Genome-based Classification of *Streptomyces Pinistramenti* sp. nov., a Novel Actinomycete Isolated From a Pine Forest Soil in Poland With a Focus on Its Biotechnological and Ecological Properties

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Abstract

A genomic-based polyphasic study was undertaken to establish the taxonomic status and biotechnological and ecological potential of a *Streptomyces* strain, isolate SF28^T, that was recovered from the litter layer in a polish *Pinus sylvestris* forest. The isolate had morphological characteristics and chemotaxonomic properties consistent with its classification in the genus *Streptomyces.* It formed long straight chains of spores with smooth surfaces, contained *LL*-diaminopimelic acid and glucose and ribose in whole-organism hydrolysates, produced major proportions of straight, *iso*- and *anteiso*- fatty acids, hexa- and octa-hydrogenated menaquinones with nine isoprenoid units and had a polar lipid pattern composed of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylmethylethanolamine, glycophospholipids and three uncharacterized components. Phylogenetic trees prepared using 16S rRNA gene and multilocus gene sequences of conserved housekeeping genes showed that the isolate formed a branch that was loosely associated with the type strains of several validly published *Streptomyces* species. A draft genome generated for the isolate was rich in natural product-biosynthetic gene clusters with the potential to produce new specialised metabolites, notably antibiotics, and stress related genes which provide an insight into how they may have adapted to the harsh conditions that prevail in acidic forest soils. A phylogenomic tree based on the genomes of the isolate and its phylogenetic neighbours confirmed that it formed a distinct lineage well separated from its closest evolutionary relatives. The isolate shared low average nucleotide index and digital DNA:DNA hybridization values with its phylogenomic neighbours and was also distinguished from them using a combination of cultural and micromorphological properties. Given this wealth of taxonomic data it is proposed that isolate SF28^T (=DSM 113360^T=PCM 3163^T) be classified in the genus *Streptomyces* as *Streptomyces pinistramenti* sp. nov. The isolate showed pronounced antimicrobial activity, especially against fungal plant pathogens.

Introduction

The genus *Streptomyces* was proposed by Waksman and Henrici (1943) for aerobic, filamentous, spore-forming actinomycetes and its formal description subsequently emended by Kämpfer (2012). The genus currently includes over 800 validly published species (http://www.bacterio.net/streptomyces.html) but remains underspeciated (Hamm et al. 2017; Sivalingam et al. 2019). Multilocus sequence analyses of concatenated, protein coding, conserved house-keeping genes and associated phenotypic properties provide a more reliable way of recognizing novel *Streptomyces* species than corresponding studies based on 16S rRNA gene sequences (Labeda et al. 2012, 2017; Zhuang et al. 2020). It is also clear that genomic-based classifications are accelerating progress in streptomycete systematics as they provide greater resolution between closely related *Streptomyces* species than corresponding trees based on single and concatenated sequences of conserved genes (Carro et al. 2018; Nouioui et al. 2018; Kusuma et al. 2021). In addition, improved metrics, such as pairwise average nucleotide index (ANI) and *in silico* DNA:DNA hybridization (DDH) values, facilitate the recognition of species boundaries (Chun et al. 2018).

Streptomycetes are a unique source of antibiotics which include many used in agriculture, medicine and veterinary practice (Barka et al. 2015; Chater, 2016; Qi et al. 2021). Members of the genus are considered to be gifted (Baltz 2017, 2019) as they have large genomes (≥ 8.0 Mbp) rich in natural product-biosynthetic gene clusters (NP-BGCs) with the potential to encode for novel and uncharacterized antibiotics of potential therapeutic value, as exemplified by *Streptomyces leeuwenhoekii* strains isolated from extreme hyper-arid Atacama desert soils (Busarakam et al. 2014; Gomez-Escribano et al. 2015; Castro et al., 2018). Novel streptomycetes from extreme biomes are proving to be a potential rich source of new bioactive molecules (Goodfellow et al. 2018; Rateb et al. 2018; Sivalingam et al.

2019; Sivakala et al., 2021) thereby underpinning the premise that abiotic conditions in extreme biomes select for strains with the capacity to synthesize novel specialised metabolites (Bull and Stach 2007; Bull 2011; Gomez-Escribano et al. 2015). However, little attention has been focused on the delineation of *Streptomyces* species isolated from coniferous forest soils (Golińska et al. 2022), exceptions include the recognition of *Streptomyces abietis* (Fuijii et al. 2013), *Streptomyces pini* (Madhaiyan et al. 2016) and *Streptomyces piniterrae* (Zhuang et al. 2020). Streptomycetes from pine forest soils are also known to be antagonistic towards fungal pathogens of pine seedlings (Golińska and Dahm, 2013). It is becoming increasingly apparent that whole-genomes of actinomycetes isolated from extreme habitats contain the stress-related genes that can provide an insight into how they adapt to harsh abiotic conditions that prevail therein (Busarakam et al. 2016; Abdel-Mageed et al. 2020; Golińska et al. 2022).

The present study, a continuation of earlier work on the diversity of filamentous actinomycetes in coniferous litter and soil (Golińska et al. 2022), was designed to establish the taxonomic provenance of a *Streptomyces* strain, isolate SF28^T, recovered from pine forest litter and to determine its ability to inhibit the growth of fungal pathogens. The isolate and its closest phylogenomic neighbours were the subject of a polyphasic study that included information drawn from whole-genome sequences. The resultant data show that the strain inhibits the growth of diverse fungal phytopathogens and belongs to a new *Streptomyces* species, designated *Streptomyces pinistramenti* sp. nov.

Materials And Methods

Isolation, maintenance and cultural conditions

Strain SF28^T was isolated from partially decomposed needles (F-horizon) under *Pinus sylvestris* trees growing on the southern slope of an inland sand dune in the Toruń Basin, Poland (52°55’37”N, 18°42’11”E) in October 2013, using a standard dilution plating procedure (Goodfellow et al. 1967) and starch-casein agar (Küster and Williams 1964) adjusted to pH 4.5 using 1M HCl. Details of the sampling site and the selective isolation procedure have been described previously (Golińska et al. 2016). The isolate was maintained on starch-casein agar slopes (pH 5.5) at room temperature and as suspensions of mycelial fragments and spores in 20% (v/v) glycerol at -80°C. Biomass for most of the chemotaxonomic and molecular systematic studies was prepared by growing the strain in flasks of yeast extract-malt extract broth (ISP2; International *Streptomyces* Project medium 2; Shirling and Gottlieb 1966), adjusted to pH 5.5, and shaken at 150 rpm for 3 weeks at 28°C. Cells were harvested by centrifugation and washed three times in sterile distilled water; biomass for the chemotaxonomic analyses was freeze dried and that for the molecular systematic studies stored at -20°C.

Phylogenetic analyses

Genomic DNA was extracted from the isolate using a GenElute^{TM} Bacterial Genomic Kit (Sigma-Aldrich, Germany) and a 16S rRNA gene amplified by PCR following procedures described by Golińska et al. (2013a,b). The PCR product was purified using a purification kit (Qiagen, Germany), according to manufacturer instructions, and a quality check made using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The purified PCR product was sequenced at the Institute of Biochemistry and Biophysics (IBB) of the Polish Academy of Sciences in Warsaw, Poland, using an ABI 3730xl Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, USA).

