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Phospholipase B enzyme expression is not associated with other virulence attributes in Candida albicans isolates from patients with human immunodeficiency virus infection

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The extracellular phospholipases of Candida albicans are considered to play a significant role in the pathogenesis of human infections. Therefore 30 clinical isolates of C. albicans from human immunodeficiency virus (HIV)-infected individuals were screened for phospholipase production in vitro (using an egg-yolk-agar medium). Two groups of six isolates with positive (group A) or deficient (group B) phospholipase activity were then analysed for phospholipase B1 (PLB1) gene expression both in egg-yolk-agar and yeast extract/peptone/dextrose (YPD) broth media. A total of four virulence attributes of these two groups were in turn characterized, namely their germ-tube formation, cell-surface-hydrophobicity (CSH), adhesion to buccal epithelial cells (ABEC) and haemolysin production, and these factors were subsequently correlated with PLB1 expression. In the phospholipase-producing isolates (group A) a positive correlation was demonstrated between phospholipase production and the degree of PLB1 expression in YPD medium (r = 0.96, P < 0.01). No such association was observed in group A isolates for PLB1 expression in egg-yolk-agar medium. Further, PLB1 expression in egg-yolk agar was less than that in YPD medium, although a positive correlation was seen between the expression levels on regression analysis (r = 0.86, P = 0.026). Surprisingly, however, no significant associations were observed in either growth media between PLB1 expression and any of the four pathogenic attributes examined (P < 0.001). A significant correlation was seen between CSH and ABEC (r = 0.74) in group A isolates. The phospholipase-deficient group B, however, demonstrated a significant correlation between the latter parameters (r = +0.50) and also between germ-tube formation and ABEC (r = −0.59), and germ-tube formation and haemolysin production (r = +0.31). It appears that in oral C. albicans isolates in HIV infection there may be no significant association between the degree of PLB1 expression and other widely recognized major virulence attributes.

INTRODUCTION

Candida albicans is a commensal fungal pathogen that colonizes the mucosal surfaces of humans in both health (MacFarlane, 1990) and disease (McCullough et al., 1996). The virulence attributes that enhance this colonization, such as the secretion of extracellular enzymes and dimorphism, have been studied by many investigators (Calderone & Fonzi, 2001). In particular the secretion of extracellular phospholipases is considered a key attribute that aids invasion of the host mucosal epithelia (Leidich et al., 1998). The phospholipases in general catalyse the hydrolysis of phospholipids, which are major components of all cell membranes (Banno et al., 1985; Salyers & Witt, 1994). Although the literature contains contradictory reports on the number and specific types of phospholipase genes that may be linked to the virulence of C. albicans (Ghannoum, 2000), the PLB1 gene appears thus far to be the single most important contributory factor for phospholipase activity of C. albicans (Ibrahim et al., 1995; Leidich et al., 1998).

Abbreviations: ABEC, adhesion to buccal epithelial cells; BEC, buccal epithelial cell; CHEF, contour-clamped homogeneous electrophoretic field; CSH, cell-surface-hydrophobicity; HIV, human immunodeficiency virus.
Phospholipase B is encoded by two genes, *PLB1* and *PLB2* (Leidich *et al.*, 1998; Sugiyama *et al.*, 1999). Targeted mutagenesis of *PLB1* in *C. albicans* strain CAI-4 has shown partial or complete attenuation of virulence in mice and a significant reduction of phospholipase B activity in comparison to the parental wild-type strain (Ibrahim *et al.*, 1995). The reintroduction of a functional *PLB1* gene into the *plb1-null* mutant restored virulence to levels similar to those observed for the parental strain in both haematogenously disseminated and oral-intraesophageal infant mouse models of candidiasis (Mukherjee *et al.*, 2001). This suggests a pathogenic role for the phospholipase enzyme in host–fungal interactions.

It is also known that in clinical isolates of *C. albicans* higher levels of extracellular phospholipase activity correlate well with a number of other pathogenic attributes of the yeast, such as adhesion (Barrett-Bee *et al.*, 1985; Ibrahim *et al.*, 1995), invasion of host tissues (Pugh & Caswion, 1975) and greater germ-tube formation. While the foregoing studies have investigated the phospholipase activity in *C. albicans* isolates from non-compromised individuals, there is only one study that has reported the enhanced expression of extracellular phospholipase in *C. albicans* isolates in human immunodeficiency virus (HIV)-infected individuals (Ribeiro *et al.*, 2004). Hence the objectives of the current study were to (a) screen 30 oral isolates of *C. albicans* from HIV-infected individuals for phospholipase activity, and correlate enzyme production in *vitro* with phospholipase B1 gene (*PLB1*) expression in egg-yolk agar and yeast extract/pptone/dextrose (YPD) broth, and (b) determine the association, if any, between phospholipase B production and the following virulence attributes of *C. albicans*: germ-tube formation, cell-surface-hydrophobicity (CSH), adhesion to buccal epithelial cells (ABEC) and haemolysin production.

