Proteolytic Activity of Clinical Candida albicans Isolates in Relation to Genotype and Strain Source

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Abstract
Proteolytic activity is regarded as one of the most important virulence factors of Candida albicans. Several authors recently demonstrated that some karyotypes and genotypes harbouring a group I self-splicing intron (CaLSU) located in the gene encoding the large rRNA subunit showed a high level of proteinase production. The aim of this study was to investigate the correlation between the level of proteinase production and the presence of the CaLSU intron in C. albicans isolates originating from the blood and respiratory tracts (sputum/pharyngeal swabs) of patients with and without oropharyngeal candidosis. The results revealed statistically significant differences in genotype distribution and the level of proteinase production between the C. albicans isolates obtained from blood and from the respiratory tract. Genotype A, without the intron, was prevalent in all groups of strains and its prevalence was higher among isolates from blood (75%) and from patients with candidosis (80%) compared with strains from colonisation (as opposed to infection) (57.8%). Isolates from blood produced significantly less proteinase than isolates from the respiratory tract (p<0.02), and this difference should be attributed to lower proteinase production of genotypes B and C from blood compared with genotypes B and C from the respiratory tract (p<0.01). The higher proteinase production of genotype B than of genotype A was found among respiratory tract isolates only. The presented data indicate that the association between proteinase production and the CaLSU intron depends on the strains’ population. Further study is needed on well-defined groups of clinical isolates to elucidate whether the observed diversity in proteinase production plays a role in the selection of strains inducing bloodstream infections.

Key words: Candida albicans; acid proteinase; group I self-splicing intron; rDNA-genotyping

Introduction

Candida albicans is an opportunistic microorganism widespread in the human population. It colonises the gastrointestinal tract of up to 70% of healthy adults and is the most frequent cause of superficial (mucosa- or skin-associated) as well as deep-seated, life-threatening mycoses. Proteolytic activity is regarded as one of the most important virulence factors of this pathogen (Schaller et al., 2005; Naglik et al., 2003). C. albicans produces at least ten iso-enzymes of aspartic proteinases (Sap1-10), of which Sap1-8 are extracellularly secreted (Hube et al., 1991; Monod et al., 1994; Monod et al., 1998). A contribution of the Saps to host tissue-invasion and the activation of inflammatory response has been postulated. The broad spectrum of Saps’ substrate specificity includes important host proteins, e.g. collagen, laminin, fibronectin, immunoglobulins, sIgA, and lactoferrin. Saps also digest some cell-surface antigens, enhance adhesion, participate in cell membrane damage, and induce inflammatory response via activation of interleukin-1β (Schaller et al., 2005; Naglik et al., 2003, Naglik et al., 2004). The expression of sap genes depends on environmental factors, e.g. pH, temperature, and presence of protein, as well as morphological transition (hypha, yeast) and switch phenotypes (opaque/white phenotype) (Hube et al., 1994). Clinical C. albicans isolates differ in the level of proteinase produced in vitro and may also show different patterns of sap expression. Elevated proteinase production has been correlated with strain invasiveness in vivo; for example, strains isolated from candidosis of the oral cavity secreted higher proteinase levels than strains isolated...
from the oral cavities of healthy individuals (Kuriyama et al., 2003; De Bernardis et al., 1992). It was demonstrated that the level of proteinase production may be connected with some genotypes of C. albicans. Taylor et al. (2005) and Tavanti et al. (2004) found that strains with distinct karyotypes differ in the level and pattern of Sap secretion. A very interesting finding is the association between a high level of proteinase production and a genotype harbouring a group I self-splicing intron (CaLSU) located in the gene encoding the large rRNA subunit (Sugita et al., 2002, Nawrot et al., 2004). C. albicans strains can be differentiated according to the presence of the CaLSU intron into three main genotypes, namely genotype A, without the intron, genotype B, harbouring the intron, and the heterozygous genotype C, possessing LSU rRNA genes with and without the intron in a single genome (Mercure et al., 1993; McCullough et al., 1999a; McCullough et al., 1999b). The intron is a target for base analogues (5-fluorocytosine), pentamidine, and also the anti-tumour glycopeptide bleomycin, which interfered with the self-splicing process (Mercure et al., 1993; Zhang et al., 2002; Jayaguru and Raghunathan, 2006). It is supposed that a high susceptibility to the factors mentioned above may be one of the reasons for the elimination of genotype B and its lower frequency among clinical isolates.