The almost complete 16S rRNA gene sequence of the isolate was compared with corresponding sequences of type strains of closely related species using the EzBioCloud server (https://www.ezbiocloud.net; Yoon et al. 2017).

Maximum-likelihood (ML) and maximum-parsimony (MP) phylogenetic trees were generated using the Single-Gene Trees Phylogeny online tool (https://www.dsmz.de/services/online-tools/single-gene-phylogenies; Meier-Kolthoff et al. 2013a) adapted for single genes. Multiple sequence alignments were generated using MUSCLE software (Edgar 2004) and a ML tree was inferred from alignments with RAxML (Stamatakis 2014) using rapid bootstrapping together with the auto maximal-relative-error (MRE) bootstrapping criterion (Pattengale et al. 2010). Similarly, a MP tree was constructed from alignments with the Tree Analysis New Technology (TNT) program (Goloboff et al. 2008) using 1000 bootstraps together with tree bisection and reconnection branch swapping and ten random sequence replicates. The sequences were checked for compositional bias using the X^2 test, as implemented in PAUP* (Swofford 2002). The neighbour-joining algorithm (Saitou and Nei 1987) and the MEGA7 software package (Kumar et al. 2016) were used to generate a phylogenetic tree and evolutionary distances were calculated using the Kimura-2-parameter model (Kimura 1980) with 1000 bootstrap repetitions (Felsenstein 1985). The root position of the trees were determined using a 16S rRNA gene sequence taken from the genome of *Kitasatospora setae* DSM 43861^T (NC_016109.1) using the SEED viewer (Aziz et al. 2012). A multilocus genome analysis based on 16S rRNA, *atpD*, *gyrB*, *recA* and *rpoB* gene sequences was carried out using an established procedure (Carro et al. 2012) and a MLSA tree generated from nearly 4000 nt using the ML algorithm. Sequence data from all of these genes for each of the tested strains have been deposited in GenBank with the accession numbers shown in **Table S1**.

Cultural, morphological and phenotypic properties

The growth and cultural properties of the isolate were recorded from tryptone-yeast extract, yeast extract-malt extract, oatmeal, inorganic salts-starch, glucose-asparagine, peptone-yeast extract-iron and tyrosine agars (ISP media 1–7; Shirling and Gottlieb 1966) and from Bennett's and modified Bennett's agar (Jones 1949), HSA5 agar (Busti et al. 2014), nutrient (Becton Dickinson, USA) and 100-fold diluted nutrient agar (Becton Dickinson, USA) and tap water (Harris 1986) agar after 4 weeks incubation at 28°C. The colour of aerial and substrate mycelia and diffusible pigments were determined by comparison against NBS/IBCC Colour Charts (Kelly 1958). Hyphal and spore chain features of the isolate were recorded on acidified ISP2 agar plates (pH 5.5), after 4 weeks at 28°C, using the coverslip technique of Kawato and Shinobu (1959). Spore arrangement and spore surface ornamentation were established by examining gold-coated dehydrated preparations with growth taken from ISP2 agar plates (pH 5.5), using the procedure described by O'Donnell et al. (1993) and a scanning electron microscope (Model 1430 V P, LEO Electron Microscopy Ltd, Cambridge, England).

The isolate was also examined for a combination of phenotypic properties. Its ability to grow over a range of pH values (pH 4–13 at single unit intervals), temperatures (4, 10, 15, 20, 25, 30, 35, 40°C) and in the presence of various NaCl concentrations (1–15 at single unit intervals) were determined using acidified ISP2 agar (Shirling and Gottlieb 1966) as the basal medium; the pH levels were achieved using KH_2PO_4/HCl (pH range 4–5), KH_2PO_4/K_2HPO_4 (0.1 M both, pH range 6–8) and K_2HPO_4/NaOH (pH range 9–13) buffer systems. Standard biochemical, degradative and physiological properties were examined using media and methods described by Williams et al. (1983), albeit with media adjusted to pH 5.5. All of the tests were carried out, in triplicate, using 12-well plates that were inoculated using a standard inoculum corresponding to 5 on the McFarland scale (Murray et al. 1999) and a multipoint inoculator (Mast Uri®Dot, Mast Group Ltd., Merseyside, UK); the inoculated plates were incubated for 3 weeks at 28°C (apart from the temperature tests). The enzymic activities of the isolate were determined, in duplicate, using API-ZYM kits (BioMerieux, France), according to the manufacturer's instructions.

Chemotaxonomy

Isolate SF28^T was examined for the presence of chemical markers using standard chromatographic methods with appropriate controls. The thin-layer chromatography (TLC) was used to determine isomers of diaminopimelic acid following Staneck and Roberts (1974) and whole-organism sugars according to Hasegawa et al. (1983). Isoprenoid quinones and polar lipids were extracted from freeze-dried cells, as described by Minnikin et al. (1984) and separated using high performed liquid chromatography (HPLC; Kroppenstedt 1985) and two-dimensional TLC (Minnikin et al. 1984), respectively. Cellular fatty acids were extracted, methylated after Miller (1982) with minor modifications from Kuykendall et al. (1988), analysed using the protocol of the Sherlock Microbial Identification (MIDI) system, version 5 (Sasser 1990) and the resultant peaks identified using the ACTIN1 3.80 database.

Whole genome sequencing and phylogenomic analyses

Genomic DNA was extracted from biomass of isolate SF28^T following growth in ISP2 broth for 7 days at 28°C using the protocol provided by MicrobesNG (Birmingham, UK; http://www.microbesng.uk) and sequenced on a MiSeq instrument (Illumina, San Diego, USA). Genomic DNA libraries were prepared at MicrobesNG using Nextera XT library preparation kits. The purity and concentration of the extracted genomic DNA was measured using the Microlab STAR handling system (Hamilton, Birmingham, UK) and libraries generated using Kapa Biosystems library quantification kits designed for Illumina instruments on a LightCycler 96 real time PCR instrument (Roche, West Sussex, UK). The libraries were sequenced following the 2 × 250 bp paired-end protocol (MicrobesNG). Reads were trimmed using Trimmomatic software version 0.30 (Bolger et al. 2014) and their quality assessed using in-house scripts from MicrobesNG; those under 1000 bp were discarded. The esultant reads were assembled into contigs using Spades 3.7 software (Bankevich et al. 2012), annotated using Prokka 1.11 (Seemann 2014) and analysed using the SEED Viewer (Aziz et al. 2012). The genome sequence of isolate SF28^T was deposited in the GenBank database under accession number JAJCXB000000000.