**METHODS**

*Candida* isolates and growth conditions. A total of 30 *C. albicans* isolates were obtained from three HIV-infected individuals (P3, P4 and P9) attending an out-patient AIDS clinic in Hong Kong during therapy sessions over a period of 1 year as described previously (Samaranayake *et al.*, 2001). The organisms were recovered using the oral rinse technique of Samaranayake *et al.* (1986) and were identified by the germ-tube test, growth at 45 °C, chlamydospore production, API 20C AUX assimilation tests (BioMérieux) and the phenotype defined using CHROMagar *Candida* plates (CHROMagar) (Odds & Bernaerts, 1994). In addition improved identification software for API20C biochemical profiles (APILAB Plus, BioMérieux) was used to exclude *Candida dubliniensis*.

The yeasts were stored in vials with multiple glass beads (Microbank, Gibco) and maintained at 4 °C during the experimental period. Purity of cultures was confirmed periodically by visualization of Gram-stained organisms and germ-tube test. The isolates were identified by the patient number and the isolate number in subscript.

**Determination and quantification of extracellular phospholipase activity by the egg-yolk-agar method.** *C. albicans* isolates were screened for their extracellular phospholipase activity by growing them on egg-yolk agar and measuring the size of the zone of precipitation by the method of Samaranayake *et al.* (1984). Briefly, the egg-yolk medium consisted of 13.0 g SDA, 11.7 g NaCl, 0.111 g CaCl2 and 10 % sterile egg yolk. The egg yolk was centrifuged at 500 g for 10 min at room temperature, and 20 ml of the supernatant was added to the sterilized medium. An 18 h culture of the test strain grown on SDA was harvested and suspended in 0.9 % NaCl to achieve an optical density of 0.5 at 520 nm using a spectrophotometer (Ultraspex III; Pharmacia LKB; Biochrom).

Extracellular phospholipase activity was detected as follows: 5 μl aliquots of the yeast suspension (approximately 10^8 yeast cells ml^-1) were inoculated onto the surface of the egg-yolk medium in quadruplicated samples, left to dry at room temperature and after incubation at 37 °C for 48 h the diameter of the precipitation zone around the colony was determined. Each experiment was carried out on two separate occasions.

The plates were read using an image analysis method (Quantimet 500+ Image Analysis System; Quantimet Image System, Leica Instruments) wherein the diameter of the colonies and precipitation zones were evaluated on a magnified scale. Phospholipase activity (so called Pz value) was determined by the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone (Samaranayake *et al.*, 1984). Thus, a Pz value of 1 indicates no activity, and less than one (<1) indicates the degree of phospholipase positivity.

**CHEF analysis for chromosomal DNA karyotyping.** *C. albicans* isolates grown in YPD broth overnight at 37 °C on a rotary shaker (150 r.p.m.) were harvested by centrifugation at 3500 g for 15 min and used in contour-clamped homogeneous electrophoretic field (CHEF) analysis. Chromosomal DNA for karyotyping by CHEF electrophoresis was extracted using Genomic DNA Plug kits (Bio-Rad) according to the manufacturer’s instructions. The chromosomes of each isolate were separated by the CHEF Mapper XA Electrophoresis System (Bio-Rad) as described by Espinel-Ingroff *et al.* (1999) with modifications. The electrophoretic conditions used were as follows: two-state mode with linear ramping factors; run time, 72 h, initial and final switching times, 30 min and 17 min, respectively; constant voltage of 3 V cm^-1; switching angle, 106°. *Hansenula wingei* and *Saccharomyces cerevisiae* chromosomes (Bio-Rad) were used as the molecular-size standard.

The resulting gel was stained with ethidium bromide for 30 min and destained in distilled water for 1 h. DNA bands were visualized and photographed under Gel Documentation system. Each specimen was analysed on at least two separate occasions. Since the results obtained from CHEF analysis demonstrated close genotypic relationships among the isolates from each individual patient we decided to develop a dendrogram using these results.

**Computer-assisted analysis of data and histogram and dendrogram generation.** The different banding patterns of CHEF fingerprinting patterns of multiple isolates of *C. albicans* were analysed using the Dendron 3.0 software program (Soll, 2000) (Technology Innovation Center). For an analysis of relationships among a number of strains the Dendron software program constructs histograms and dendrograms by the unweighted pair group method with the software program demonstrated close genotypic relationships among the isolates from each individual patient we decided to develop a dendrogram using these results.
grouped, with a branch-point corresponding to the \( S_{AB} \). The program then searches for the strain-strain or strain-unit pair with the next highest \( S_{AB} \) and groups them, with a branch point corresponding to that \( S_{AB} \). The process continues to include all strains. A unit can be two or more strains, and a branch-point for a unit-strain or unit-unit is determined by the mean \( S_{AB} \) between each member of the unit and another strain or unit. Therefore, branch-points involving a unit are not as accurate as \( S_{AB} \) values calculated for two strains.

