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Streptomyces ansochromogenes Tur-10 produces a substance with antifungal bioactivity

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ABSTRACT. The increased incidence of fungal infections and the development of drug resistance have led to the search for microorganisms capable of producing bioactive metabolites with antifungal activity. Among these microorganisms, *Streptomyces* spp are distinguished mainly owing to their potential to secrete bioactive molecules. The aim of this study was to evaluate the production of secondary metabolites by *Streptomyces* sp TUR-10 against 12 fungal clinical isolates (yeast and filamentous fungi). In the preliminary screening, *Streptomyces* sp TUR-10 showed activity against 75% of the clinical isolates, and was selected for fermentation. In this assay, we tested three different media (MPE, M1, and ISP-4) for 96 h at pH 7.0 and 30°C for the production of bioactive metabolites. Increased production of bioactive compounds was observed when using the MPE medium for 48 h, with good activity against *Candida pelliculosa*. The minimum inhibitory concentration showed significant antifungal activity values ranging from 15.6 to 250 µg/mL. The actinobacterium was characterized by *16S rRNA* analysis

and the pattern suggested that the isolate studied belonged to the species *Streptomyces ansochromogenes*. The biotechnological potential of this strain was also demonstrated by the detection of the *nrps* and *pks* genes. These results indicate the production of secondary metabolites of biotechnological interest by actinobacteria from the rhizosphere, suggesting great potential for further research.

Key words: *Actinomycetes*; Antifungal activity; Antimicrobial activity; *pks* gene; *nrps* gene; *Streptomyces ansochromogenes*

INTRODUCTION

The indiscriminate use of synthetic antifungals has increasingly promoted the emergence of pathogens with resistance profiles. Attention to the irrational use of drugs derives from the fact that, according to the World Health Organization (WHO, 2010), more than 50% of all medicines are incorrectly prescribed, distributed, and sold; and more than 50% of patients use them incorrectly (Gaash, 2008).

Studies have been conducted that take into account the growing number of infections, both fungal and bacterial, in order to obtain new pharmacological compounds that are less toxic to human beings and that show effective activity against resistant microorganisms (Kitouni et al., 2005).

Among the filamentous and yeast-like fungi of clinical interest that cause mycosis, *Cryptococcus*, *Candida albicans*, *Aspergillus*, *Tricophytum*, *Cladosporium*, and *Microsporum* account for 90% of fungal infections in human beings, and are most commonly isolated in hospital environments. Their occurrence is often increased in patients with compromised immune functions caused by human immunodeficiency virus, chemotherapy treatments, and transplantations, or even those who undergo invasive procedures or use a broad-spectrum antimicrobial agent (Leiva et al., 2004).

A variety of natural products have been studied, a large part of which are secondary metabolites produced by microorganisms, and many have biotechnological applications. Among these microorganisms, the actinobacteria are a major source of bioactive metabolites with antimicrobial activity (Malviya et al., 2009).

The genus *Streptomyces* is noteworthy owing to its frequent occurrence in soil, and is widely known for its ability to produce different types of bioactive substances such as antibiotic agents. About 75% of natural antibiotic agents are produced by *Streptomyces* spp (Nikodinovic et al., 2003).

In studies previously carried out by our research group, we observed that *Streptomyces* sp TUR-10 has characteristics that are important for the antagonism of pathogenic microorganisms (Bernardo, 2012). Therefore, it was deemed appropriate to conduct this study in order to evaluate its antifungal activity against various clinical isolates.

MATERIAL AND METHODS

Microorganisms

Streptomyces sp TUR-10 was isolated from the rhizosphere of *Terminalia fagifolia*, which belongs to the Caatinga biome and is deposited at the Microorganism Culture Collection of Departamento de Antibióticos of Universidade Federal de Pernambuco (UFPEDA).

The following fungi were supplied by the URM Collection at the Department of Mycology (DM/UFPE): *Candida pelliculosa* (URM 6281); *Candida albicans* (URM 6401); *Candida guilliermondii* (URM 6403); *Candida parapsilosis* (URM 6431); *Candida glabrata* (URM 6392); *C. albicans* (URM 6395); *Epidermophyton floccosum* (URM 3182); *Fusarium solani* (URM 5903); *Trichophyton rubrum* (URM 5908); *Microsporum gypseum* (URM 6199); *Trichophyton mentagrophytes* (URM 6272); and *Aspergillus niger* (URM 6642).