The genome sequences of isolate SF28^T, the type strains of closely related *Streptomyces* species and *Kitasatospora setae* DSM 43861^T were uploaded onto the Type (Strain) Genome Server (TYGS; Meier-Kolthoff and Göker 2019) and compared using the MASH algorithm which allows a fast approximation of intergenomic relatedness between strains (Ondov et al. 2016). A phylogenomic tree was inferred with FastME 2.1.4 (Lefort et al. 2015) from GBDP distances calculated from the genome sequences and branch lengths scaled using the GBDP distance formula d_5 (Meier-Kolthoff et al. 2013a); GBDP pseudo-bootstrap support values above branches on the tree were based on 100 replications. The tree was rooted at the midpoint (Farris 1972). Average nucleotide identity (ANI; Rodriguez and Konstantinidis 2016) and digital DNA:DNA hybridization (dDDH; Meier-Kolthoff et al. 2013b) values between genomes of the isolate and its closest phylogenomic neighbours were determined using the online resource from the Rodriguez and Konstantinidis group (http://enve-omics.gatach.edu/) and the GGDC web server (http://ggdc.dsmz.de/ggdc), respectively.

Genome analyses

The presence of BGCs in the genomes of the isolate and its phylogenomic neighbours was investigated using anti-SMASH 6.0 software with “strict” detection criteria and extra features, including KnownClusterBlast, ClusterBlast, SubClusterBlast, MIBiG cluster comparison, ActiveSiteFinder, RREFinder, Cluster Pfam analyses, Pfam-based GO term annotation and TIGRFam analysis (Blin et al. 2021). The distribution of functional gene classes and the presence of stress response genes potentially involved in adaptation to harsh environmental conditions in the genome of isolate SF28^T were analyzed using the RAST-SEED webserver at https://rast.nmpdr.org/

Antimicrobial screens

The isolate was tested for its ability to inhibit the growth of *Bacillus subtilis* PCM 2021, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Micrococcus luteus* ATCC 10240, *Pseudomonas aeruginosa* ATCC 10145 and *Staphylococcus aureus* ATCC 25923 using a standard agar plug assay (Fiedler 2004). It was grown on ISP2 agar (Shirling and Gottlieb 1966) for 3 weeks at 28°C when agar plugs (ø = 5 mm) were taken from the plates and placed in square Petri dishes (Sterilin, UK). Overnight cultures of the reference strains (50 µL) grown at 37^{o}C were used to seed 25 mL of Luria Bertani broth (LB, Becton Dickinson, USA) to an optical density (OD) of 0.6 prior to diluting them to an OD of 0.0125 with 100 mL of LB broth and the same volume of nutrient agar (Becton Dickinson, USA). The resultant preparations were thoroughly mixed and poured into the square Petri dishes containing the plugs and incubated for 24 h at 37°C; inhibition zones around the agar plugs were recorded in millimetres. All of the tests were carried out in triplicate.

A co-culture method described by Świecimska et al. (2021) was used to determine the ability of the isolate to inhibit the growth of fungal and fungal-like plant and human pathogens. Briefly, the isolate was streaked as a line across one side of Potato Dextrose Agar (PDA, Becton Dickinson, USA) plates which were incubated for 14 days at 28°C. The discs (ø = 8 mm) of pathogens grown on PDA in Petri plates for 7-14 days were placed on the opposite side of the plates inoculated with the isolate and the preparations incubated for 7 days in the case of *Alternaria alternata* IOR 1783, *Fusarium culmorum* and *Fusarium oxysporum* (isolated from pine roots), *Fusarium culmorum* D and *Fusarium graminearum* D (both isolated from wheat), *Phytophthora plurivora* (isolated from the rhizosphere of oak), *Rhizoctonia solani* (isolated from pine roots) and *Sclerotina sclerotiorum* IOR 2242, for 14 days for *Fusarium poae* A and *Fusarium tricinctum* A (isolated from wheat), *Botritis cinerea* IOR 1873, *Colletotrichum acutatum* IOR 2153, *Fusarium culmorum* IOR 2333, *Fusarium oxysporum* IOR 342, *Phytophtora cactorum* IOR 1925, and for 21 days for *Fusarium graminearum* A and *Fusarium oxysporum* D (isolated from wheat), *Fusarium solani* IOR 825, *Phytophthora cryptogea* IOR 2080, *Phytophthora megasperma* IOR 404 and *Phoma lingam* IOR 2284. The human pathogens, *Trichophyton erinacei* DSM 25374^{T} and *Trichophyton thuringense* DSM 25373^{T} were incubated for 14 days. All tests were carried out in triplicate at 28^{o}C; the positive controls were cultures of the pathogens grown under the same incubation conditions. Inhibition (I) of pathogen growth was calculated using the formula: I (%)= (C-T/C) x100, where C is the diameter of pathogen growth in the control sample and T the diameter of the pathogen growth in each of the co-culture samples.

Results And Discussion

Isolate SF28^{T} showed chemotaxonomic, cultural and morphological properties consistent with its classification in the genus *Streptomyces* (Kämpfer 2012; Nouioui et al. 2018). The organism was found to be aerobic, Gram-stain positive, formed an extensively branched substrate mycelium and aerial hyphae that differentiated into long chains of smooth surfaced spores (Fig. 1), grew from pH 4 to 12, from 4 to 30 ^{o}C and in the presence of up to 11%, w/v NaCl. Whole-organism hydrolysates of the isolate contained *LL*-A2pm, glucose and ribose, the predominant isoprenologues were hexa- and octa-hydrogenated menaquinones with nine isoprene units (74.1 and 23.1%, respectively) and the polar lipid pattern consisted of diphosphatidylglycerol, glycophospholipids, phosphatidylethanolamine (diagnostic component), phosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidylinositol, and 3 unknown lipids, as shown in **Figure S1**. The fatty acid profile contained major proportions of *anteiso*-$C_{15:0}$ (26.2%), $C_{16:0}$ (20.0%), *iso*-$C_{16:0}$ (11.0%) and *anteiso*-$C_{17:0}$ (11.5%), smaller proportions (> 11%) of $C_{14:0}$ (1.4%), $C_{15:0}$ (2.4%), 9 methyl-$C_{16:0}$ (1.0%), $C_{15:0}$ cis 9 (5.3%), *anteiso*-$C_{15:0}$ 2OH (3.0%), *anteiso*-$C_{17:1}$ c (1.6%), *iso*-$C_{14:0}$ (1.7%), *iso*-$C_{15:0}$ (8.1%) and *iso*-$C_{17:0}$ (3.1%), and traces of cyclo $C_{17:0}$ and *iso*-$C_{16:1}$ H.

The isolate grew well on ISP2, ISP6, Bennett's and modified Bennett's agar, moderately well on ISP1 and 3 and nutrient agar but poorly or not at all on the remaining media, as shown in **Table S2**.

Phylogeny

The almost complete 16S rRNA gene sequence generated for isolate SF28^T (1414 [nt]) was deposited in Genbank (accession number: OK576049). The isolate forms a branch in a well-supported subclade in the ML/MP trees which includes the type strains of 22 *Streptomyces* species few of which are closely related based on low bootstrap values. Isolate SF28^T is most closely related to the type strains of *Streptomyces kronopolitis* (Liu et al. 2016), *Streptomyces chattanoogensis* (Burns and Holtman 1959) and *Streptomyces lydicus* (De Boer et al. 1956), showing 16S rRNA gene sequence similarities with them of 99.3% (10 nt differences), 99.2% (12 nt differences) and 99.2% (12 nt differences), respectively (**Fig. S2**). The corresponding sequences between the isolate and the type strains of the remaining *Streptomyces* species ranged from 98.0 (28 nt differences) to 98.9% (15 nt differences; **Table S3**). The isolate and the *S. kronopolitis* strain formed a well-supported branch in the neighbour-joining tree that was loosely associated with the other *Streptomyces* strains (**Fig. S3**).

