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Posted Date: 29 February 2024

doi: 10.20944/preprints202402.1660.v1

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Article

Taxonomic Identification and Antagonistic Mechanism of *Streptomyces luomodiensis* sp. nov. against Phytopathogenic Fungi

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Abstract: Banana wilt caused by *Fusarium oxysporum* f. sp. *ubense* tropical race 4 (Foc TR4) is a devastating fungal disease. Biocontrol strategies owned immense potential in inhibiting the spread of Foc TR4. Here, thirty actinobacteria were isolated from soils and screened for their antagonistic activity against Foc TR4. Strain SCA4-21^T exhibited broad-spectrum antifungal activity against nine phytopathogenic fungi including Foc TR4. The strain was identified as the genus *Streptomyces* according to phenotypic characteristics and 16S rRNA gene phylogenetic analysis. Multi-locus sequence analysis based on five house-keeping gene alleles (*atpD*, *gyrB*, *recA*, *rpoB*, and *trpB*) revealed that strain SCA4-21^T clustered into *S. hygroscopicus* subsp. *hygroscopicus* NBRC 13472^T with 100% of bootstrap value. Average nucleotide identity and dDDH were 98.49% and 44.30% above the threshold of novel species. Hence, strain SCA 4-21^T represented a novel species within the genus *Streptomyces*, named as *Streptomyces luomodiensis* sp. nov.. The type strain is SCA4-21^T (=GDMCC4.340^T=JCM36555^T). By the CAZymes analysis, 348 carbohydrate-active enzymes were detected, including 15 chitinases and 8 β -1,3-glucanases. The fermentation broth of strain SCA4-21^T showed high activities of chitinase and β -1,3-glucanase, contributing to the antifungal activity. Our results showed an innovative potential biocontrol agent for managing plant fungal diseases, specifically banana *fusarium* wilt.

Keywords: Broad-spectrum antifungal activity; Taxonomic identification; CAZymes analysis; *Streptomyces luomodiensis* sp. nov. Fungal cell wall degradation

1. Introduction

Bananas and plantains are important fruit and staple food crop in tropics and subtropics [1]. However, the world banana industry is suffering from the serious harm of *Fusarium* wilt disease, caused by the soil-borne fungi *Fusarium oxysporum* f. sp. *ubense* race 1 (Foc 1). The strain destroyed the major cultivar of the Gros Michel banana in Central America and Caribbean during the mid-20th century [2]. Although the detriment of Foc 1 was overcome by the resistant Cavendish cultivars, a new strain known as Foc tropical race 4 (Foc TR4) was found and rapidly spread [3]. Foc TR4 can infect almost all banana cultivars. Long survival ability in soil makes it difficult to be controlled [3]. Utilizing antagonistic microbes to control Foc TR4 is considered as a promising strategy due to environmentally friendly and high effective advantages [4].

Actinobacteria ubiquitously distributed in the soil environment. They are considered as an ideal candidate of excellent biocontrol agents [5,6]. Actinobacteria successfully colonizing the plant rhizosphere provide a biocontrol potential for soil-borne disease [7]. *Streptomyces* is the largest genus of the phylum actinobacteria [8]. They are Gram-positive, aerobic, filamentous bacteria with around

70% content of G + C in their genomes [9]. The traditional method of DNA-DNA hybridization allowed the classification for prokaryote, serious shortcomings limited its application such as a time-consuming procedure and operational feasibility [10]. Currently, identification of *Streptomyces* primarily relies on the combination of phenotypic and genotypic characteristics. 16S rDNA did not provide a sufficient resolution for the species-level identification. Average nucleotide identity (ANI) provided an efficient method to identify the level of species [10]. Until now, more than 1200 of *Streptomyces* species are classified according to the LPSN-List of Prokaryotic names with Standing in Nomenclature (<https://www.bacterio.net/>). However, there still lacks strong adaptability and high antimicrobial activity of *Streptomyces* in agricultural practice.

Streptomyces are also massive reservoirs of bioactive substances such as antibiotics, enzymes, and siderophore [11]. Two-thirds of known antibiotics are derived from *Streptomyces* [12]. Moreover, *Streptomyces* also participate in the cycling of carbon due to their ability to secreting many carbohydrate-active enzymes (CAZymes) These enzymes can facilitate the biodegradation of remains from animals, plants, and fungi [7]. CAZymes are classified into six major families according to the functional characteristics [13]. The members of glycoside hydrolase family contain chitinase and β -1,3-glucanase[14,15]. Chitinase and β -1,3-glucanase are responsible for antifungal activity of *Streptomyces* sp. 5-4 [16] and *Streptomyces griseus* (Anitha and Rebeeth, 2009). However, the antifungal mechanism of *Streptomyces* is still unclear due to the physiological property difference of diverse members.

In this study, thirty actinobacteria were isolated from a hot-dry valley of Huili County, Sichuan Province, China. Their antagonistic activity was investigated against Foc TR4. The broad-spectrum antifungal activity was also detected against the selected eight phytopathogenic fungi. Strain SCA4-21^T with the strong inhibition activity was identified by the phenotypic and genotypic characteristics. The antifungal mechanism was further investigated by comparing the genome of SCA4-21^T. Our results provide an accurate method for identifying the species *Streptomyces* and a potential biological agent for managing banana *Fusarium* wilt.

2. Materials and Methods

2.1. Isolation of Actinobacteria

The soil samples were collected from the Luomodi Village in a hot-dry valley of Huili County, Sichuan Province, China. Actinobacteria were isolated using a serial dilution method on the starch-casein medium (SCA, 10 g of soluble starch, 0.3 g of casein, 2.0 g of KNO₃, 2.0 g of NaCl, 2.0 g of K₂HPO₄, 0.05 g of MgSO₄·7H₂O, 0.02 g of CaCO₃, 0.01 g of FeSO₄·H₂O, and 18 g of agar in 1 L sterile ddH₂O, pH 7.0-7.4). The addition of potassium dichromate (50mg/L) and nystatin (50mg/L) was used to eliminate the microbial pollution [17]. Briefly, soil samples were sieved through a 0.6-mm mesh. One gram sample was dissolved completely in 9 mL of sterile water. After incubated at 55°C for 20 min, the soil homogenate was diluted with sterile water into 10⁻¹, 10⁻², 10⁻³ and poured to the SCA plates. The plates were cultured at 28°C for 7 d, the colonies with different shapes and colors were picked up and further purified on ISP2 (yeast extract-malt extract agar). All the purified isolates were preserved in the slant agar medium at 4°C and 30 % of glycerol (v/v) at -80°C, respectively.

2.2. Screening of Antagonistic Actinobacteria against Foc TR4

The antifungal activity of actinobacteria were screened against Foc TR4 using the plate confrontation method [18]. Namely, these isolates were inoculated at four symmetrical points 2.5 cm away from the center of potato dextrose agar (PDA) plates. After 2 d, the mycelial disc (0.5cm diameter) of Foc TR4 was inoculated into the center of the PDA plate. The mycelial disc (0.5cm diameter) of Foc TR4 only was used to a control. The experiment was set up in triplicate. After incubation at 28°C for 7 d, the diameters of Foc TR4 were measured. The antifungal activity of actinobacteria was calculated according to the following formula: MI = [(C-T)/C] × 100%, where C and T represented the growth diameters of the control and the treatment group, respectively. Strain

SCA4-21 (the type strain SCA4-21^T) was selected to be investigated further due to the strong antifungal activity against Foc TR4.

