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MOLECULAR AND ANTIGENIC CHARACTERIZATION OF TWO UNIQUE EPITOPES FROM THE PHOSPHOLIPASE B1 MOLECULE OF CANDIDA ALBICANS

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MOLECULAR AND ANTIGENIC CHARACTERIZATION OF TWO UNIQUE EPITOPES FROM THE PHOSPHOLIPASE B1 MOLECULE OF *CANDIDA ALBICANS*.

By

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ABSTRACT

MOLECULAR AND ANTIGENIC CHARACTERIZATION ON TWO UNIQUE EPITOPES FROM THE PHOSPHOLIPASE B1 MOLECULE OF *CANDIDA ALBICANS*

By

Eric J. Tarcha

Diagnosis of systemic candidiasis, an opportunistic fungal infection caused by *Candida* spp. including *C. albicans*, has been problematic due to the lack of pathognomonic clinical symptoms, and lack of highly specific and sensitive diagnostic assays. Recent studies have suggested that the Phospholipase B1 molecule of *C. albicans* (caPlb1) could be utilized as a diagnostic marker for candidiasis. In our study two unique epitopes of caPlb1(Pep1 and Pep2), identified using clustal analysis of caPlb1 with other fungal Plb1 molecules, were analyzed to determine their fidelity as highly specific and sensitive molecular markers. A 768 base pair fragment from the *caPLB1* gene containing Pep1 and Pep2 was sequenced from five isolates of *C. albicans*. There was 100% homology of the deduced amino acid sequences from these isolates. A bacterial expression system was optimized to express Pep1 from nested PCR amplicons. Immunoblotting of Pep1 was perfromed using sera from a patient with systemic candidiasis. Cross reactivity between the patient sera and the *E.coli* proteins in the Pep1 expression samples was observed. Further immunoblotting experiments, with purified Pep1 and Pep2, should be performed to fully demonstrate their diagnostic potential.

This thesis is dedicated to my parents, James and Diana Tarcha, whose hard work and support carried and inspired me throughout my undergraduate and graduate career at MSU.

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INTRODUCTION

Candida albicans

Candidiasis is a fungal infection caused by species in the genus *Candida*, primarily *Candida albicans* (Odds 1988). *C. albicans* is an opportunistic fungal pathogen found as a part of the normal animal flora. Hippocrates, in his "Epidemics", described thrush, a form of oral candidiasis, as a clinical condition as early as 400 BC. Throughout history studies have supported Hippocrates' observation by showing a correlation between candidiasis and previously debilitated patients (Galen 200 AD, Rosen von Rosenstein 1771 and Underwood 1784). Berg was the first to define, in 1846, the relationship between the thrush fungus and mouth abscesses and hence the pathogenesis of this yeast.

Candida albicans can be isolated from various areas of the human body, but it is most readily isolated from the alimentary tract and mucocutaneous regions, with the gastrointestinal tract being the most common source of candidal infections (Cole *et al.*, 1996, Greenfield, 1991, McCullough *et al.*,1996, Thomas, 1993). Colonies of *C. albicans* grown on Sabroud's agar after three days at 25°C are white to cream colored, smooth, and glistening. After time the colonies turn waxy and soft, smooth or wrinkled, with or without mycelial fringes due to hyphal formation. Microscopically, colonies are composed mainly of oval-to-elongate yeast cells of various sizes that can undergo three different morphogenic processes, blastospore formation, psuedohyphal formation and hyphal formation (Kwon-Chung and Bennett, 1992). The parasitic phenotypes of *C. albicans* can be demonstrated using hematoxylin and eosin, Gridley, or Gomori-Methenamine

Silver (GMS) stained sections in which they are usually observed as masses of intra- and extracellular mycelial and yeast elements (Rippon, 1998).

Candidal Infections

Candida albicans is the major opportunistic pathogen in a group of several pathogenic *Candida* species known to cause candidiasis mainly in immunocompromised individuals (Segal and Elad, 1998, McCullough *et al.*, 1996). Because it is part of the normal human flora, the pathogenesis of this yeast involves one or more predisposing factors which impair the immune response to the pathogen (metabolic diseases, HIV/AIDS, immunosuppressive chemotherapy), produce an imbalance in the flora in favor of the yeasts (use of broad spectrum antibiotics), or disrupt the cutaneous and mucocutaneous barriers of infection (surgery and intravenous catheters) (Segal and Elad, 1998). Appearance of the infection and macroscopic lesions are greatly influenced by the site of the infection and the competence of the host's immune system to fight the pathogen.

Candidal infections can be acute or chronic with nonspecific clinical manifestations and can be divided into two broad categories: superficial and systemic (Ashman *et al.*, 1990). Superficial candidal infections are the result of invasion of the host's outer cutaneous and mucocutaneous layers by the organism. Topical mucocutaneous and cutaneous lesions are often characterized as grayish-plaques surrounded by edema, while deeper lesions of the mucosa, such as those of the esophagus and digestive tract, are observed as ulcerated areas with the organisms reaching the submucosa and blood

vessels causing hematogenous dissemination (Kwon-Chung and Bennett, 1992, Odds 1988). Superficial infections can be classified into infections of mucocutaneous involvement and those of cutaneous involvement. Mucocutaneous candidal infections include vaginitis, balanitis, alimentary tract infections, chronic mucocutaneous candidiasis, and oral infections such as thrush, glossitis, stomatitis, cheilitis, and perleche (Reichart *et al.*, 2000, Thomas, 1993). Cutaneous candidiasis involves infections of the skin and nails mainly paronychia, onychomycosis, diaper disease, candidal granuloma, and intertriginous candidiasis (Chapman and Danielle, 1994). T

The spectrum of clinical entities of systemic and deep-seated candidiasis are variable. The clinical features of systemic candidiasis are non-specific. Although fever is the most common clinical presentation of candidemia, it is not pathognomonic (Thomas, 1993). Presentations vary from isolated candidemia with little or no clinical manifestations to severe septic-like illnesses with multiple organ failure (Chapman and Daniel, 1994). Almost any organ can be affected, but disseminated candidiasis is mainly reported in the gastrointestinal tract, kidneys, eyes, liver and central nervous system (Sobel and Vasquez, 1990). Candidiasis of the gastrointestinal tract can be localized on the esophageal mucosa, stomach and/or large and small intestine. The frequency of esophagitis has increased since the emergence of HIV and AIDS, with 10-30% of AIDS patients that showed symptoms of oral candidiasis having esophagitis (Segal and Elad, 1998). Infections of the stomach and intestines, which are observed as white plaques and ulcerations, are common in cancer patients, mainly due to

treatment with antibiotics (switching the flora to favor the fungus) and anti-cancer chemotherapy (suppression of the immune system). These infections are usually the source of disseminated candidal infections. Renal candidiasis can originate from an ascending urinary tract infection or from hematogenously disseminated organisms (Lundstrom and Sobel, 2001). Intravenous and intraperitoneal catheterizations are an important source of renal candidal infections. Ocular infections caused by *Candida spp.* can be found in both the outer and inner eye. Inner eye infections are usually the result of hematogenous dissemination, whereas direct introduction of the fungus into the eye by surgery or injury can lead to outer eye infections (keratitis). Ocular candidiasis has also been reported as a secondary infection to chemotherapeutic treatment for other infections (Segal and Elad, 1998). Hepatosplenic candidiasis is most commonly seen in individuals with leukemia due to their neutropenic conditions. Candidiasis of the central nervous system is uncommon, but found primarily in AIDS patients and pre-term infants. This type of infection is usually reported after disseminated candidiasis, and primarily involves the meninges causing meningitis (Lipton *et al.* 1984, Fernandez *et al.* 2000).

Pathogenicity of *Candida albicans*

The pathogenicity of *C. albicans* has been extensively explored. Several important virulence factors have been identified. These include; 1) the ability of *C. albicans* to adhere to host tissues (Calderone *et al.*, 2000, Chafin *et al.*, 1998, Jong *et al.*, 2001, de Repentigny *et al.*, 2000), 2) the production of proteolytic and lipolytic enzymes such as the secretory aspartyl proteases and the

phospholipases which seem to facilitate tissue penetration and invasion (Fallon et al., 1997, Ghannoum, 2000), 3) the yeast to hyphal morphogenesis that assists tissue penetration (Cutler, 1991, Davis *et al.*, 2000, Phan, Belanger and Filler, 2000, San-Blas *et al.*, 2000) and 4) immunomodulatory effects of various fungal constituents that impair the host's immune response (Fidel, Vazquez and Sobel, 1999, Lui *et al.*, 2001, Noverr *et al.*, 2001). Many breakthroughs in understanding the pathogenesis of candidiasis have been made in the past ten years, though it is still not fully understood. It has been found that adhesion molecules allowing adherence to different types of tissues as well as being able to bind mucins secreted by mucous and salivary cells, to be initial factors in dissemination of *C. albicans* (de Repentigny *et al.*, 2000, Calderone *et al.*, 2000). These adhesion molecules, which are localized on the fungal cell wall, allow for the yeasts to bind the host's tissues, colonize, and start the process of invasion. Invasion of the host tissues is also facilitated by the release of proteolytic enzymes such as aspartyl proteases that break up the integument. A breach in any vascularized tissue, the gut mucosal for example, allows for further dissemination and colonization by *C. albicans*. As colonization and invasion proceeds, the yeast to hyphal transition takes place. The production of hyphae, along with the release of phospholipase molecules, allows for the mechanical and enzymatic disruption and penetration of host tissues. Hyphal development also helps to protect the fungus from the host's cellular immune system as the organism is too large to be phagocytized, as well as by producing. Factors such as molecular mimicry of host molecules and phenotypic switching are being

studied as important virulence targets for anti-*C. albicans* drug development (Soll, 1992, Perez-Martin, Uria, and Johnson, 1999).

Immunity to *Candida*

The interactions between *C. albicans* and the host's immune system during infection are still under investigation. This topic has garnered a considerable amount of attention and research, yet these studies have been inconclusive. Studies that have evaluated the immune response during candidal infections found that there are different immune mechanisms of defense against the mucocutaneous/cutaneous forms versus the deep-seated forms of candidiasis, although these differences are not clear-cut (Ashman *et al.*, 1989, Greenfield, 1992, Fidel, Vazquez, and Sobel, 1999).

