# Trailing end-point phenotype antibiotic-sensitive strains of *Candida albicans* produce different amounts of aspartyl peptidases

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## Abstract

*Candida albicans* is an opportunistic fungal pathogen that causes severe systemic infections in immunosuppressed individuals. *C. albicans* resistance to antifungal drugs is a severe problem in patients receiving prolonged therapy. Moreover, trailing yeast growth**,** which is defined as a resistant MIC after 48 h of incubation with triazole antifungal agents but a susceptible MIC after 24 h, has been noted in tests of antifungal susceptibility against some *C. albicans* isolates. In this context, we recently noticed this phenomenon in our routine susceptibility tests with fluconazole/itraconazole and *C. albicans* clinical isolates. In the present study, we investigated the production of cell-associated and secreted aspartyl peptidases (Saps) in six trailing clinical isolates of *C. albicans*, since this class of hydrolytic enzymes is a well-known virulence factor expressed by this fungal pathogen. Sap2, which is the best-studied member of the Sap family, was detected by flow cytometry on the cell surface of yeasts and as a 43-kDa polypeptide in the culture supernatant, as demonstrated by Western blotting assay using an anti-Sap1-3 polyclonal antibody. Released aspartyl peptidase activity was measured with BSA hydrolysis and inhibited by pepstatin A, showing distinct amounts of proteolytic activity ranging from 5.7 (strain 44B) to 133.2 (strain 11) arbitrary units. Taken together, our results showed that trailing clinical isolates of *C. albicans* produced different amounts of both cellular and secreted aspartyl-type peptidases, suggesting that this phenotypic feature did not generate a regular pattern regarding the expression of Sap.

Key words: *Candida albicans*; Trailing growth; Secreted aspartyl peptidases; Sap2

## Introduction

*Candida albicans* is commonly found on the mucosal surfaces of the gastrointestinal and genitourinary tracts in many healthy people. However, it can also cause opportunistic infections, especially in immunocompromised, cancer and transplant patients (1,2). *C. albicans* possesses a number of virulence attributes that enable the organism to cause disseminated infections in susceptible hosts and that also favor the persistence and colonization of the host tissues. Putative virulence factors of *C. albicans* include cell wall adhesins, phenotypic switching, hypha formation, thigmotropism, and secretion of hydrolytic enzymes, such as phospholipases and proteolytic enzymes (3,4). Among these virulence factors, enzymes with proteolytic activity continue to attract the attention of many investigators who study the pathogenesis of candidiasis. Secreted aspartyl peptidases (Saps), encoded by the *Sap* gene family with

10 members (*Sap1* to *Sap10*), appear to play a major role in *C. albicans* virulence during fungal infections (reviewed in Ref. 4).

The infections caused by *Candida* spp result in increased length of hospital stay and medical costs, which constitute an important public health problem (5). Fluconazole and other antifungal azoles have proved to be effective for the treatment of mucosal and systemic *Candida* infections; however, they lack fungicidal activity and treatment failures are common in severely compromised patients. Such failures have been associated with the emergence of azole-resistant strains of *C. albicans* during treatment, and resistance has been shown to be a direct consequence of the daily cumulative doses of azoles (6-8). *In vitro*, azoles not only fail to kill but, in a number of clinical isolates, also fail to truly suppress growth, resulting in trailing growth in broth microdilution assays. Among fungal pathogens, *in vivo* and *in vitro* acquired resistance to azoles and other drugs has been shown to be inducible and rapid but transient, and has not been associated with plasmids or other transferable genetic elements, but is thought to involve primarily mutations and genetic or epigenetic rearrangements (9,10). Interestingly, a positive correlation of peptidase activity with antifungal susceptibility was demonstrated in *C. albicans*. For example, *C. albicans* isolates from the HIV-positive group that were characterized by higher levels of aspartyl-type proteolytic activity were also less susceptible to the widely used azole antifungal agents ketoconazole and fluconazole (11). It has been thought that the proteolytic activity increases resistance to azole group antifungal agents especially in immunosuppressed patients (12).