2.3. Measurement of a Broad-Spectrum Antifungal Activity

The broad-spectrum antagonistic activity of strain SCA4-21^T were determined using the plate confrontation method as described above. The selected eight pathogenic fungi include *Curvularia fallax* (ATCC 34598), *Colletrichum litchi* Trag (ATCC MYA-456), *Colletotrichum gloeosporioides* (ATCC 36351), *Colletotrichum acutatum* (ATCC 56815), *Colletotrichum fragariae* (ATCC 58718), *Colletotrichum gloeosporioides* (Penz) Saec (ATCC 36351), *Fusarium oxysporum* f. sp. *cucumerinum* Owen (ATCC 204378), *FusaHum graminearum* (DSM 21803). The pathogens were kept in the Institute of Tropical Bioscience and Biotechnology, China Academy of Tropical Agricultural Sciences, Haikou, China.

2.4. Cultural and Morphological Characteristics of Strain SCA4-21^T

Strain SCA4-21^T was inoculated on eight media including ISP2, ISP3 (oatmeal agar), ISP4 (inorganic salts-starch agar), ISP5 (glycerol-asparagine agar), ISP6 (peptone yeast-iron agar), ISP7 (tyrosine agar) and Gause No. 1 agar at 28°C for 7 d. The culture characteristics were observed such as the growth of aerial and substrate hyphae and the production of soluble pigment. The spore chain and spore morphology of strain SCA4-21^T were observed by a scanning electron microscope (SEM, Zeiss ΣIGMA, Germany) after 7 and 21 d of growth on ISP2 medium according to our previous method [19]. Mycelial blocks (5 mm diameter) were cut from plates of strain SCA4-21^T using a sterile scalpel. The samples were fixed with 2.5% of glutaraldehyde at 4°C for overnight and were rinsed twice with phosphoric acid buffer solution (PBS, 0.1 mol/L, pH 7.0) The materials were dehydrated in a graded ethanol solution (70, 80, 90, 95, and 100%) for 2 min, respectively. The specimens were soaked in fresh t-Butyl alcohol overnight. Dehydrated samples were freeze-dried and metal-coated for SEM.

2.5. Physiological and Biochemical Characteristics of Strain SCA4-21^T

The physiological characteristics of strain SCA4-21^T were determined such as the utilization of nitrogen and carbon as well as the tolerance to NaCl (0-11%, w/v), temperature (16-50°C) and pH (4-10). We also measured its biochemical features, including urease, esterase (Tween-20, Tween-40, and Tween-60), gelatin liquefaction, starch hydrolysis, nitrate reduction, siderophore and antibiotics-sensitivity test [19].

2.6. Chemotaxonomic Characteristics of SCA4-21^T

Strain SCA4-21^T was inoculated in the IPS2 liquid culture medium and shaken at 180 rpm/min and 28°C for 5 d. The thallus was collected by the centrifugation at 12000 rpm for 5 min. The precipitate was freeze-dried. The cellular fatty acids were determined by a gas chromatography (Agilent 6890) using the Sherlock Microbial Identification System (MIS) software [20]. The composition of cell-wall diaminopimelic acid (DAP) was analyzed according to the method of [21]. Respiration quinone was performed as the description of Minnikin et al. [22].

2.7. Phylogenetic Analysis of Strain SCA4-21^T

Strain SCA4-21^T was inoculated in the ISP2 liquid medium and shaken at 180 rpm/min and 28°C for 3 d. DNA was extracted using the bacterial genome rapid DNA kit (RN43, Aidlab Co., Ltd., Beijing, China). The 16S rRNA sequence was amplified using a pair of the common primers 27F (50-AGAGTTTGATCCTGGCTCAG-30) and 1492R (50-GGTTACCTTGTTACGACTT-30) [23]. The PCR reaction system was set according to Wang et al. [24]. After sequencing, the multiple alignment of 16S rRNA sequence was carried out using EzBioCloud (<https://www.ezbiocloud.net/>) [25] and CLUSTALW within BioEdit 7.0.5.3 [26]. Phylogenetic trees were constructed using neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) by MEGA7.0, respectively [27]. Multi-locus sequence analysis (MLSA) was performed based on five-housekeeping genes including

atpD, *gyrB*, *recA*, *rpoB* and *trpB* [28]. These housekeeping genes of strain SCA4-21^T were exacted from the sequenced genome. The homologue sequences of five-housekeeping genes from other species were downloaded from the GenBank (<https://www.ncbi.nlm.nih.gov/>) and Ezbiocloud (<https://www.ezbiocloud.net/>). The phylogenetic trees were constructed using the above method. The evolutionary distance was calculated by using the Kimura two-parameter (K2P) model for nucleotide sequences [26]. The sequence Genbank accession numbers of these housekeeping genes were shown in the Supplementary Table S1.

2.8. Genome sequencing and Feature Analysis of Strain SCA4-21^T

Strain SCA4-21^T was cultured in the yeast malt extract agar (ISP2) liquid medium at 28°C for 3 d. Mycelia of strain SCA4-21^T were collected by centrifugation and frozen in nitrogen. The whold genome was sequenced using Illumina Hiseq + PacBio sequencing platform (Majorbio Bio-Pharm Technology Co., Ltd, Shanghai, China). Raw reads were trimmed and filtered using the Trimmomatic (v 0.36) software [29]. The clean reads were assembled using the Unicycler (v 0.4.8) software [30]. The protein-coding genes were predicted using the Glimmer (v3.02) [31], Prodigal (v 2.6.3) [32] and GeneMarkS (v 4.3) (Besemer and Borodovsky, 2005) [33]. Genome annotation was performed by the Prokaryotic Genome Annotation Pipeline of NCBI (Tatusova et al., 2016) [34]. Carbohydrate-active enzymes (CAZyme) were first predicted using the online CAZy v 6 software (Cantarel et al., 2009) [35].

2.9. The overall Genome Related Indexes

To further identify the species of strain SCA4-21^T, the overall genome related indexes (OGRI) such as average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) between strain SCA4-21^T and its closely related species were calculated by the online OrthoANiU (<http://www.ezbiocloud.net>) [36] and the Genome-to-Genome Distance Calculator (GGDC) version 3.0 (<http://ggdc.dsmz.de>) [37], respectively.

2.10. Determination of Antifungal Activity of Strain SCA4-21^T Fermentation Broth

Antifungal activity of fermentation broth of *Streptomyces* sp. SCA4-21^T was determined by the agar well diffusion method [38]. Briefly, strain SCA4-21^T was inoculated into 100 mL of liquid YE medium and cultured at 28°C and 180rpm/min for 7 d. Culture supernatant was harvested from the culture media of strain SCA4-21^T by centrifugation at 2000 rpm for 20 min, followed by filtration through 0.22- μ m sterile filter (Millipore, Bedford, MA, United States). 100 μ L of sterile supernatant was aliquoted into each symmetrical well 2.5 cm away from the center of the PDA plate. The mycelial disc (5 mm diameter) of Foc TR4 was placed on the center of PDA plate. Sterilized H₂O was used as a control. Three replicates were set for each experiment. After incubating at 28°C for 5-7 d, antifungal activity of fermentation broth was calculated using the above formula.

