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RESEARCH ARTICLE

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Identification and antifungal activity of an actinomycete strain against *Alternaria* spp.

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Abstract

Alternaria alternata (Fries) Keissler is a phytopathogenic fungus responsible for tobacco brown spot disease. This study aims to evaluate the antifungal activity of strain 163 against *A. alternata* and clarify its taxonomic status. The evaluation of the antifungal activity of strain 163 and its bacteria-free filtrate of fermentation broth was done through measuring the diameters of inhibition zones, and testing the antimicrobial spectrum and the inhibition effect on mycelial growth *in vitro*. The biocontrol activity of the bacteria-free filtrate *in vivo* was evaluated by using detached tobacco leaves method and assaying the inhibition rate to disease incidence in growth chamber. A polyphasic approach was taken in the identification of strain 163. The bacterial strain 163 showed inhibitory effect *in vitro* against *A. alternata.* The bacteria-free filtrate of the strain 163 fermentation broth showed a 56.7% inhibition rate in a detached leaf assay. In growth chamber conditions, it showed greater biocontrol activity when applied before plants being inoculated with *A. alternata* than after, the inhibition rate being 46.05%. Investigations into the morphological, cultural, physiological and biochemical properties of strain 163 found it to be most similar to *Streptomyces microflavus.* Its classification into cell wall type I and sugar type C further confirmed its *Streptomyces* characteristics. Construction of a phylogenetic tree based on 16S rDNA verified that strain 163 was most closely related to *Streptomyces microflavus.* From polyphasic taxonomical analysis, strain 163 was found to be identical to *S. microflavus.*

Additional key words: antagonistic actinomycete; tobacco brown spot; polyphasic taxonomical; *Streptomyces microflavus.*

Introduction

The genus *Alternaria* contains many notorious plant pathogens. Around 90% of the *Alternaria* spp. reported to date can facultatively parasitize different species of plants, causing huge agricultural losses (Cui *et al*., 2005). Tobacco brown spot, a disease caused by *Alternaria alternata* (Fries) Keissler, has brought about enormous economic losses of tobacco leaf production since its first report in 1892 (Stavely & Main, 1970; Lamondia, 2001). Resistant varieties and chemical fungicides, especially the latter, have been commonly used in the attempt to reduce losses caused by the disease. Though these fungicides can effectively control the spread of the disease especially in the beginning, the increasing prevalence of the disease is observed all over the world. The decline of control efficacy of fungicides might be related to the emergence of fungicide resistance (Luo *et al*., 2009).

In these circumstances, biocontrol using the activity of microorganisms or their metabolites, has become the method of choice to combat plant diseases and pests (Cook, 1993; El Karkouri *et al*., 2010). Actinomycetes are an exceptionally prolific source of secondary metabolites and extracellular enzymes with interesting antibiosis (Berdy, 2005; Tu, 2008). Some researchers have been engaged in using actinomycetes as replacements or supplements to chemical fungi-

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Abbreviations used: BF (bacteria-free filtrate); CK (control check); CSA (conidial suspension of *A. alternata*); GNA (Gause No. 1 agar); GW (Gause wheat bran); IR (inhibition rate); ISP (International Streptomyces Project); PDA (potato dextrose agar).

cides for the control of diseases caused by *Alternaria* spp. (Chu *et al*., 2004; Wu *et al*., 2004; He *et al*., 2005; Luo *et al*., 2010; Tian *et al*., 2012). However, despite great initial optimism and extensive research efforts, progress in achieving commercial, large-scale usage of biological agents has been slow (Gao & Wu, 2008). What's more, the number of terrestrial antibiotics seems currently to approach a saturation curve with an apparent limit in the near future. So, some new isolation strategies have been adopted to obtain valuable natural antifungal metabolites (Iwai & Omura, 1992). Isolation of novel micro-organisms and their metabolites from remote or environmentally hostile areas of the world suggests that exploration of new habitats might continue to yield useful organisms (Moncheva *et al*., 2002; Saadoun & Gharaibeh, 2003).