Recently, Ribeiro (13) noticed the trailing phenomenon in routine susceptibility tests with fluconazole/itraconazole and *C. albicans* isolated from different hospitals from Rio de Janeiro, Brazil. In the present study, we report the measurement of cell-associated and released aspartyl-type peptidases in six clinical isolates of *C. albicans* that presented the trailing phenomenon in terms of fluconazole and itraconazole susceptibility *in vitro*.

## Material and Methods

### *Candida albicans* strains

The following *C. albicans* strains were employed in this study: *C. albicans* ATCC strain (code number 36802) and six clinical isolates (Table 1) from patients who were attended at the Laboratório de Microbiologia e Micologia, Instituto Estadual de Hematologia Arthur de Siqueira Cavalcanti (HemoRio), Rio de Janeiro, Brazil (13). In order to minimize the genetic changes over time, each strain was frozen in glycerol and stored at -80°C. These stocks were used for all experiments.

### Broth microdilution susceptibility testing method

The fungi were cultured on Sabouraud-dextrose (Oxoid, England) agar plates for 48 h at 37°C. The minimum inhibitory concentrations (MICs) of fluconazole and itraconazole for the *C. albicans* isolates were determined by the microdilution broth method (14) according to the guidelines of the Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards) (15), document M27-A. The MIC was read spectrophotometrically and the end point calculated as a 50% reduction in absorbance as compared to the growth in the control well. Trailing growth was defined as a resistant MIC after 48 h of incubation but a susceptible MIC after 24 h of incubation.

### Culture conditions and evaluation of cell growth

*C. albicans* strains were inoculated into 1.2% (w/v) yeast carbon base (YCB) medium (HiMedia Laboratories Ltd., India) supplemented with 0.1% (w/v) bovine serum albumin (BSA), pH 5.0, and grown at 37°C for 48 h under slight agitation (100 rpm), which is a well-known Sap-inducing condition (16,17). Cell growth was estimated by counting the yeast cells in a Neubauer chamber.

### Yeast and cell-free culture supernatants

The cultures were harvested by centrifugation at 4000 *g* for 10 min at 4°C and the spent culture media were filtered through a 0.22-μm membrane (Millipore, Brazil) and used to measure the extracellular peptidase activity. Alternatively, each cell-free culture supernatant was concentrated 25-fold in a 10,000-molecular weight cut-off Centricon micropartition system (AMICON, USA) to be employed in Western blotting assays. The same volume of YCB-BSA medium was also concentrated and used as a control (17). Conversely, yeast cells were washed twice in cold phosphate-buffered saline (PBS, 150 mM NaCl, 20 mM sodium phosphate buffer, pH 7.2) and immediately processed by flow cytometry analysis as described below.

### Protein profile

Cell-free culture supernatants of *C. albicans* strains were treated with an equal volume of SDS-PAGE sample buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol, and 0.002% bromophenol blue) containing 10% β-mercaptoethanol, followed by heating at 100°C for 5 min. Proteins (10 μg of each sample) were analyzed on 10% SDS-PAGE by the method described by Laemmli (18). Electrophoresis was carried out at 100 V for 90 min at 4°C, and the gels were silver-stained. Prior to electrophoresis, Gibco BRL (USA) molecular mass standards were boiled in SDS-PAGE sample buffer and then applied to the same gel.

### Quantitative proteolytic activity assay measured with BSA

Proteolytic activity was measured spectrophotometrically using BSA as substrate, according to the method described by Buroker-Kilgore and Wang (19) and modified by Silva et al. (20). Initially, each spent culture medium

was incubated for 20 min at 37°C in the presence or in the absence of 10 µM pepstatin A (Sigma, USA), an aspartyl peptidase inhibitor. To assay the remaining proteolytic activity, BSA (0.5 mg/mL), 20 mM sodium citrate, pH 4.0, and 40 µL of each culture supernatant were added to a microcentrifuge tube (350 µL) and incubated for 2 h at 37°C. After this incubation, three aliquots (100 µL each) of the reaction mixture were transferred to wells on a microtiter plate containing 50 µL of water and 100 µL of a Coomassie solution (0.025% Coomassie brilliant blue G-250, 11.75% ethanol, and 21.25% phosphoric acid). After 10 min to allow dye binding, the plate was read on a Thermomax Molecular Device microplate reader at an absorbance of 595 nm. One unit of proteolytic activity was defined as the amount of enzyme that caused an increase of 0.001 in the absorbance under standard assay conditions. The proteolytic activity is expressed as arbitrary units (20).

