Revista Brasileira de Biociências
Brazilian Journal of Biosciences

ISSN 1980-4849 (on-line) / 1679-2343 (print)

ARTICLE

Antifungal activity of actinobacteria against fungus isolates of clinical importance

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Available online at http://www.ufrgs.br/seerbio/ojs/index.php/rbb/article/view/2762
Received: September 8 2013 Received after review: December 13 2013 Accepted: December 16 2013

ABSTRACT: (Antifungal activity of actinobacteria against fungus isolates of clinical importance). The escalating use of antifungal agents and long-term treatment approaches has led to an increased prevalence of fungus species that are resistant to the most common antifungal drugs. Actinobacteria produce a wide variety of bioactive secondary metabolites. This study investigated actinomycete isolates with the potential to produce bioactive compounds against dermatophyte fungus species and *Candida* spp. of clinical importance. Antifungal activity of actinomycetes was assessed using the double-layer agar technique. Active isolates were cultivated in starch casein broth (SCB) at 30 ºC for 8 days. Aliquots were retrieved at 24-h intervals and centrifuged to obtain extracts. Extract activity was assessed using the well-diffusion method. No dermatophyte fungus isolate was inhibited in the double-layer assay, although isolates 1S, R18(6) and 6(2) were active against all *Candida* spp. used in the assay. The well-diffusion method revealed that isolate R18(6) inhibited the six *Candida* spp. in a 72-h growth period in SCB broth, showing good potential to yield a compound with antifungal activity.
Key words: bioactive compounds, *Candida* spp., dermatophyte fungi.

RESUMO: (Atividade antifúngica de actinobactérias contra fungos isolados de importância clínica).O uso crescente de antifúngicos e os tratamentos prolongados vêm aumentando a incidência de fungos resistentes às drogas antifúngicas comumente utilizadas. As actinobactérias são conhecidas por produzirem uma grande variedade de metabólitos secundários bioativos e este trabalho teve como objetivo selecionar isolados de actinomicetos com potencial para produção de compostos bioativos contra fungos dermatófitos e espécies de *Candida* de importância clínica. A atividade antifúngica dos actinomicetos foi avaliada pela técnica da dupla camada. Os isolados que apresentaram atividade foram cultivados em caldo amido caseína (AC) à temperatura de 30 ºC por oito dias e foram retiradas e centrifugadas alíquotas a cada 24h, para obtenção do extrato. A atividade dos extratos foi avaliada através da técnica de difusão em poço. Nenhum dos isolados de fungos dermatófitos foi inibido no ensaio de dupla camada e os isolados 1S, R18(6) e 6(2) mostraram atividade frente todas as espécies de *Candida* testadas. No ensaio de difusão em poço com os extratos, o isolado R18(6) inibiu as seis espécies de *Candida* em 72h de crescimento em caldo AC e mostrou grande potencial para obtenção de composto com atividade antifúngica.
Palavras-chave: compostos biativos, *Candida* spp., fungos dermatófitos.

INTRODUCTION

The occurrence of fungal infections in humans has increased significantly in the past 30 years (Lass-Flörl 2009). Among the various antimicrobial agents currently marketed, antifungal substances comprise a comparatively small but nevertheless important group of medicinal drugs with a key role in the treatment of a variety of mycoses (Thakur *et al*. 2007). Compared to antibacterial agents, the number of antifungal compounds approved for use in humans is quite limited, a circumstance explained by the high toxicity of these substances to the hosts. However, it comes as no surprise that the use of antifungal drugs has led to the selection of resistant fungi populations. As described by Rex *et al.* (1995), resistant strains find their way to hosts in three colonization and infection scenarios: (i) exposure to an initially susceptible strain that subsequently mutates and becomes resistant; (ii) exposure to a number of strains of which one is resistant and eventually is the only one to thrive, resisting the presence of antifungal drugs in the host organism; and (iii) exposure to an inherently resistant strain. The escalating use of antifungal agents in long-term treatment strategies has raised the prevalence of fungus strains that are resistant to the most commonly prescribed antifungal agents (Selmecki *et al*. 2009). Additionally, the larger number of opportunistic infections in immunosuppressed patients, together with the growing resistance exhibited by these fungus species, poses a considerable challenge to the pharmaceutical industry in the search for safe and efficient antifungal drugs (Dhanasekaran *et al*. 2008).