In dendrogram construction, the data for two \( C. albicans \) isolates or two banding patterns (lanes A and B) can be represented by the binary values 0 and 1, where 0 indicates no band at a position and 1 indicates a band at that position. The similarity coefficient (\( S_{AB} \)) for the pair of strains, A and B, was calculated by the formula:

\[
S_{AB} = 1 - [\sqrt{(b + c)/(a + b + c)}]
\]

where \( a \) is the number of bands common to both lanes A and B (coded as 1,1), \( b \) is the number of bands in lane A with no counterpart in lane B (coded as 1,0) and \( c \) is the number of bands in lane B with no counterpart in lane A (coded as 0,1). The pair of strains with the highest \( S_{AB} \) value is grouped into a unit with a branch-point corresponding to its \( S_{AB} \) value.

**Selection criteria for \( C. albicans \) isolates to determine PLB1 expression and the study of virulence attributes.** Six isolates of \( C. albicans \) (Group A; P3,1, P3,2, P3,4, P3,5, P3,6, P3,7, P3,8) with low Pz values (high degree of phospholipase production) and six isolates (Group B; P3,1, P3,4, P3,5, P3,6, P3,7, P3,8) with a Pz value of 1 (phospholipase-negative) were chosen from the initial group of 30 isolates to (a) determine an association between Pz value and PLB1 gene expression in two growth media, i.e. egg-yolk agar and YPD broth, (b) study their phenotypic attributes, namely germ-tube production, CSH, ABEC and haemolysin production, and (c) determine any correlation between PLB1 gene expression and each of the virulence attributes studied.

**RT-PCR.** As RT-PCR is a highly sensitive and accurate method for studying the expression of low-copy-number genes we employed semi-quantitative RT-PCR to study the expression patterns of PLB1. For this investigation the 12 \( C. albicans \) isolates were grown in two different growth media: (a) egg-yolk-agar medium, in which phospholipase production was determined, and (b) YPD medium (1% yeast extract, 2% peptone and 2% glucose).

(a) Egg-yolk-agar medium: \( C. albicans \) cells \((1 \times 10^8 \text{ cells ml}^{-1})\) were grown on egg-yolk-agar plates at 37 °C for 5 days, at which time the cells were harvested for total RNA extraction.

(b) YPD medium: For the extraction of the RNA the \( Candida \) isolates were grown in YPD broth (Difco) for 16 h at 37 °C and 150 r.p.m. After the incubation period the resultant culture was diluted 1 : 50 in YPD broth and grown to an optical density of 0.6–1.0 at 520 nm for a further period. The culture was then centrifuged at 14000 g and the pellet directly used for total RNA extraction. Total RNA was extracted from the 12 \( C. albicans \) isolates by SV Total RNA isolation system (Promega) according to the manufacturer’s instructions.

The conditions for RT-PCR were optimized with regard to the amount of RNA in the RT reaction, the amount of cDNA in the PCR and the ratio of primer pairs in the PCR. The number of amplification cycles was chosen within the linear range of the reaction. cDNA was synthesized in ratio of primer pairs in the PCR. The number of amplification cycles was of RNA in the RT reaction, the amount of cDNA in the PCR and the

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The RT-PCR was performed in a reaction of 50 μl containing 50 mM KCl, 20 mM Tris/HCl, 1.5 mM MgCl2, 200 μM of dNTPs, 50 pmol of

**Genomic PCR.** Genomic DNA from \( C. albicans \) isolates was extracted by a Wizard Genomic DNA Purification kit (Promega) according to the manufacturer’s instructions. The primers used were PLB1-1 and PLB1-2 and EFB1F and EFB1R, the PCR conditions were identical to RT-PCR and the cyclic parameters included 5 min of denaturation at 94 °C and then 24 cycles of 95 °C for 2 min, 55 °C for 2 min, and final extension at 72 °C for 10 min.

The cyclic parameters for PCR amplification with primers PLB1-3 and PLB1-4 were 5 min of denaturation at 94 °C and then 35 cycles of 95 °C for 2 min, 50 °C for 2 min and 72 °C for 2 min, and final extension at 72 °C for 10 min, and the PCR conditions were identical to RT-PCR. The PCR products were examined on a 1.5% agarose gel with ethidium bromide staining. The gel image was captured with a CCD camera (Bio-Rad). The ratio of phospholipase B to \( EFB1 \) gene expression was determined by the Quantity One Quantification Software (Bio-Rad).

**Dermatophyte assays.** Dermatophyte production in \( C. albicans \) was measured by using a slightly modified method described by Ibrahim et al. (1995). A germ tube is defined as a blastospore having a slender, cylindrical apical extension at least one blastospore diameter in length. Briefly, yeast grown overnight in an SDA plate were harvested and a cell suspension of 5 × 10⁷ cells ml⁻¹ was prepared in sterile PBS. A 250 μl quantity of the yeast cell suspension was added to 500 μl of fetal calf serum (Globepharm) and incubated at 37 °C for 2 h. The tube was then vortexed (Thermolyne Maxi Mix II type 37600 mixer) for 10 s.