Cell-mediated immunity (CMI), mainly the CD4+ T cells, has been shown to play a large role in the defense of mucocutaneous/cutaneous forms of candidiasis (Ogawa *et al.*, 1998, Nawrot *et al.,* 2000). This is emphasized by the fact that individuals who are affected by mucocutaneous/cutaneous candidiasis are mainly patients with some kind of CMI deficiency (Greenfield, 1992, Segal and Elad, 1998). HIV/AIDS patients and chronic mucocutaneous candidiasis patients are examples of people with CMI deficiencies that are highly susceptible to these types of infections. It has been noted that, although CMI deficient patients are susceptible to mucocutaneous/cutaneous candidiasis they are rarely affected by systemic infections unless other predisposing factors are involved (Ashman and Papadimitriou, 1995). Conversely, systemic candidiasis is usually observed in patients with severe neutropenia. Therefore, it is evident that

polymorphonuclear cells (PMNs) are important in protection against systemic candidal infections (Ashman *et al.*, 1989, Greenfield, 1992). Induced neutropenia in animal models of candidal infections has been shown to increase susceptibility to systemic candidal infections (Segal and Elad, 1998). CMI involving CD4+ T-cells has also been reported in the defense against systemic candidiasis immunity. However, it seems that phagocytic cells play a greater role in controlling *C. albicans* systemic infections.

The role of antibody protection in candidal infections remains a controversial subject. The link that relates a defective CMI or neutropenic state and an invasive infection is weakened by the fact that these conditions may also result in a decrease of antibody levels (Casadevall, 1995, De Bernardis *et al.*, 2000). Therefore, it is not truly understood what role antibody protection plays during invasive infections. Theories about the mechanisms of protection that antibodies provide during candidal infections have been suggested. For instance, anti-candidal antibodies may contribute to the hosts defense by direct candicidal activity, preventing attachment, providing opsonins that enhance phagocytosis, binding immunomodulatory molecules, neutralizing extracellular proteases and phospholipases, and inhibiting yeast-to-hyphae transition (Casadevall, 1995). Although data concerning the immunity of systemic and mucocutaneous/cutaneous candidiasis has contributed to our understanding of the complex interactions between *C. albicans* and the host, much of it has been obtained from animal models and *in vitro* studies. Therefore, there is still much

more to be done to further define and characterize the humoral and cellular immunity triggered by *Candida* spp during human infection.

Incidence of Candidiasis

The incidence of systemic candidiasis continues to grow at a dramatic pace. During a four-year period, *Candida* species emerged as fourth most common septicemia and 8% of all nosocomial bloodstream infections in a nation wide hospital survey (Kalin and Petrini, 1996). More recently, a study reported that candidal infections were associated with the highest crude mortality when compared to other nosocomial blood born infections (Edmond *et al.*, 1999). Mortality rates in immunocompromised patients with systemic candidiasis were found to be 38-50% even with therapy (Pfaller, 1996, Espinel-Ingroff, 1997).

Treatment of Candidiasis

The treatment of systemic candidiasis caused by *C. albicans* currently includes a number of toxic antifungal agents such as amphotericin B, fluconazole, ketoconazole, miconazole and flucytosine (Thomas, 1993, Hadley and Karchmer, 1995, Pfaller, 1996, Martin, 1999, Rex *et al.*, 2000). Due to the difficulty encountered in diagnosing systemic infections, often therapy for systemic candidiasis is provided based on the patient's clinical symptoms without a definitive laboratory diagnosis. This practice has led to the increase in anti-fungal resistance in non-*C. albicans* species (Rex *et al.*, 2000).

Diagnosis of Candidiasis

Complications in diagnosing systemic candidiasis exist because of the lack of pathognomonic clinical symptoms presented during infection and the lack of sensitive and specific diagnostic assays for invasive candidiasis (Verweij *et al.* 1998, Vincent *et al.* 1998, Bucheidt, 2000). High mortality rates and the administration of empirical anti-fungal chemotherapy are problems clinicians have had as the result of the difficulties in diagnosing this disease (Matthews, 1996, Maenza and Merz, 1997). Development of fast, highly specific and sensitive assays for the early and rapid diagnosis of systemic candidiasis could allow for the early initiation of specific therapy. Early initiation of therapy could improve the ability of clinicians to manage this important infection, reducing mortality and anti-fungal resistance.

The current diagnostic methods for disseminated and deep-seated candidiasis lack the specificity and sensitivity that is required to readily diagnose this infection (Lew, 1989, Harley and Dummer, 1994, Matthews, 1996). In the past fifty years, the isolation of *Candida* in blood culture systems and demonstration of the pathogen in clinical specimens have been the primary tools in diagnosing invasive candidiasis (Lew, 1989, Harley and Dummer, 1994). However, due to their insensitivity, there is concern about the large number of false-negative results with the blood culture systems (Maenza and Merz 1997, Segal and Elad 1998, Verweij *et al.* 1998). Due to the sub optimal sensitivity of the culture systems, the development of novel non-culture based methods for the early specific detection of candidemia are currently under investigation.

Some of the proposed methods to diagnose deep-seated candidiasis involve non-culture based approaches such as immunodiagnostics, detection of candidal DNA sequences and detection of candidal metabolites.

At least two types of immunodiagnostic techniques have been explored. The first system detects the presence of circulating candidal antigens in a patient's serum or other body fluids and the second is an antibody detection system against immunoreactive antigens. Latex agglutination, radioimmunoassy and enzyme-linked immunoassays (ELISA) have been used to detect circulating cell wall or cytoplasmic candidal antigens such as mannans, aspartyl proteases, heat shock proteins and enolase enzymes (Bennett, 1987, Bougnoux *et al.*, 1990, Gutierrez *et al.*, 1993, Harley and Dummer, 1994, Martinez *et al.*, 1998, Verweij *et al.* 1998). Although some of these are promising, issues of low sensitivity, lack of specificity, and cost effectiveness have deterred their implementation.

The detection of circulating anti-candidal antibodies has also been investigated. Anti-candidal enolase antibodies and anti- candidal mannan antibodies have been detected in sera of patients with invasive candidiasis. However, low levels of these antibodies in severely immunocompromised patients have limited their use in diagnostics (Na and Song, 1999, Sendid *et al.*, 1999). The detection of biochemical metabolites released by *Candida* spp. has also been investigated with some success. Metabolites such as D-arabinitol and 1,3-β-D-glucan have shown some promise as putative markers. Most of these

metabolite assays are still under investigation (Obayashi *et al.* 1995, Richardson and Ellis, 2000).

The use of molecular techniques to detect candidal DNA sequences are currently being examined as possible tools that could rapidly detect invasive diseases caused by *C. albicans*. Amplification by PCR of candidal DNA sequences and detection of specific sequences with DNA probes in hybridization assays have been studied (Steffan *et al.*, 1997, Gottfredsson, Cox and Perfect, 1998, Reiss *et al.*, 1998, Wahyuningsih *et al.*, 2000). These assays have proved to be fast, specific and highly sensitive, although their high sensitivity has posed a potential disadvantage. Because *C. albicans* is part of the normal flora in mammalian hosts, contamination by commensal *Candida* species could lead to false positives in these types of assays. In addition, the amplification of circulating DNA from dead/degraded candidal cells makes these approaches unsuitable to monitor treatment.

Phospholipase and *Candida albicans*

It was recently found that the phospholipase protein of *C. albicans* is an important virulence factor in the pathogenesis of candidiasis and could possible be used as a diagnostic marker (Ghannoum, 2000). Phospholipases are a grouping of enzymes that have the capacity to hydrolyze one or more ester linkages in phospholipids (Streyer, 1995). Because most of these enzymes target specific ester bonds in their phospholipid substrates, identifying letters such as A, B, C, and D are used to indicate the specific bond that is cleaved.

Phospholipases have been suggested as virulence determinants in bacteria, protozoans as well as fungi (Long-Krug *et al.*, 1985, Saffer and Schwartzman, 1991, Titball, 1993). Pathogens that produce enzymes that hydrolyze phospholipids and proteins are more likely to be involved in membrane disruption process during host cell invasion because these molecules target the major chemical constituents of the host cell envelopes. Organisms that release phospholipases during invasion of the host's tissues may rely on the molecules ability to destroy constituents of the cellular membranes allowing for further attachment and penetration of the tissues. Hence phospholipases were suggested as important virulence determinants (Ghannoum, 2000).

To investigate the role of phospholipases as virulence factors during candidal infections, Ibrahim *et al.* (1995) compared the abilities of pathogenic blood isolates versus commensal isolates of *C. albicans* to produce phospholipase. They also compared the pathogenicity of clinical isolates that vary in levels of phospholipase production in a murine model of disseminated candidiasis. Their studies found that the blood isolates produced significantly higher levels of phospholipase than the commensal isolates. They also found that only extracellular phospholipase activity was predictive of mortality among six other virulence factors examined. Although these findings indicate that phospholipases might be important in candidal pathogenesis, they do not prove association between the production of phospholipase and virulence. The fact that all the isolates were not genetically related does not rule out the possibility that the difference in pathogenicity is due to factors other than phospholipase

activity. To further investigate the role of phospholipase in pathogenicity of candidiasis, the genes that encode for the phospholipases in *C. albicans* have been cloned and sequenced. So far, three genes that encode for *C. albicans*' phospholipases named ca*PLB1*, ca*PLB2*, and ca*PLD*, have been cloned and sequenced (Mirbod *et al.*, 1995, Leidich *et al.*, 1998, Kanoh *et al.*, 1998, Sugiyama *et al.*, 1999).

Phospholipase B1 (Plb1) is the dominant phospholipase secreted by *C. albicans* (Ghannoum, 2000). This molecule was first purified by Mirbod *et al.* (1995) and the gene (ca*PLB1*) was cloned and sequenced by Leidich *et al.* (1998). Nucleotide analysis revealed an open reading frame of 1818 bp that predicted a protein of 605 amino acid residues (Leidich *et al.*, 1998). Comparisons of this sequence with other phospholipase sequences from Genbank revealed significant homology. The cloning of ca*PLB1* allowed for construction of a ca*PLB1*-deficient mutant strain by gene knockout. These mutants in turn were used to investigate the role of this molecule in the virulence of *C. albicans.* Comparison of the pathogenicity of an isogenic strain pair of *C. albicans,* which differ only in the ability to secrete Plb1, was studied in two different murine models of candidiasis. The virulent properties of the mutant strain lacking the ability to secrete Plb1 were considerably attenuated, showing that Plb1 is indeed a putative virulence factor (Leidich *et al.*, 1998). In order to prove that Plb1 is actively expressed during infection, immunoelectron microscopic studies were performed using tissues harvested from mice challenged with *C. albicans* and Plb1-antiserum (Ghannoum, 2000). This study

revealed that Plb1 is expressed and secreted into host tissue during infection and mainly located at the hyphal tips of the invading cells (Ghannoum, 2000). Based on this research, the Plb1 molecule has garnered interest as a potential diagnostic markers for invasive candidal infections.