The CaLSU has been intensively studied by molecular biologist investigating the self-splicing phenomenon (e.g. Mercure et al., 1997; Zhang and Leibowitz, 2001; Xiao et al., 2005) and has also been used in many evolutionary and epidemiological studies as a valuable molecular marker (Lott et al., 1999; Pujol et al., 2002; Blignaut et al., 2002). Recently, several authors reported the distribution of intron-based genotypes in different groups of clinical isolates of C. albicans (Tamura et al., 2001, Millar et al., 2002; Karahan, 2004; Karahan and Akar, 2005; Qi et al., 2005; Millar et al., 2005; Girish Kumar et al., 2006), and some of them postulated association of genotype A with strain invasiveness.

In this study we tested the correlation between the level of proteinase production and the presence of the CaLSU intron in two groups of C. albicans isolates, one originating from blood and the second from the respiratory tracts of patients with haematological malignancies.

**Experimental**

**Materials and Methods**

**Strains.** The study was performed on 206 strains, of which 112 were C. albicans blood isolates obtained during the 2002 Belgian Candidemia Survey and preserved in the BCCM-IHEM Collection and 94 were clinical isolates obtained from the respiratory tracts (54 from throat swabs and 40 from sputum) of patients hospitalised in the Department of Haematology of Wroclaw Medical University in the years 2000–2002. Thirty of the respiratory isolates were obtained from patients with clinical signs of pharyngeal mycoses and 64 originated from non-symptomatic patients regarded as colonised. The isolates were identified using the ID32C tests (bioMérieux) and stored at −70°C.

**DNA extraction.** For DNA extraction, yeast cells were cultivated in YPG medium (2% yeast extract, 2% peptone, and 2% glucose) at 30°C for 18 hours. Cellular DNA was extracted according to Rose et al. (1990) with some modification. Briefly, cells were collected by centrifugation (1 min, 14 000 rpm) and resuspended in 200 µl of solution containing 0.5% zymolase in 50 mM Tris-HCl buffer (pH 7.5), 25 mM EDTA, and 1% mercaptoethanol. After 40 minutes of incubation at 37°C, 200 µl of lysis solution with 1% SDS and 0.2 M NaOH, 240 µl of 3 M sodium acetate was added. The mixture was briefly vortexed and centrifuged for 15 min (14 000 rpm, 4°C). The supernatant was transferred to a new microcentrifuge tube. The DNA was precipitated by adding an equal volume of isopropanol and collected by centrifugation (14 000 rpm, 4°C, 15 min). The DNA precipitate was washed with 70% ethanol, dried, suspended in 100 µl of water with RNase (300 µg/ml), and stored in −20°C.

**Detection of CaLSU by PCR.** The PCR assay was performed with the primer pair CA-INT-L (5'-ATA GGA GTA GTC GGC AAA ATA GAT CCG TAA-3') and CA-INT-R (5'CCT TGG CTG TGG TTT CGC AGG GAA GTC GGC AAA ATA GAT CCG TAA-3') and CA-INT-R (5'CCT TGG CTG TGG TTT CGC TAG ATA GTA GAT-3'), described previously by McCullough et al. (1999b). The reactions were performed with a DNA ENGINE PTC-200 thermal cycler (MJ Research, USA). DNA samples were denatured at 94°C for 3 min before 30 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 4 min with a final extension at 72°C for 4 min following the last cycle. The expected PCR products were single bands of size 450 bp and 840 bp for genotypes A and B, respectively, and two bands for genotype C (450 bp and 840 bp). Electrophoresis in 2% agarose gel visualised in UV light after ethidium bromide staining was used (Fig. 1).

**Detection of proteolytic activity.** For the proteolytic activity assay the Candida isolates were cultured on Staib’s medium with 5% casein at 28°C for 7 days. The supernatants were removed by centrifugation (3000 rpm, 30 min), adjusted to pH 6.5 (using 0.1 M NaOH), sterilised by filtration, and stored frozen at −20°C. Five hundred µl of 0.2 M sodium citrate-HCl buffer (pH 3.3) and 700 µl of 0.5% haemoglobin (used as a substrate) were added to 200 µl of the supernatant (for each strain in triplicate). After 1 hour of incubation at 37°C the reaction was stopped by adding...
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5% TCA. At the same time, control tests were carried out with buffer, TCA (added at the beginning of incubation), haemoglobin, and appropriate supernatant. After incubation, the samples were centrifuged at 4000 rpm for 30 min and the protein concentration was measured spectrophotometrically at 280 nm (Remold et al., 1968). One arbitrary unit of enzyme activity was defined as a 0.1 extinction increase at 280 nm and was calculated for 1 litre of medium. The proteolytic activities of the culture supernatants from five selected strains were additionally tested after exposure to different concentrations of pepstatin A (Sigma), an inhibitor of aspartic proteinases. The buffer and the culture supernatants were mixed with pepstatine A at final concentrations of 0.025–0.8 µM and incubated for 15 min at 37°C. After incubation, the substrate (haemoglobin) was added and the proteolytic activity was determine as described above.