The main objectives of this study were to evaluate the antifungal activity against *A. alternata* of strain 163, which was isolated from the primitive forest soil of the Changbai Mountain (China), and identify strain 163 by a polyphasic approach.

Material and methods

Antifungal activity of actinomycete strain 163

The antifungal activity of strain 163 against *A. alternata* (kindly provided by Associate Researcher Kong FY, Tobacco Research Institute of Chinese Academy of Agricultural Sciences) *in vitro* was evaluated by measuring the diameters of inhibition zones and testing the antimicrobial spectrum. *A. alternata* was grown on potato dextrose agar (PDA) at 28°C for 120 h. The conidial suspension of *A. alternata* (CSA) was prepared and adjusted to 10^5-10^6 cfu mL^{-1}, and was used as inoculum in the following experiments.

Strain 163 was incubated on Gause No. 1 agar (GNA) slants at 28°C for 120 h. The spore suspension was prepared and adjusted to 10^6-10^8 cfu mL^{-1}. A 100-µL spore suspension was streaked onto GNA plates, and the plates were incubated at 28°C for 120 h. Mycelial disks of strain 163 (7 mm) were punched from the plates and used as inocula for the PDA plates, which had previously been seeded with CSA (600 µL/150 mL PDA). These plates were then incubated at 28°C for 72 h and inhibition zone diameters were recorded.

The antimicrobial spectrum was investigated using the dual culture technique. The pathogens were incubated at 28°C for 120 h on PDA plates. Then, mycelial disks (7 mm) were punched from the periphery of the colonies and used as inocula. Next, the inocula were transferred to the center of fresh PDA plates, and three mycelial disks of strain 163 were placed equidistantly (2.0 cm from each pathogen colony) around the mycelial disks of the test pathogens. After incubation of the plates at 28°C for 72 h, the widths between the pathogen and mycelial disks of strain 163 were recorded.

Antifungal activity of the bacteria-free filtrate

The antifungal activity of the fermentation broth of strain 163 against *A. alternata* was evaluated *in vitro* by measuring the diameters of inhibition zones, testing the antimicrobial spectrum and the inhibition effect on mycelial growth. Three mycelial disks of strain 163, prepared as described above, were inoculated into Gause wheat bran (GW) medium (10 g wheat bran, 13.3 g soluble starch, 6.7 g glucose, 0.5 g NaCl, 0.5 g $MgSO_4$, 0.5 g K_2HPO_4, 0.05 g $FeSO_4$, 1 g KNO_3 and 1000 mL distilled water, pH 7.2-7.4; 40 mL per 250 mL flask), and incubated under 150 rpm at 28°C for 96 h. The fermentation broth was centrifuged at 6,000 rpm for 15 min and residual mycelium and spores in the supernatant were removed with a bacterial filter (0.22 µm). The bacteria-free filtrate (BF) was used in the following experiments. The plates seeded with *A. alternata* were prepared as described above. A sterilized Oxford cup was placed in the center of each seeded plate and 100-µL BF was poured into it. After incubation at 28°C for 72 h, the inhibition zone diameters were measured.

The antimicrobial spectrum was tested using the same method. The inocula used for the seeded plates were the conidia or mycelial suspensions of other test pathogens (see Table 1).

The inhibition effect of the fermentation broth on mycelial growth was evaluated using the poison media technique. BF was evenly mixed with molten PDA medium so that 0, 10, 20, 40, 80, 160, 320 and 640-fold dilutions were obtained and a 15-mL toxic plate was prepared per 9-cm Petri dish. The mycelial disks of *A. alternata* were transferred to the center of the toxic plate. After incubation at 28°C for 72, 120 and 168 h, the colony diameters were measured. A non-toxic plate was used as a control. In all of these tests,