Antifungal activity of *Streptomyces* sp TUR-10

Preliminary bioassay

To evaluate the antifungal activity of *Streptomyces* sp TUR-10, we used the agar block assay (Ichikawa et al., 1971). For this, the actinobacteria were cultivated in ISP-4 medium (Shirling and Gottlieb, 1966) and arginine yeast agar (AYA) (Nonomura and Ohara, 1969) at 37°C. After 5 days, 10-mm agar blocks were placed on Sabouraud (SAB) plates containing yeast and filamentous fungi [Clinical Laboratory and Standards Institute (CLSI), 2008]. Plates were re-incubated at 30° ± 2°C and the inhibition zones were re-measured after 48 h for yeasts and after 120 h for filamentous fungi. The experiment was performed in triplicate.

Secondary assay

Streptomyces sp TUR-10 was cultivated in the media ISP-4 (Soluble starch 20g/L, K_2HPO_4 2g/L, $MgSO_4$ $7H_2O$ 2g/L, NaCl 2g/L, $(NH_4)_2SO_4$ 4g/L, $CaCO_3$ 4g/L); M1 (glucose 10g/L, soy powder 10g/L, NaCl 5g/L, $CaCo_3$ 1g/L); MPE (glucose 20g/L, soy powder 20g/L, NaCl 5g/L, $CaCo_3$ 2g/L) (Kawamura et al., 1976), and stirred at 200 rpm and 37°C for 48 h. After this period, 10% (v/v) was transferred to Erlenmeyer flasks containing 50 mL medium. Every 24 h, a 1-mL aliquot was taken to evaluate the dry weight, antimicrobial activity, and pH. For determining dry weight, samples were centrifuged and the tubes containing the cell mass were returned to the incubator for drying and weighing. For evaluating the antifungal activity of secondary metabolites, a disc diffusion test was performed. Fifty-microliter aliquots fermented liquid were placed on SAB plates containing yeast and filamentous fungi (CLSI, 2008). The plates were incubated at 30°C for 48-120 h. The disc diffusion test was performed in triplicate and the results were determined through the arithmetic average of the inhibition zone diameters in millimeters (CLSI, 2008).

Extraction of bioactive metabolites

Streptomyces sp TUR-10 was cultivated in the MPE medium at 37°C for 48 h at 200 rpm. Extraction of metabolites from the cell mass and liquid was then performed. The cell mass was treated with acetone, ethanol, and pure methanol at pH 2.0, 7.0, and 9.0, and for the metabolic liquid we used ethyl acetate, chloroform, and pure petroleum ether at the same pH values mentioned above. The pH was then adjusted to 7.0, and the antifungal activity was assessed using the disc diffusion test (Porto et al., 1996).

Determination of minimum inhibitory concentration (MIC)

The MIC of the extracts was determined using the macrodilution technique, according

to the CLSI (2008) guidelines. The extracts were tested at concentrations ranging from 3.9 to 1000 µg/mL. Amphotericin B concentrations were 7.8 to 1000 µg/mL. The minimum fungicidal concentration was determined by a subculture in the assay of MIC in a SAB medium. Plates were incubated at 30°C from 48 to 120 h (Koneman et al., 2006).

Identification of actinobacteria

Classical taxonomy

For identifying micromorphology, *Streptomyces* sp TUR-10 was inoculated as a wide streak on the ISP-4 culture medium, coverslips were inserted at 45° inclination on the streak to induce growth of aerial mycelia on the coverslip, and the culture medium was incubated at 37°C for 5-10 days. A coverslip was removed to observe the formation of the spores-sporophores chains under an optical microscope (Medilux, MDL-150-BAI) at 40X objective, according to the technique proposed by Shirling and Gottlieb (1966).