With evidence that Plb1 is an important virulence factor in candidiasis, additional studies were performed to investigate the presence of anti-caPlb1 in patients with and without invasive candidiasis. Western blot analysis was performed using sera from healthy individuals and patients with invasive candidiasis against purified Plb1. This study revealed that anti-caPlb1 antibodies were present in sera obtained from patients with invasive candidiasis, but were not detectable in sera obtained from healthy volunteers. This study also showed the immunogenic capabilities of caPlb1 in humans. Preliminary data from experiments evaluating the levels of anti-Plb1 in patients with invasive candidal infections from different C*andida* species and in healthy individuals, showed that the sera from patients with invasive candidiasis contained significantly higher levels of anti-Plb1 antibodies in comparisons to the other tested sera. Although there was a degree of cross reaction between sera from patients with *C. albicans* infections and other non-*C. albicans* species, these results support the idea that Plb1 could be used as a possible diagnostic marker for candidiasis.

Epitope mapping of immunodominant antigens from infectious organisms, with the purpose of finding unique peptides (or antibodies against these peptides) to be used as specific diagnostic markers of infection, has been investigated with moderate success in microorganisms such as *Treponema*

pallidum, herpes simplex virus type 2, hepatitis C virus, *Streptococcus pyogenes*, *Mycobacterium kansasii* and *Rhodococcus equi* (Osborne *et al.*, 1995, Norton, Heuzenroeder and Manning, 1996, Baughn *et al.*, 1996, Marsden *et al.*, 1998, Naito *et al.*, 1998, Vanniasinkam, Barton, and Heuzenroder, 2001). Matthews *et al.* (1991) first performed epitope mapping of candidal antigens in 1991. This group developed a limited assay using a rabbit antiserum probe against a specific epitope on the heat shock 90 protein of *C. albicans* . Epitope mapping using clustal analysis was applied to the Plb1 molecule of *C. albicans* in order to identify unique immunogenic peptides not present in other fungal Plb1s. It was proposed that the use of these epitopes or monoclonal antibodies against these peptides in a serological assay would increase the specificity and sensitivity of these assays compared to the current serodiagnostic assays. The advantage of using unique epitopes in these tests lies with the fact that using a monoclonal antibody to these epitopes may greatly enhance the sensitivity and specificity of any Plb1 antigen detection assays and the peptides could be used as the substrate in an ELISA to measure anti-caPlb1 antibody levels in infected patients.

The identification of unique epitopes in the Plb1 molecule of *C. albicans* was done by aligning the predicted amino acid sequence of candidal Plb1 against those of known fungal Plbs. The alignment was visually inspected for regions unique to *C. albicans.* Several conserved regions were found and two unique variable regions (Pep1 ^{155}Met-^{193}Lys, and Pep2 ^{321}Glu-^{347}Ile). These variable regions were present in the same location in all the aligned amino acid

sequences indicating that they may be specific for their fungal Plb. The antigenic potential of these peptides were determined using the ANTHERPROT software that details factors such as hydrophilicity, hydrophobicity, and solvent accessibility. Both Pep1 and Pep2 had profiles indicative of potential antigenicity, which in turn suggests that the host's immune system has access to these regions.

Research Study

Based on the uniqueness of Pep1 and Pep2, and their potential as diagnostic markers, the objectives of this study were threefold.

1.) To demonstrate that the Pep1 and Pep2 amino acid sequences are conserved among *C. albicans* isolates.

2.) To optimize a bacterial expression system to express both Pep1 and Pep2, which ensures an unlimited source of these peptides for later studies.

3.) To investigate the antigenicity of the expressed peptides by Western blotting with sera from patients with systemic candidiasis.

Materials and Methods

Organisms

Four clinical isolates and one reference strain of *C. albicans* were obtained from Dr. Mahmoud Ghannoum of the Center for Medical Mycology, Case Western University in Cleveland Ohio. The four clinical isolates used in this study (A-25, A-46, A-54, and A-55) were isolated from blood cultures in patients with systemic candidiasis. The reference strain, ATCC #36082, was originally obtained from the American Type Culture Collection (Rockville, MD). All isolates were inoculated on Sabouraud agar slants (10 g/L tryptone, 40 g/L glucose, 15 g/L Difco agar) and then incubated and maintained at 25°C.

DNA Isolation

The genomic DNA in all five *C. albicans* isolates was obtained by a modified boiling method as per Steffan *et al.* (1997). Each isolate was streaked for single colony isolation on Sabouraud agar plates and incubated at 25°C for 72 hours. For each isolate, a single colony was picked with a sterile toothpick and resuspended in 50 μL of sterile Tris/EDTA (TE) buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) in a 1.5 mL microcentrifuge tube. The tubes were placed in a boiling water bath for 5 minutes to lyse the cells. After boiling, 250 μL of sterile TE buffer was used to dilute each sample. The samples were stored at -20°C until needed.

Polymerase Chain Reaction Amplification of a 768 Base Pair Fragment of the *caPLB1* Gene that Contains the Two Putative Unique Regions

Primers were designed to amplify a 768 bp fragment of the *caPLB1* gene, which contained the sequences that encode both Pep1 and Pep2 (Primer Set 1, Table 1 and Figure 1). These primers were synthesized by IDT, Coralville, IA. The primers were diluted to a concentration of 5.0 pMol/μL and maintained at -20°C. Genomic DNA from each of the five *C. albicans* isolates was used as the template DNA in amplification reactions. *Aspergillus fumigatus* DNA was used as a fungal control to be certain the primers would not amplify a similar sized product from other fungi. A negative control using ddH_2O was included. For each isolate and the controls, a twenty-five μL total volume PCR was set-up in 0.2 mL PCR reaction tubes (Perkin Elmer, Foster City, CA). Each reaction consisted of: 2.5 μL 10X PCR Gold Buffer™ (no $MgCl_2$) (Perkin Elmer), 1.5 μL 25mM $MgCl_2$ solution (Perkin Elmer), 2 μL 10 mM dNTP mix (Perkin Elmer), 5 pmoles of forward primer, 5 pmoles of reverse primer, 0.25 μL AmpliTaq Gold™(Perkin Elmer), 5.0 μL DNA template, and sterile ddH_2O to Q. S. to 25 μL. PCR amplification was performed with a Perkin Elmer GeneAmp® 9700 thermocycler with the following cycling parameters: 5 minutes at 95°C followed by 35 cycles of 1 minute at 94°C, 2 minutes at 50°C, and 3 minutes at 72°C followed by 1 cycle of 1 minute at 95°C, 2 minutes at 50°C, and 10 minutes at 72°C.

The reactions were electrophoresed in a 1.2% agarose TAE (50X: 48.4 g/L tris base, 11.4 mL/L glacial acetic acid, and 20mL/L of 0.5 M EDTA) gel containing 0.5 μg/ mL of ethidium bromide for 45 minutes at 150V, visualized and documented on a Gel Doc 1000 system (Bio-Rad, Hercules, CA).

Table 1: Primer sets used to amplify the caPLB1 specific regions.

Primer Set	Region Amplified	Forward Primer (5'- 3')	Reverse Primer (5'- 3')
1	768 bp *caPLB1*	GGGTTATCAGGTGGATCG	TTGGCCATCTTCTCCACC
2	Pep1	ATGTTATCTCAAGGTTTG	TTTGCTGGCAACTTGATT
3	Pep2	GAAGCGGTGTTGTCTATC	TGGATCTACCAAAATATC

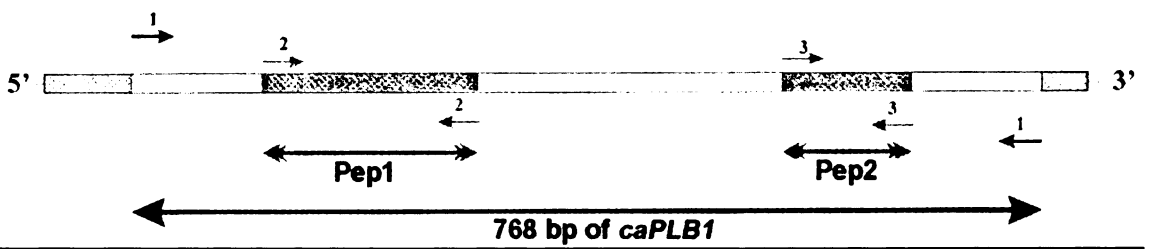

Figure 1: Schematic representation of primer sets used to amplify regions within the *caPLB1* gene.
Primer set one amplifies the 768 bp region that contains the sequences which encode both the *C. abicans*-specific peptides Pep1 and Pep2. Primer set two amplifies a 117 bp fragment that encodes for a 39 amino acid peptide, Pep1. Primer set three amplifies an 81 bp fragment that encodes a 27 amino acid peptide, Pep2.

Cloning of the 768 Base Pair Fragment of the *caPLB1* Gene

The 768 bp amplicon of the *caPLB1* gene from each of the five isolates was cloned separately. The 768 bp fragments were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). The amplicons were ligated into the pCR 4-TOPO vector as per the manufacturer's protocol. Briefly, the amplicons were first ligated into the vector and then transformed into TOP10 *E. coli* cells. The cells were plated on Luria-Bertani Kanamycin (LBK) agar (10g/L of NaCl, 10g/L tryptone, 5g/L yeast extract, 15 g/L agar, 50 ìg/mL Kanamycin, pH 7.0) for selection of positive clones and incubated overnight at 37°C. Putative positive clones (20 for A-55 and 10 for A-25, A-46, A-54, ATCC #36082) were picked and plated again on LBK agar plates and incubated overnight at 37°C. The plates were stored at 4°C until needed for plasmid isolation and long-term storage.

Long-Term Storage of Clones

Glycerol stocks were made of each clone for long-term storage at -80°C. Positive clones were streaked onto LBK agar plates for isolation of single colonies, and incubated overnight at 37°C. A single colony of each clone was resuspended in a sterile tube containing 2.0 mL of LBK broth and incubated at 37°C with shaking until the cultures reached mid-log phase. Eight hundred and fifty micro-liters of each culture was mixed with 150 μL of sterile glycerol and stored at -80°C in a 1.5 mL cryovial.

Plasmid DNA Isolation

For each *C. albicans* isolate, five putative positve clones whose plasmids contained the 768 bp amplicon were grown in a broth culture and used for plasmid DNA isolation. Briefly, each clone was streaked onto LBK agar plates for isolation of single colonies, and grown overnight at 37°C. A single colony from each clone was resuspended in a sterile tube containing 2.0 mL of LBK broth and incubated at 37°C with shaking until the cultures reached mid-log phase. These cultures were centrifuged at 5000 x g for 5 minutes at 25°C to pellet the cells. The pelleted cells were used for plasmid DNA isolation using the S.N.A.P. Miniprep Kit (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. The resulting purified plasmid DNA was resuspended to a volume of 60 μL with ddH_2O and stored at -20°C.