Secondary microbial metabolites represent a major source of compounds with remarkable chemical struc-

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tures and potent biological activity (Arasu *et al*. 2009). Bacteria belonging to the actinomyctes group exhibit several physiological and metabolic attributes, such as the production of extracellular enzymes and of a variety of secondary metabolites (Ventura *et al.* 2007). Their apparently unlimited ability to produce bioactive compounds makes actinomycetes especially useful in the development of new medicinal drugs. Thousands of these compounds have been isolated and characterized, and several are used in the treatment of diseases in humans as well as in veterinary medicine and agriculture (Arasu *et al.* 2008). These microorganisms are responsible for the production of more than half of the commercially available bioactive compounds, including antibiotics, immunosuppressant drugs, antitumor agents and enzymes (Qin *et al.* 2009). Considering the importance of new active compounds against fungi, the aim of this study was to obtain actinomycete strains with the potential to produce bioactive compounds against dermatophyte fungi and *Candida* spp. with clinical importance.

MATERIALS AND METHODS

Samples

Twenty-five actinomycete isolates were used in the antagonism assay against six *Candida* spp. (*Candida albicans* – ATCC10231*, Candida glabrata* – CG01*, Candida krusei* – CK03*, Candida parapsilosis* – CP06*, Candida tropicalis* – CT08 and *Candida dubliniensis* - 24P), and 40 were used in the assay to test for activity against five dermatophyte fungi (*Epidermophyton floccosum* – EF31*, Microsporum gypseum* -MGY45*, Microsporum canis* – MCW3*, Trichophyton metagrophytes* – TMW1 and *Trichophyton rubrum* – TRW2). All actinomycetes used were provided by the Laboratory of Environmental Microbiology. Department of Microbiology, Immunology and Parasitology, Federal University of Rio Grande do Sul (UFRGS), Brazil. All fungal isolates used were isolated were obtained from the culture collections of the Laboratory of Applied Mycological Research, UFRGS.

Fungal susceptibility profiling

The disk-diffusion method was used to assess the susceptibility of *Candida* isolates to the antifungal agents fluconazole and nystatin (BIO-RAD, Brazil). The assay was conducted in accordance with the standards defined by CSLI M44-A (2004), with modifications. Mueller-Hilton agar with 2% glycose were used, in which a 0.5 McFarland scale cell suspension was inoculated. The antifungal were placed on the surface of the agar disk and incubated at 30 ºC for 24 h and 48 h. After incubation, all disks were analyzed.

Evaluation of antifungal activity

Antifungal activity was assessed using the double-layer method. Actinomycete isolates were inoculated using the spot-inoculation method onto plates with mineral salts-starch-casein-agar (SCA) medium (10.0 g starch, 0.3 g casein, 2.0 g K_2HPO_4, 2.0 g NaCl, 2.0 g KNO_3, 0.05 g $MgSO_4 .7H_2O$, 0.01 g $Fe_2(SO4)_3.6H_2O$, 15.0 g agar) and incubated at 30 ºC for 10 days. Dermatophyte fungus and yeast isolates were inoculated in Sabouraud dextrose agar and incubated at 30 ºC for seven days and 48 h, respectively. All assays were conducted so that the incubation periods of fungi and actinomycete isolates would end concurrently.