Statistica for Windows (StatSoft, Inc., 1997) was used for statistical evaluations. Student’s t-test was used and differences were considered significant when the value of \( p \) was less than or equal to 0.05.

Results

The expected PCR products were obtained for genotypes A and B (single bands of size 450 bp and 840 bp, respectively), but for genotype C an additional band of 690 bp was observed (Fig. 1). To test the stability of the unexpected product, PCR with the DNA of the strains with genotype C was performed at annealing temperatures of 52.6°C, 59.6°C, and 68.6°C (Fig. 2). The changes in the annealing temperature did not result in increased intensity of the observed band. One of the genotype C isolates was found to display a band of 430–435 bp instead of the typical 450 bp.

The results of CaLSU-based genotyping are presented in Table I. In the investigated group of strains, genotype A, without the intron, dominated (145/206 strains, 70.4%), whereas the CaLSU was fully present (genotype B) in 41 (19.9%) strains and partially present (genotype C) in 20 (9.7%). Among the blood isolates, 84 were classified as type A (75%), 20 as type B (17.9%), and 8 as type C (7.1%). Of the respiratory tract (RT) isolates, genotype A was identified in 61 strains (64.89%), genotype B in 21 (22.3%), and genotype C in 12 (12.76%). When we analysed the strains isolated from infection and from colonisation separately, elevated percentages of genotype A (80%) and lower levels of genotypes B (10%) and C (10%) were observed among the strains obtained from infection. Statistically significant differences in genotype distribution were found between the isolates from blood and the isolates colonising the RT (\( p = 0.035 \)). The proteolytic activities of all the RT isolates and of 59 blood isolates (20 strains with genotype B, 8 with genotype C, and 31 randomly selected strains with genotype A) were tested. The proteolytic activities of the strains isolated from the blood ranged from 10 to 802 U/l (medium: 315.9 U/l) and were significantly lower than...
those obtained from the respiratory tract \((p<0.02)\) (Fig. 3). Comparison of strains of genotypes A, B, and C did not reveal statistically significant differences when we analysed isolates from blood and the total group (the sum of blood and respiratory tract isolates). Nevertheless, when analysing RT isolates we found statistically significant higher activity of genotype B \((p<0.05)\) than of genotype A. In addition, when comparing the activities of genotypes A, B, and C isolated from blood with the respective genotypes isolated from the respiratory tract, we observed significantly lower activities of genotypes B and C from blood \((p<0.005)\), but no significant differences in the activity of strains with genotype A \((p>0.5)\) were observed. Comparison of the activities of RT-infecting and RT-colonising isolates of \(C.\) albicans revealed no significant differences when the whole groups as well as particular genotypes were analysed \((p>0.2)\), but the differences between the activities of genotypes A and B and between A and C among the isolates from infection were significant (Fig. 4). Statistically significant differences were also found between the activities of RT-colonising strains and strains from blood \((p<0.01)\), between genotype B from RT (colonising as well as infecting strains) and genotype B from blood \((p=0.011\) and \(p=0.048\), respectively), and between genotype C from RT (colonising as well as infecting strains) and genotype C from blood \((p=0.0024\) and \(p=0.019\), respectively).

**Discussion**

The results presented in this study reveal statistically significant differences in the distribution of genotypes and in the level of proteinase production among \(C.\) albicans isolates from blood and from the respiratory tract. An association between the presence of the CaLSU intron and high proteinase secretion has been confirmed.