Table 1. Antimicrobial spectrum of actinomycete strain 163

Pathogens[1]	Inhibition zone (mm)	
	Strain 163[2]	BF[3]
Alternaria alternata (Fries) Keissler	11.3 ± 0.76	25.7 ± 1.26
Alternaria alternata f. sp. *mali*	8.0 ± 1.00	25.0 ± 2.64
Alternaria solani (Ell. et Mar) Jones et Grout.	10.7 ± 1.25	36.0 ± 1.80
Bipolaris maydis (Nisikado et Miyake) Shoem.	—	—
Bipolaris sorokiniana (Sacc.) Shoem	—	—
Colletotrichum capsici	—	—
Colletotrichum destructivum	—	—
Colletotrichum lagenarium (Pass.) Ell. et Halst	—	—
Colletotrichum orbiculare (Berk. et Mont.) Arx	—	—
Exserohilum turcicum (Pass.) Leonard & Suggs	—	—
Fusarium graminearum Schw.	—	—
Fusarium moniliforme	4.0 ± 1.00	8.5 ± 1.80
Fusarium oxysporum f. sp. *cucumarinum* Owen.	—	—
Fusarium oxysporium f. sp. melonis (Leach) Syn.	—	—
Rhizoctonia solani Kühn	—	—
Verticillium dahliae Kleb	—	—

[1] All the pathogens used in the study, except *A. alternata*, were isolated, identified and preserved by the Plant Pathology Laboratory of College of Plant Protection, Shenyang Agricultural University. [2] Values indicate the space between the pathogen and the mycelial disk of strain 163, as determined using the dual culture technique. [3] Diameter of the inhibition zone (including the diameter of the Oxford cup). BF: bacteria-free filtrate. All values are the means ± SD of triplicate experiments. —: no activity.

the antifungal activity was expressed as the mean of three repeat experiments.

Biocontrol activity of BF on detached tobacco leaves

Biocontrol activity was evaluated by a simple *in vivo* assay using detached tobacco leaves. BF and CSA were mixed at a 1:1 ratio to be used as an inoculum. The method of inoculation used by Fang *et al*. (2002) was adopted with some adjustments. Tobacco (susceptible *Nicotiana tabaccum* cv. NC89) leaves were gently stabbed with a needle to create 10 holes as inoculation sites in each leaf. A 10-µL drop of admixture inoculum was spotted onto each site and incubated at 28°C in moist conditions under a 12 h light/dark cycle. An admixture of CSA and fermentation medium supernatant was used as control. Each leaf was considered as one treatment and each treatment was replicated four times. Necrotic spots on the leaves were counted 120 h after inoculation and the inhibition rate was calculated as %IR = [(N1 – N2) / N1] * 100, where N1 and N2 are the average number of necrotic spots in control and in treatment, respectively.

Biocontrol activity of BF in the growth chamber

The growth chamber experimental schedule included two foliar spray treatments with BF, *i.e.* 48 h before and 48 h after inoculating plant pathogens. The inoculation method used by Shen *et al.* (2009) was adopted with some adjustments. At the 7-leaf stage, 3 leaves nearest to the ground were gently stabbed to create 10 inoculation sites. Cotton balls (diameter 5-mm) were dipped into the CSA for 2 min, and then lightly placed on the sites to incubate the plants at 25-28°C for 48 h in a moist chamber. Water was used as control and 5 plants were considered one treatment and each treatment was replicated three times. The disease incidence was investigated when these plants had been incubated for 7 more days in the growth chamber and the inhibition rate was calculated using the following formula: %IR = [(I1 – I2) / I1] * 100, where I1 and I2 are average disease incidence in control and in treatment, respectively. The data were subjected to one-way analysis of variance, and treatment means were compared to the control at $p < 0.05$ by Tukey's post-hoc test. Statistical analysis was done with SPSS version 16.0 (IBM, Armonk, NY, USA).