2.11. CAZymes Analysis of Fermentation Broth of Strain SCA4-21^T

The activities of CAZymes, namely chitinase and glucanase, were measured. Namely, A 5-mm block of strain SCA4-21^T was inoculated into 100 mL of liquid YE medium and shaken at 28°C and 180 rpm for 7 d. Culture filtrates were collected from the culture media of strain SCA4-21^T by centrifugation at 2000 rpm for 20 min. The activities of chitinase and β -1,3-glucanase were determined using the Chitinase Activity Assay Kit (Beijing Solarbio Science & Technology Co., Ltd.) and the Microorganism β -1,3-glucanase ELISA Kit (Jiangsu Meibiao Biotechnology Co., Ltd.), respectively. The amount of chitinase that breaks down chitin to produce 1 μ g of N-acetylglucosamine per milliliter of sample solution per minute at 37°C is defined as one unit of chitinase activity. One unit of β -1,3-glucanase activity is expressed as the amount of enzyme that converts 1 μ mol of substrate into product per milliliter of sample solution per minute at 37°C. All experiments were repeated three times.

3. Results

3.1. Isolation and Antifungal Activity Screening of Actinobacteria against Foc TR4

Thirty actinobacteria were isolated from soil samples in the dry hot valley and designated as SCA4-1 to SCA4-30 (Figure 1A). The antifungal activities of these isolates against Foc TR4 were analyzed using the plate confrontation method. Seven strains showed antifungal activity against Foc TR4. Among them, SCA4-21^T (the type strain SCA4-21^T) has the highest percentage of mycelial inhibition (74.22 ± 1.23), followed by SCA4-6 (54.95 ± 0.07), SCA4-14 (52.62 ± 0.19), SCA4-1 (51.31 ± 0.11), SCA4-17 (46.46 ± 0.07), SCA4-8 (43.23 ± 0.17) and SCA4-27 (28.69 ± 0.13). Therefore, strain SCA4-21^T was selected for the following experiment (Figure 1B).

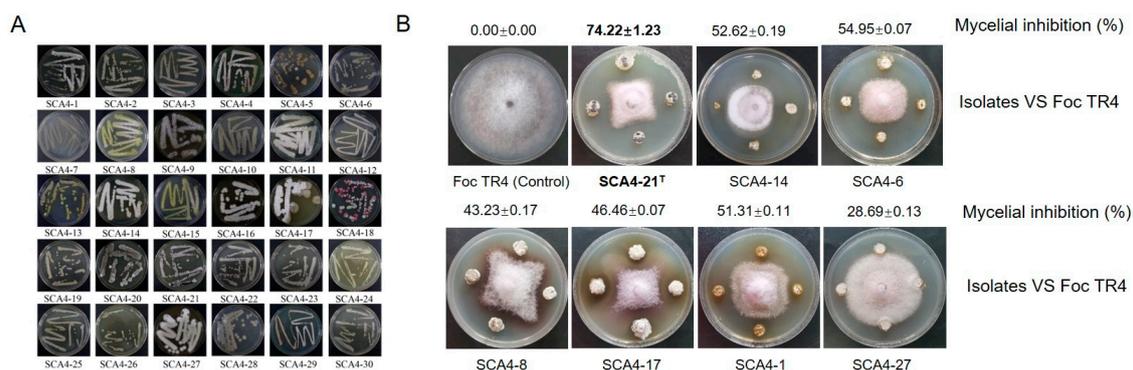


Figure 1. Isolation of actinobacteria and screening of their antagonistic activity against Foc TR4 (A) Thirty actinobacteria were isolated from wheat rhizosphere soil samples. (B) Seven actinobacteria exhibited antifungal activity against Foc TR4.

3.2. Strain SCA4-21^T Exhibited a Broad-Spectrum Antifungal Activity

Compared to the control group, strain SCA4-21^T exhibited a strong broad-spectrum antifungal activity. The highest inhibition rates were 84.96% and 86.63% for *C. litchi* Trag and *C. fallax*, respectively (Figure 2). The lowest mycelial inhibition rate of against *C. gloeosporioides* (Penz) Saec at 70.05%, followed by *C. acutatum* (73.04%), *C. fragariae* (74.78%), *F. oxysporum* f. sp *cucumerinum* (75.21%), *F. graminearum* Sehwa (78.63%) and *C. gloeosporioides* (78.73%).

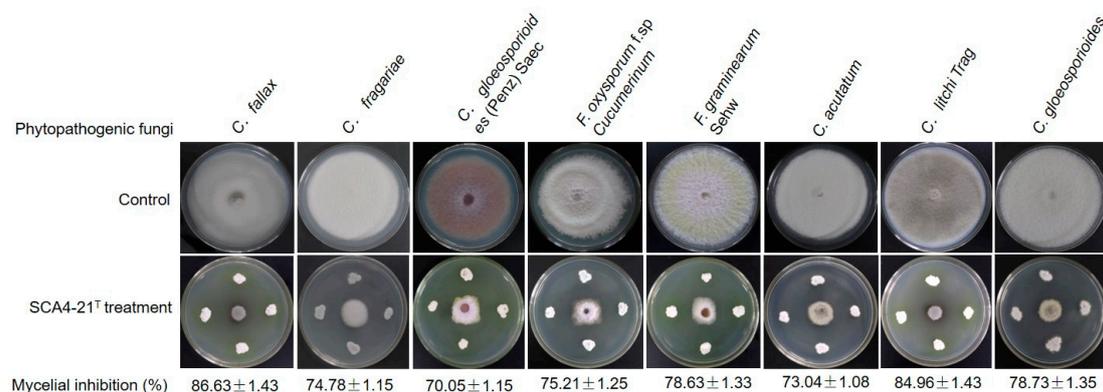


Figure 2. Strain SCA4-21^T exhibited broad-spectrum antifungal activity against 8 other Phytopathogenic fungi.

3.3. Cultural and Morphological Characteristics of Strain SCA4-21^T

Strain SCA4-21^T was an aerobic and Gram-positive bacterium. It could grow well on the ISP2, PDA and Gause's no.1 plates, followed by ISP5-7. The colors of colonies on different media exhibited

white, cream, pure white, grey white and silver grey. Strain SCA4-21^T did not produce soluble pigment on the six media, including the ISP2-6 and Gause's no.1. It produced light-brown and light-yellow pigment on ISP7 and PDA plates, respectively (Figure 3A and Table 1). Morphological observations of strain SCA4-21^T on ISP2 plates revealed the presence of branched aerial mycelia, a spiral spore chain, and cylindrical spores with a shrinkage surface. On 7-day-old plates, abundant aerial mycelia and spore chains were observed, while on 21-day-old plates, a large number of mature spores detached from spore chains were observed (Figure 3B). On ISP3, PDA and Gause's no.1 plates, these spirals gradually merged into dark masses of spores as they aged. This phenomenon was frequently observed in the members of *Streptomyces hygrosopicus* and *Streptomyces iranensis* groups [39,40]. The cultural and morphological characteristics of strain SCA4-21^T were consistent with those of the genus *Streptomyces*.

Table 1. Cultural characteristics of strain SCA4-21^T on eight media.

Culture Medium	Aerial Hyphae	Vegetative Mycelium	Soluble Pigment	Colony Characteristics	Growth Conditions
ISP2	White	Sand yellow	None	Wrinkled, hard	+++
ISP3	Cream	Light ivory	None	Wrinkled, hard	+
ISP4	Pure white	Cream	None	compact, wrinkle-free	+
ISP5	Pure white	Cream	None	compact, wrinkle-free	++
ISP6	Pure white	Cream	None	Loose, wrinkle-free	++
ISP7	Grey white	Sand yellow	Light brown	Loose, wrinkle-free	++
PDA	Pure white	Lemon yellow	Light yellow	Loose, wrinkle-free	+++
Gause's no.1	Silver grey	Silver grey	None	Loose, powery	+++

Note: “+++” represented that the strain grew well; “+ +” represented the general growth of the strain; “+” represented that the strain can grow.