Sequencing the Clones Containing the 768 Base Pair Fragment of the *caPLB1* Gene

Sequencing of the *caPLB* 768 bp fragment from the five *C. albicans* isolates was done on an ABI Prism 310 Genetic Analyzer (Perkin Elmer, Foster City, CA) using ABI Prisms' Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) to facilitate the sequencing reactions. Three clones each, were sequenced for isolates A-25, A-46, A-54, and A-55, while 5 clones were sequenced for ATCC #36082 isolate. All clones were sequenced using both the M13 forward and M13 reverse universal primers. Sequencing reactions were prepared using the ABI PRISM™ BigDye™ Terminator Cycle Sequencing

Ready Reaction Kit with AmpliTaq® DNA Polymerase (TRRM)(Perkin Elmer). The sequencing reactions were set-up in 0.2mL MicroAmp® tubes (Perkin Elmer). To each reaction 4.0 μL of TRRM, 1.0 μL of primer (3.2 pMol/μL), and 5.0 μL of template plasmid DNA were added. A GeneAmp® 9700 thermal cycler was programmed to repeat the following thermal profile for 25 cycles: 96°C for 10 seconds, 50°C for 5 seconds, 60 °C for 4 minutes. After the 25th cycle the reactions were cooled to 4°C and purified for sequencing.

The sequencing reactions were purified using an ethanol precipitation methodology to remove the excess of dye terminators in the reactions. Briefly, each 10 μL reaction was added to a 0.5 mL Genetic Analyzer sample tube (Perkin Elmer) containing 32 μL of room temperature 95% ethanol and 6 μL of sterile ddH_2O. The products were allowed to precipitate at room temperature for 15 minutes. After the precipitation the tubes were centrifuged at full speed for 30 minutes at 25°C. The supernatant was carefully aspirated from each tube and discarded, taking care not to disturb the pellets. The open tubes were placed in a vacuum centrifuge and allowed to dry for 5 minutes. The dried pellets were resuspended in 18μL of template suppression reagent (Perkin Elmer). The tubes were then heated for 2 minutes at 95°C to denature the double stranded DNA. Immediately following the 95°C incubation the tubes were placed on ice for 10 minutes. The tubes were vortexed and centrifuged briefly at full speed at 25°C. The samples were loaded onto an ABI Prism 310 Genetic Analyzer and run on a 61 cm x 50 μm internally uncoated capillary using POP-6 polymer and 310 Genetic Analyzer Buffer with EDTA (Perkin Elmer). The samples were run

using the Seq POP6 (1mL) E run module, the DT POP6 {BD Set-Any Primer} dye set/primer file, and the raw data was analyzed using a matrix that was constructed on the same 310 for analyzing BigDye™ terminator chemistry.

PCR Amplification of the 117 Base Pair Pep1 and the 81 Base Pair Pep2 Fragments

Nested primers were designed to amplify the 117 bp and 81 bp fragments of the *caPLB1* gene, which encode for the peptides Pep1 and Pep2 respectively (Primer Set 2 and 3, Table 1 and Figure 1). The primers were designed to allow the resulting amplicons to be in frame for expression when ligated into the expression vector. These primers were synthesized by IDT, Coralville, IA. The primers were diluted to a concentration of 5.0 pMol/μL. Plasmid DNA from the cloned 768 bp *caPLB1* fragment of the ATCC # 36082 isolate, clones #2 and #3, was used as the template DNA for the amplification reactions. A negative reagent control using ddH_2O to replace the template genomic DNA was included. The reactions contained the same concentrations of reagents as previously described in the amplification of the 768 bp fragment except that the amount of template DNA was reduced to 0.5 μL per reaction. The following cycling parameters were used: 5 minutes at 95°C followed by 25 cycles of 30 seconds at 94°C, 30 seconds at 50°C, and 30 seconds at 72°C followed by 1 cycle of 30 seconds at 94°C, 30 seconds at 50°C, and 10 minutes at 72°C. The reactions were then electrophoresed in a 1.5% agarose TAE gel containing 0.5 μg/mL of

ethidium bromide for 30 minutes at 150V, visualized and documented on a Gel Doc 1000 system.

Cloning of the 117 Base Pair Pep1 and the 81 Base Pair Pep2 Amplicons for Expression

Both of the 117 bp Pep1 and 81 bp Pep2 amplicons were cloned for the purpose of expressing the encoded peptides. The PCR parameters and primers outlined above were used to amplify both fragments from the 768 bp plasmid DNA of ATCC #36082 clone #2. The resulting amplicons were electrophoresed in a 1.0% low melting agarose TAE gel containing 0.5 μg/mL of ethidium bromide for 30 minutes at 150V to gel purify the amplified fragments. The gel was documented on a Gel Doc 1000 system and the bands of interest were excised from the gel. The low melting agarose containing the PCR products was incubated at 65°C until the gel was melted. The mixture was vortexed and placed in a 37°C heating block and used for the ligation reaction. For cloning and expression, the pBAD TOPO TA Expression Kit (Invitrogen, Carlsbad, CA) was utilized. The pBAD-TOPO 4.1 kb vector utilizes an arabinose promoter for expression within *E. coli.* The cloning was as per the manufacturer's instructions. Briefly, the amplicons were ligated into the expression vector, transformed into TOP10 competent *E. coli* cells, plated on Luria-Bertani Ampicillin (LBA) agar (10g/L of NaCl, 10g/L tryptone, 5g/L yeast extract, 15 g/L agar, 80 ìg/mL Ampicillin, pH 7.0) for selection of positive cl ones and incubated overnight at 37°C. Putative positive clones (20 for each Pep1 and Pep2) were

picked and plated again on LBA agar plates and grown overnight at 37°C. The plates were stored at 4°C until use for plasmid isolation, long-term storage, and expression. The positive control expression vector containing the sequence for the β-Galactosidase protein was also transformed into TOP10 cells as per the instruction manual and used later as a control during the expression experiments.

Long-Term Storage, Plasmid DNA Isolation and Sequencing of Pep1 and Pep2 Clones

Long-term storage of the Pep1 and Pep2 clones was done as described before. However, the LBK agar plates and LBK broth were replaced with LBA agar plates and LBA broth. Plasmid DNA isolation of 5 putative positive Pep1 and Pep2 clones was done as described before. The Pep1 and Pep2 clones were sequenced to evaluate if the amplicons were ligated into the vector in the correct orientation, as well as to verify if they were in frame with the vector for proper expression. Five clones were sequenced for Pep1 and Pep2 using the pBAD Forward Primer (Invitrogen, Carlsbad, CA) as described above.

Pilot Expression of the Pep1 Fusion Protein

A Pep1 clone, whose plasmid was in frame, and a positive control β-Galactosidase clone were selected for pilot expression experiments. The clones were streaked onto LBA agar plates for isolation of single colonies, and incubated overnight at 37°C. A single colony of the Pep1 clone was used to

inoculate 2 mL of LBA broth in a 50 mL conical tube. The same protocol was used for a positive control colony. The cultures were incubated overnight at 37°C with shaking until the cells reached to an OD_{600} of ~1.5. Six 50 mL conical tubes were properly labeled, 1 mL and 10 mL of LBA broth was added to each tube. Tubes 1-5 were inoculated with 0.1 mL of the overnight Pep1 culture and the positive control tube was inoculated with 0.1 mL of the overnight β-Galactosidase culture. The tubes were incubated at 37°C with shaking until they reached mid-log phase (an OD_{600} of ~0.5). Meanwhile, four 10-fold serial dilutions of 20% L-arabinose were made with ddH_2O using aseptic technique, to be used later as the stock solutions for induction of expression. Once mid-log phased was reached, a 1.0 mL aliquot of cells was removed from each tube and placed in separate 1.5 mL microcentrifuge tubes. These tubes were centrifuged at full speed for 30 seconds at 25°C to pellet the cells. The supernatant was removed from each tube and discarded. The pellets were frozen at -20°C until used for expression analysis. These samples were taken at the zero time point prior to induction of expression with L-arabinose. The previously prepared stock solutions of L-arabinose were then added to the six 9.0 mL cultures (Table 2) to induce expression of the fusion proteins. These cultures were incubated at 37°C while being rotated for 4 hours. After incubation, 1.0 mL aliquots were taken again from each culture and treated the same as the zero point samples. These samples were used to evaluate expression by SDS-PAGE and Immunoblotting.

TABLE 2: Volumes and concentrations of L-arabinose used for pilot expression of Pep1 and the positive control.

Tube	Stock Solution	Volume (mL)	Final Concentration
1	0.002 %	0.09	0.00002%
2	0.02%	0.09	0.0002%
3	0.2%	0.09	0.002%
4	2.0%	0.09	0.02%
5	20.0%	0.09	0.2%
Pos. Cont.	2.0%	0.09	0.02%

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To evaluate the expression of the fusion proteins, SDS-PAGE was used to prepare the samples for Coomassie staining and immunoblotting. Expression samples (frozen cell pellets) and Positope (Invitrogen, Carlsbad, CA) blotting control were thawed at 25°C for 30 minutes and then treated with 100 iL of 1X Laemmli sample buffer (BioRad, Hercules, CA). The samples were then placed in a boiling water bath for 5 minutes. The samples were allowed to cool at room temperature. 5 μL of each sample, 10 μL of Positope, and 10 μL of Prestained SDS-PAGE Broad Range Standard (BioRad) were loaded into a 4%/12% stacked SDS-Polyacrylamide Gel [Two 12% Resolving Gels (2.5 mL 1.5M Tris-HCl pH 8.8, 100 μL 10% SDS, 4.0 mL 30% Acrylamide/Bis (29:1) Stock, 50 μL 10% ammonium persulfate, 5.0 μL TEMED, and 3.35 mL ddH_2O) and Two 4%

Stacking Gels (2.5 mL 0.5M Tris-HCl pH 6.8, 100 μL 10% SDS, 1.3 mL 30% Acrylamide/Bis Stock, 50 μL 10% ammonium persulfate, 10.0 μL TEMED, and 6.1 mL ddH_2O)] in duplicate. 1X Tris/Glycine/SDS Buffer (BioRad) was used in the Mini Protean II gel system (BioRad) for the SDS-PAGE. Before the start of the electrophoresis, two drops of 0.1% Bromophenol Blue was added to the center chamber. The samples were then electrophoresed at 150V until the dye front ran off the gel. Note: Some samples were run on 4%/14% stacked SDS-PAGE gels.

Coomassie Staining SDS-PA Gels

Gels were placed in Coomassie Blue Stain (0.1 g Coomassie blue, 40 mL methanol, 10 mL acetic acid, 50 mL ddH_2O) for 45 minutes with gentle shaking. Gels were then placed in Destain 1 (40 mL methanol, 40 mL acetic acid, 320 mL ddH_2O) for 20 minutes 2X with gentle shaking. After the second wash in Destain 1, gels were placed in Destain 2 (80 mL methanol, 40 mL acetic acid, 280 mL ddH_2O) with gentle shaking. The gels were stored in Destain 2 until they could be documented.