Identification of strain 163

Investigations into the cultural and taxonomic characteristics of strain 163 were carried out by the methods of the International Streptomyces Project (ISP) recommended by Shirling & Gottlieb (1966) and cited by Xu *et al*. (2007). The morphological characteristics of the spores and aerial mycelia of strain 163 grown on GNA media at 28°C for 120 h were examined by scanning electron microscopy (Xu *et al*., 2007). The physiological and biochemical characteristics were evaluated according to the method presented in Buchanan & Gibbens (1984).

The amino acid composition of the cell wall and whole-cell sugars were analyzed by thin layer chromatography, using the method recommended by Hasegawa *et al*. (1983) and Xu *et al*. (2007). For determination of the 16S rDNA sequence of strain 163, genomic DNA was isolated using the method described in Sambrook & Russell (2001). PCR amplification and DNA sequencing reactions were performed by Takara Biotechnology Co. Ltd (Dalian, China). The amplification reaction was performed using the TaKaRa 16S rDNA Bacterial Identification PCR Kit (Code No. D310). DNA fragment (~1.5 kb) was excised from the gel and purified using a TaKaRa Agarose Gel DNA Purification Kit Version 2.0 (Code No. DV805A). DNA sequencing reaction was performed using the primers: Seq Forward, Seq Reverse and Seq Internal (Gao *et al*., 2012). The obtained DNA sequence was aligned with reference sequences obtained from the GenBank database (NCBI) using the ClustalX 1.83 software (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). Phylogenetic analysis was performed according to the neighbor-joining method using the MEGA 4.1 software (http://www.megasoftware.net/), and was based on 1,000 bootstrap repetitions of the neighbor-joining dataset.

Results

Antifungal activity of actinomycete strain 163

Both strain 163 and its fermentation broth supernatant had a significant inhibitory effect on *A. alternata*, with inhibition zone diameters of 30.3 ± 2.02 and 25.2 ± 1.26 mm, respectively (Fig. 1). The antimicrobial spectrum showed that strain 163 had an inhibitory effect on *A. alternata*, *Alternaria solani* and *Alternaria alternata* f. sp. *mali*, but only a slight effect or no effect on the other pathogens tested (Table 1).

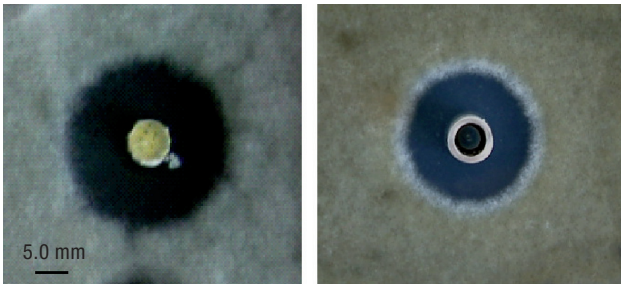

Figure 1. Example of the inhibition zone of strain 163 (left) and BF (right) against *Alternaria alternata.*

Fig. 2 shows that with the dilution fold set, the inhibition rate of the BF decreased with time. With time set, the inhibition rate of the BF fell with the increase of the dilution fold. At a dilution fold of 80 and a working time of 120 hours, the inhibition rate of the BF against the mycelial growth of *A. alternata* was still up to 45.7%. It is therefore safe to say that the BF had inhibitory effect on the mycelial growth. We also found that the mycelia of *A. alternata* in the toxic plates appeared as sparse villi, whereas those in the control were dense. Based on these results, we speculated that the fermentation broth contained antimicrobial substances with activity against *Alternaria* spp.

Biocontrol activity of BF on detached tobacco leaves and in growth chamber

The presence of BF significantly reduced the total number of lesions on detached leaves. Necrotic lesions appeared at 75.0% of inoculated sites on the control leaves and the inhibition rate of the BF against tobacco brown spot was 56.7%.

In growth chamber tests, bioassays of tobacco plants indicated that chlorotic lesions caused by *A. alternata* appeared around the infection sites 3-4 days after the inoculation and some of these lesions became necrotic after 7 days. The disease incidence (both chlorotic and necrotic) of the treated plants was much lower than that of the control (Table 2). The biocontrol activity of BF was greater applied before *A. alternata* inoculation than after, the inhibition rate being 46.05% and 34.79% respectively.

