.54%, 82.65%, 84.02%. This value is significantly lower than the widely accepted threshold for describing prokaryote species (95–96%; Kim *et al.* 2014). The dDDH value between strain TRM63209^T and the phylogenetically related strain *Streptomyces bungoensis* DSM 41781^T, *Streptomyces phyllanthi* PA1-07^T, *Streptomyces longwoodensis* DSM 41677^T and *Streptomyces caeruleatus* NRRL B-24802 was respectively determined to be 26.30%, 25.10%, 26.20%, 29.50%. These significantly lower than the 70% threshold value for delineation of prokaryotic genomic species (Wayne *et al.* 1987). It is thus proposed that strain TRM63209^T can be differentiated from closely related *Streptomyces* species and represents a novel species. The supernatant of strain TRM63209^T inhibited biofilm formation by both *C. albicans*, with inhibition ratios over 40% (**Table S2**). The anti-SMASH biosynthetic gene cluster prediction tool was used to investigate the draft genome sequence of strain TRM63209^T and found one type I, two type III polyketide biosynthetic gene clusters, five nonribosomal peptide synthetase biosynthetic gene clusters and one NRPS-like fragment. In addition, five terpene, three siderophore, three Class I lanthipeptide clusters like nisin, one Non-alpha poly-amino acids like e-Polylysin(NAPAA), one ectoine, one Arylpolyene, one Other unspecified ribosomally synthesised and post-translationally modified peptide product (RiPP), one Redox-cofactors such as PQQ (NC_021985:1458906–1494876), one Oligosaccharide, two siderophore, one melamin and one indole biosynthetic gene clusters were detected. Numbers of secondary metabolite-associated gene clusters in TRM63209^T in comparison to other species in the family that is shown in Table S4. A product of one of these clusters may be involved in the antibiofilm activity observed. Through anti-SMASH analysis, the **7-prenylisatin** antibiotic biosynthesis gene cluster can be found, which can effectively inhibit the growth of fungi and the similarity to 60%, whereby the strain TRM63209^T inhibits the formation of BF may be associated with this gene cluster (Liang D). In summary, the sequencing of the genome of strain TRM63209^T further clarified the evolutionary relationship between strains and will guide the screening for active secondary metabolites.

Description of Streptomyces blattellae

Streptomyces blattellae (blat.tel'lae. N.L. gen. n. blattellae of the cockroach genus Blattella).

Aerobic, Gram-stain positive actinomycete. Forms abundant aerial mycelium, occasionally twisted, which differentiates into spiral spore chains. Each spore is oval-shaped with a smooth surface. The strain grew well and developed more abundant aerial mycelia on ISP3 and ISP2 than on ISP 1, ISP 4, ISP 5, NA and Gause's synthetic agar no. 1, but poor growth was observed on ISP7 and Czapek's medium. Growth was observed at 5–55, with 0–20 % (w/v) NaCl and at pH 4.0–12.0 and was found to grow optimally at 28°C, pH 7 and in the presence of 1% (w/v) NaCl. The whole-cell sugars of strain TRM 63209^T were rhamnose, ribose, xylose, mannose galactose and glucose, and the principal phospholipids were found to be diphosphatidylglycerol(DPG), Phos-phatidylethanolamine(PE), phosphatidylcholine(PC),phosphatidylinositol mannoside(PIM), phosphatidylinositol(PI) and an unknown phospholipid(L). The diagnostic cell wall amino acid was identified as LL-diaminopimelic acid. The predominant menaquinone was found to be MK-9(H6), MK-9(H2), MK-9(H8), MK-10(H2). The major cellular fatty acids were identified asiso-C16:0, 16:0, anteiso-C15:0, anteiso-C17:0, iso-C15:0, Sum In Feature 3 and Summed Feature 3. The DNA G + C content of the strain was determined to be 70.2 mol %.

The type strain, TRM63209^T (CCTCC AA 2018093^T = TRM63209^T), was isolated from the *in vivo* of an *Blattella germanica* in Tarim University, Alar City, Xinjiang Province, The GenBank/ EMBL/ DDBJ accession numbers for the genome and 16S rRNA gene sequence of strain TRM63209T are WJBG00000000 and MK795724, respectively.

Declarations

Author contributions

GML contributed to performing the experiments and writing the initial draft. HZ and ZFX contributed to the guidance of experimental operations. LLY contributed to the morphological analyzes. HZ and LLZ contributed to reagents, instrumentation and the financial support for this work. All authors approved the manuscript.

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Compliance with ethical standards

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

Ethical approval This article does not contain any studies with human participants and/or animals performed by any of the authors. The formal consent is not required in this study

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Tables

Table 1 Growth and cultural characteristics of strain TRM63209^{T}

Culture medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigments
Tryptone—yeast extract agar medium ISP 1	++	White; moderate	Colorless	None
Yeast extract/malt extract ISP 2	+++	White; Abundant	Light yellow	None
Oatmeal ISP 3	+++	White; Abundant	Colorless	None
Inorganic salts/starch ISP 4	++	White; moderate	Colorless	None
Glycerol/asparagine ISP 5	++	White; moderate	Colorless	Light yellow
Peptone/yeast extract/iron agar ISP 6	+	No aerial mycelium	None	Greenish White
Tyrosine Agar Medium Base ISP 7	+	No aerial mycelium	None	None
Nutrient Agar	++	White; moderate	Colorless	None
Gauze's No. 1	++	White; moderate	Colorless	
Czapek's agar	+	No aerial mycelium	None	None