Molecular taxonomy

DNA extraction was performed on the culture grown in ISP-2 liquid medium for 16 h at 37°C. Subsequently, the sample was centrifuged (3 min at 9168 *g*) and DNA extraction was performed using the Wizard Genomic DNA Purification kit (Promega®), according to manufacturer instructions. The DNA sequence was assessed by electrophoresis on agarose gel and the *16S rRNA* gene was amplified by polymerase chain reaction using universal oligonucleotides (Table 1).

Table 1. Oligonucleotides selected for amplifying *16S rRNA*, *pks*, and *nrps*.

Gene	Oligonucleotides	Sequence 5'-3'	Fragment size (bp)	Reference
16S rRNA	fD1	AGAGTTTGATCCTGGCTCAG	1500	Weisburg et al. (1991)
	rD1	AAGGAGGTGATCCAGCC		
pks	ksα	TSGRCTACRTCAACGGSCACGG	500	Jenke-Kodama et al. (2005)
	ksβ	TACSAGTCSWTCGCCTGGTTC		
nrps	A3F	GCSTACSYSATSTACACSTCSGG	700	Jenke-Kodama et al. (2005)
	A7R	SASGTCVCCSGTSCGGTAS		

The reaction mixture comprised 50 ng DNA, 5 pmol of each oligonucleotide, 200 mM dNTP, 1.5 mM $MgCl_2$, 1X buffer, and 1 U Platinum Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA), to a 25-µL final volume. The reaction conditions were: 5 min of denaturation at 94°C, followed by 25 cycles of 1 min at 94°C, 30 s at 52°C, and 2 min at 72°C; and a final extension of 10 min at 72°C. The amplification product was analyzed by electrophoresis on 1.2% agarose gel, and the sample was sent for sequencing. The sample was sequenced by Macrogen (Seoul, Korea) and this sequence was compared with all sequences in GenBank using the BLAST software from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The sequence was aligned with the Clustal software and the phylogenetic tree was constructed using Mega 5.5. Topology was assessed by bootstrap analysis (1000 resamplings).

Assessing the actinobacteria potential by amplification of the non-ribosomal peptide synthetase (*nrps)* and polyketide synthase (*pks*) genes

For amplifying sequences of the *nrps* and *pks* genes, we used the degenerate oligonucleotides described in Table 1. The reaction mixture comprised 50 ng DNA, 5 pmol of each oligonucleotide, 200 mM dNTP, 1.5 mM $MgCl_2$, 1X buffer, and 1 U Platinum Taq DNA polymerase (Invitrogen Life Technologies), to a 25-µL final volume. The reaction conditions were: 5 min denaturation at 94°C followed by 30 cycles of 35 s at 94°C, 40s at 55°C, and 2 min at 72°C. These cycles were followed by a final extension of 7 min at 72°C. The amplification product was analyzed by electrophoresis on 1.2% agarose gel (González et al., 2005).

RESULTS

The results of the preliminary bioassay showed good antimicrobial activity for 75% of the clinical isolates tested, with inhibition zones ranging from 20 mm for *A. niger* (URM 6642) and *C. albicans* (URM 6401) to 28 mm for *C. guilliermondii* (URM 6403). There was no significant activity for *C. glabrata* (URM 6392), *T. rubrum* (URM 5908), and *F. solani* (URM 5903) (Figure 1).