### Immunoblotting assay

The extracellular polypeptides present in the concentrated supernatant of each *C. albicans* strain were separated by 10% SDS-PAGE and then electrophoretically transferred at 4°C at 100 V/300 mA for 2 h to a nitrocellulose membrane. The membrane was blocked in 5% low-fat dried milk in TBS (150 mM NaCl, 10 mM Tris, pH 7.4) containing 0.5% Tween 20 (TBS/Tween) for 1 h at 25°C. The membrane was washed three times (10 min each) with TBS/Tween and then incubated with the anti-Sap1-3 polyclonal antibody raised against *C. albicans* (kindly provided by Dr. Nina Agabian, University of California, San Francisco, CA, USA) at 1:1000 dilution for 1 h (16). The secondary antibody used was peroxidase-conjugated goat anti-rabbit IgG at 1:5000. Immunoblots were exposed to X-ray film after reaction with ECL reagents for chemiluminescence (17).

### Flow cytometry analysis

The yeasts ($5.0 \times 10^6$ cells) used for these experiments were fixed at 4°C in 4% paraformaldehyde in PBS, pH 7.2, for 20 min, followed by extensive washing in the same buffer. These fixed cells maintained their morphological integrity, as indicated by light microscope observation. They were incubated for 1 h at 25°C with a 1:250 dilution of rabbit anti-Sap1-3 antibody, and then incubated for an additional hour with a 1:200 dilution of fluorescein-isothiocyanate-labeled goat anti-rabbit IgG. For flow cytometry analysis, these cells were examined in an EPICS ELITE flow cytometer (Coulter Electronics, USA) equipped with a 15-mW argon laser emitting at 488 nm. Untreated cells and those treated only with the anti-Sap and secondary antibody were used as controls. Each experimental population was then mapped by using a two-parameter histogram of forward-angle light scatter versus side scatter. The mapped population (N = 10,000) was then analyzed for log green fluorescence by using a single-parameter histogram (17).

### Statistical analysis

All the experiments, using the same batch culture of each strain, were repeated at least three times and each was performed in triplicate. Representative images of these experiments are shown. Data were analyzed statistically by the Student *t*-test and P values of 0.05 or less were considered to be statistically significant.

## Results and Discussion

Invasive *Candida* infections continue to cause major problems of morbidity and mortality in a diverse range of debilitated and immunocompromised hosts (21-23). There is concern about the possibility that the empirical use of fluconazole will lead to the emergence of fluconazole-resistant or fluconazole-tolerant *Candida* isolates. Moreover, the term "trailing" has been used to describe the reduced but persistent growth that some isolates of *C. albicans* and *C. tropicalis* exhibit at drug concentrations above the MIC in broth dilution tests with azole antifungal agents, such as fluconazole and itraconazole (24). This trailing growth can be so great as to make an isolate that appears susceptible after 24 h of incubation appear resistant at 48 h. In this respect, we recently noticed the trailing phenomenon in our routine susceptibility tests with fluconazole and itraconazole and *C. albicans* clinical isolates (13). The nature of the trailing phenomenon is unknown, as is its contribution to the perception of resistance *in vitro* and the ultimate possibility of *in vivo* resistance translation (25). It has been recently suggested that trailing may be due, at least in part, to the ability of *C. albicans* isolates to up-regulate, in response to drug exposure, the transcription of genes encoding the azole drug target, lanosterol demethylase (ERG11), the terbinafine target, squalene epoxidase (ERG1) or the azole and terbinafine efflux transporters (CDR1, CDR2 and MDR1) (25). These same mechanisms, as well as point mutations in ERG11, have been implicated in the development of azole drug resistance in *C. albicans* (26,27). Furthermore, a simple explanation for the interpretation of the trailing event is that adaptation to a toxic compound can force a longer lag phase in *C. albicans* growth, since adaptation is a time-spanning process.