Multilocus sequence analyses of single copies of conserved housekeeping genes provide greater resolution between closely related streptomycetes than corresponding 16S rRNA gene sequence studies as they are based on comparisons of many more nucleotide sequences (Labeda et al. 2012, 2017; van der Aart et al. 2019). In the present study, the isolate was assigned to a subcluster that was supported by a 64% bootstrap value (Fig. 2). This taxon encompassed the type strains of 10 *Streptomyces* species, eight of which featured in the subclade defined in the 16S rRNA gene tree. Isolate SF28^T was most closely related to the type strains of *S. chattanoogensis, Streptomyces inhibens* (Jin et al. 2019), *S. kronopolitis* and *S. lydicus* sharing nucleotide sequence similarities with them of 96.2%, 96.0%, 95.9% and 95.8% (**Table S4**). The MLSA evolutionary distances between the isolate and the type strains of the most closely related *Streptomyces* species ranged from 0.039 to 0.124 (**Table S5**), values well above the species threshold of 0.007 used to distinguish between closely related strains, including streptomycetes (Rong and Huang 2012, 2014). Based on these data isolate SF28^T is not closely related to the *S. chattanoogensis, S. inhibens* and *S. kronopolitis* strains as it shows evolutionary distance values with them of 0.039, 0.040 and 0.042, respectively. A corresponding distance score of 0.43 was recorded between the isolate and the type strains of *Streptomyces celluloflavus* (Nishimura et al 1953; Madhaiyan et al. 2020), and *S. lyidicus*.

Greater confidence can be placed on the topology of phylogenomic trees than in corresponding 16S rRNA and MLSA trees as they are based on millions not thousands of unit characters (Nouioui et al. 2018). It is evident from the phylogenomic tree (Fig. 3) that the sequence generated for isolate SF28^T and corresponding data available on the type strains of its phylogenetic relatives show that the isolate forms a singleton in a well-supported subclade which includes the type strains of 14 *Streptomyces* species, including those representing *S. chattanoogensis, S. kronopolitis* and *S. lyidicus*.

Comparison of genomes

The draft genome sequence of isolate SF28^T has been deposited in GenBank (accession number JAJCXB000000000). It is composed of 133 contigs, has 6594 protein coding genes, 74 RNA genes and L50 and N50 scores of 21 and 117612, respectively. The total genome size is 7.85 Mbp and the digital (d) G+C content 71.5%. The major classes of functional gene clusters in the genome of the isolate are associated with amino acids and derivatives (441), carbohydrates (297) and protein (237) metabolism, as shown in **Figure S4**. In general, the

genome of the isolate is of a similar size to those of its evolutionary neighbours, as exemplified by *S. chattanoogensis* NRRL ISP-5002^T (9.1 Mbp; Burns and Holtman 1959), *S. celluloflavus* NRRL B-2493^T (8.65 Mbp; Madhaiyan et al. 2020), *Streptomyces decoyicus* NRRL 2666^T (8.6 Mbp; Kumar and Goodfellow 2010), *S. inhibens* NEAU-D10^T (9.5 Mbp; Jin et al. 2019), *S. kronopolitis* NEAU-ML8^T (7.8 Mbp; Liu et al. 2016), *S. lydicus* ATCC 25470^T (7.9 Mbp; De Boer et al. 1956) and *S. piniterrae* jys28^T (8.5 Mbp; Zhuang et al. 2020).

The dDDH relatedness values between the isolate and its evolutionary relatives falls within the range 21.7 to 28.7% (**Table S6**), which is well below the 70% the threshold for the assignment of bacterial strains to the same species (Wayne et al. 1987). This Table shows that the corresponding ANI similarities range from 80.5 to 85.1%, similarities below the recommended threshold (95-96%) for species delineation (Richter and Rosselló-Móra 2009; Lee et al. 2016). These results indicate that isolate SF28^T represents a new *Streptomyces* species that is only loosely associated with its evolutionary relatives. It seems likely that the position of the isolate and its closest phylogenetic/phylogenenomic relatives will only be settled by the addition of new species to this unstable part of *Streptomyces* gene trees. Other *Streptomyces* species found to form distinct lineages in *Streptomyces* gene trees include *Streptomyces adelaidensis* (Kaewkla et al. 2021), *Streptomyces leeuwenhoekii* (Busarakam et al. 2014) and *Streptomyces tardus* (Králová et al. 2021).

Phenotypic properties

Closely related species can usually be distinguished using a broad-range of phenotypic properties (Komaki and Tamura, 2019; Kusuma et al. 2021). Cultural and morphological properties have been shown to be particularly predictive in this respect, as exemplified in extensive phylogenetic analyses of *Streptomyces* species (Labeda et al. 2012, 2017). In the present study, isolate SF28^T was examined for cultural and key morphological features, and for its ability to metabolize a broad range of carbon and nitrogen sources, enzymes and growth characteristics. It is particularly encouraging that identical results were recorded for the triplicated cultures, as shown in Table 1 and the species description. Comparison of some of these properties with corresponding data acquired for *S. decoyicus* NRRL 2666^T, its closest phylogenomic neighbour based on dDDH similarity, can be distinguished readily using cultural and morphological properties, as well as by associated phenotypic properties that were also recorded using media and methods described by Williams et al. (1983). It is significant that these strains show different properties when grown on oatmeal agar and exhibit markedly different spore chain morphologies (Table 2). In addition, only the isolate grows at 10°C and at pH 4.0, and 5.0; it also shows more activity than *S. decoyicus* NRRL 2666^T when grown on sole carbon sources.

Table 1

Phenotypic features which separate isolate SF28^T from the type strain of *S. decoyicus*

	Isolate SF28^T	*S. decoyicus** NRRL 2666^T
Pigments formed on oatmeal agar:		
Aerial spore mass	Bluish grey	Grey, becoming black and moist
Substrate mycelium	Brownish grey	Deep yellow
Diffusible pigment	Greyish yellow	None
Morphology:		
Spore chains	Rectiflexibles	Spiral
Degradation of:		
Xylan, xanthine	+	-
Growth on sole carbon sources (1% w/v):		
Adonitol, amygdalin, *meso*-erythritol, inulin, α-lactose, D-raffinose, L-rhamnose, D-salicin	+	-
D-Xylose	-	+
Tolerance tests		
Growth tests:		
pH 4 and 5	+	-
10°C	+	-
Growth on presence of:		
NaCl 13% w/v	-	+
DNA G+C content (%)	71.5	70.9
Genome size (Mbp)	7.85	8.63

*Properties of *S. decoyicus* strain are from Kumar and Goodfellow (2010). DNA G+C content and genome size are from GenBank. Both strains formed spores with smooth surfaces, degraded adenine, gelatin, casein, hypoxanthine, L-tyrosine and uric acid, used D-arabitol, D-cellobiose, dextrin, D-fructose, D-galactose, glycogen, D-glucose, glycerol, *myo*-inositol, D-maltose, D-melezitose, D-ribose, sucrose and D-trehalose as a sole carbon sources, and L-alanine, L- glutamic acid, L-histidine, L-*iso*leucine, DL-methionine, L-phenylalanine, L-threonine and L-valine as sole nitrogen sources, and grew at pH 9 and 10 and 30°C. None of tested strains hydrolysed allantoin, degraded starch or used L-arabinose as a sole carbon source.