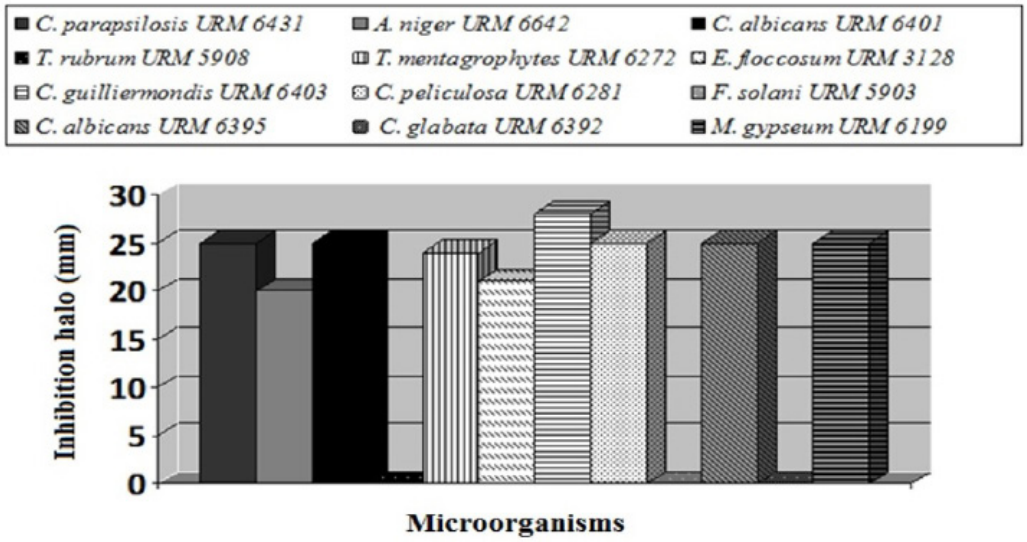

Figure 1. Antifungal activity of *Streptomyces* sp TUR-10 against pathogenic fungi detected by the preliminary bioassay in ISP-4 medium. Values of the inhibition zone are in millimeters.

The assessment of metabolite production was performed by means of the secondary assay using different culture media. Increased production of bioactive compound was observed when using the MPE medium for 48 h of fermentation. This medium was useful both for metabolite production and for microorganism growth (biomass of 0.23 g) (Table 2).

Ethyl acetate and acetone extracts (pH 7) showed antimicrobial activity with zones of 20 and 22 mm, respectively, for *C. pelliculosa* (URM 6281).

MIC was defined as the minimum extract concentration that caused 100% inhibition of visible microorganism growth (turbidity). The ethyl acetate extract exhibited a MIC value of 15.6 µg/mL for *C. guilliermondii* (URM 6403), *C. pelliculosa* (URM 6281), and *C. parapsilosis* (URM 6431). Table 3 shows all the MIC values of the crude extracts tested on the various fungal isolates.

Table 2. Assessment of antifungal activity of *Streptomyces* sp TUR-10 (mm) during 48 h of fermentation in different culture media.

TUR-10	MPE	M1	ISP-4
Clinical isolates			
C. albicans URM 6401	++	+	-
C. albicans URM 6395	+++	+	-
C. pelliculosa URM 6281	+	+	-
C. guilliermondii URM 6403	+++	+	-
C. parapsilosis URM 6431	+++	+	-
E. floccosum URM 3182	++	+	-
M. gypseum URM 6199	++	+	-
T. mentagrophytes URM 6272	++	+	-
A. niger URM 6642	++	+	-

Table 3. Values of minimum inhibitory concentration of the crude extracts and the positive control (amphotericin B) against fungi.

Clinical isolates	MIC and MFC (µg/mL) of ethyl acetate extract		MIC and MFC (µg/mL) of acetone extract		MIC and MFC (µg/mL) of amphotericin B	
	MIC	MFC	MIC	MFC	MIC	MFC
C. albicans URM 6401	250.00	500.00	125.00	250.00	7.8	15.6
C. guilliermondii URM 6403	15.6	31.25	62.5	125.00	7.8	15.6
C. pelliculosa URM 6281	15.6	31.25	15.6	31.25	7.8	15.6
C. albicans URM 6395	31.25	62.5	125.00	250.00	7.8	15.6
C. parapsilosis URM 6431	15.6	31.25	15.6	31.25	7.8	15.6
E. floccosum URM 3182	25.00	250.00	250.00	500.00	15.6	31.25
A. níger URM 6642	31.25	62.5	125.00	250.00	15.6	31.25
M. gypseum URM 6199	31.25	62.5	125.00	250.00	15.6	31.25
T. mentagrophytes URM 6272	1000.00	1000.00	1000.00	1000.00	31.25	62.5

Amphotericin B showed a MIC ranging from 7.8 to 31.25 µg/mL. We determined amphotericin B resistance to be a concentration requirement of ≥4 µg/mL (CLSI, 2008).