**Table 1.** Characteristics of fluconazole/itraconazole trailing strains of *Candida albicans* used in this study.

| Code | Day of isolation (day/month/year) | Site of isolation | Patient information | |
|------|-----------------------------------|-------------------|---------|------|
| | | | Gender | Age |
| 2A | 02/10/2002 | Blood | Male | – |
| 9 | 21/03/2003 | Blood | Male | 36 years |
| 11 | 30/01/2003 | Blood | Male | 46 years |
| 44A | 29/10/2003 | Gastric washing | Female | 2 years |
| 44B | 29/10/2003 | Gastric washing | Female | 2 years |
| 78 | 12/10/2004 | Sputum | Male | 65 years |

*C. albicans* has developed several virulence mechanisms, which facilitate its invasion of host tissues and the evasion of host defense mechanisms. Virulence factors that contribute to this process are the hydrolytic enzymes, some of which are extracellularly released by the fungus and may play a central role in the pathogenesis of candidiasis. In the present study, we selected six trailing strains of *C. albicans* (Table 1, Figure 1) and a non-trailing ATCC strain (36802) in order to evaluate the activity of aspartyl peptidases, which are well-recognized virulence factors expressed during different phases of *C. albicans*-host interaction, including adhesion, nutrition, proliferation, differentiation, and escape of host immune response (reviewed in Ref. 4). Initially, we investigated the capacity of these seven *C. albicans* strains to grow *in vitro* in YCB medium containing BSA as the sole nitrogen source. Under these culture conditions, *Candida* strains might produce extracellular peptidases to degrade BSA molecules in order to obtain free amino acids. By
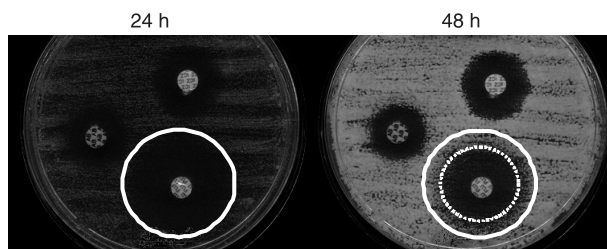
means of SDS-PAGE, we showed that all strains were able to hydrolyze soluble BSA at different intensity, generating low molecular mass polypeptides (Figure 2).

BSA cleavage during the growth of *C. albicans* denotes the production of surface and/or released proteolytic activity(ies). In this context, we measured peptidase activity in both cell-free culture supernatants and in intact yeast cells after growth of the trailing strains in YCB-BSA medium for 48 h. Our results demonstrated that yeasts secreted different amounts of pepstatin-sensitive protease activity into the extracellular environment, in which strain 11 was the highest producer, followed by 78, 2A and ATCC strains (Figure 3A). Strains 9, 44A, and 44B presented the lowest amount of secreted proteolytic activity (Figure 3A). As