Identification of actinomycete strain 163

Strain 163 grew at 15-28°C and pH 5-12 and the aerial hyphae were straight or flexible, with more than 20 cylindrical spores per chain (Fig. 3). Other growth,

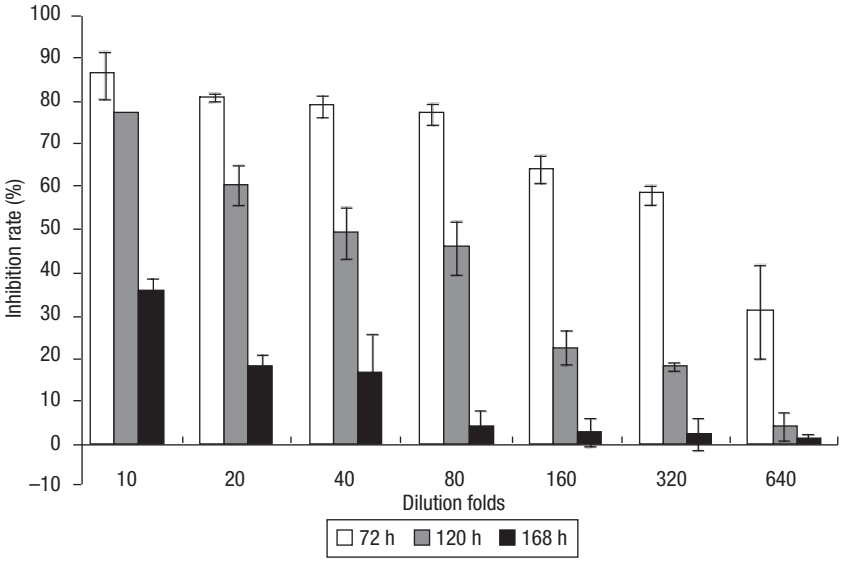

Figure 2. The inhibitory effect of BF on mycelial growth of *Alternaria alternata.*

physiological and biochemical characteristics are summarized in Table 3. Comparison of the cultural, morphological, physiological and biochemical characteristics of strain 163 with those of other *Streptomyces* spp. revealed that most of the characteristics of strain 163 were consistent with those of *Streptomyces microflavus*, except that it could not hydrolyze starch or grow on cellulose.

Thin-layer chromatography analysis of the whole-cell hydrolysate of strain 163 revealed LL-diaminopimelic acid and glycine, but no sugar component. It was confirmed that strain 163 could be classified as cell wall type I and sugar type C. Based on these taxonomic characteristics, strain 163 was assigned to the genus *Streptomyces*.

The partial 16S rDNA sequence of strain 163, 1424 bp in length, was deposited in the NCBI database under accession number GU108458. A BLAST search of this sequence found 100% nucleotide identity with the 16S rDNA sequence of *S. microflavus*. A phylogenetic tree was constructed based on the percent differences in the genetic relationships between *Streptomyces* species and strains, which suggested that strain 163 must be closely related to *S. microflavus* (Fig. 4). Strain 163 was then confirmed to be identical to *S. microflavus* based on polyphasic taxonomical analysis.

Table 2. Biocontrol activity of BF against tobacco brown spot in the growth chamber

Treatment[2]	Disease incidence[1]	
	Foliar spraying prior to inoculation	Inoculation prior to foliar spraying
BF	29.67 ± 3.51[a]	35.00 ± 5.29[a]
CK (water)	55.00 ± 17.58[b]	53.67 ± 8.08[a]

[1] Values show disease incidence mean (%) and standard deviation. Values followed by different letters are significantly different at the 5% level. [2] BF: bacteria-free filtrate of strain 163; CK: control check.