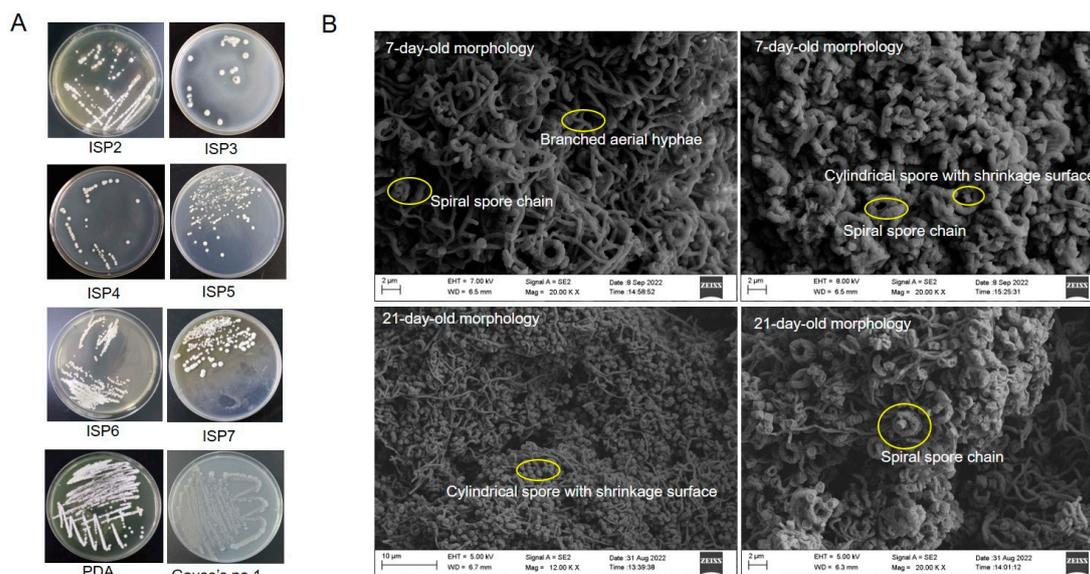


Figure 3. Cultural and morphological characteristics of strain SCA4-21^T (A) Cultural characteristics of strain SCA4-21^T cultured on eight different media, including ISP2, ISP3, ISP4, ISP5, ISP6, ISP7, PDA, and Gause's no.1 medium. (B) Morphological characteristics of strain SCA4-21^T cultured on ISP2 plates for 7d and 14 d at 28 °C.

3.4. Physiological and Biochemical Characteristics of Strain SCA4-21^T

The growth of strain SCA4-21^T was observed at pH 6.0 to 9.0 (optimal at 7%), at temperature 16–46°C (optimal at 28°C) and with NaCl of 0% to 3% (w/v) (optimal at 2%). It could degrade starch, urea and Tween-20, while producing melanoid pigment and siderophores (Figure 4A). It could not degrade gelatin, Tween-40, Tween-60, nitrate and cellulose, nor produce H₂S (Supplementary Table S2). The strain demonstrated resistance to ten antibiotics, namely furazolidone, compound sulfamethoxazole, polymyxin B, vancomycin, erythromycin, minocycline, kanamycin, gentamicin, ceftriaxone and cefuroxime. However, it was sensitive to eleven antibiotics, including clindamycin, chloramphenicol, penicillin, cefepime, cefotaxime, cefamandole, midecamycin, carbenicillin, ampicillin, benzylpenicillin and piperacillin (Figure 4B and Supplementary Table S2). In addition, as shown in Supplementary Table S3, the isolate was able utilize D-mannose, D-trehalose, sorbitol, D-fructose, lactose, mannitol and xylan as carbon sources, but could not use L-arabose, and raffinose. Strain SCA4-21^T used L-phenylalanine, L-asparagine, L-methionine, L-valine, L-histidine, L-tryptophan, D-fibrinose, L-hydroxylungine, and so on, but did not utilize anhydrous inositol, L-glutamic acid, L-tyrosine, and arginine.

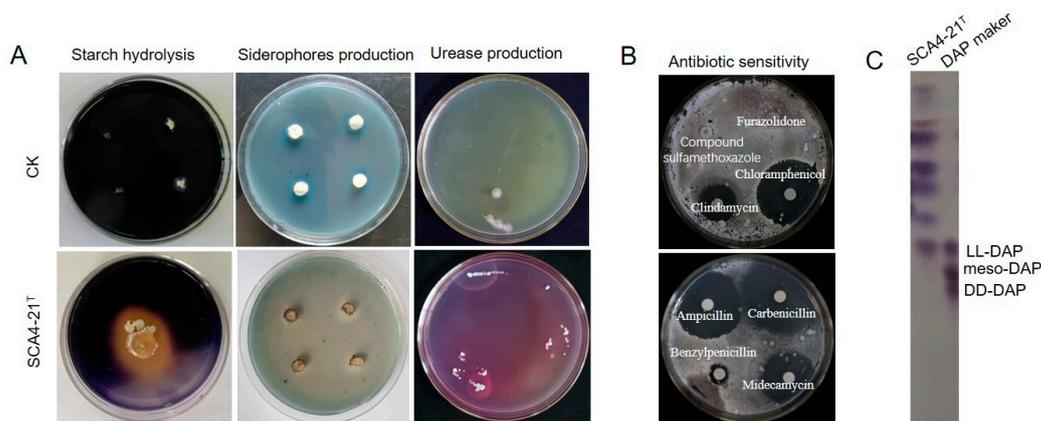


Figure 4. Physiological and chemotaxonomic characteristics of strain SCA4-21^T (A) Compared with the control strain, strain SCA4-21^T could produce amylase, siderophores and urease. (B) Antibiotic

sensitivity experiment. The strain was resistant to some antibiotics such as furazolidone, compound sulfamethoxazole, but sensitive to other antibiotics like clindamycin, chloramphenicol, carbenicillin, midecamycin, ampicillin, benzylpenicillin. (C) Composition analysis of DAP in cell wall. The cell wall of strain SCA4-21^T mainly contained LL-DAP.

3.5. Chemotaxonomic Characteristics of Strain SCA4-21^T

The chemotaxonomic characteristics of strain SCA4-21^T were listed in Table 2. The dominant menaquinones were MK9 (H₈) (65.50 %) and MK10(H₂) (34.50%) (Supplementary Figure S1). The dominant fatty-acid compositions of the isolate consisted of anteiso-C_{15:0} (34.78%) and C_{16:0} (19.89%). The presence of LL-DAP in the peptidoglycan of cell wall was considered as one of the effective methods for the identification of the genus *Streptomyces* [41]. The cell wall of strain SCA4-21^T largely contained LL-diaminopimelic acid (LL-DAP) (Figure 4C), which made the rapid identification of this strain as *Streptomyces*.

Table 2. Chemotaxonomic characteristics of strain SCA4-21^T.