Fungi grown in Petri plates were used to prepare the spore suspensions. For that, 3 mL of 0.8% sterile saline was added to plates containing the dermatophyte fungi, and the spores were removed using a Drigalski loop. The mixture was transferred to sterile glass test tubes and the final spore concentration was adjusted to 5×10^4 spores/mL. Inoculums of yeast strains were standardized using the 0.5 McFarland scale (1.5×10^8 cells/mL). Of these suspensions, a 1 mL aliquot was retrieved and homogenized in 9 mL melted Sabouraud dextrose agar. This mixture was poured on the plates containing cultured actinomycetes. Plates were incubated at 30 ºC for seven days and 48 h, for filamentous fungi and yeasts, respectively. Then, the presence of inhibition halos was examined. The antibiosis index (AI) was calculated as the mean inhibition halo divided by the mean colony diameter (Rosato *et al*. 1981).

Submerged cultures

Actinomycetes with antifungal potential were selected to produce an active compound in submerged culture. Extracts were prepared in two steps. First, the pre-inoculum was prepared, in which the antagonist isolate was inoculated in 50 mL starch casein broth (SCB) and incubated for 48 h at 30 ºC with constant shaking at 100 rpm. After, a 5 mL aliquot was transferred to conical 250 mL vials containing 50 mL of the SCB medium. Cultures were grown for eight days under the same conditions as the pre-inoculum. Aliquots from the culture were retrieved every 24 h to assess antimicrobial activity; the aliquots were centrifuged for 10 min at 13,000 rpm to separate the cells and obtain a cell-free extract.

Antifungal activity of the crude extract

Antifungal activity of the crude extract was assessed using the well-diffusion method. By means of a sterile cork borer, wells were punctured in plates containing Sabouraud dextrose agar previously seeded with one of the *Candida* isolates. One hundred microliters (100 µL) of supernatant of each isolate was added in each well. The plates were incubated at 8-10 °C for 16 h for the diffusion of the bioactive compound, and then incubated at 30 ºC for 48 h. After the incubation period, inhibition halos were measured and the antibiosis index calculated.

RESULTS

Of the 25 actinomycete isolates used in the double-layer assay with the six *Candida* spp., 11 (44%) inhibited

growth of at least one of the yeasts used (Table 1) and no antagonism was recorded with dermatophyte fungi. Therefore, the search for active compounds against dermatophyte fungi was discontinued for the time being.

Of the 11 actinomycete isolates showing antifungal activity against yeast species, one inhibited only one (16.6%) *Candida* species, three inhibited the growth of three (50%) species, two showed activity against four (66.6%), and two others inhibited five (83.3%) species (Table 1). Three of the actinomycete isolates (R18(S), 1S and 6(2)) inhibited the growth of all six (100%) *Candida* spp. used (Table 1).

Actinomycete isolates R18(6), 1S and 6(2), which exhibited antifungal activity against the six *Candida* spp. used in the double-layer assay, were grown in submerged culture. The antifungal activity of the metabolic liquid was assessed according to the well-diffusion method. Isolates 1S and 6(2) did not inhibit the growth of yeasts when grown in SCB. Isolate R18(6) exhibited inhibition halos against all six *Candida* spp. (Fig. 1). As early as 24 h after the beginning of growth and until 96 h, the compound produced by isolate R18(6) inhibited the growth of *C. dubliniensis, C. parapsilosis, C. krusei* and *C. tropicalis*. Inhibition of *C. albicans* and *C. glabrata* was observed after 72 h of growth.

The fungal susceptibility profiling assay was carried out to evaluate the susceptibility of *Candida* spp. isolates to the antifungal agents fluconazole and nystatin, using the agar disk-diffusion assay. The results showed that five of the six *Candida* isolates were resistant to fluconazole. In contrast, five of the isolates exhibited intermediate susceptibility to nystatin (Table 2).