A total of 300 cells seen in contiguous fields were then examined at ×400 magnification using a drop of the cell suspension placed in an improved Neubauer haemocytometer chamber (Hawksley). Yeast cells with a germ tube that had no constriction at the junction between the cells were considered as germ tube-positive. Any remaining yeast cells clumped with germ tubes and pseudohyphae were excluded. The percentage of germ tube-positive cells was then calculated. The experiment was repeated on three separate occasions with duplicate determinations.

**Cell-surface-hydrophobicity.** The CSH of \( Candida \) cells was measured using the method described by Sweet et al. (1987). Briefly, yeast isolates were grown on SDA for 18 h at 37 °C and a loopful of this culture was transferred into 10 ml of saliva ion buffer (Clark et al., 1978) and washed twice. Saliva ion buffer was prepared by dissolving the following
constituents in 1:51 of distilled water: 1 mM K$_2$HPO$_4$ (0.136 g), 50 mM KCl (3.727 g), 1 mM CaCl$_2$ (0.111 g), 0.1 mM MgCl$_2$ (0.02 g). This solution was filtered and stored in sterile 250 ml flasks and used in the experiments within 1 week of preparation. The yeast suspension thus prepared was then resuspended in saliva ion buffer to an absorbance of 1.0 ± 0.2 measured at 520 nm.

For each organism tested, 5 ml of suspension was added to two glass test tubes, representing one test and one control. In addition, a test and a control were prepared from the suspending medium alone as spectrophotometer blanks. One millilitre of xylene was added to each test and control were prepared from the suspending medium alone as spectrophotometer blanks. One millilitre of xylene was added to each test suspension. The test and the controls were placed in a water bath at 37 °C for 10 min to equilibrate, then taken in turn and vortex-mixed for 30 s and returned to the water bath for a further 30 min to allow the immiscible xylene and aqueous phases to separate. The lower aqueous phase of the sample was removed carefully using a pipette and transferred to a clean test tube. Any traces of contaminating xylene that may have formed. The hydrophobicity was expressed as the percentage reduction in optical density of the test suspension compared to the control. Thus, the greater the change in absorbance, the greater the shift in yeasts from the bulk medium to the interface, i.e. the more hydrophobic the yeast isolate. The assay was conducted in duplicate on three separate occasions for each yeast tested. Suspensions without xylene were used as the negative controls.

Adhesion to buccal epithelial cells. The candidal adhesion assay was conducted as described by Kimura & Pearall (1978). Human buccal epithelial cells (BECs) were used for the adhesion assay. The cells were collected by gently rubbing the cheek mucosa of four healthy adult volunteers (two males, two females; age range, 25–30 years) with sterile swabs and then resuspending the cells by rotating the swabs in 10 ml PBS. To obviate variations in quality, the BECs were collected between 10:00 and 11:00 am (after breakfast and routine oral hygiene). The individual samples were then washed twice in PBS (by centrifugation at 2500 g for 10 min each time), pooled immediately and resuspended to a concentration of 1 × 10$^6$ cells ml$^{-1}$ in PBS using haemocytometer quantification. The donors were healthy and not taking any medications including antibiotics.

To explain the method briefly, equal volumes of BECs (1 × 10$^6$ cells ml$^{-1}$) and yeast cell suspension (1 × 10$^5$ cells ml$^{-1}$) were mixed and incubated at 37 °C for 1 h on a shaking incubator (Lab-Line Orbit Incubator-Shaker, Melrose) at 75 r.p.m. The cells were then filtered with a manifold filter apparatus through 12 μm pore polycarbonate filters (Millipore). The filters were washed with 70 ml of PBS to get rid of unattached yeasts, removed carefully and then pressed gently onto glass slides cleaned with chromic acid. Afterwards the filters were carefully peeled off, thus leaving most of the BECs and the attached yeasts on the glass slide. The cells were air-dried and then stained by Gram’s method. The number of yeasts attached to 50 epithelial cells was counted microscopically at a magnification of ×400. Counting was undertaken randomly without prior knowledge of the source of the sample, and only uniform, unfolded epithelial cells were included.

Haemolysin production. Haemolysin production was determined by the modified plate assay described by Luo et al. (2001). In brief, a loopful of the stock culture was streaked onto SDA and incubated at 37 °C for 18 h. The resultant cultures were harvested and washed with sterile saline, and a yeast suspension with an inoculum size of 10$^8$ cells ml$^{-1}$ was prepared using an improved Neubauer haemocytometer chamber (Hawksley). Ten microlitres of this suspension was spot inoculated on a sugar-enriched sheep blood agar medium so as to yield a circular inoculation site of about 5 mm in diameter. The median was prepared by adding 7 ml of fresh sheep blood (Haemostat, Dixon) to 100 ml of SDA supplemented with 3 % glucose (final concentration, w/v; Merck). The final pH of the medium was 5.6 ± 0.2. The plates were incubated at 37 °C in 5 % CO$_2$ for 48 h.