Table 2

Antimicrobial activity of isolate SF28^T against fungal and fungal-like organisms evaluated using the co-cultured method.

Fungi and fungi-like organisms	I [%]
Plant pathogens	
Alternaria alternata IOR 1783	85.6 ± 2.1
Botritis cinerea IOR 1873	82.4 ± 1.4
Chalara fraxinea	61.8 ± 0
Fusarium culmorum	91.9 ± 2.1
Fusarium culmorum D	91.0 ± 0.6
Fusarium graminearum A	91.3 ± 2.3
Fusarium graminearum D	92.5 ± 2.0
Fusarium oxysporum IOR 342	80.3 ± 0
Fusarium poae A	58.1 ± 2.1
Fusarium solani IOR 825	88.9 ± 1.4
Fusarium tricinctum A	50.2 ± 2.1
Phoma lingam IOR 2284	89.7 ± 0
Phytophthora cactorum IOR 1925	90.5 ± 0
Phytophthora cryptogea IOR 2080	60.2 ± 0.7
Phytophthora megasperma IOR 404	52.1 ± 2.8
Phytophthora plurivora	92.2 ± 0
Rhizoctonia solani	94.4 ± 0.6
Sclerotina sclerotiorum IOR 2242	89.3 ± 0.7
Human pathogens	
Trichophyton erinacei DSM 25374	92.0 ± 0
T. thuringense DSM 25373	78.7 ± 2.3
I; % inhibition of fungal growth.	

The isolate can be distinguished from the type strains of *Streptomyces* species listed in **Table S6** as they show different cultural features on oatmeal agar and form spiral chains of spores, albeit ones with smooth surfaces (Kämpfer, 2012; Liu et al. 2016; Kamaki and Tamura 2019; Madhaiyan et al. 2020). It can also be separated from the *S. chattanoogensis, S. hygroscopicus* subsp. *glebosus*, *S. inhibens* and *S. piniterrae* strains as they form spiny, rudged or wrinkled spores in spiral chains (Kämpfer 2012; Jin et al. 2019; Zhuang et al. 2020).

Antimicrobial activity

Isolate SF28^T formed zones of inhibition against the *B. subtilis* (9.7 ± 1.2 mm), *E.coli* (3.0 ± 1.0 mm), *K. pneumoniae* (2.0 ±0.0 mm), *M. luteus* (8.0 ±0.1 mm), *P. aeruginosa* (3.0 ± 0.1 mm) and *S. aureus* (6.2 ± 0.6 mm) in the agar plug assays. Similar results have been recorded by members of novel *Streptomyces* species isolated from natural habitats, including extreme hyper-arid Atacama Desert soils (Sharma et al. 2014; Goodfellow et al. 2017; Abdelkader et al. 2018; Le Roes-Hill et al. 2018). Similarly, the isolate inhibited the growth of diverse fungal pathogens, including representatives of several *Fusarium* and *Phythophthora* species, as shown in Table 2. These results provide further evidence that novel *Streptomyces* species from different habitats, notably from plant rhizospheres and living plant tissues, produce new natural products, especially antifungal antibiotics which inhibit the growth of phytopathogens (Zhao et al. 2017; Singh and Dubey 2018; Qi et al. 2019; Peng et al. 2020) and which thereby show promise as biocontrol agents (Cao et al .2020). It is also interesting that the isolate showed pronounced activity against representatives of the two *Trichophyton* species (Table 2).

Genome mining

AntiSMASH predicts BGCs and potential products based on the percentage of genes from the closest known bioclusters showing significant BLAST hits against corresponding clusters in the genomes of strains under consideration (Blin et al. 2021). The genome of isolate SF28^T was found to contain 29 BGCs, notably ones predicted to encode for druggable molecules such as non-ribosomal peptide synthetases (Table 3). Eleven of the bioclusters showed at least 50% gene identity with known compounds, as exemplified by those associated with the synthesis of anantin C (75% gene identity), a peptide antagonist of the atrial natriuretic factor(Tietz et al. 2017), desferrioxamine E (100% gene identity), a siderophore which forms stable hexadentate complexes with ferric ions (Barona-Gómez et al. 2004), ectoine (100% gene identity), which protects against osmotic stress and desiccation (Prabhu et al. 2004), ethylenediaminesuccinic acid hydroxyarginine (EDHA) (100% gene identity), a second line siderophore (Spohn et al. 2018), and lugdunomycin (74% gene identity), a novel aromatic polyketide (Wu et al. 2019). Similarly, several bioclusters are predicted to encode for a range of products, as exemplified by those involved in the synthesis of alkylresorcinol (100% gene identity) which has multiple biological functions (Funabashi et al. 2008; Nakano et al. 2012), a heat-stable antifungal factor (75% gene identity) that inhibits the growth of diverse fungi (Yu et al. 2007) and the tallysomycins (TLMs) (60% gene identity), antitumor antibiotics (Tao et al. 2007).

Table 3

The distribution of BGCs in the genome of isolate SF28^T

Gene type	Product	Span (nt)	Gene similarity (%)	Most similar biosynthetic gene cluster	Similarity (%)
Ectoine	Ectoine	123,024-133,437	100	*Streptomyces kasugaensis* BCRC 12349	100
Lassopeptide	Anantin C	1-14,4	75	*Geodermatophilus siccatus* DSM 45419	25
				Streptomyces cyaneogriseus subsp. *noncyanogenus*	25
				Streptomyces leeuwenhoekii	25
				Streptomyces kasugaensis BCRC 12349	25
NAPAA	Eethylenediaminesuccinic acid hydroxyarginine (EDHA)	1-38,693	100	*Kitasatospora* sp. CB02891	37
NRPS/ Type 1 PKS (T1PKS)	Tallysomycin A	9,621-84,434	60	*Actinosynnema mirum* DSM 43827	39
NRPS/ Type 1 PKS (T1PKS)	Heat-stable antifungal factor	130,781-204,146	75	*Streptomyces* sp. CS090A	30
				Streptomyces fulvissimus DSM 40593	30
Siderophore	Desferrioxamine E	71,733-81,169	100	*Streptomyces* sp. MOE7	83
				Streptomyces lydicus GS93	83
				Streptomyces lydicus 103	83
				Streptomyces lydicus WYEC 108	83
				Streptomyces sp. RPA4-5	83
				Streptomyces platensis ATCC 23948	83
				Streptomyces sp. NEAU-S7GS2	83