Characteristic	SCA4-21
Major menaquinones (%)	
MK9(H ₈)	65.50
MK10(H ₂)	34.50
Major fatty acids (>0.5%)	
iso-C _{13:0}	0.83
anteiso-C _{13:0}	0.59
iso-C _{14:0}	8.21
C _{14:0}	3.34
iso-C _{15:0}	9.67
anteiso-C _{15:0}	34.78
C _{16:0}	19.89
iso-C _{16:1} H	tr
iso-C _{16:0}	8.16
anteiso-C _{16:0}	ND
iso-C _{17:0}	1.4
anteiso-C _{17:0} ω _{9c}	0.53
anteiso-C _{17:0}	5.93
C _{17:0} cyclo	1.78
C _{17:0}	0.75
iso-C _{18:0}	tr
Summed feature 3*	0.54
Summed feature 9*	tr
Main amino acid of the cell wall	LL-diaminopimelic acid

Note: percentages of the total fatty acids and fatty acids amounting to less than 0.5% in all strains were not shown. Major components (> 10.0%) were highlighted in bold. tr, trace amount (< 0.5%); ND, not detected. * Summed features represented the groups of two fatty acids that cannot be separated by gas-liquid chromatography with the MIDI system. Summed feature 3* comprised C_{16:1} ω_{6c} and/or C_{16:1} ω_{7c}, Summed feature 9* comprised iso-C_{17:1} ω_{9c} and/or C_{16:0} 10-methyl.

3.6. Phylogenetic Analysis of 16S rRNA and House-Keeping Genes

The EzBioCloud analysis of 16S rRNA gene sequence (1524 bp) of strain SCA4-21^T revealed that the strain shared the highest similarity to *S. iranensis* HM 35^T (99.45%), *S. rapamycinicus* NRRL B-5491^T (99.35%) and *S. hygroscopicus* subsp. *hygroscopicus* NBRC 13472^T (99.17%). NJ and MP trees based on the complete 16S rRNA gene sequence revealed strain SCA4-21^T formed a subclade with *S. iranensis* HM35^T with the bootstrap values of 61% and 36%, respectively, indicating that the strain belongs to the genus *Streptomyces* (Figure 5A and Supplementary Figure S2). However, ML tree showed that strain SCA4-21^T formed a subclade with *S. iranensis* HM35^T and *S. rapamycinicus* NRRL B-5491^T with a bootstrap value of 63% (Supplementary Figure S3). The above results indicated that it is difficult to distinguish this strain from its closely related species using the 16S rRNA gene.

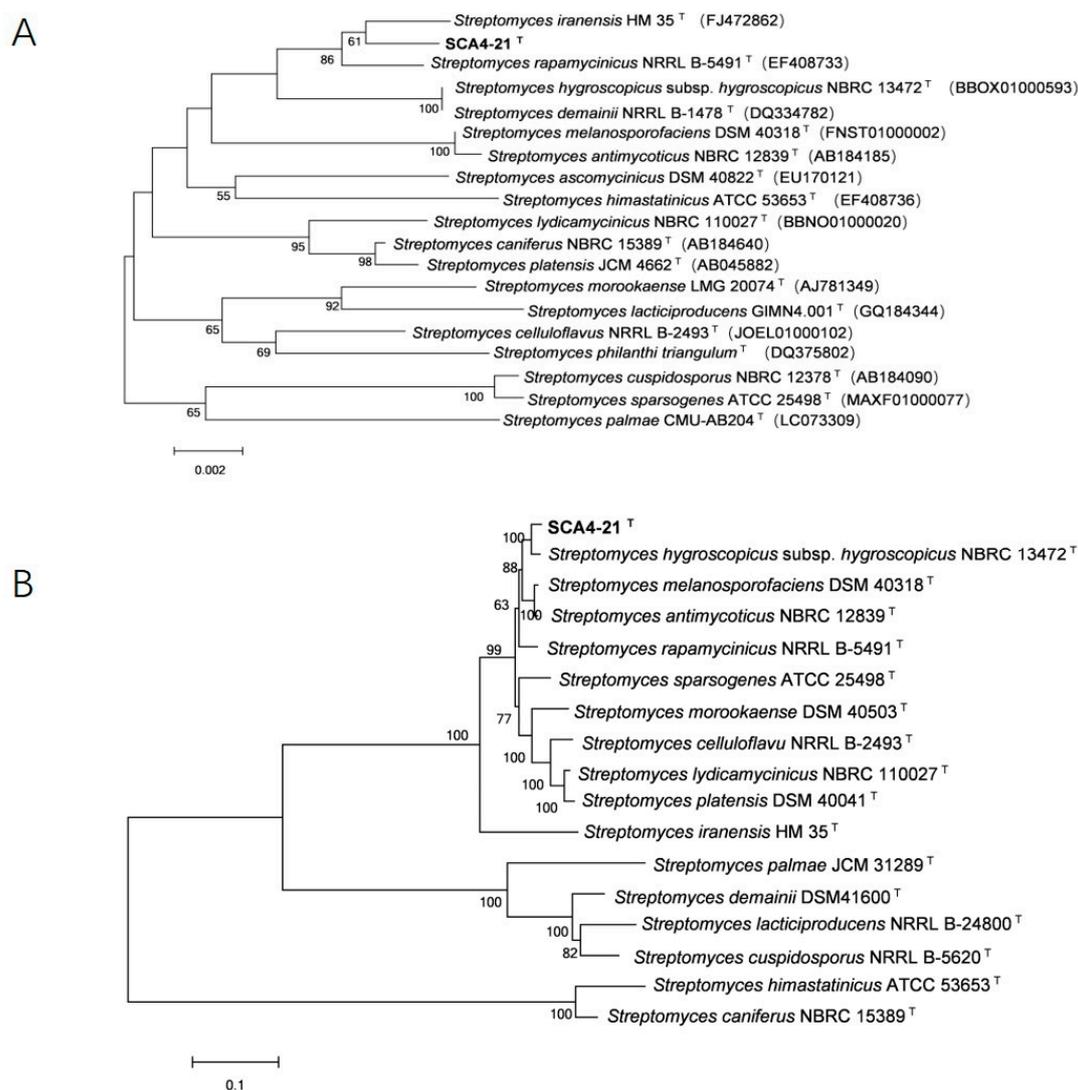


Figure 5. The phylogenetic trees of strain SCA4-21^T (A) Construction of phylogenetic tree based on the complete 16S rRNA sequences. Accession numbers of the selected genes were listed in brackets. (B) Construction of phylogenetic tree based on concatenated five house-keeping genes (*atpD*, *gyrB*, *recA*, *rpoB*, and *trpB*). Strain names and accession numbers were listed in Table S1. Bootstrap percentages (based on 1000 replications) were shown at branching points. Bar, 0.002 or 0.1 substitutions per nucleotide position.

Therefore, to assign strain SCA4-21^T to species, MLSA was performed using five house-keeping genes *atpD*, *gyrB*, *recA*, *rpoB*, *trpB*. The phylogenetic trees using all three algorithms showed that SCA4-21^T formed a well-delineated subclade with *Streptomyces hygroscopicae* subsp. *Hygroscopicus*

NBRC 1372^T with bootstrap values of 100% (Figure 5B and Supplementary Figure S4, S5). Our results also approved that five house-keeping genes *atpD*, *gyrB*, *recA*, *rpoB*, *trpB* could distinguish closely related *Streptomyces* species. The MLSA distance between strain SCA4-21^T and other related species of *Streptomyces* ranged from 0.021-1.092 (Table 3), which was much higher than the threshold of 0.007 for the delineation of bacterial species [42]. The above data indicated that strain SCA4-21^T represents a new species of *Streptomyces*.