Discussion

Polyoxin, originating from *Streptomyces cacaoi* var. *asoensis*, is the most effective agricultural antibiotic in the prevention and treatment of fungal diseases caused by *Alternaria* spp. However, with the emergence of resistant strains, its application has become extre-

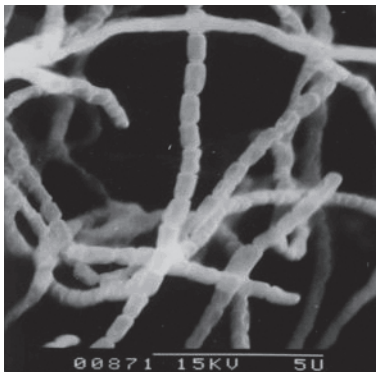

Figure 3. Scanning electron micrograph showing the spore chains of strain 163 (8,000×).

Table 3. Growth, physiological and biochemical characteristics of strain 163

Growth characteristics			
Medium	**Substrate mycelium**	**Aerial mycelium**	**Diffusible pigment**
ISP-2	Clove brown	Clove brown	Light brown
ISP-4	Lotus-seed white	Grayish white	None
ISP-5	Grayish white	Grayish white	None
Gause No. 1 agar	Lychee-pulp white	Lychee-pulp white	None
Glucose-asparagine agar	Lotus-seed white	Lotus-seed white	None
Czaper's agar	Lychee-pulp white	Lychee-pulp white	None
Potato glucose agar	Light gray	Light gray	None
Carbon utilization		**Enzyme properties and metabolic production**	
L-arabinose	+	Catalase	+
D-fructose	+	Urease	+
Galactose	+	Esterase	+
Glucose	+	Gelatin liquefaction	+
Amylomaltose	+	Hydrolyze starch	–
D-xylose	+	Grow on cellulose	–
D-mannitol	+	Peptonization	+
Sucrose	–	Coagulation	–
Inositol	–	Methyl red test	–
Raffinose	–	Voges-Proskauer test	–
Control	–	H_2S production	–
		Nitrate reduction	–

+: positive result. –: negative result.

mely limited (Hwang & Yun, 1986; Gafur *et al*., 1998; Tanaka *et al*., 2002). The urgent demand for new anti-*Alternaria* antibiotics in agriculture has enforced the search for metabolites in so-far-undisturbed habitats. The contrast between the inhibition effect on the three *Alternaria* spp. and the slight or no effect on other pathogens tested *in vitro* indicated that the antagonistic action of strain 163 against *Alternaria* spp. may be specific; however further studies into its inhibitory effect against other *Alternaria* spp., which cause agriculturally serious fungal diseases, are now needed. Meanwhile, the early identification by paper chromatography (data not shown) found that the active substance present in the BF of 163 may be one of polyene antibiotics, different from polyoxin, a nucleoside antibiotic. This brings hope for the solution to the polyoxin resistance problem.

The antifungal activity of biocontrol agents is usually evaluated first under *in vitro* conditions, and these tests do not tell us how various environmental factors influence biocontrol agents under agricultural conditions (Nobutaka, 2008). Therefore, it is of great necessity to carry out a growth chamber test or field trial to test the practical abilities of biocontrol agents in preventing diseases. The BF was assayed on detached leaves and in the growth chamber, but the results showed a lower suppression level than expected. The data also indicated the inhibition rate on detached leaves is higher than in growth chamber tests. The difference between inhibition rates agrees with Yuan & Crawford's (1995) view of no clear correlation between *in vitro* antagonism of a biocontrol candidate and disease suppression *in vivo*. The reasons may be that plant disease suppression by biocontrol agents is governed by a multitude of factors. The influence of these factors varies with the type of biocontrol agent, plant cultivar and the nature of the pathogen targeted for control (Schroth & Hancock, 1981). Besides, there are different action modes of biocontrol candidate, such as inhibition of the pathogen by antimicrobial compounds (antibiosis), and induction of plant resistance mechanisms (Whipps, 2000).

From the findings of polyphasic taxonomical analyses, strain 163 was classified along with *S. microflavus*. It has been reported that *S. microflavus* has potential applications in medicine, pharmacology and in the production of microbial fertilizer.