Gene type	Product	Span (nt)	Gene similarity (%)	Most similar biosynthetic gene cluster	Similarity (%)
				Streptomyces lydicus strain A02	83
				Streptomyces sp. GS7	83
				Streptomyces sp. 2323.1	83
Type 2 PKS (T2PKS)	Lugdunomycin	37,499-80,898	74	*Streptomyces* sp. CB02414	36
Type 2 PKS (T2PKS)	Spore pigment	84,575-157,09	83	*Streptomyces* sp. PAMC 26508	23
				Streptomyces microflavus CG 893	23
				Streptomyces pratensis ATCC 33331	23
				Streptomyces sp. Root1304	23
Type 3 PKS (T3PKS)	Naringenin	213,991-241,849	100	*Streptomyces griseorubens* JSD-1	26
Type 3 PKS (T3PKS)/ Terpene	Alkylresorcinol	59,464-105,812	100	*Nonomuraea* sp. WYY166	22
Terpene/Lanthipeptide class I/NRPS	Hopene	221,191-291,727	69	*Streptomyces platensis* ATCC 23948	100

The SEED analyzes (Aziz et al. 2012) show that genome of isolate SF28^T contains 61 putative stress related genes, notably those linked to cold and heat shock responses, DNA repair and oxidative stress (**Table S7, Fig. S4**,). This complement of genes included *cspC* and *cspE* which express for cold shock proteins (Etchegaray and Inouye 1999) and chaperone genes such as *clpB, clpC, clpX* and *hrcA* which are involved in responses to heat shock (Li et al. 2011). In turn, genes such as *betA, betB* and *proU* are involved in the uptake of betaine and choline, metabolites, which help in responses to osmotic stress (Boncompagni et al. 1999; Nau-Wagner et al. 2012), as do enzymes expressed by genes *katE* and *soxR* (Normand et al. 2012; Golińska et al. 2020), and the products of genes *trx* and *trxR* (Kim et al. 2008). The genome of the isolate also included genes such as *RecF, RecO and RecR*, *UvrD* which are associated with DNA repair and stabilizing (Hickson 2003; Kang and Blaser 2006). The detection of *CoxG* gene which encodes for a subunit of carbon monoxide dehydrogenase suggest that the isolate may be able to adapt to chemolitotrophic lifestyle by using carbon monoxide as a carbon and energy source (Lorite et al. 2000), as is the case with the type strains of *Streptomyces thermocarboxydovorans* and *Streptomyces thermocarboxydus* (Kim et al. 1998). The genome of the isolate is also rich in genes that express for DNA polymerase Sigma factors, as shown in Table S7. Some of these genes, such as *sigB*, which is involved in osmotic stress, is upregulated under

acidic conditions (Kim et al. 2008). These authors also showed that this phenomenon applied to heat shock genes, including ones found in the genome of the isolate, notably ones supressing for proteins belonging to the DnaK family and chaperones, such as GroEL2. It is also interesting that the genome of the isolate contained gene *atpA*, which is involved in a transmembrane protein transport system, is known to enhance survival of bacteria under acidic conditions (Guan and Liu 2020). These results provide further evidence that pH is a major factor governing the survival and distribution of streptomycetes in acidic soils (Williams et al. 1971; Goodfellow and Williams 1983).

Conclusions

The isolate showed antimicrobial activity, notably against fungal pathogens, had a large genome rich in BGCs predicted to encode for a broad range of specialised metabolites, especially putatively new antibiotics and stress related genes, notably ones associated with adaptation to acidic conditions. It is also evident from the sequence data that isolate SF28^T forms a distinct lineage within the evolutionary variation occupied by *Streptomyces* species. It is loosely associated with its closest phylogenetic/phylogenomic neighbours, a point underlined by corresponding low ANI and dDDH similarities. It can also be distinguished from these organisms using key cultural and micromorphological properties. Consequently, the isolate is considered to represents a novel *Streptomyces* species for which the name *Streptomyces pinistramenti* sp. nov. is proposed.

Description of Streptomyces pinistramenti **sp. nov.**

Streptomyces pinistramenti (pi.ni.stra.men'ti. L. fem. n. *pinus*, pine tree; L. neut. n. *stramentum*, litter; N.L. gen. n. *pinistramenti*, of pine litter)

Aerobic, Gram stain-positive actinomycete which forms an extensively branched substrate mycelium and aerial hyphae that differentiate into long chains of smooth surfaced spores. Grows from 4 to 30°C, optimally around 25°C, from pH 4-12, abundantly from pH 5-11, and in the presence of 0-11% NaCl w/v, optimally without NaCl. Grows well on yeast extract-malt extract agar forming a brownish grey substrate mycelium, a greenish grey aerial mycelium and a greyish yellow diffusible pigment. Hydrolyses aesculin and arbutin, but not allantoin or urea. Nitrate is reduced. Adenine, casein, gelatin, hypoxanthine, Tweens 40 and 60, L-tyrosine, uric acid, xanthine and xylan are degraded, but not chitin, elastin, guanine, starch, Tweens 20 or 80. Positive for cystine, leucine and valine arylamidases, α- chymotrypsin, acid- and alkaline- phosphatases, α-galactosidase, β-glucosidase, α-mannosidase, naphthol-AS-BI-phosphohydrolase and trypsin, but negative for esterase (C4), esterase lipase (C8), α-fucosidase, β-galactosidase, N-*acetyl*-β-glucosaminidase α-glucosidase, β-glucuronidase and lipase (C14) (API-ZYM tests). Adonitol, amygdalin, D-arabitol, D-cellobiose, dextrin, *meso*-erythritol, D-fructose, D-galactose, D-glucosamine, D-glucose, glycerol, glycogen, *meso*-inositol, inulin, D-lactose, D-maltose, D-mannitol, D-melezitose, D-melibiose, α- and β-*methyl*-D-glucosides, D-raffinose, L-rhamnose, D-ribose, D-salicin, D-sucrose, D-trehalose and xylitol are metabolized as a sole carbon sources, but not L-arabinose, L-arabitol, D-glucuronic acid or D-xylose (all at 1% w/v). Metabolizes acetate, citrate, fumarate, hippurate, propionate, pyruvate and succinate, but not adipate, benzoate, butyrate or oxalate (sodium salts) or *para*-hydroxybenzoic acid (all at 0.1% w/v). L-alanine, L-arginine, L-asparagine, L-cysteine, ethanolamine, L-glutamic acid, L-histidine, L-hydroxyproline, L-*iso*leucine, L-methionine, L-phenylalanine, L-serine, L-threonine and L-valine are used as sole nitrogen sources, but not acetamide or L-aspartic acid (all at 0.1% w/v).

The cell wall peptidoglycan contains *LL*-diaminopimelic acid, the whole organism sugars are glucose and ribose, the major fatty acids are *anteiso*-$C_{15:0}$, $C_{16:0}$, *iso*-$C_{16:0}$ and *anteiso*-$C_{17:0}$, the predominant menaquinones MK-9 (H_6)

and MK-9 (H_8), and the major polar lipids diphosphatidylglycerol, glycophospholipids, phosphatidylethanolamine (diagnostic component), phosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidylinositol, and 3 unknown lipids.

The type strain SF28^T (= DSM 113360^T = PCM 3163^T) was isolated from partially decomposed needles under *Pinus sylvestris* trees growing on the southern slope of an inland sand dune in the Toruń Basin, Poland.