**Figure 1.** Representative culture of trailing growth observed in *Candida albicans*. Strain 11 was tested against fluconazole, amphotericin B and 5-fluorocytosine. The white circles show the characteristic trailing growth, which is defined as a resistant MIC after 48 h (dotted circle) of incubation with triazole antifungal agents but a susceptible MIC after 24 h (full circle), in the presence of fluconazole after 24 and 48 h of incubation. The same result was observed when itraconazole and all other trailing strains were tested (data not shown).
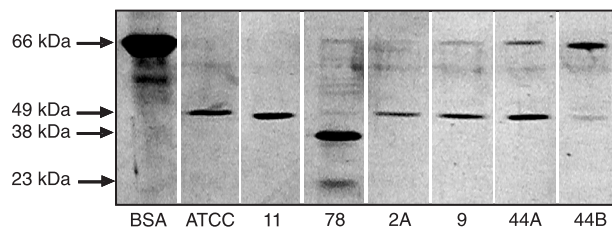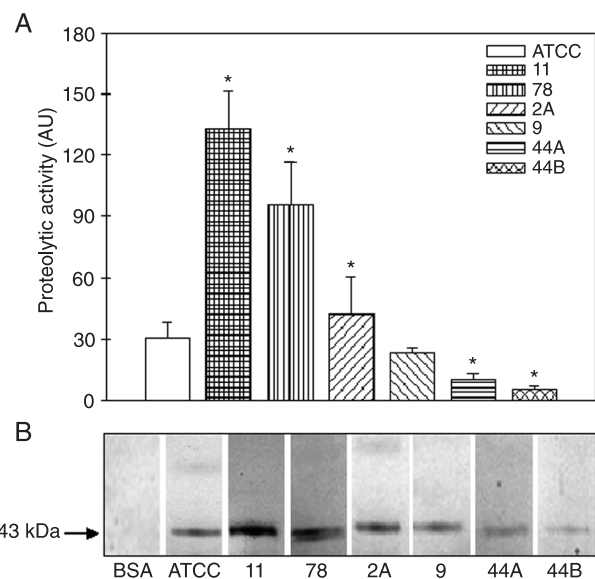


**Figure 2.** Albumin degradation profiles from different strains of *Candida albicans*. ATCC strain 36802 and six trailing clinical strains (designated 2A, 9, 11, 44A, 44B, and 78) were grown in YCB medium supplemented with 0.1% BSA (as the nitrogen source) at 37°C under slight agitation. After 48 h of growth, the cultures were harvested and filtered and the spent culture media were then analyzed by SDS-PAGE to demonstrate the cleavage of soluble BSA. A control in which the culture medium was collected before the yeast inoculation is shown (lane BSA). The gel was silver-stained and the numbers on the left indicate the relative molecular mass of standards, reported in kilodaltons (kDa).



**Figure 3.** *A*, Measurement of secreted aspartyl peptidase activity on BSA in clinical isolates of *Candida albicans*. After growth in YCB-BSA medium for 48 h at 37°C under slight agitation, the cultures were harvested and filtered and the spent culture media were then tested to degrade soluble BSA. Each cell-free culture supernatant was supplemented with BSA and 10 mM sodium citrate, pH 4.0, and subsequently incubated at 37°C for 2 h. The proteolytic activity was determined as described in Material and Methods and is reported as arbitrary units (AU). The values are reported as the means ± SEM of three independent experiments, which were performed in triplicate. Asterisks denote trailing clinical isolates that showed a rate of substrate hydrolysis significantly different from the ATCC strain (*$P < 0.05$, Student *t*-test). *B*, Western blotting analysis showing the detection of Sap protein in each *C. albicans* isolate. The proteins in the spent culture media were separated by SDS-PAGE and transferred to a nitrocellulose membrane. A control in which the culture medium was collected before the yeast inoculation is shown (lane BSA). The blot was sequentially incubated with rabbit anti-Sap1-3 polyclonal antibody at 1:1000, peroxidase-conjugated goat anti-rabbit IgG at 1:5000 and then developed with enhanced chemiluminescence reagents. The molecular mass of the reactive polypeptide, reported as kilodaltons (kDa), is given on the left.

expected, pepstatin A at 10 µM completely inhibited the extracellular peptidase activity measured in all *C. albicans* strains, showing that the peptidases produced belong to the aspartyl peptidase class (data not shown). Anti-Sap1-3 polyclonal antibody recognized, in a Western blotting assay, only one secreted polypeptide of 43 kDa in all *C. albicans* strains (Figure 3B). As previously reported by White and Agabian (16), the 43-kDa component corresponds to the Sap2 isoenzyme. Finally, flow cytometry analysis using the anti-Sap1-3 antibody detected a comparable level of surface aspartyl peptidase in all trailing strains, except for isolate 9 that presented half the amount of Sap (Figure 4).