DISCUSSION

Fluconazole is a triazole antifungal agent that is well tolerated and safe, and its clinical action against most strains of *Candida* spp. is considered good. However, the increased use of this drug has led to the selection of resistant strains (Santos Jr. *et al*. 2005). Additionally, some species, such as *C. glabrata* and *C. krusei*, are naturally less susceptible to fluconazole (Dovigo *et al*. 2009). In the present study, only the isolate of *C. parapsilosis* was susceptible to fluconazole. Wingter *et al*. (2007) observed a 10% resistance to fluconazole and 2% resistance to nystatin in *Candida* spp. isolated from HIV+ patients. Loss of susceptibility to currently marketed drugs prescribed to treat infections caused by *Candida* spp. has been reported in various studies (Colombo *et al.* 2006, Mujica *et al.* 2004, Favalessa *et al.* 2010), and the observations in the present study concord with these findings.

Research on the biological properties of actinomycetes has shown that these bacteria are potential candidates for the production of antifungal compounds. Bachiega *et al.* (2005) reported that 20.3% (13/64) of the actinomycete isolates studied were active against *C. albicans*. More recently, Gandotra *et al.* (2012) observed that 33.3 (3/9) of *Streptomyces* spp. analyzed showed some degree of activity against *Candida* spp. isolates. Additionally, several studies have proved that actinomycetes exhibit antifungal properties, especially against *C. albicans*, with values ranging between 4.9% (101/2041) (Hong *et al.* 2009) and 12.5% (1/8) (Susithra *et al*. 2009). In the present study, the double-layer assay showed that 44% of the isolates (11/25) had an antifungal effect on the yeast strains tested. Compared to results reported elsewhere, this proportion is comparatively high, and shows good promise for the production of bioactive compounds that inhibit clinically important *Candida* spp.

None of the actinomycete isolates assayed exhibited activity against dermatophyte fungi. The search

Table 1. Antibiosis index values obtained in the double-layer assay for actinomycete isolates against six different *Candida* spp. isolates.

Actinomycetes	*C. dubliniensis*	*C. albicans*	*C. parapsilosis*	*C. krusei*	*C. glabrata*	*C. tropicalis*
1s	0.33(0)*	0.31(0.07)	0.81(0.07)	1.83(0.35)	0.42(0)	1.00(0.56)
R18(6)	1.28(0.14)	0.58(0.42)	1.50(0.35)	1.27(0.07)	0.93(0.07)	1.15(0.21)
6(4)	0	0.44(0.28)	0.11(0)	0.33(0.14)	0.36(0.21)	0.37(0.14)
R(11)6	0.78(0.35)	0.75(0.49)	0.23(0.14)	0.36(0.35)	0	1.87(0.35)
24(3)	0	0.33(0)	0	0.10(0.07)	0.55(0.07)	0.15(0.07)
2(4)	0	0.27(0)	0	0	0.33(0)	0.13(0.07)
6(2)	0.90(0.07)	0.80(0)	1.00(0.14)	0.50(0.07)	1.25(0.35)	0.53(0.28)
8(4)	0	0.33(0)	0	0	0	0
27(3)	0	0.29(0)	0	0.16(0.14)	0	0.22(0.07)
5(3)	0	0.30(0)	0	0	0.50(0.14)	0.07(0)
iso5(5)	1.30(0.07)	0.27(0.14)	0	0.50(0.14)	1.00(0.14)	0

*. Standard deviation.

Table 2. Results obtained with the fungal susceptibility assay for *Candida* isolates.

Samples	Fluconazole	Nystatina
C. dubliniensis	R	I
C. albicans	R	I
C. glabrata	R	S
C. krusei	R	I
C. parapsilosis	S	I
C. tropicalis	R	I

Abbreviations: R, resistant; I, intermediary; S, susceptible.