Novel streptomycetes isolated from extreme habitats are a rich source of new specialised metabolites, including antibiotics (Rateb et al. 2018; Sivalingam et al. 2019; Sivakala et al. 2021). It is therefore surprising that streptomycetes known to be common in coniferous forest soils (Golińska et al. 2022) have received little attention, especially since they have been shown to be antagonistic to fungal pathogens (Golińska and Dahm 2013; Cao et al. 2020) and can form mutualistic associations with the pine beetle, *Dendroctonus frontalis* (Strzelczyk and Szpotański 1989; Scott et al. 2008). It is also interesting that *S. piniterrae* jys28^T, an isolate from the rhizosphere soil of *Pinus yunnanensis*, contains a putative gene cluster that encode for the synthesis of heliquinomycins which belong to rubromycin family of compounds (Zhuang et al. 2020). Consequently, the discovery that *S. pinistramenti* SF28^T shows pronounced activity against diverse fungal plant pathogens provides further evidence that novel *Streptomyces* species isolated from coniferous forest soils merit greater attention as a source of new bioactive metabolites. It is also interesting that S. *pinistramenti* SF28^T and *S. piniterrae* jys28^T are associated with strains that produce novel antibiotics, as exemplified by *S. decoyicus* NRRL ISP-5087^T which produces psicofuranine, a purine nucleoside antibiotic which shows antibacterial and antitumor activity (Eble et al. 1959; Varva et al 1959).

Abbreviations

A2pm, diaminopimelic acid; ANI, average nucleotide identity; BGCs, biosynthetic gene clusters; BLAST, Basic Local Alignment Search Tool; dDDH, digital DNA–DNA hybridization; DNA, deoxyribonucleic acid; DSMZ, German Collection of Microorganisms and Cell Cultures; GGDC, genome to genome distance calculator; HPLC, high-performance liquid chromatography; IBB, Institute of Biochemistry and Biophysics; ISP, International *Streptomyces* Project; MIDI, Microbial Identification System; ML, maximum-likelihood; MP, maximum-parsimony; MRE, maximal-relative-error; PCM, Polish Collection of Microorganisms; PUAP, Phylogenetic Analysis Using Parsimony; RAST, Rapid Annotation using Subsystem Technology; rRNA, ribosomal RNA; S., *Streptomyces*; TLC, thin-layer chromatography; TNT, Tree Analysis New Technology; TYGS, Type Strain Genome Server

Declarations

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Author Contributions

PG and MG conceived the study, PG designed it and MŚ carried out all the experiments and was responsible for the data analyses. MŚ wrote the first draft of the manuscript which was revised by PG and MG. The final version of the manuscript was approved by all of the authors.

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Data Availability

The GenBank accession numbers for the 16S rRNA gene and the whole-genome sequences of strains SF28^{T} are OK576049 and JAJCXB000000000, respectively.

Conflict of Interest

The authors declare that they do not have any conflicts of interest.

Ethical statement

This article does not contain any studies with human participants and/or animals performed by any authors. Formal consent is not required in this study.

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Figures

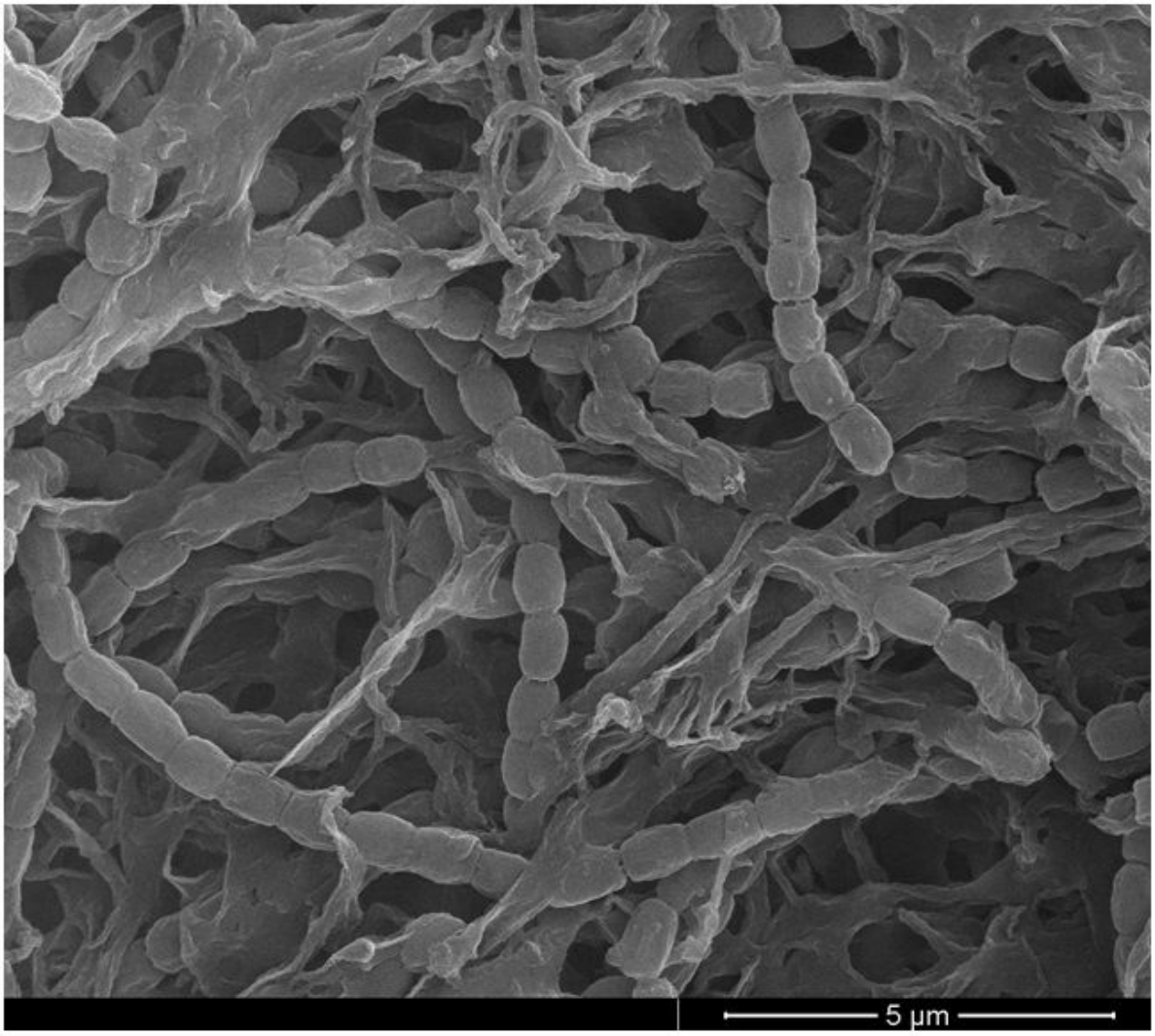

Figure 1

Scanning electron micrograph of isolate SF28^T showing straight chains of cylindrical spores with smooth surfaces following growth on ISP 2 agar for 4 weeks at 28 °C. Bar, 5 µm