Table 3. MLSA distance values for the selected strains.

Strains	MLSA distance (Kimura two-parameter)																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
1																		
2	0.1																	
3	87	0.0	0.1															
4	50	53	0.0	0.1	0.0													
5	21	88	48	0.0	0.1	0.0	0.0											
6	0.6	0.7	0.7	0.6														
7	79	14	05	60	0.0	0.1	0.0	0.0	0.6	0.0								
8	40	80	41	40	76	0.0	0.1	0.0	0.0	0.6	0.0							
9	0.0	0.1	0.0	0.0	0.6	0.0												
10	39	78	41	40	77	08	1.0	1.1	1.1	1.0								
11	92	36	00	77	02	04	94	0.0	0.2	0.0	0.0	0.7	0.0	0.0	1.0			
12	0.0	0.2	0.0	0.0	0.7	0.0	0.0	1.0										
13	92	26	93	92	10	89	88	99	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
14	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.1	1.0									
15	38	91	39	25	80	29	27	05	40	0.0	0.2	0.0	0.0	0.7	0.0	0.0	1.1	0.0
16	0.0	0.2	0.0	0.0	0.7	0.0	0.0	1.1	0.0	1.0	0.0	0.0						
17	98	32	98	96	15	93	93	02	18	50	0.0	0.2	0.0	0.0	0.7	0.0	0.0	1.1
18	0.0	0.2	0.0	0.0	0.7	0.0	0.0	1.1	0.0	1.0	0.0	0.0						
19	94	31	94	94	28	86	89	20	47	57	53	0.0	0.2	0.0	0.0	0.7	0.0	0.0
20	0.0	0.2	0.0	0.0	0.7	0.0	0.0	1.1	0.0	1.0	0.0	0.0						
21	93	21	89	91	11	88	87	20	87	41	93	89	0.0	0.2	0.0	0.0	0.7	0.0
22	0.7	0.7	0.7	0.7	0.1	0.7	0.7	1.1	0.7	1.1	0.7	0.7	0.7					
23	54	35	29	38	19	17	18	86	34	30	36	39	13					
24	0.7	0.7	0.7	0.7	0.2	0.7	0.7	1.2	0.7	1.1	0.7	0.7	0.7	0.2				
25	31	44	34	35	76	25	27	34	45	78	47	61	35	97				
26	0.7	0.7	0.7	0.7	0.0	0.6	0.6	1.1	0.7	1.0	0.7	0.7	0.7	0.1	0.2			
27	14	26	08	07	84	96	94	33	23	91	32	47	24	09	98			

17	0.0	0.2	0.0	0.0	0.6	0.0	0.0	1.0	0.0	1.0	0.1	0.1	0.0	0.7	0.7	0.6
	65	04	68	63	85	67	68	85	94	31	00	03	92	32	29	72

Strains: 1. SCA4-21^T, 2. *Streptomyces iranensis* HM 35^T, 3. *Streptomyces rapamycinicus* NRRL B-5491^T, 4. *Streptomyces hygroscopicus* subsp.hydroscopicus NBRC 13472^T, 5. *Streptomyces demainii* DSM41600^T, 6. *Streptomyces melanosporofaciens* DSM 40318^T, 7. *Streptomyces antimycoticus* NBRC 12839^T, 8. *Streptomyces himastatinicus* ATCC 53653^T, 9. *Streptomyces lydicamycinicus* NBRC 110027^T, 10. *Streptomyces caniferus* NBRC 15389^T, 11. *Streptomyces platensis* DSM 40041^T, 12. *Streptomyces celluloflavu* NRRL B-2493^T, 13. *Streptomyces morookaense* DSM 40503^T, 14. *Streptomyces laticiproducens* NRRL B-24800^T, 15. *Streptomyces palmae* JCM 31289^T, 16. *Streptomyces cuspidosporus* NRRL B-5620^T, 17. *Streptomyces sparsogenes* ATCC 25498^T.

3.7. Genome Sequencing and Feature Analysis of Strain SCA4-21^T

The complete genome of strain SCA4-21^T was sequenced and assembled into a circular chromosome of 10,044,493 bp with 71.48% of GC content (Figure 6A). Genomic features of strain SCA4-21^T were list in Table 4. The genome contained 8246 protein-coding genes (CDS) with 8,847,075 bp of gene total length, 64 tRNA genes, 18 rRNA genes, 134 sRNA genes, 1436 Repeat genes, 80 CRISPR-Cas genes and 6 Is genes. Functional analysis revealed that 7548, 3460 and 4391 genes were annotated to KEGG COG and GO categories, respectively (Figure 6B, 6C). In COG function classification, most of predicted CDS related to metabolism (47.4%), followed by cellular processes and signaling (20.2%) and information storage and processing (19.7%). 12.6% of CDS are poorly characterized (Supplementary Table S4).

Table 4. Genome features of strain SCA4-21^T.

Feature	Chromosome characteristics
Genome size (bp)	10,044,493
Chromosome No.	1
Plasmid No.	0
GC Content (%)	71.48
Depth	454.86
Protein-coding genes No.	8246
Gene total length (bp)	8,847,075
Gene average length (bp)	1072.89
Gene density	0.82
tRNA genes No.	64
Type of tRNAs No.	20
rRNA genes No.	18
16S rRNA No.	6
23S rRNA No.	6
5S rRNA No.	6
sRNA No.	134
Repeat No.	1436
CRISPR-Cas No.	80
Is No.	6
Genes assigned to COG (bp)	7548
Genes assigned to KEGG (bp)	4930

the genome of strain SCA4-21^T encoded 348 carbohydrate-active enzymes including 36 auxiliary activities (AAs), 6 carbohydrate-binding modules (CBMs), 74 carbohydrate esterases (CEs), 155 glycoside hydrolases (GHs), 65 glycosyl transferases (GTs), and 12 polysaccharide lyases (PLs). The proportion of CAZyme was 4.22% in strain SCA4-21^T genome (Figure 7B). Family and gene ID of strain SCA4-21^T CAZyme were list in Supplementary Table S5. AAs were divided into nine families, namely AA1, AA2, AA3, AA3_2, AA4, AA5, AA6, AA7, and AA10. CBMs contained four families: CBM2, CBM13, CBM35, and CBM66. CEs included eleven families: CE1, CE2, EC3, EC4, EC7, EC8, EC9, EC10, EC12, EC14, and EC15. GTs were classified into fifteen families: GT1, GT2_Glycos_transf_2, GT2_Glyco_tranf_2_3, GT4, GT5, GT9, GT20, GT28, GT35, GT39, GT41, GT76, GT81, GT83, and GT87. PLs were divided into eleven families: PL1, PL1_5, PL1_6, PL3_4, PL7_3, PL8, PL9, PL9_3, PL11, PL26, and PL31. GHs were classified into eighty families: GH1, GH2, GH3, GH4, GH5_1, GH5_8, GH5_18, GH5_19, GH5_40, GH5_43, GH6, GH8, GH9, GH10, GH11, GH12, GH13_3, GH13_9, GH13_10, GH13_11, GH13_13, GH13_16, GH13_26, GH13_30, GH13_32, GH15, GH16, GH18, GH19, GH20, GH23, GH25, GH26, GH27, GH30_5, GH30_7, GH31, GH33, GH35, GH36, GH42, GH43, GH43_3, GH43_5, 1 GH43_10, GH43_24, GH43_26, GH43_34, GH44, GH46, GH48, GH51, GH54, GH55, GH62, GH63, GH64, GH65, GH67, GH74, GH76, GH77, GH78, GH85, GH87, GH89, GH92, GH93, GH95, GH106, GH109, GH113, GH114, GH127, GH128, GH135, GH145, GH146, GH152, and GH154. Fifteen Chitinase (EC 3.2.1.14) were identified in GH18, GH19, GH23, and GH48 families. Additionally, eight β -1,3-glucanases (EC 3.2.1.39) were found in GH16, GH55, GH64 and GH128 families (Figure 7D). Chitinase and β -1,3-glucanases are often found to degrade the cell walls of plant pathogenic fungi [44,45]. Therefore, we speculated that chitinase and β -1,3-glucanases were responsible for antifungal activity of strain SCA4-21^T.