Immunoblotting

After electrophoresis, the 4% stacking gel was removed from the resolving gel. The SDS-PAGE gels were placed in Transfer Buffer for equilibration (3.03 g/L Tris base, 1.07 g/L glycine, 200 mL/L methanol, pH 8.3 in ddH_2O) for 10 minutes prior to electro-transfer. The proteins were electro-transferred in transfer buffer to a 0.45 μm pore Protran Nitrocellulose Membrane (Schleicher and Schuell, Keene, NH) using a Mini Trans-Blot Cell (BioRad, Hercules, CA) for

1 hour at 100V with the cell surrounded by ice. Once transfer was completed, the gel was coomassie stained to visualize the effectiveness of the transfer and the membrane was placed in 15 mL of Blocking Buffer [5% nonfat dry milk w/v in TBST (20 mL/L 0.5M Tris-HCl pH 8.0, 8.8 g/L NaCl, 0.05% Tween 20 in ddH $_2$O)] and incubated at room temperature for 1 hour with gentle shaking. The membrane was then washed 2x with TBST for 10 minutes each wash. Membranes incubated with Anti-V5-HRP antibodies (Invitrogen, Carlsbad, CA) were placed in a container with 15 mL of Anti-V5-HRP antibody diluted 1:5000 in dilution buffer (1% nonfat dry milk w/v in TBST) for 1 hour at 25°C with gentle shaking. Membranes incubated with Anti-β-Galactosidase primary antibodies (Invitrogen) were placed in a container with 15 mL of Anti-β-Galactosidase antibodies diluted 1:5000 in dilution buffer for 1 hour at 25°C with gentle shaking. Membranes incubated with Anti-V5 (Invitrogen) primary antibodies were placed in a container with 15 mL of Anti-V5 antibodies diluted 1:5000 and/or 1:1000 in dilution buffer for 1 hour at 25°C and/or at 4°C with gentle shaking. Membranes incubated with sera from patients with systemic candidiasis (Dr. Ghannoum, Cleveland, OH) were placed in a container with 15 mL of sera from a patient with systemic candidiasis diluted 1:1000 and/or 1:5000 overnight at 4°C with gentle shaking. Membranes incubated with Anti-V5 primary antibodies were incubated with 15 mL of Goat Anti-Mouse IgG Alkaline Phosphatase conjugated secondary antibodies (Sigma, St. Louis, MO) diluted 1:10000 in dilution buffer for 1 hour at 25°C with gentle shaking. Membranes incubated with β-Galactosidase primary antibodies were incubated with 15 mL of Donkey Anti-Rabbit IgG Alkaline

Phosphatase conjugated secondary antibodies (Promega, Madison, WI) diluted 1:10000 in dilution buffer for 1 hour at 25°C with gentle shaking. Membranes incubated with sera from patients with systemic candidiasis were incubated with 15 mL of Goat Anti-Human IgG Alkaline Phosphatase conjugated secondary antibodies (Sigma) diluted 1:10000 in dilution buffer for 1 hour at 25°C with gentle shaking. Horse Radish Peroxidase detection was performed with the HRP Conjugate Substrate Kit (BioRad). Alkaline Phosphatase detection was done with the Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega).

RESULTS

Amplification of a 768 Base Pair Fragment of the *caPLB1* Gene Containing the Putative Unique Regions, Pep1 and Pep2

Genomic DNA was isolated from each of the four clinical isolates (A-25, A-46, A-54, and A-55) and one reference strain (ATCC #36082) of *C. albicans*. The genomic DNA from each isolate was used as the template to amplify by PCR a 768 bp fragment of the ca*PLB*1 gene that contains the two unique epitopes Pep1 and Pep2. Aliquots of the amplification reactions were electrophoresed in an ethidium bromide stained agarose gel to visual the size of the resulting amplicons (Figure 2). It was observed that amplicons approximately 770 bp in size were amplified from each of the five isolates. The fungal control reaction (*A. fumigatus* DNA) showed non-specific amplification of smaller products. There was no amplification in the negative control. The same parameters were used for subsequent amplifications of the 768 bp fragments for cloning purposes.

Sequencing of the Clones Containing the 768 *caPLB1* Fragment

To determine variability among the amino acid sequences, the *caPLB1* 768 bp fragments from the five *C. albicans* isolates were cloned and then sequenced. For isolate A-55, clones 1, 3 and 4 were sequenced.

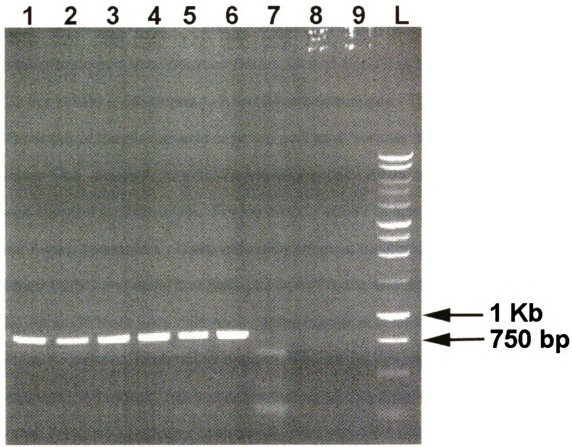

Figure 2: 768 base pair amplicons of the *caPLB1* gene.
A 1.2% agarose gel showing the resulting amplicons from a PCR amplification of the 768 bp fragments in the five *C. albicans* isolates. Lane 1 is the A46 isolate, lane 2 the A55 isolate, lane 3 the A54 isolate, lane 4 the A25 isolate, lanes 5 and 6 the ATCC 36082 strain, lane 7 the fungal control, lanes 8 and 9 the negative reagent controls and lane L is the 1 Kb DNA Ladder.

There was one difference at the nucleic acid between clones 1/3 and clone 4 at base pair 543 (C to T) and between clones 3/4 and 1 at base pair 639 (T to C). For isolate A-25, clones 1, 3 and 4 were sequenced. There was one difference at the nucleic acid level between clones 1/4 and clone 3 at base pair 543 (T to C). For isolate A-54, clones 1, 2 and 3 were sequenced. There were no differences at the nucleic acid or amino acid level between these clones. For isolate A-46, clones 6, 15 and 17 were sequenced. All the sequenced clones were identical for this isolate. For the ATCC #36082 isolate, clones 1, 2, 3, 5 and 7 were sequenced. There were differences at the nucleic acid level between clones 1/2/5/7 and clone 3 at base pair 444 (T to C), and at base pair 447 (A to G). Although there were differences at the nucleic acid level in some of the clones sequenced, none of the changes affected the predicted amino acid sequence. An aligned, representative nucleic acid sequence for each isolate is shown in Figure 3. It was observed that the deduced amino acid sequence was highly conserved among all the *C. albicans* isolates in this region(Figure 4).