The presence of a distinct translucent halo around the inoculum site, viewed with transmitted light, indicated positive haemolytic activity. The diameters of the zones of lysis and the colonies were measured with the aid of a computerized image analysis system (Quantimet 500 Qwin; Leica) (Luo et al., 2001) and the ratios (equal to or larger than 1) were used as a haemolytic index to represent the intensity of the haemolysin production by different isolates of Candida. The assay was conducted in quadruplicate on two separate occasions for each yeast isolate tested.

Statistical analysis. Statistical analysis was conducted by ANOVA (SPSS; Statistical Package for Social Sciences, Version 10.1) to determine significant differences in extracellular phospholipase production between the 30 C. albicans isolates obtained from three HIV-infected individuals. Simple linear regression analysis was used to determine the relationship between (a) Pz value and PLB1 gene expression in two growth media and (b) PLB1 gene expression in egg-yolk-agar and YPD media.

The results were also analysed using the Student’s t-test, in particular to determine significant differences of phospholipase production if any between the Candida isolates belonging to the two groups, A and B. Post hoc (Student-Newman-Keuls) tests were carried out to investigate significant differences in the two groups of Candida isolates for each of the virulence attributes studied. Spearman correlation analysis was used to determine associations between PLB1 gene expression and the different pathogenic attributes studied.

RESULTS AND DISCUSSION

Extracellular phospholipase production by C. albicans

In the current study, of the 30 isolates of C. albicans tested, 24 (80 %) were phospholipase-positive according to the plate assay. The range of Pz values observed was 0.15–1.0 (Pz value of 1 indicates no detectable phospholipase activity) (Fig. 1). The overall mean Pz for the group of isolates was 0.506, with no significant statistical difference in phospholipase production between the 30 C. albicans isolates. A similar result (79 %) was reported for 41 oral C. albicans isolates by Samaranayake et al. (1984) using an identical plate assay. A marginally higher phospholipase positivity (89 %) was also reported for clinical oral C. albicans isolates by Kothavade & Panthaki (1998), while Kantarcioğlu & Yucel (2002) observed that 56 of the 60 (93.3 %) clinical C. albicans strains isolated from the oral cavity, respiratory tract, blood and urogenital systems of non-immunocompromised individuals were phospholipase producers. These data imply that a vast majority of clinical C. albicans isolates are phospholipase producers.

In a recent study Ribeiro et al. (2004) showed using 239 oral and vaginal C. albicans strains from HIV-positive patients that significantly higher quantities of phospholipase were produced in isolates from this group than from HIV-negative individuals. Furthermore, isolates obtained from HIV-positive patients who were not on anti-retroviral therapy produced higher levels of the enzyme than those from HIV-
positive women using HAART (highly active antiretroviral therapy) with protease inhibitors (Ribeiro et al., 2004). Others have also reported differences in extracellular phospholipase activity in Candida from different ecological sites or disease states. For instance, Ibrahim et al. (1995) compared 11 blood isolates and an equal number of commensal isolates and reported higher activity in blood isolates. Furthermore, two recent publications have documented that C. albicans isolates from respiratory tract infections produce significantly larger amounts of phospholipase than those from blood (Borst & Fluit, 2003; Ribeiro et al., 2004). These data in general indicate that the isolation site of C. albicans as well as the disease state of the patient may be an important factor in dictating phospholipase activity.

Genetic relatedness among the tested C. albicans isolates

In order to determine the genetic relatedness between the yeast isolates, a factor which may confound the phenotypic association data, we conducted CHEF analysis of all 30 isolates. Variations of the chromosome band patterns were seen for all C. albicans isolates using this technique. As there were differences in the intensities of the generated bands and variations in the chromosomal separation profiles (Fig. 2A–C) we further evaluated the information using a dendrogram based on $S_{AB}$ values generated for the group of 30 C. albicans isolates (Fig. 3). This clearly indicated genetic heterogeneity among this group of Candida isolates and only two isolates, both from patient P4 (P4.5 and P4.8), were genetically identical.

Detection of phospholipase activity using egg-yolk agar

The egg-yolk-agar plate method for the detection of phospholipase activity in C. albicans was first described by Price et al. (1982). Since then the extracellular phospholipases of C. albicans isolates have been detected by a number of investigators using this methodology (Samaranayake et al., 1984; Ibrahim et al., 1995; Borst & Fluit, 2003). However, this method has drawbacks as C. albicans may also contain lipase in addition to phospholipase. Fu et al. (1997), using a S. cerevisiae strain transformed with a C. albicans genomic library, demonstrated the presence of a lipase gene using the egg-yolk medium of Price et al. (1982). Furthermore, since egg yolk contains large amounts of triglycerides and cholesterol apart from phospholipids (Fu et al., 1997), this method cannot be considered as an exclusive test for detecting phospholipase activity. Therefore the absence of a significant difference in phospholipase production amongst the majority of isolates that we noted could either be due to the poor sensitivity of the agar plate method or alternatively reflect relatively low variations in enzyme production amongst this group of isolates from an HIV-infected cohort.