+++ good, ++moderate, + weak, none—not observed

Table 2 Differential characteristics between strain TRM63209T and phylogenetically related species of the genus *Streptomyces*. Strains: 1, TRM63209(T); 2, *Streptomyces bungoensis* DSM 41781(T); 3, *Streptomyces phyllanthi* PA1-07(T); 4, *Streptomyces longwoodensis* DSM 41677(T); 5, *Streptomyces caeruleatus* NRRL B-24802(T). Data for reference strains were taken from Eguchi T et al. (1993), Pittayakhajonwut P et al. (2016), Prosser B L T et al. (1976) and Zhu HH et al. (2011). + Positive; – negative; AD; not shown. PIM phosphatidylinositol mannoside, PG phosphatidylglycerol, PI phosphatidylinositol, PE phosphatidylethanolamine, APL aminophospholipids, AL aminolipids, DPG diphosphatidylglycerol

Characteristic	1	2	3	4
Spore chains	Spiral	Spiral	Short and compact Spiral	Short Spiral
Spore shape	oval-shaped, with a smooth surface	moderately short with simple branches spiny surface	oval-shaped with arugose surface	smooth
Growth temperature (optimum temperature)	5-55(28)	5-55 (28)	20-40(28–30)	10-45 (28)
Optimal pH for growth	4-12(8)	4-12(7)	5-9	7
NaCl tolerance (%, w/v)	0-20(1)	1-15(7-10)	5	5
Utilization as sole carbon source				
Cellobiose	+	+	+	-
D-fructose	-	+	+	-
Glucose	-	+	+	+
Raffifinose	+	+	+	-
Ribose	ND	ND	-	ND
Inositol	-	-	+	-
L-arabinose	-	+	+	+
L-rhamnose	+	-	+	-
Xylose	-	-	+	+
Catalase production	+	+	ND	ND
Oxidase reaction	+	+	+	ND
Starch hydrolysis	+	-	ND	+
Cellulose hydrolysis	+	+	ND	-
Polar lipids components	DPG PE PG PI PIM	ND	DPG PE PIM	ND
DNA G + C content (mol %)	70.2%	70.3%	71.0%	73.0%

+ positive; - negative; ND; not no data available

Figures

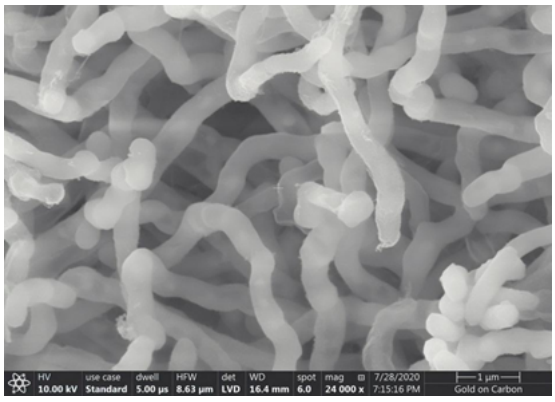

Figure 1

SEM image of strain TRM63209T grown on ISP3 at 37 °C for 7 days

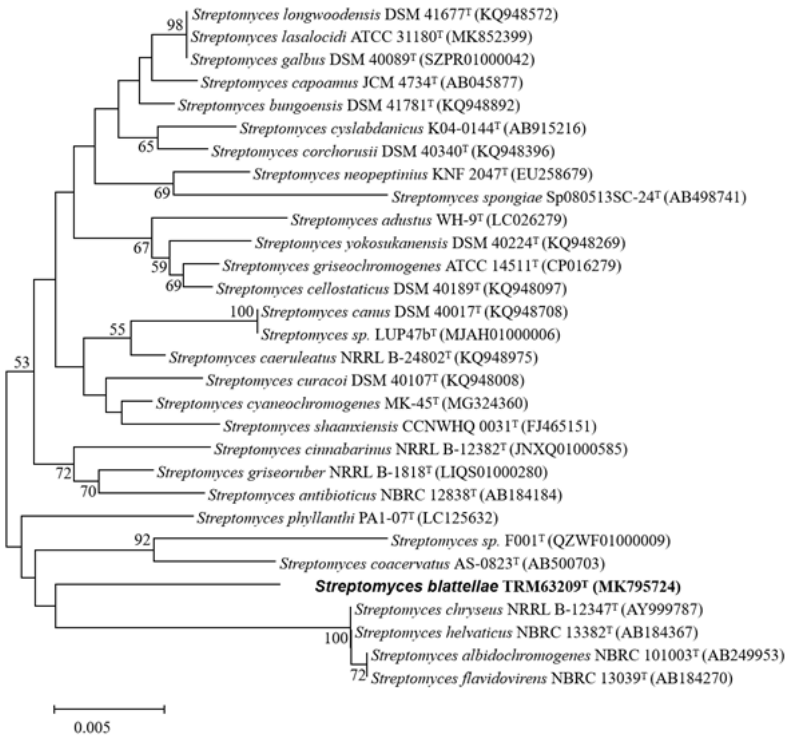

Figure 2

Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain TRM63209T and other closely related species of Streptomyces. Numbers at the nodes are percentage bootstrap values based on 1000 resampled datasets; only values > 50% are shown. Bar, 0.0050 substitutions per nucleotide position

Supplementary Files

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

- Revisionsupplement.docx