Amplification of the 117 Base Pair Pep1 and 81 Base Pair Pep2 Fragments

Nested primers were designed to amplify the 117 bp Pep1 and 81 bp Pep2 fragments by PCR for cloning into an expression vector. To verify amplification of the expected products, aliquots of each reaction were electrophoresed in an ethidium bromide stained agarose gel. Amplicons

```
          10                                                        60
A55   GGGTTATCAG GTGGATCGTG GTTAGTTGGA AGTCTTGCAA GTAATAATTT GAACTCTGTT
A54   GGGTTATCAG GTGGATCGTG GTTAGTTGGA AGTCTTGCAA GTAATAATTT GAACTCTGTT
A46   GGGTTATCAG GTGGATCGTG GTTAGTTGGA AGTCTTGCAA GTAATAATTT GAACTCTGTT
ATCC  GGGTTATCAG GTGGATCGTG GTTAGTTGGA AGTCTTGCAA GTAATAATTT GAACTCTGTT
A25   GGGTTATCAG GTGGATCGTG GTTAGTTGGA AGTCTTGCAA GTAATAATTT GAACTCTGTT
          70                                                       120
A55   GATGACATGT TATCTCAAGG TTTGTGGGAA CTCACACACT CATTTCTTAG TTACTATGGC
A54   GATGACATGT TATCTCAAGG TTTGTGGGAA CTCACACACT CATTTCTTAG TTACTATGGC
A46   GATGACATGT TATCTCAAGG TTTGTGGGAA CTCACACACT CATTTCTTAG TTACTATGGC
ATCC  GATGACATGT TATCTCAAGG TTTGTGGGAA CTCACACACT CATTTCTTAG TTACTATGGC
A25   GATGACATGT TATCTCAAGG TTTGTGGGAA CTCACACACT CATTTCTTAG TTACTATGGC
         130                                                       180
A55   ATTGAACATC CTATAAAGCA AGTTGAAGAA TGGGTTAATG TTGGTAATCA AGTTGCCAGC
A54   ATTGAACATC CTATAAAGCA AGTTGAAGAA TGGGTTAATG TTGGTAATCA AGTTGCCAGC
A46   ATTGAACATC CTATAAAGCA AGTTGAAGAA TGGGTTAATG TTGGTAATCA AGTTGCCAGC
ATCC  ATTGAACATC CTATAAAGCA AGTTGAAGAA TGGGTTAATG TTGGTAATCA AGTTGCCAGC
A25   ATTGAACATC CTATAAAGCA AGTTGAAGAA TGGGTTAATG TTGGTAATCA AGTTGCCAGC
         190                                                       240
A55   AAAAGAAATG CAAATTTTAA CGTTTCTTTA ACTGACATAT ATGGAAGACT ATTAAGTTAC
A54   AAAAGAAATG CAAATTTTAA CGTTTCTTTA ACTGACATAT ATGGAAGACT ATTAAGTTAC
A46   AAAAGAAATG CAAATTTTAA CGTTTCTTTA ACTGACATAT ATGGAAGACT ATTAAGTTAC
ATCC  AAAAGAAATG CAAATTTTAA CGTTTCTTTA ACTGACATAT ATGGAAGACT ATTAAGTTAC
A25   AAAAGAAATG CAAATTTTAA CGTTTCTTTA ACTGACATAT ATGGAAGACT ATTAAGTTAC
         250                                                       300
A55   CCTTTGCTCA CAAACACAGA AGACGAAGGT GATGCTTATT TGTGGTCTGA TGTAACGAGT
A54   CCTTTGCTCA CAAACACAGA AGACGAAGGT GATGCTTATT TGTGGTCTGA TGTAACGAGT
A46   CCTTTGCTCA CAAACACAGA AGACGAAGGT GATGCTTATT TGTGGTCTGA TGTAACGAGT
ATCC  CCTTTGCTCA CAAACACAGA AGACGAAGGT GATGCTTATT AGTGGTCTGA TGTAACGAGT
A25   CCTTTGCTCA CAAACACAGA AGACGAAGGT GATGCTTATT AGTGGTCTGA TGTAACGAGT
         310                                                       360
A55   GCTTCAAACT TCCAAAGCCA CCAAATGCCG TTTCCAATAT TGATCAGTGA TGGTAGAGCT
A54   GCTTCAAACT TCCAAAGCCA CCAAATGCCG TTTCCAATAT TGATCAGTGA TGGTAGAGCT
A46   GCTTCAAACT TCCAAAGCCA CCAAATGCCG TTTCCAATAT TGATCAGTGA TGGTAGAGCT
ATCC  GCTTCAAACT TCCAAAGCCA CCAAATGCCG TTTCCAATAT TGATCAGTGA TGGTAGAGCT
A25   GCTTCAAACT TCCAAAGCCA CCAAATGCCG TTTCCAATAT TGATCAGTGA TGGTAGAGCT
         370                                                       420
A55   CCAGATACTA CCATTATTAA TCTCAACTCC ACTGTGATTG AATTAACTCC ATACGAATTT
A54   CCAGATACTA CCATTATTAA TCTCAACTCC ACTGTGATTG AATTAACTCC ATACGAATTT
A46   CCAGATACTA CCATTATTAA TCTCAACTCC ACTGTGATTG AATTAACTCC ATACGAATTT
ATCC  CCAGATACTA CCATTATTAA TCTCAACTCC ACTGTGATTG AATTAACTCC ATACGAATTT
A25   CCAGATACTA CCATTATTAA TCTCAACTCC ACTGTGATTG AATTAACTCC ATACGAATTT
         430                                                       480
A55   GGTTCATGGG ATCCTAGTTT AAATGAATTT GTTGACACTA GATATTTGGG AACAAAATTA
A54   GGTTCATGGG ATCCTAGTTT AAATGAATTT GTTGACACTA GATATTTGGG AACAAAATTA
A46   GGTTCATGGG ATCCTAGTTT AAATGAATTT GTTGACACTA GATATTTGGG AACAAAATTA
ATCC  GGTTCATGGG ATCCTAGTTT AAACGAGTTT GTTGACACTA GATATTTGGG AACAAAATTA
A25   GGTTCATGGG ATCCTAGTTT AAATGAATTT GTTGACACTA GATATTTGGG AACAAAATTA
         490                                                       540
A55   GACAATGGTC GTCCAACTGG TAAGTGTTAT AATGGATTTG ACAATGCTGG GTTTTTCATG
A54   GACAATGGTC GTCCAACTGG TAAGTGTTAT AATGGATTTG ACAATGCTGG GTTTTTCATG
A46   GACAATGGTC GTCCAACTGG TAAGTGTTAT AATGGATTTG ACAATGCTGG GTTTTTCATG
ATCC  GACAATGGTC GTCCAACTGG TAAGTGTTAT AATGGATTTG ACAATGCTGG GTTTTTCATG
A25   GACAATGGTC GTCCAACTGG TAAGTGTTAT AATGGATTTG ACAATGCTGG GTTTTTCATG
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          550                                                        600
A55   GGCACTTCTT CTGCTCTTTT CAACGAAGCG GTGTTGTCTA TCACTGAAGC TAACATACCT
A54   GGCACTTCTT CTGCTCTTTT CAACGAAGCG GTGTTGTCTA TCACTGAAGC TAACATACCT
A46   GGTACTTCTT CTGCTCTTTT CAACGAAGCG GTGTTGTCTA TCACTGAAGC TAACATACCT
ATCC  GGTACTTCTT CTGCTCTTTT CAACGAAGCG GTGTTGTCTA TCACTGAAGC TAACATACCT
A25   GGTACTTCTT CTGCTCTTTT CAACGAAGCG GTGTTGTCTA TCACTGAAGC TAACATACCT
          610                                                        660
A55   AGCTTTTTAA AAGATATAAT TGATGATATT TTGGTAGACC CAATTCTCAA GTCCAACATA
A54   AGCTTTTTAA AAGATATAAT TGATGATATT TTGGTAGATC CAATTCTCAA GTCCAACATA
A46   AGCTTTTTAA AAGATATAAT TGATGATATT TTGGTAGATC CAATTCTCAA GTCCAACATA
ATCC  AGCTTTTTAA AAGATATAAT TGATGATATT TTGGTAGATC CAATTCTCAA GTCCAACATA
A25   AGCTTTTTAA AAGATATAAT TGATGATATT TTGGTAGATC CAATTCTCAA GTCCAACATA
          670                                                        720
A55   GACGTGTCTG CTTACAATCC AAATCCATTT TTCAAAAGTT CTGGCAGCAA TACTGCTATT
A54   GACGTGTCTG CTTACAATCC AAATCCATTT TTCAAAAGTT CTGGCAGCAA TACTGCTATT
A46   GACGTGTCTG CTTACAATCC AAATCCATTT TTCAAAAGTT CTGGCAGCAA TACTGCTATT
ATCC  GACGTGTCTG CTTACAATCC AAATCCATTT TTCAAAAGTT CTGGCAGCAA TACTGCTATT
A25   GACGTGTCTG CTTACAATCC AAATCCATTT TTCAAAAGTT CTGGCAGCAA TACTGCTATT
          730
A55   TCTCAATCAA AAAATCTTTA TTTAGTTGAC GGTGGAGAAG ATGGCCAA
A54   TCTCAATCAA AAAATCTTTA TTTAGTTGAC GGTGGAGAAG ATGGCCAA
A46   TCTCAATCAA AAAATCTTTA TTTAGTTGAC GGTGGAGAAG ATGGCCAA
ATCC  TCTCAATCAA AAAATCTTTA TTTAGTTGAC GGTGGAGAAG ATGGCCAA
A25   TCTCAATCAA AAAATCTTTA TTTAGTTGAC GGTGGAGAAG ATGGCCAA
```

Figure 3: Representative sequences of the 768 bp region from each *C. albicans* isolate.
The sequences have been aligned and the differences are highlighted.

A.

```
                1          11         21         31         41
ATCC Pep1       MLSQGLWELTHSFLSYYGIEHPIKQVEEWVNVGNQVASK
A55 Pep1        MLSQGLWELTHSFLSYYGIEHPIKQVEEWVNVGNQVASK
A54 Pep1        MLSQGLWELTHSFLSYYGIEHPIKQVEEWVNVGNQVASK
A46 Pep1        MLSQGLWELTHSFLSYYGIEHPIKQVEEWVNVGNQVASK
A25 Pep1        MLSQGLWELTHSFLSYYGIEHPIKQVEEWVNVGNQVASK
Consensus       mlsqglwelthsflsyygiehpikqveewvnvgnqvask
```

B.

```
                1          11         21
ATCC Pep2       EAVLSITEANIPSFLKDIIDDILVDP
A55 Pep2        EAVLSITEANIPSFLKDIIDDILVDP
A54 Pep2        EAVLSITEANIPSFLKDIIDDILVDP
A46 Pep2        EAVLSITEANIPSFLKDIIDDILVDP
A25 Pep2        EAVLSITEANIPSFLKDIIDDILVDP
Consensus       eavlsiteanipsflkdiiddilvdp
```

Figure 4: Deduced amino acid sequences of the Pep1 and Pep2.

A. The alignment of the deduced amino acid sequences of the Pep1 epitope in the five isolates of *C. albicans* used in this study.

B. The alignment of the deduced amino acid sequences of the Pep2 epitope in the five isolates of *C. albicans* used in this study.

approximately 120 bp in size were observed in the reactions using Pep1 primers and approximately 80 bp in size in the reactions using Pep2 primers (Figure 5). There was not amplification in the negative reagent control. The same parameters were used for subsequent amplifications of the Pep1 and Pep2 fragments for cloning into an expression vector.

Sequencing of the Clones Containing the Pep1 Fragment

Pep1 and Pep2 fragments were cloned into an expression vector and sequenced to determine if the inserts were in frame for expression. Pep1 clones 2 and 3 were found to be in frame in the expression vector (Figure 6).

Evaluation of the Pep1 Fusion Protein Expression

Pilot expression of the Pep1 fusion protein was evaluated using SDS-PAGE and Immunoblotting. Coomassie stained SDS-polyacrylamide gels revealed the presence of bands at numerous molecular weights in the cell-lysate expression samples. A prominent band at 130 kD was detected in the β-Galactosidase positive expression control post expression samples, but it was not observed in the pre- induction expression samples (Figure 7). There were no prominent bands at the expected molecular weight of 6-8 kD that might indicate expression in the Pep1 fusion protein post-expression samples (Figure 7).

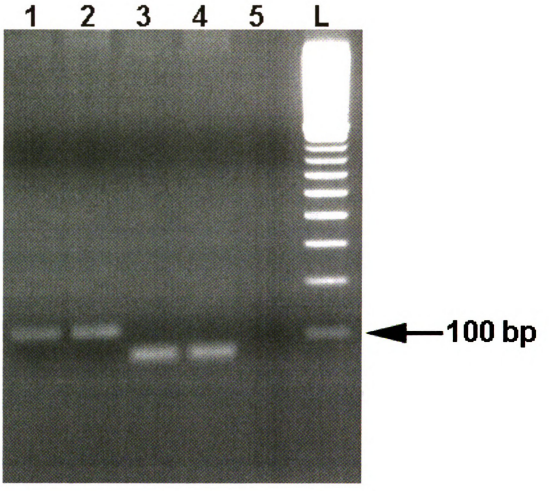


Figure 5: PCR amplification of the Pep1 and Pep2 fragments.
A 1.5% agarose gel showing the resulting amplicons from a PCR amplification of the Pep1 and Pep2 fragments. Lanes 1 and 2 show the Pep1 amplicons using plasmid DNA from ATCC 768bp clones 2 and 3 respectively. Lanes 3 and 4 show Pep2 amplicons using plasmid DNA from ATCC 768bp clones 2 and 3 respectively. Lane 5 shows the negative reagent control. Lane L is the 100 bp PCR Marker.