The extracellular peptidases of eukaryotic microbial pathogens have attracted the attention of many laboratories because of their potential role in pathogenesis. They facilitate the penetration into the host organism and counteract its defense system. A variety of aspartic peptidases are also produced and secreted by *C. albicans*. Although there are several members of this family, probably individually expressed at various stages of the infection process and playing different roles, Sap2 appears to be the major secretory aspartic peptidase in log-phase *Candida* cells (16,28). Saps may play an important role in the pathogenicity of candidiasis and their hydrolytic activity probably has a number of possible functions in addition to the simple role of digesting molecules for nutrition (3,4,29). Sap2, as the best-studied member of this group of hydrolytic enzymes, contributes to host tissue invasion by digesting or destroying cell membranes and by degrading host surface molecules. There is also some evidence that Sap2 is able to attack cells and molecules of the host immune system to avoid or resist antimicrobial activity (reviewed in Ref. 4). Furthermore, Sap2 may also contribute to the pathogenesis of inflammatory mucosal lesions in an *in vitro* model of vaginal candidiasis based on reconstituted human vaginal epithelium, by inducing the up-regulation of epithelial proinflammatory cytokines (29).

Taken together, our results show that the 24-h incubation period of the standard antibiotic sensitivity test is not sufficient for the satisfactory growth of trailing strains. Compounding this problem, *C. albicans* resistance to antifungal drugs is a major problem in patients receiving prolonged therapy. Strains of *C. albicans* vary in virulence, but, as is the case for all yeast infections, the balance between transient or commensal colonization and parasitism is dependent on the physiological status of the host. We also demonstrated that trailing clinical isolates of *C. albicans* produced different amounts of both cellular and extracellular aspartyl-like peptidases, suggesting that this phenotypic
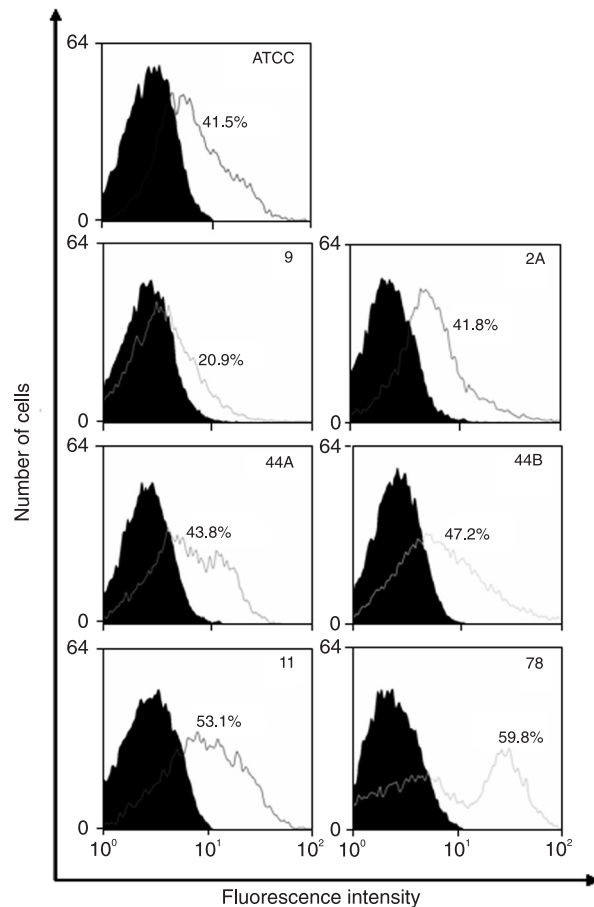


**Figure 4.** Flow cytometric analysis showing the anti-Sap1-3 antibody binding to *Candida albicans*. After growth in YCB-BSA medium for 48 h at 37°C under slight agitation, the yeast cells were washed in PBS, fixed in paraformaldehyde and then incubated in the absence (black curves) and in the presence (gray curves) of anti-Sap1-3 antibody at 1:250 dilution for 1 h. Subsequently, the cells were incubated for 1 h with a 1:200 dilution of FITC-labeled goat anti-rabbit IgG and examined in an EPICS ELITE flow cytometer. The numbers indicate the percentage of cells in each population that was marked with the anti-Sap antibody.

feature did not generate a regular pattern regarding the expression of Sap.

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