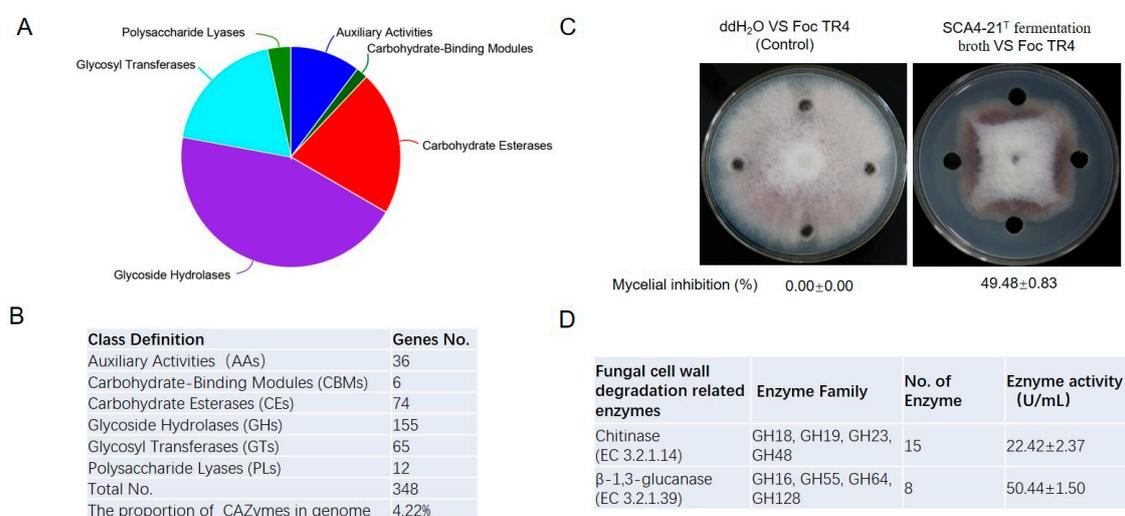


Figure 7. Carbohydrate-active enzymes (CAZymes) analysis of strain SCA4-21^T (A) Pie chart of CAZymes (B) The number of genes in different families of CAZymes. (C) Determination of antifungal activity in fermentation broth of SCA4-21^T. (D) Evaluation of chitinase and β -1,3-glucanase activity in fermentation broth of SCA4-21^T.

3.10. Antifungal Activity and CAZymes Analysis of Fermentation Broth of Strain SCA4-21^T

To confirm the above speculation, the inhibition percentage of fermentation broth from strain SCA4-21^T on Foc TR4 mycelia was initially measured. Our findings indicated that the fermentation broth from strain SCA4-21^T displayed an inhibition rate of $49.48 \pm 0.83\%$ on Foc TR4 mycelia (Figure 7C). By determination, the activities of chitinase and β -1,3-glucanase were 22.42 ± 2.37 U/mL and 50.44 ± 1.50 U/mL in the fermentation broth of strain SCA4-21^T, respectively (Figure 7D). These results provided evidence for the involvement of two enzymes in the antifungal activity of strain SCA4-21^T.

3.11. Description of *Streptomyces luomodiensis* sp. nov.

Streptomyces luomodiensis (lu.mo.di.en'sis. N.L. masc./fem. adj. luomodiensis of Luomodi, a village in China, referring to the place where the type strain was first isolated).

Gram-positive, aerobic and non-motile actinobacteria forming branched substrate and aerial mycelia which differentiates into spiral spore chain. Spores are cylindrical with shrinkage surface. Abundant growth occurs on all ISP, PDA and Gause's no.1 plates, and the colonies are white, pure or grey white, silver grey, cream. Growth appears at 20-46°C with optimum 28 °C, at pH 6-9 with optimum 7.0 and 0-3% (w/v) NaCl concentration with optimum 2%. Soluble pigment forms on ISP7 and PDA media. They degrade urea, Tween 20 and starch, while producing melanoid pigment and siderophores. They do not degrade gelatin, Tween 40, Tween 60, nitrate, or cellulose nor produce H₂S. D-mannose, D-trehalose, sorbitol, D-fructose, lactose, mannitol, and xylan are utilized as sole source of carbon, L-phenylalanine, L-asparagine, L-methionine, L-valine, L-histidine, L-tryptophan, D-fibrinose, and L-hydroxylungine are utilized as sole source of nitrogen. The predominant menaquinones are MK9 (H8) and MK10 (H2). Major fatty acids (>10.0%) are anteiso-C15:0 (34.78%) and C16:0 (19.89%). Cell wall hydrolysates contain LL-diaminopimelic acid (LL-DAP), but they do not contain meso-DAP or DD-DAP.

The type strain, SCA4-21^T (=GDMCC4.340^T=JCM36555^T), was isolated from soil of Luomodi Village, Huili County, Sichuan Province, China. The genome of type strain is characterized by a size of 10,044,493 bp and a G+C content of 71.48 mol%. Raw reads of strain SCA4-21^T were deposited to SRA database with the accession number SRP303585, 16S rRNA gene and complete genome sequence were submitted to GenBank with accession numbers OQ352835 and CP117522, respectively.

4. Discussion

Banana *Fusarium* wilt caused by Foc TR4 is a destructive fungal disease. More than 80% of bananas and plantains are susceptible to Foc TR4 [2]. The utilization of antagonistic microorganisms for controlling banana *fusarium* wilt has received the increasing attention due to its safety and efficacy. To enhance the effectiveness of biological control, it is crucial to screen broad-spectrum and novel antagonistic microorganisms as biocontrol agents [46]. Unique environments often harbor an abundance of novel microorganisms [47]. Here, thirty actinobacteria displaying diverse morphologies were isolated from the dry hot valley of Huili County (Figure 1A). Among them, seven actinobacteria exhibited antagonistic activity against Foc TR4 (Figure 1B). Especially, strain SCA4-21^T showed the most antifungal activity against Foc TR4 and other eight phytopathogenic fungi (Figure 2B).