For the foregoing reasons and to reconfirm and characterize enzyme production in more detail we further studied the degree of phospholipase B gene (PLB1) expression in a select group of six C. albicans isolates that exhibited the highest extracellular phospholipase activity levels (group A) and in an identical number that were apparently void of the enzyme (group B) as determined by the plate assay method. For this purpose we used a semi-quantitative RT-PCR technique to amplify PLB1 homologous fragments using mRNA extracted from the 12 C. albicans isolates grown in an egg-yolk-agar medium.

Comparative analysis of PLB1 expression in phospholipase-positive and -negative C. albicans isolates in egg-yolk agar and YPD broth medium

Egg-yolk agar. As described in Methods, all primers designed for this experiment were from the conserved regions of the published PLB1 sequence. RT-PCR analysis of total RNA of C. albicans with primers PLB1-1 and PLB1-2 yielded an amplicon that was of the expected size (808 bp) and had the expected characteristics with regard to the location of primers and the published sequence (U59710) of PLB1 gene. Further, a BLAST search of the nucleotide sequence of the PCR products resulted in complete identity with the published sequence of PLB1 and a high-probability
match with the PLB1 gene of other fungi, confirming that the amplified gene was indeed *C. albicans* PLB1.

To accurately evaluate the degree of PLB1 expression, a house-keeping gene, elongation factor *C. albicans* (EFB1), was used as an internal mRNA control (Schaller *et al.*, 1998) in the same PCR mix, and the expression of PLB1 was measured against the internal control. In order to detect the contamination of genomic DNA in RT-PCR, the primers were designed to amplify the region of the EFB1 gene containing the intronic region (Schaller *et al.*, 1998). RT-PCR generated a 526 bp band for EFB1 cDNA, but not a

Fig. 2. Electrophoretic karyotypes of three groups of ten *C. albicans* isolates (numbered 1–10) obtained from each of the three HIV-infected individuals P3 (A), P4 (B) and P9 (C). M1, *S. cerevisiae*; M2, *H. wingei*; ATCC, *C. albicans* ATCC 90028.
861 bp band, which is the amplification product of \textit{EFB1} genomic DNA, indicating the absence of DNA contamination in the mRNA samples used (Fig. 4). The RT-PCR results indicated two different expression patterns for \textit{PLB1} and a similar degree of \textit{EFB1} expression for all the \textit{C. albicans} isolates tested. \textit{Candida} isolates in group B did not exhibit \textit{PLB1} expression, while isolates in group A showed almost uniform \textit{PLB1} expression (Fig. 4).

As the disparity in \textit{PLB1} gene expression in these two groups of isolates could be due to a nucleotide polymorphism in the locus that corresponded to the 3' end of \textit{PLB1-1/PLB1-2} (that could subsequently affect RT-PCR), we designed another set of primers according to the published \textit{PLB1} sequence (U59710) as described in NCBI. RT-PCR experiments were thus repeated with identical cDNA derived from groups that yielded both positive and negative results with the first set of primers. The results indicated that the isolates that showed no \textit{PLB1} expression with the second set of primers (PLB1-3 and PLB1-4) were also negative for the first set of primers (PLB1-1 and PLB1-2) and vice versa (Fig. 5).

Since the results for \textit{PLB1} gene expression in egg-yolk-agar medium were not definitive we further analysed the \textit{PLB1} gene expression in YPD medium using the same methodology described above. These results are shown in Figs 6 and 7. On regression analysis it was evident that the Pz value (plate method) correlated well with the degree of \textit{PLB1} expression in the YPD medium ($r = 0.96$, $P < 0.01$) (Fig. 8), whereas no such association was observed for \textit{PLB1} expression in egg-yolk-agar medium and the Pz value. The findings indicate that the \textit{PLB1} gene is better expressed in YPD medium than in egg-yolk-agar medium. Alternatively, as RT-PCR is a very sensitive method, it may be that \textit{PLB2} or other phospholipase B genes could also be expressed simultaneously and the activity of their corresponding proteins was detected in the experiments.

Furthermore, when \textit{PLB1} gene expression in both egg-yolk-agar and YPD media was assessed semi-quantitatively using the Quantifier software, the results indicated that in YPD medium (Table 1), and on regression analysis a positive correlation ($r = 0.8651$, $P = 0.026$) between \textit{PLB1} expression in the two growth media was observed (Fig. 9). A study by Hoover et al. (1998) demonstrated that \textit{C. albicans} virulence in HIV infection and C. \textit{albicans} virulence in HIV infection.
PLB1 expression is likely to be regulated by growth parameters such as pH and temperature of YPD medium and also the morphogenetic transition of the yeast. In the current study C. albicans isolates were grown for 5 days in egg-yolk agar while in comparison the yeasts were grown in YPD medium only for a short period of about 20 h. We also observed that PLB1 expression was more defined with derivatives from YPD medium growth than from the egg-yolk agar medium, an observation confirmed by gel analysis (Figs 4 and 6). A further disadvantage of the egg-yolk-agar medium was that phospholipase-positive isolates grew well in comparison to phospholipase-negative isolates with poor growth. Therefore phospholipase-negative C. albicans isolates had to be harvested from a large number of egg-yolk-agar plates for the extraction of mRNA. Consequently it could be argued that for direct comparison of mRNA levels in phospholipase-positive and -negative isolates YPD medium is better than egg-yolk agar.