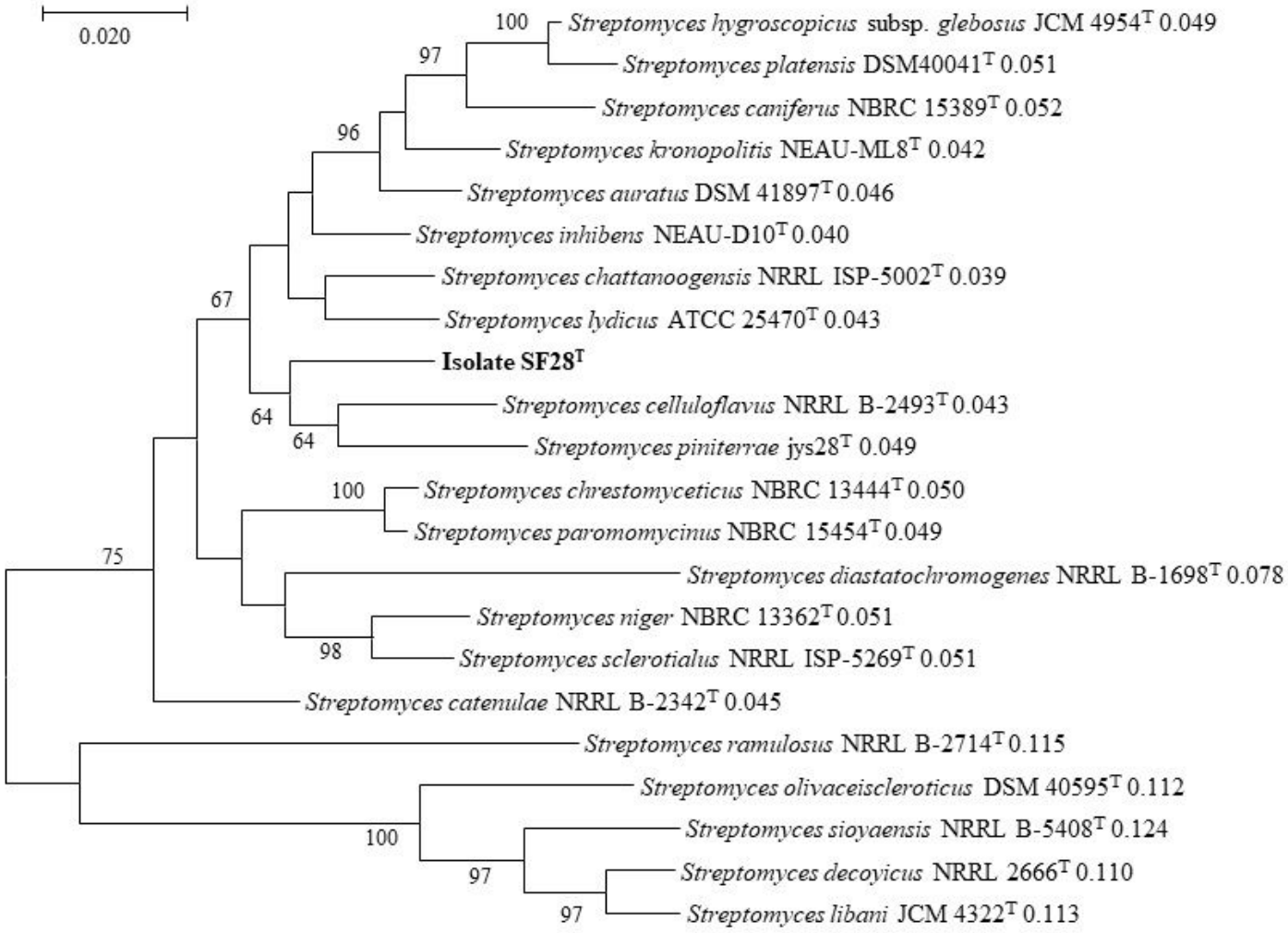

Figure 2

Maximum-likelihood tree based on MLSA analysis of concatenated sequences from the 16S rRNA gene and five housekeeping genes (*atpD*, *gyrB*, *recA, rpoB* and *trpB)* showing relationships between isolate SF28^T and the type strains of related *Streptomyces* species. The numbers at the nodes are bootstrap support values over 60%. Bar, 0.020 substitutions per nucleotide position.

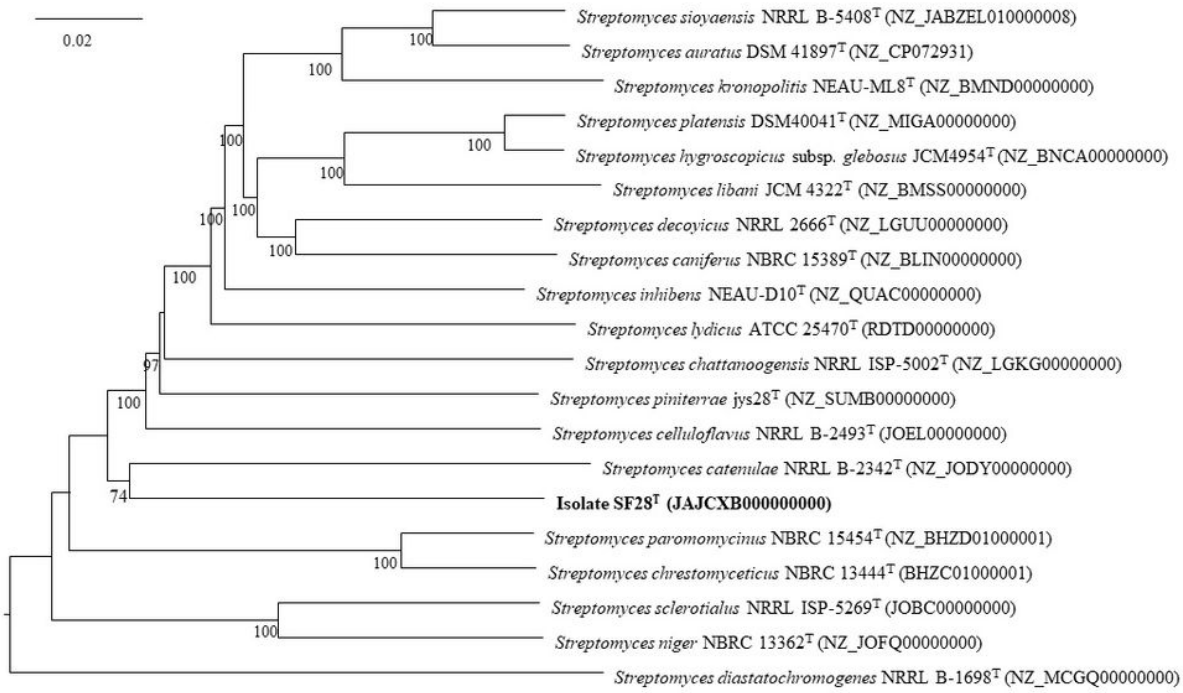

Figure 3

Phylogenomic tree showing relationships between isolate SF28^T and the type strains of the most closely related *Streptomyces* species constructed using the TYGS server. The numbers above the branches are GBDP pseudo-bootstrap support values greater than 60% from 100 replications with an average branch support of 95.4% The tree was rooted at the midpoint (Farris, 1962)

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