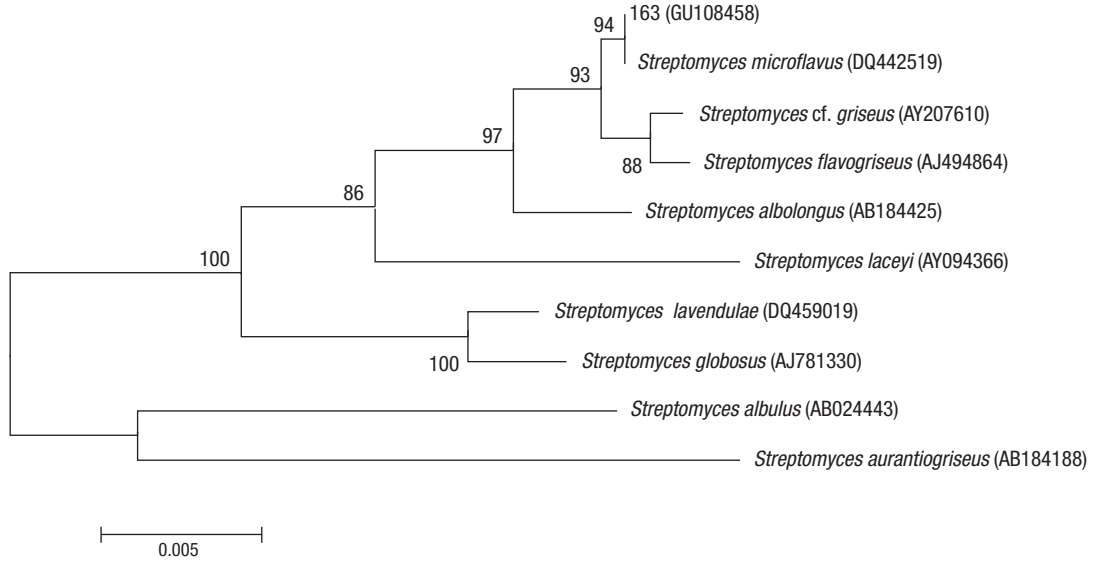

Figure 4. Phylogenetic localization of *Streptomyces* sp. 163 based on 16S rDNA sequences. The length of each pair of branches represents the distance between sequence pairs. The number at each branch point indicates the percentage of bootstrap support based on a neighbor-joining analysis of 1,000 resampled datasets. The scale bar indicates 0.005 substitutions per nucleotide position.

Fattiviracin FV-8, purified from the culture broth of *S. microflavus* strain No. 2445, showed potent antiviral activities against HIV-1, HSV-1 and VZV, etc. (Ei-Sayed *et al*., 2001). It was reported that *S. microflavus* alone or in combination with other *Streptomyces* spp. could effectively reduce the emissions of short chain volatile fatty acids (Huang *et al*., 2007). In addition, Wen *et al*. (2010) isolated compounds 1, 2, 3, 4 and 5 from the fermentation broth of *S. microflavus* strain neau3, and found that, in particular compound 1, had antiparasitic potential, exhibiting acaricidal and nematocidal activities. However, there is little information regarding the activity of *S. microflavus* against plant diseases in agricultural production, except for one report that a marine strain of *S. microflavus* (MB-97) could inhibit *Penicillium purpurogenum*, a soybean deleterious rhizospheric microorganism (Hu *et al*., 2002). To our knowledge, our study is the first to report *S. microflavus* as a promising and specific antagonistic actinomycete against *Alternaria* spp.

To sum up, in this study we screened and obtained one promising isolate, strain 163, which has an obvious inhibition effect on *Alternaria* spp. From polyphasic analysis, strain 163 was identified as *Streptomyces microflavus*. The antifungal activity of strain 163 detected in our study highlights the importance of actinomycete strains as candidates for the biological control of *Alternaria* spp. Further purification and structural elucidation of the active substance present in the BF of strain 163 are in progress.

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