**Table 1. PLB1 gene expression and in vitro phospholipase production levels in six C. albicans isolates measured using quantification software**

<table>
<thead>
<tr>
<th>Candida isolate no.</th>
<th>PLB1 gene expression*</th>
<th>Phospholipase activity (Pz)†</th>
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<tbody>
<tr>
<td></td>
<td>Egg-yolk agar</td>
<td>YPD medium</td>
</tr>
<tr>
<td>P3-1</td>
<td>0.95</td>
<td>2.08</td>
</tr>
<tr>
<td>P3-2</td>
<td>1.07</td>
<td>1.74</td>
</tr>
<tr>
<td>P4-1</td>
<td>0.76</td>
<td>1.14</td>
</tr>
<tr>
<td>P9-1</td>
<td>0.93</td>
<td>2.36</td>
</tr>
<tr>
<td>P9-2</td>
<td>1.30</td>
<td>4.02</td>
</tr>
<tr>
<td>P9-3</td>
<td>1.68</td>
<td>3.85</td>
</tr>
</tbody>
</table>

*Measured in comparison to the control gene, EFB1. Results are mean values of two experiments.
†Determined by the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone when grown in egg-yolk agar. This assay was conducted on two separate occasions with quadruplicate samples. Values are presented as mean ± SD.

**Fig. 5.** RT-PCR of PLB1 gene expression in C. albicans isolates grown in egg-yolk-agar medium using primers PLB1-3 and PLB1-4. P3, P4 and P9 are three HIV-infected individuals and subscript numbers indicate the isolate number from each patient. P3-1, P3-2, P4-1, P9-1, P9-2 and P9-3 are phospholipase-positive strains, while the other six are phospholipase-negative. The two right most lanes show two controls: one positive, C. albicans ATCC 90028, and one negative, H2O. M, marker lane.

**Fig. 6.** Semi-quantitative RT-PCR of PLB1 gene expression in the 12 C. albicans isolates grown in YPD broth using primers PLB1-1 and PLB1-2, and EFB1F and EFB1R as internal controls. P3, P4 and P9 are three HIV-infected individuals and subscript numbers indicate the isolate number from each patient. P3-1, P3-2, P4-1, P9-1, P9-2 and P9-3 are phospholipase-positive strains, while the other six are phospholipase-negative. The two right most lanes show two controls: one positive, C. albicans ATCC 90028, and one negative, H2O. M, marker lane.

**Fig. 7.** RT-PCR of PLB1 gene expression in C. albicans isolates grown in YPD broth using primers PLB1-3 and PLB1-4. P3, P4 and P9 are three HIV-infected individuals and subscript numbers indicate the isolate number from each patient. P3-1, P3-2, P4-1, P9-1, P9-2 and P9-3 are phospholipase-positive strains, while the other six are phospholipase-negative. The two right most lanes show two controls: one positive, C. albicans ATCC 90028, and one negative, H2O. M, marker lane.
Regression analysis demonstrated a significant positive correlation between these two parameters ($r = 0.96, P < 0.01$).

On further analysis of the genomic DNA of the phospholipase-positive and -deficient isolates, using the primer combination PLB1-1 and PLB1-2, the presence of the $PLB1$ gene expression (in comparison to a control $EFB1$ gene). Regression analysis demonstrated a significant positive correlation between these two parameters ($r = 0.86, P = 0.026$).

Since $PLB1$ was not expressed in the phospholipase-negative isolates irrespective of the growth medium (YPD or egg-yolk-agar) we conducted an essential further control experiment to demonstrate that the detection of the $EFB1$ fragment in the earlier experiments (Figs 4 and 6) was not due to contamination (of genomic DNA or cDNA from other sources). To determine this we once again repeated reverse transcription on all isolates without the addition of the reverse transcriptase enzyme and then carried out PCR using $EFB1$ primers (EFB1F and EFB1R). The results did not demonstrate any amplified products, confirming the purity of the RNA samples (no contamination with genomic DNA or cDNA from other sources) obtained from the 12 test $Candida$ isolates (data not shown).

DNA expression in $C. albicans$

Furthermore, we are unable to provide a definitive explanation for the variable expression of the genomic DNA seen in the 12 $Candida$ isolates (Fig. 10). However, since the DNA was extracted from all 12 isolates in an identical manner (by Wizard Genomic DNA Purification kits) this discrepancy is unlikely to be due to the quality or the quantity of the extracted DNA samples. To further confirm this result we investigated the expression of another house-keeping gene $EFB1$ (891 bp) on genomic DNA of both the ‘negative’ and ‘positive’ $Candida$ isolates using the same experimental conditions (Fig. 11). The results demonstrated a similar level of $EFB1$ expression for all 12 isolates, proving that the low detection of $PLB1$ in the ‘negative’ isolates relative to the ‘positive’ isolates is indeed not a technical problem. Therefore we conclude that the genomic $PLB1$ expression in the ‘negative’ isolates is much lower in comparison to the ‘positive’ isolates.