Fungal cell walls are mainly composed of polysaccharides, accounting for 80-90% of their dry weight. carbohydrate-active enzymes (CAZymes) are the most important enzymes for polysaccharide degradation. The functional genes encoding CAZymes participate in fungal cell wall degradation. CAZymes analysis demonstrate that the genome of strain SCA4-21^T contains 348 CAZymes, accounting for 4.22% of total genes (Figure 7A, 7B). In most bacterial genomes, CAZymes typically comprise less than 2% of total genes [13]. The genome of strain SCA4-21^T exhibited a high percentage of CAZymes, suggesting its strong potential for degrading fungal cell walls. Specifically, the GHs family of CAZymes in strain SCA4-21^T genome contained fifteen chitinase genes and eight β -1,3-glucanase genes (Figure 7D). These enzymes are responsible for catalyzing the hydrolysis of chitin and β -1,3-glucan, which are the major structural components of fungal cell walls. Accumulated data indicate that chitinases and β -1,3-glucanases produced by biocontrol microbes and plants can inhibit fungal growth [48,49]. The study conducted by Arora et al. [45] showed that the chitinase and β -1,3-glucanase produced by *Fluorescent pseudomonad* can inhibit the growth of *Rhizoctonia solani*. Aktuganov et al. demonstrated that two enzymes chitinase and β -1,3-glucanase purified from *Paenibacillus ehimensis* IB-X-b have wide-range antifungal activity by degrading fungal mycelia cell walls [14]. Anitha and Rebeeth reported that chitinase extracted from *streptomyces griseus* can inhibit soil-borne plant pathogens such as *Fusarium oxysporum*, *Alternaria alternate*, *Rhizoctonia solani*, and *Fusarium solani* [50]. Chitinase and β -1,3-glucanase produced by *Streptomyces cavourensis* SY224

contributes to the biocontrol of *anthracnose* in pepper [51]. In the present study, the fermentation broth of strain SCA4-21^T has high levels of chitinase and β -1,3-glucanase activities (Figure 7C). These findings suggest that these two enzymes play a crucial role in the antifungal activity of strain SCA4-21^T against Foc TR4.

A complete database of 16S rRNA sequences is available for the type strains of prokaryotic species, allowing to rapidly identify strains at the genus level. Nonetheless, the phylogeny of 16S rRNA gene is difficult to distinguish closely related species because of the high level of sequence conservation [10,52,53]. The gene clusters of *atpD*, *gyrB*, *recA*, *rpoB*, *trpB* have a higher level of sequence divergence than that of 16S rRNA genes. MLSA based on these five house-keeping genes had been used as an alternative phylogenetic marker in species discrimination of *Streptomyces* [19, 54-56]. In our present study, strain SCA4-21^T exhibited the highest similarity to *S. iranensis* HM 35^T (99.45%), *S. rapamycinicus* NRRL B-5491^T (99.35%), and *S. hygroscopicus* subsp. *hygroscopicus* NBRC 13472^T (99.17%). However, the 16S rRNA phylogenetic analysis revealed that strain SCA4-21^T clustered with *S. iranensis* HM35^T in the NJ and MP trees, with bootstrap values of 61% and 36% respectively (Figure 5A, Supplementary Figure 2S) while it clustered with *S. iranensis* HM35^T and *S. rapamycinicus* NRRL B-5491^T in the ML tree (Supplementary Figure 3S). Consequently, the phylogeny of 16S rRNA gene was unable to effectively differentiate strain SCA4-21^T from its closely related species. Therefore, MLSA of this strain based on five housekeeping genes (*atpD*, *gyrB*, *recA*, *rpoB*, and *trpB*) were performed. The NJ, MP and ML phylogenetic trees all consistently indicated that strain SCA4-21^T had the highest bootstrap values and formed a well-delineated subclade with *S. hygroscopicus* subsp. *hygroscopicus* NBRC 13472^T (Figure 5B, Supplementary Figure 4S, 5S). Therefore, strain SCA4-21^T is closely related to *S. hygroscopicus* subsp. *hygroscopicus* NBRC 13472^T.

After the identification of most close species, the similarity alignment of genome is required to assign the isolate to a known or unknown species [10]. As a gold standard for identifying prokaryotic species, the traditional DNA-DNA hybridization (DDH) is time-consuming and labor-intensive, with a low inter-laboratory reproducibility [53]. With a rapid development of sequencing technology, it has become possible to directly use the alignment of genome sequences to classify the species. The overall genome related indexes (OGRI) such as average nucleotide identity (ANI) and digital DDH (dDDH) have been widely used to replace the traditional DDH to identify different species [37,53,57]. The ANI and dDDH values for accepted species boundary are around 95-96% and 70%, respectively [43]. In this study, ANI and dDDH values are calculated between strain SCA4-21^T and the adjacent six strains in the phylogenetic trees of 16S rRNA gene or MLSA. By calculation, the genome of strain 4-21^T has 91.26% of ANI and 44.3% of dDDH with *S. hygroscopicus* subsp. *hygroscopicus* NBRC 13472^T (Table 5). Both of values are below the threshold, indicating that strain SCA4-21^T represents a novel species of *Streptomyces*. Our results further demonstrate that the combination of MLSA and ANI/dDDH can accurately assign bacterial isolates to species. Polyphasic identification method showed that strain SCA4-21^T represented a novel species within the genus *Streptomyces*. Our research results offer an accurate method for identifying the species *Streptomyces* and a novel potential biocontrol agent for biocontrol of plant fungal diseases.

5. Conclusions

Thirty actinobacteria were isolated and screened for their antagonistic activity against Foc TR4. Strain SCA4-21^T exhibited a strong antifungal activity. Based on the morphological, physiological, biochemical and chemotaxonomic characteristics, strain SCA4-21^T was identified as genus *Streptomyces*. The genome of strain SCA4-21^T was subjected to complete genome sequencing and assembly, resulting in a 10,044,493 base pair chromosome. This chromosome encodes 8,246 protein-coding genes, with a GC content of 71.48%. CAZy Analysis revealed the presence of 348 carbohydrate-active enzymes, which accounted for 4.22% of the total genes. These included 15 chitinases and 8 β -1, 3-glucanases related to fungal cell wall degradation. The phylogenetic trees of 16S rRNA do not distinguish the close species *Streptomyces*. MLSA showed that strain SCA4-21^T clustered with *S. hygroscopicus* subsp. *hygroscopicus* NBRC 13472^T with over the species threshold, suggesting that strain SCA4-21^T represents a novel species. ANI and dDDH values are calculated to

further verify the conclusion. The strain is named after *Streptomyces luomodiensis* sp. nov.. The type strain is SCA4-21^T (=GDMCC4.340^T=JCM 34963^T).

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: **Dengfeng Qi:** Conceptualization, Methodology, Investigation, Formal analysis, Writing - review & editing. **Qiao Liu:** Methodology, Investigation, Formal analysis, Writing - original draft. **Liangping Zou, Miaoyi Zhang, Yufeng Chen, Junting Feng:** Methodology, Resources, Visualization. **Dengbo Zhou, Lu Zhang:** Resources, Writing - review & editing. **Kai Li, Yankun Zhao, Yongzan Wei:** Investigation, Formal analysis. **Jianghui Xie, Wei Wang:** Writing - review & editing, Project administration, Funding acquisition.

Funding: This work was supported by the Natural Science Foundation of Hainan (322QN417, 322RC660, 321RC543), the Central Public-interest Scientific Institution Basal Research Fund (1630052022006; CATASCXTD202309), the Hainan Science and Technology Association young talent lifting project (QCQTXM202203), and Chinese Academy of Tropical Agricultural Sciences for Science and Technology Innovation Team of National Tropical Agricultural Science Center (CATASCXTD202312).

Acknowledgments: We are grateful to Zhufeng Gao and Tianyu Li for their help in this work.

Declaration of competing interest: The authors declare that they have no known competing financial interest or personal relationships that could have influenced the work reported in this manuscript.

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