Genotype A was prevalent among all the tested groups of \(C.\) albicans strains, and the percentage of strains with genotype A was higher in blood isolates...
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(75%) and in strains infecting the RT (80%) compared with strains colonising the RT (57.8%). Similarly, Karahan et al. (2004) showed a prevalence of genotype A in strains isolated from blood and concluded that this intron-less genotype may be more invasive and more prone to induce blood-stream infection than genotypes carrying the intron. To our knowledge, all reports on blood isolates showed at least a 50% participation of genotype A, although the prevalence of this genotype was also reported in different, non-invasive groups of C. albicans isolates. For example, Qi et al. (2005) found genotype A in up to 90% of isolates from the oral cavities of healthy carriers, Karahan et al. (2004) found it in 44.6% of non-invasive clinical isolates, and in our previous study (Nawrot et al., 2004) we found 43–53% of genotype A among strains colonising the gastrointestinal tract in diabetic children. Taking all these reports into account, we suppose that in some populations there may be a selective pressure which favours genotype A colonisation and infection of the human body, especially invasion of the bloodstream. Very good candidates for such selective factors are anti-microbial and anti-tumour drugs, such as pentamidine and bleomycin, which showed antifungal activity almost exclusively to intron-harbouring strains.

The published data also demonstrate differences in the distribution of intron-possessing genotypes. McCullough et al. (1999a), analysing the global genotype diversity of C. albicans, showed an increasing prevalence of group C after 1990 (17%) compared with isolates collected before 1990 (5.5%). Currently, studies performed on local strain collections reported different participation of genotype C (e.g. 5.8% by Qi et al., 2005; 34.5% by Karahan et al., 2004). In our study we found genotype C in 7% of the invasive isolates and in 12.8% of the non-invasive, which strongly demonstrates that the divergence in the distribution of genotype C also depends on the infected body-site. According to the definition by McCullough et al. (1999b), genotype C strains are heterozygous, and PCR with primers flanking the fragment of rDNA including CaLSU yields two bands (450 bp without the intron and 840 bp with the intron). Recently, Qi et al. (2005) reported an additional, third PCR product. However, in our experiments we also observed the band described by these authors as a PCR product. Moreover, such a band of different intensity is also visible in most studies by other authors (e.g. McCullough et al., 1999b; Tamura et al., 2001; Karahan et al., 2004; Millar et al., 2001). To test whether the observed band is really a PCR product, we performed several PCRs at different annealing temperatures (Fig. 2), assuming that the amount of the product should be enhanced. Unfortunately, we did not observe increased intensity of this band, which rather suggests its artificial origin. What is interesting is that among the genotype C isolates tested in this study we found one with an atypical band of approximately 430 bp instead of 450 bp. Recently, Karahan et al., 2005, described eight subtypes of genotype A distinguished on the basis of sequence diversity and RFLP analysis. Because of an 18-bp insertion, four of these subtypes differed in the length of the PCR product. Our observations suggest that genotype C strains also show diversity in the discussed fragment of LSU rDNA.

![Fig. 4. Proteolytic activity of strains isolated from infection and from colonisation of the respiratory tract.](image-url)
Apart from genotype distribution, the investigated populations of *C. albicans* isolates showed significant differences in the level of produced proteinase. The isolates were cultured under the same conditions and underwent the same environmental pressure, so the levels of produced enzymes should be attributed to the micro-organisms’ properties. The blood isolates produced less enzyme than isolates from the RT and, moreover, strains with genotypes B and C from blood produced lower proteinase than genotypes B and C from the RT. According to Hube (Hube et al., 1994), Sap2 is the main iso-enzyme produced on protein-containing media in 30–37°C. Recent data (Kuryama et al., 2003) showed that particular strains cultured under such conditions often differ in the pattern and level of isoenzymes produced and that these differences are connected with karyotype. Previously we found a significantly higher level of proteinase production in genotype B isolates (Nawrot et al., 2004). In the current study, this correlation was observed only among the RT isolates, especially those from infection. These data indicate that the association between proteinase production and the CaLSU intron depends on the strains’ population. The CaLSU is regarded as a quite stable molecular marker, and its high frequency has been observed in some *C. albicans* clades (Lott et al., 1999; Pujol et al., 2002). Therefore, the association between the presence of the intron and proteinase production most probably reflects an association between karyotype and the regulatory mechanisms involved in the expression of *sap* genes. It is not clear if the observed differences in proteinase production may play a role in selecting strains inducing bloodstream infections. Further study is needed on well-defined groups of clinical isolates employing advanced molecular technology to learn about the epidemiological mechanisms and to characterise the properties of invasive *C. albicans* isolates.

### Literature


Pujol C., M. Pfaller and D.R. Soll. 2002. Ca3 fingerprinting of *Candida albicans* bloodstream isolates from the United States,
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