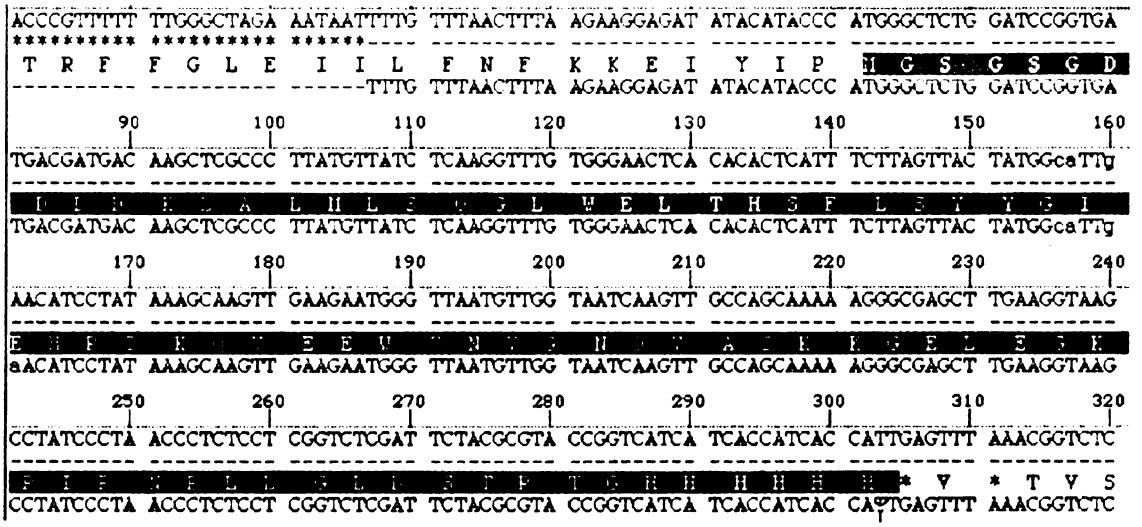
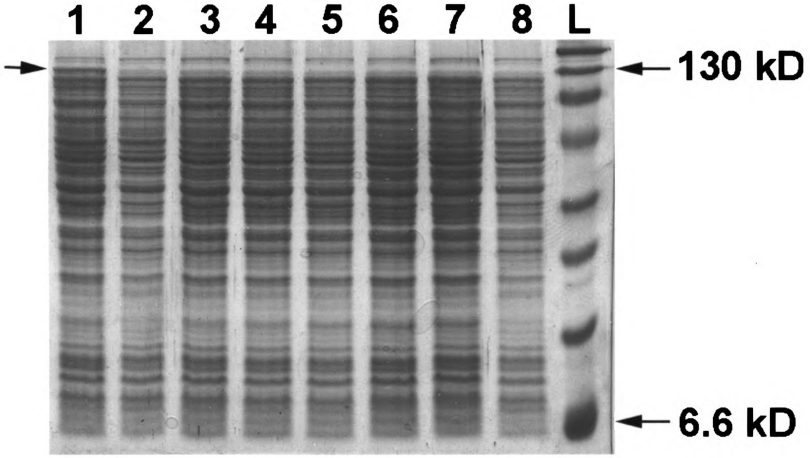


Figure 6: Alignment of Pep1 clones showing deduced amino acid sequence of the expression product.
Pep1 clones 2 and 3 are aligned and the insert is in frame with the arms of the vector. The deduced amino acid sequence for the Pep1 fusion protein is highlighted.

Figure 7: Pilot expression of the Pep1 fusion protein.
SDS-PAGE gel stained with Coomassie Blue of 130 kD β-Galactosidase positive expression control (Lane 1), pre-induction expression sample (Lane 2), Pep1 expression samples with decreasing amount of L-arabinose (Lanes 3-7), Pep1 pre-induction expression sample (Lanes 8) and Protein Standard (L). Numbers on the right indicate the molecular weights of the proteins in the standard. The arrow on the left designates the expression of the 130 kD β - Galactosidase expression control.


1
2
3
4
5
6
7
8
L
130 kD
6.6 kD

Immunoblotting with Anti-V5-HRP antibodies (Invitrogen, Carlsbad, CA), that are specific for the V5 epitope on the expressed fusion proteins, detected the 50 kD antibody control Positope™ protein (Invitrogen) but none of the expressed proteins (Figure 8). The same results were observed after re-expression of both Pep1 and the β -Galactosidase positive expression control.

The expression protocol was re-evaluated by expressing and detecting the products of two clones of the β -Galactosidase positive control. Coomassie stained SDS-polyacrylamide gels showed again the presence of the 130 kD band in the post-expression samples. Immunoblotting using polyclonal primary anti-bodies against β -Galactosidase detected bands at the same 130 kD position in all lanes. Stronger bands were always observed in the post-expression samples (Figure 9).

New, non-conjugated anti-V5 primary antibodies (Invitrogen, Carlsbad, CA) were used to detect expressed products in the original Pep1 and Beta-Galactosidase expression samples. These antibodies detected the 50 kD antibody control Positope™ protein (Invitrogen) as well as the 130 kD β -Galactosidase expression control. In an effort to detect the Pep1 fusion protein in the post-expression samples, the primary antibody dilution was reduced to 1:1000, primary antibody incubation time was increased and the amount of sample was increased. These changes resulted in the detection of the putative Pep1 fusion protein. Immunoblotting with these new parameters detected the Positope™ 50 kD antibody control protein (Invitrogen), the 130 kD β -

Galactosidase expression control and the ~6.5 kD putative Pep1 fusion protein (Figure 10).

Immunoblotting with Sera from Patients with Systemic Candidiasis

In order to investigate the antigenicity of the expressed putative Pep1 fusion protein, post-expression samples and a TOP10 *E. coli* cell lysate control were subjected to SDS-PAGE and immunoblotting with sera from patients with systemic candidiasis. Sera from patient with systemic candidiasis, designated as CMM 20, was used at a 1:1000 and 1:5000 dilution. Bands were detected at multiple molecular weights in both the expression samples and the TOP10 *E. coli* cell lysate control. A band was detected at the expected 6.5 kD molecular weight of the Pep1 fusion protein in all samples (Figure 11).

Figure 8: Immunoblot with anti-V5-HRP antibodies.
Immunoblot analysis of the 130 kD β -Galactosidase expression control and pre-expression sample (Lane 1 and 2), Pep1 expression samples with decreasing amount of L-arabinose and Pep1 pre- induction expression sample (Lanes 3-8), Positope™ antibody control protein (Lane 9), and protein standard (Lane 10) after incubation with anti-V5-HRP antibodies. Numbers on the right indicate the molecular weight of the proteins in the standard. Note the presence of only one band in the lane with the Positope™ antibody control protein.

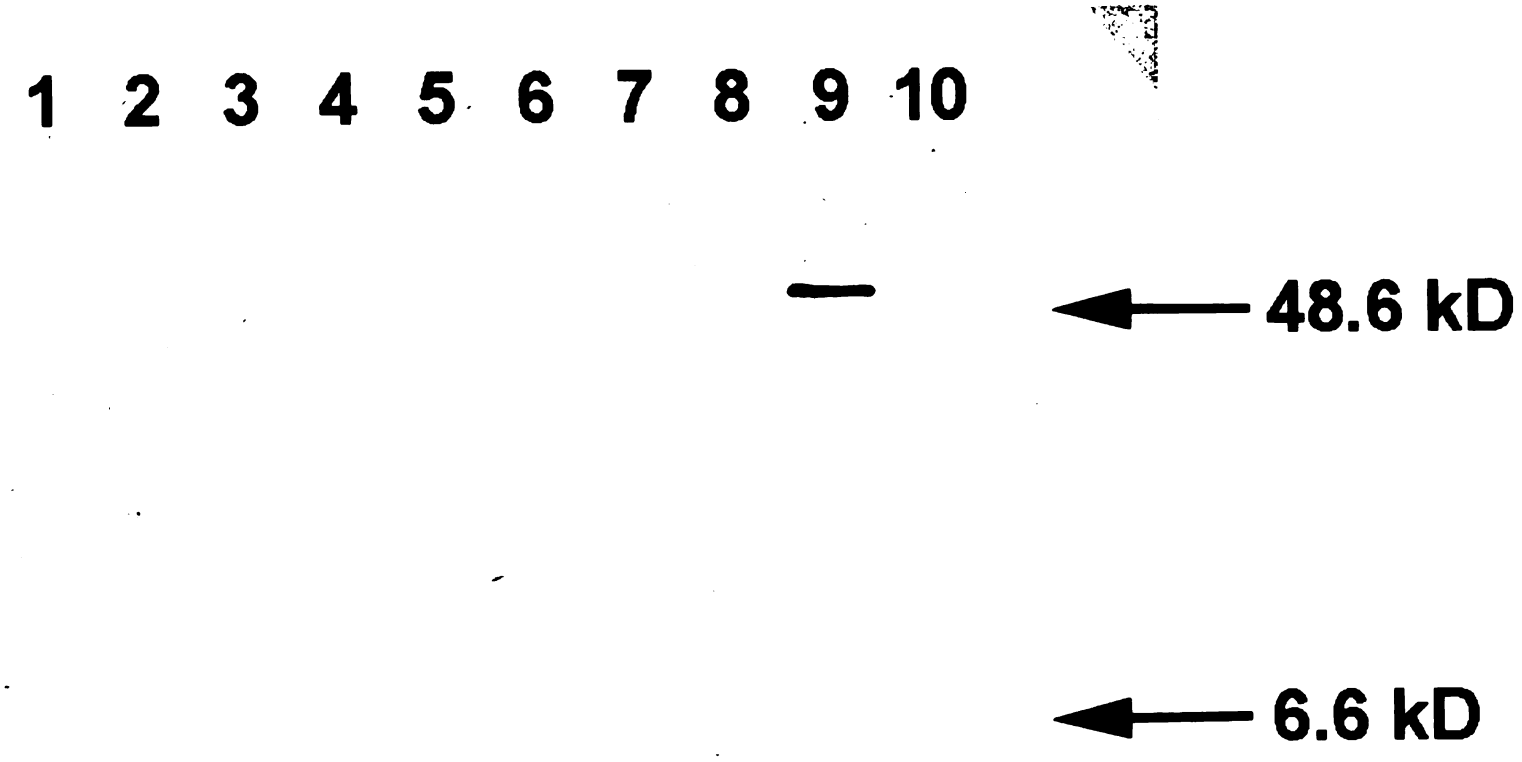

1 2 3 4 5 6 7 8 9 10
48.6 kD
6.6 kD

Figure 9: Immunoblot of 130 kD β-Galactosidase expression.
Immunoblot of 130 kD β-Galactosidase expression controls (Lanes 1-4), pre-induction expression samples of the β -Galactosidase expression controls (Lanes 5-8), and protein standard (Lane L) after incubation with polyclonal anti- β -Galactosidase primary antibodies and AP conjugated anti-rabbit IgG secondary antibodies. Number on the right indicates the molecular weight of protein in standard. Note the increase of expression of the β -Galactosidase molecule in the post expression samples when compared to the basal levels of expression in the pre-induction expression samples.