PCR products obtained from RT-PCR and genomic PCR were purified and partially sequenced from both directions. Bi-directional sequence analysis indicated that they all contained the same nucleotide sequences. BLASTN analysis further revealed that these sequences were identical to the published sequences of $C. albicans$ PLB1 (U59710) and homologous with nucleotide sequences of $Candida glabrata$ (AF498581), $Torulaspora delbrueckii$ (D32134), $S. cerevisiae$ (NC_001145), $Pichia jadinii$ (AB114901), $Schizosaccharomyces pombe$ (AB005603), $Cryptococcus bacillisporus$ (AJ302038) and $Debaryomyces hansenii$ (CR382137).

Most recent studies document that candidiasis is caused by many factors interacting in a complex manner to varying degrees to cause disease (Calderone & Fonzi, 2001). One of these, the ability of the yeast to adhere to various host surfaces...
is an important event in colonization and pathogenesis. The relative cell-surface-hydrophobicity (CSH) of the organism is also an important contributory physical force involved in the latter process (Douglas, 1987).

A positive correlation between CSH and adherence to epithelial cells in a large number of C. albicans has been reported by Macura (1987). In our earlier investigations we reported a significant positive correlation between CSH and adhesion to cultured cell surfaces with Candida krusei isolates (Samaranayake et al., 1995), and also a significant correlation between the adhesion of Candida parapsilosis to both human BECs and acrylic strips, as well as their relative CSH (Panagoda & Samaranayake, 1998). Although CSH and adhesion of the yeasts appear to act in tandem there are no documented studies to date on the relationship of the latter characteristics of Candida and other major pathogenic markers such as extracellular enzyme production.

**Association of PLB1 expression with virulent attributes of C. albicans**

When the results of the four virulent attributes studied here were pooled and analysed, no significant intra-species differences in any of the four phenotypic traits (germ-tube production, ABEC, CSH and haemolysin) were noted. The data for all four virulence attributes examined for the phospholipase-positive (group A) isolates also failed to produce a significant association with PLB1 expression in either egg-yolk-agar or YPD broth media ($P < 0.001$). On further analysis of the characteristics of groups A and B, C. albicans isolates belonging to the phospholipase enzyme-positive group A demonstrated a significant positive correlation between CSH and ABEC ($r = +0.74, P < 0.05$). On the other hand phospholipase-negative group B demonstrated significant correlations between germ-tube production and ABEC ($r = -0.59$), germ-tube production and haemolysin production ($r = +0.31$) as well as CSH and ABEC ($r = +0.50$). We are unable to explain these anomalous results between group A and group B strains. However, it is possible that further studies with a larger number of phospholipase-positive and phospholipase-deficient isolates may clarify this issue.

In an earlier investigation Barrett-Bee et al. (1985) observed correlations between extracellular phospholipase production, epithelial cell adherence and mortality in mice infected with C. albicans isolates that produced the highest enzyme activity. Ibrahim et al. (1995) showed that blood isolates of C. albicans produce significantly more phospholipase than oral commensal isolates from healthy individuals and that the blood isolates express a greater degree of germ-tube formation, higher adherence and more damage to endothelial cells than the commensal isolates. Interestingly, however, the current results do not appear to support the postulated association between phospholipase production and adhesion of yeast to BECs, CSH or germ-tube production of Candida as reported by previous workers.

When the effect of PLB1 gene expression on virulence was evaluated in a murine model of haematogenously disseminated candidiasis a C. albicans strain deleted for the PLB1 gene demonstrated no detectable difference in germ-tube production, growth rate and adherence to cultured endothelial or epithelial cell monolayers relative to the isogenic parental strain (Leidich et al., 1998). However, since previous workers have also reported a correlation between in vitro phospholipase activity and virulence (Barrett-Bee et al., 1985; Kothavade & Panthaki, 1998), the isolates in our study that expressed a higher level of PLB1 mRNA could be hypothesized to be more virulent than their counterparts.

In the present study we have attempted to obtain evidence of any correlations between phospholipase B production and other putative virulence factors in C. albicans isolates obtained from HIV-infected individuals. Although individual C. albicans isolates belonging to the phospholipase-positive group (group A) showed higher numerical values for the Pz value and a positive correlation for the evaluated PLB1 gene expression, no such association emerged for any of the virulence factors investigated. To conclude, while demonstrating a close correlation between extracellular phospholipase production and PLB1 gene expression in C. albicans, our data also suggest that phospholipase enzyme secretion may not necessarily be an accompaniment to the expression of other virulent features of this ubiquitous pathogen.

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REFERENCES