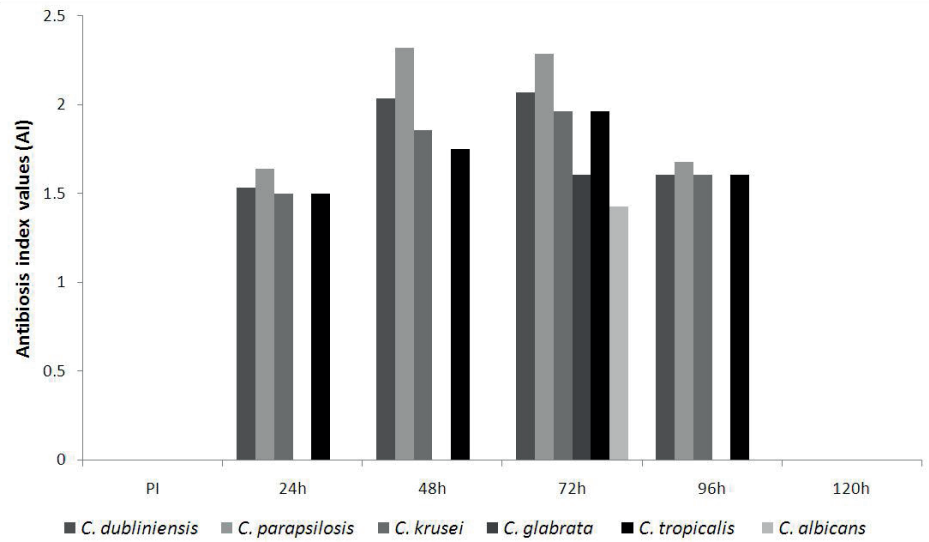

Figure 1. Antibiosis index (AI) obtained for isolate R18(6) in SCA broth at 30 ºC against six different *Candida* spp. isolates (PI, pre-inoculum).

for antifungal substances active against dermatophyte fungi has faced considerable obstacles. Augustine *et al.* (2005) tested 218 actinomycete isolates, and observed that only one (0.45%) exhibited activity against fungi of this group. Similarly, of the 100 isolates tested by Lakshmipathy and Kannabiran (2009), only 3% were active against dermatophyte fungus species. In turn, Deepa *et al.* (2011) isolated 30 actinomycetes from soils, of which five (16%) exhibited activity against *Microsporum* spp. However, these authors observed that, by sorting these bioactive compounds by thin-layer chromatography, the Rf values obtained were similar to those exhibited by commercially available antifungal compounds, which may indicate that these compounds are responsible for the antifungal action of these isolates.

Of the extracts produced in submerged culture, isolates 1S and 6(2) did not retain their antifungal activity as detected in the double-layer assay. Research has shown that the production of antibiotic compounds is more efficient in solid culture media, compared with submerged media, in which activity may decrease or even cease completely. Thakur *et al.* (2007) reported that, of 65 isolates that showed antibacterial activity in solid medium, 15 failed to do so in liquid medium. Similar results were described by other authors (Salamoni *et al.* 2010, Anibou *et al*. 2008). The production of antibiotic compounds in liquid media is generally low, and the detection of bioactive compounds requires high concentrations of them (Oliveira *et al.* 2010).

Isolate R18(6), when grown in SCA at 30 ºC, retained its antifungal activity observed in the double-layer assay, inhibiting the six *Candida* spp. used when inhibition was assessed for the 72-h growth period. However, analysis of the data obtained here (Figure 1) suggests that isolate R18(6) produces more than one bioactive compound, since it was only after 72 h of growth that *C. albicans* and *C. glabrata* were also inhibited by it, in addition to the other *Candida* spp., which may be due to the production of a second active compound. Previous studies have found more than one molecule with antimicrobial activity in bacterial isolates of the actinomycete group (Badji 2006, Boudjella *et al.* 2006, Smaoui *et al.* 2012).

The study conducted by Oliveira *et al.* (2010), using the same isolate R18(6), showed that it has potential in the biocontrol of phytopathogens (filamentous fungi and bacteria) that affect tomato plantations. In the present study, only R18(6) inhibited all *Candida* spp. used, when grown in submerged medium. Therefore, this isolate can be considered an efficient target for use in the production of antifungal compounds against *Candida* spp. with clinical importance, and further studies should be conducted to isolate and characterize its bioactive molecule.

ACKNOWLEDGEMENTS

The authors are grateful to CNPq and CAPES for the scholarship and grants that supported this work.

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