The bacterial strain was identified as belonging to the genus *Streptomyces*, which has the morphological characteristic of forming chains with long spiral spores. In addition, we conducted a molecular analysis of the *16S rRNA* gene. A 1474-bp fragment was sequenced and it showed a similarity above 96% to *Streptomyces ansochromogenes* NBRC 13665 (Figure 2).

In this study, we also investigated the presence of the *nrps* and *pks* genes because they are involved in the biosynthesis of important antibiotic agents produced by actinobacteria, and *Streptomyces* sp TUR-10 amplifies both these genes. The amplification products were demonstrated by the presence of the 500-bp *pks* and 700-bp *nrps* genes.

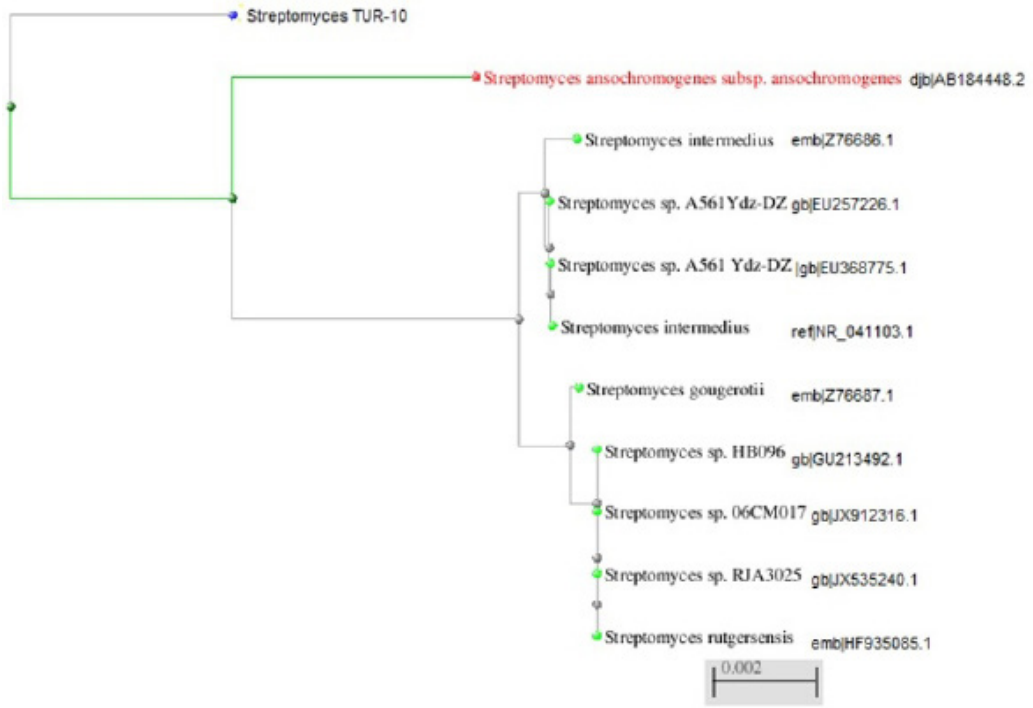

Figure 2. Neighbor-joining analysis based on the partial sequence of *16S rDNA*, showing phylogenetic relationships between *Streptomyces* sp TUR-10 and other species of *Streptomyces*. Bar, sequence divergence of 0.2%.

DISCUSSION

Soil represents a vast reservoir of microorganisms, a large part of which are found in the rhizosphere and have the ability to produce bioactive secondary metabolites. The production of antimicrobial agents is an important feature of the genus *Streptomyces*; they produce antiviral, antitumor, and immunosuppressive substances (Procópio et al., 2012).

Actinobacteria stand out owing to their broad activity in the face of various microorganisms, which means that they have great potential for applications in numerous areas of health and agriculture (Mukherjee and Sen, 2006; Zhao et al., 2011). In this study, the strain *Streptomyces* sp TUR-10, isolated from the rhizosphere of *T. fagifolia* (Caatinga biome), was identified by classical and molecular taxonomy as *S. ansochromogenes* TUR-10. The actinobacteria show significant antifungal activity. Reports in the literature show that *S. ansochromogenes* produces a number of antibiotic agents belonging to different chemical classes and with different structures (Chen et al., 2000; Tan et al., 2002; Liu et al., 2005). According to Li et al. (2012), *S. ansochromogenes* has strong antifungal activity that arises from chemical components such as nikkomycin Z and polyoxin, which are nucleoside peptide antibiotics that are strong competitive inhibitors of chitin synthesis.

It is known that fungal infections have a progressive nature and can present potentially severe stages in immunocompromised patients. According to Bassetti et al. (2011), several studies demonstrate a significant increase in candidiasis caused by fungi mainly involving

the genus *Candida*, and particularly *C. albicans*. However, in the last 10 years, this etiologic profile has changed markedly, and increasing rates of candidiasis caused by other species such as *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, and *C. krusei* have been reported worldwide. Thus, there is an urgent need to discover new drugs that are capable of controlling the growth of these microorganisms.

In our quality assessment of the assay on agar blocks, we found that ISP-4 medium was the best for determining antimicrobial activity. Several reports have shown that media containing starch are more suitable for antifungal activity than media containing glycerol and glucose (Nonoh et al., 2010; Shahat et al., 2011), which corroborates the fact that ISP-4, which contains starch, presented larger inhibition zones when compared with AYA medium, which comprises glycerol and glucose, in this study.

In this research, the MPE medium positively influenced the growth and production of secondary metabolites. Several studies have reported that the production of bioactive metabolites may be influenced by culture medium, time, and pH. Wang et al. (2010) observed increased antimicrobial activity in different strains of *Streptomyces* spp when pH reached a value of around 7.0. Bonfim (2008), working with endophytic *Streptomyces*, also found better activity in the face of yeasts for 48 h of fermentation with inhibition zones ranging from 20 to 23 mm. The MPE medium has concentrations of carbon and nitrogen in its composition. The presence of these components may influence the production of secondary metabolites by *Streptomyces* spp and, therefore, result in larger inhibition zones (Ismet et al., 2004).

The solvents ethyl acetate and acetone were effective for extracting the metabolites that are active against the fungal isolates. Arasu et al. (2013) observed that the MIC of the ethyl acetate extract of *Streptomyces* sp AP-123 was 125 µg/mL when used against *A. niger* and *E. floccosum.* The results obtained in the current study demonstrate that *S. ansochromogenes* TUR-10 is effective against the fungal isolates tested and presents better results than those reported in the literature for *Candida* and *A. niger*, suggesting great fungicide potential.

Non-ribosomal peptides and polyketides represent important classes of compounds produced by bacteria, fungi, and plants. They include compounds with very diverse structures deriving from secondary metabolism, such as antibiotics, toxins, siderophores, and immunosuppressive agents. The production of these compounds is directly associated with the multifunctional enzymes PKS I, PKS II and NRPS (Hill, 2005).

The *nrps* and *pks* genes are involved in the synthesis of numerous biologically active compounds of great biotechnological importance. However, there are still few studies involving the search for these genes in actinobacteria. Xi et al. (2012) estimated that 27.5% of the isolates tested that belonged to the genus *Streptomyces* (34 isolates) showed antimicrobial activity, and it has been shown that the strains where both *pks* and *nrps* genes were identified produced a significantly higher number of secondary metabolites.

Zhong et al. (2013) demonstrated the assembly and features of secondary metabolite biosynthetic gene clusters with *pks* and *nrps* in *S. ansochromogenes*. In the current study, we provided evidence for the presence of both genes (*nrps* and *pks*) in the strain *S. ansochromogenes* TUR-10, suggesting that the production of the secondary metabolites might be performed by two metabolic routes. The peptide compounds produced by the non-ribosomal pathway have a broad spectrum of activity and great importance for the pharmaceutical industry. Some examples of this class of compounds are vancomycin, penicillin, and bleomycin (an antitumor substance) (Jenke-Kodama et al., 2005).

The data presented in this paper suggest that *Streptomyces ansochromogenes* TUR-10

produces metabolites with antifungal activity, which are worthy of further research. New experiments should be conducted to investigate the active ingredients produced by this microorganism.

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