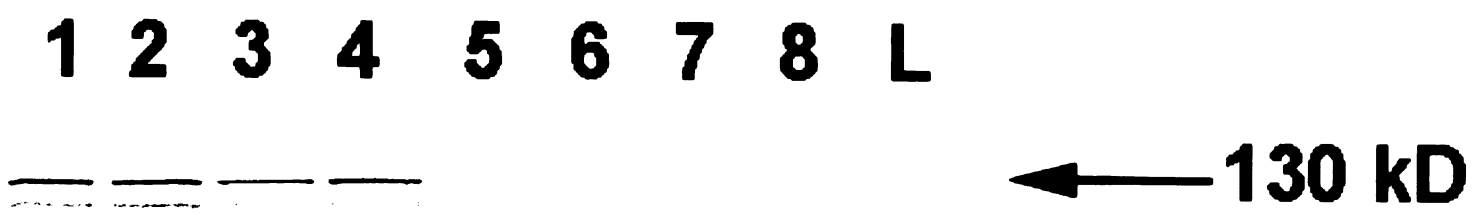

1 2 3 4 5 6 7 8 L
130 kD

Figure 10: Immunoblot of Pep1 expression with anti-V5 primary antibodies and AP conjugated anti-mouse IgG secondary antibodies.
Immunoblot analysis of Pep1 post expression samples (Lanes 1-3), Pep1 pre-induction sample (Lane 4), β -Galactosidase expression control (Lane 5), pre-induction samples of the β -Galactosidase expression control (Lane 6), Positope™ antibody control protein (Lane 7), and protein standard (Lane L) after incubation with anti-V5 primary antibodies and AP conjugated anti-mouse IgG secondary antibodies. Numbers on the right indicate the molecular weight of the proteins in the standard. The asterisk (*) indicates the β -Galactosidase expression control being detected and the double asterisks (**) indicates the Pep1 fusion protein being detected.

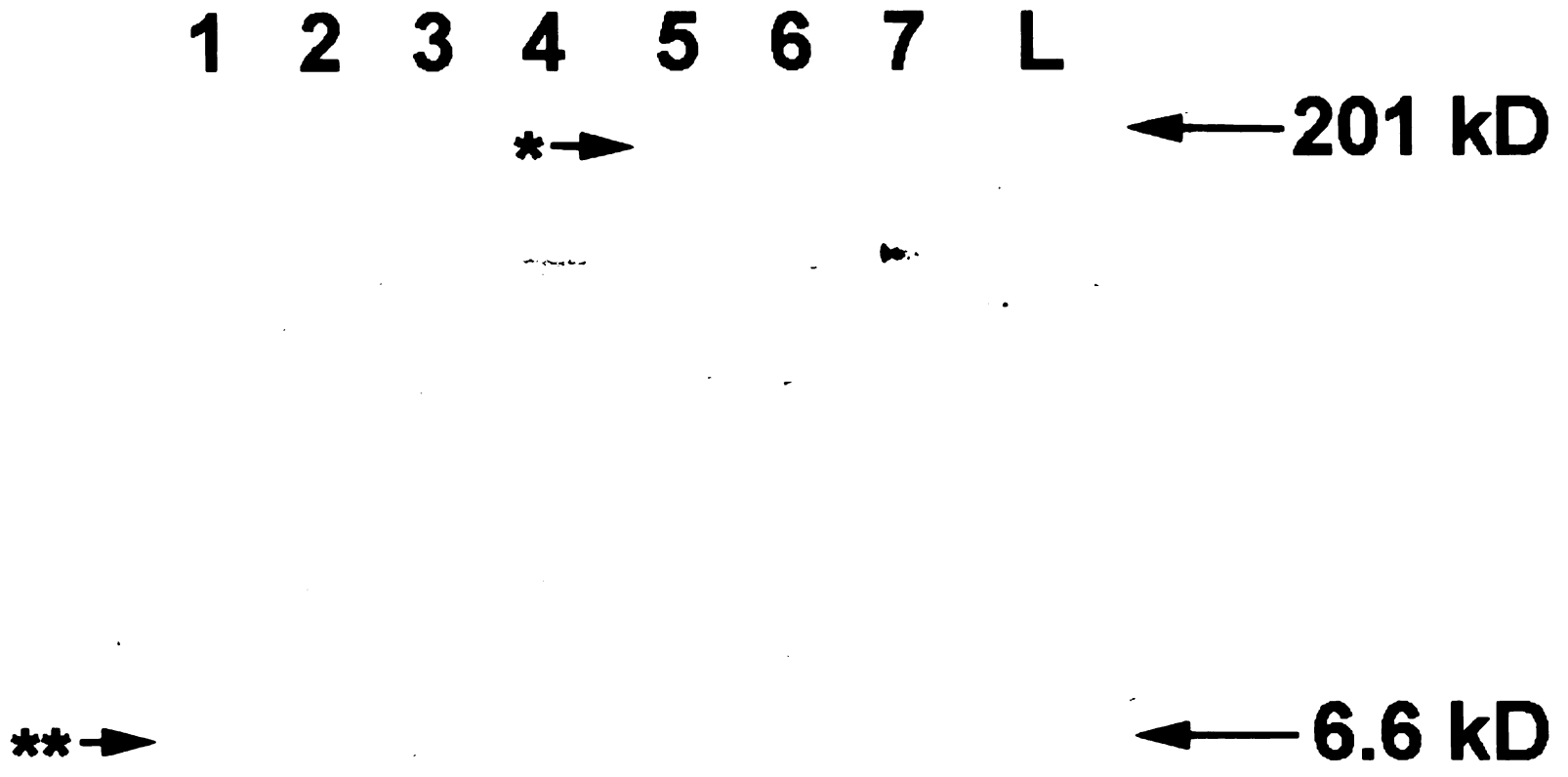

1 2 3 4 5 6 7 L
201 kD
*
**
6.6 kD

Figure 11: Immunoblot of Pep1 samples with sera from a patient with systemic candidiasis and AP conjugated anti-Human IgG secondary antibodies.
Immunoblot analysis of TOP10 *E. coli* cell lysate control (Lane 1), decreasing amounts of Pep1 expression samples (Lanes 2-4) and protein standard (Lane 5) after incubation with a 1:1000 dilution of sera from a patient with systemic candidiasis and AP conjugated anti-Human IgG secondary antibodies. Numbers on the right indicate the molecular weight of the proteins in the standard.

20.6 kD
6.6 kD
1
2
3
4
5

DISCUSSION

The need for new, highly specific and sensitive assays to diagnose disseminated candidiasis has received enormous attention (Lew, 1989, Matthews, 1996, Verweij *et al.*, 1998, Vincent *et al.*, 1998). Factors that have contributed to this include the lack of sensitivity and specificity of the current diagnostic markers, the lack of specific clinical symptoms of systemic candidiasis, and the high mortality rate associated with this disease. Investigations into non-culture based methods for diagnosing systemic candidiasis using traditional method have been difficult. This has led researchers to explore novel molecular and serological approaches (Vewiej et al., 1998, Reiss et al., 1998). However, these newly developed assays have lacked the sensitivity and specificity required in clinical laboratories. Recent findings suggested that the Phosphlipase B1 molecule of *Candida albicans* could be used as a diagnostic marker of systemic candidiasis. Certain advantageous properties of *C. albicans* Plb1 make it an attractive marker. These properties include the fact that Plb1 is a naturally secreted antigenic protein, isolates from patients with systemic candidiasis secrete higher levels of Plb1 in comparison to commensal isolates, and the gene and its encoded protein has been purified and well defined (Ghannoum, 2000).

Epitope mapping has been successfully applied by researchers in the development of serological diagnostic markers for infectious diseases (Baughn *et al.*, 1996, Marsden *et al.*, 1998, Naito *et al.*, 1998, Vanniasinkam, Barton, and Heuzenroder, 2001). We took advantage of this approach to align and map

known fungal Plb1s to define unique regions within the molecules. In order to develop Plb1 as a unique marker for systemic candidiasis, unique regions in the amino acid sequence of *C. albicans* Plb1 that were not present in other fungal Plb1 molecules were identified. These unique epitopes are proposed to be used in developing new serological tests, to improve old antigen or antibody detection assays, or their DNA sequences used as the basis for novel nucleic acid based assays. An advantage in using epitopes specific for *C. albicans* in a diagnostic assay is that these peptides could be used to make monoclonal antibodies as well. These monoclonal antibodies could be later used in a highly specific antigen detection assay and the peptides in highly specific antibody detection assays. This could circumvent the possible cross reaction between the whole Plb1 molecule/anti-Plb1 polyclonal sera and other fungal Plb1s/anti-fungal Plb1 antibodies.

In order for these epitopes to be used in the development of new assays, several characteristics should be required. These include:

1.) Their amino acid sequences would have to be conserved among all clinical isolates of *C. albicans.*

2.) An unlimited source of these peptides must be available.

3.) They must be antigenic to the host immune systems.

In our study we a) found that the amino acid sequences of the unique epitopes (Pep1 and Pep2) are conserved among five clinical isolates of *C. albicans*, b) optimized a bacterial expression system for Pep1 to provide an

unlimited source of this peptide, and c) performed initial antigenicity testing of the Pep1 peptide.

In order to use one of the unique epitopes in diagnostic assays, it was important to determine the variability of their amino acid sequences among clinical isolates of *Candida albicans*. Our strategy was to determine if the amino acid sequence were too variable in these regions to be used as specific peptides for *C. albicans*. If these peptides or monoclonal antibodies to these peptides were too variable they could not be used to consistently diagnose infections caused by *C. albicans*. Our analysis of the amino acid sequence of Pep1 and Pep2 in four clinical isolates and one reference strain of *C. albicans* showed that the 768 base pair region within the *caPLB1* gene contains the sequence for the two unique peptides. Although differences were observed at the nucleic acid level, none of the base changes resulted in a change in the deduced amino acid sequence of the peptides. The fact that the amino acid sequences of these regions were conserved in the five isolates used in this study, indicated to us that it is likely that these regions would be highly conserved in most clinical isolates of *C. albicans*. Therefore, they could be exploited as specific markers.

The possibility of producing unlimited amounts of these peptides is financially attractive. If these peptides, or monoclonal antibodies made against these peptides, are to be used in assays in clinical laboratories, a dependable source of these peptides would be needed. Two potential sources of purified peptides could be a) commercially synthesized peptides and b) recombinantly expressed peptides. Commercial synthesis of the peptides is an expensive route

to obtaining large amounts of the peptides; therefore we optimized a bacterial expression system to express Pep1. Once a set of recombinant clones that express the peptides was established, large amounts of purified peptide could be obtained relatively inexpensively. In our study both unique regions were successfully amplified using sets of nested primers and their amplicons were cloned into an expression vector. These clones were sequenced in order to determine if there were any mutations were induced during amplification of the inserts and to determine if they were in frame with the vector for proper expression. Their characterization showed that the PCR amplification of Pep1 and Pep2 did not result in any mutations and that the inserts were indeed in frame with the vector.

The optimization of the system to express the Pep1 fusion protein proved to be a tedious task. Expression of the Pep1 fusion protein was evaluated using SDS-PAGE and immunoblotting. Initial analysis of Coomassie stained SDS-PAGE gels of the expression samples indicated that the expression system was working properly. Although there were no visible bands that were indicative of Pep1 expression, there was an intense band observed in the expression control sample, which expressed a 130 kD β-Galactosidase protein. Immunoblotting of these expression samples gave contrasting results. Blots reacted with the anti-V5-HRP antibodies did not indicate that there was expression taking place. After performing multiple experiments to determine the reason for the contrasting results it was found that there was a problem with the anti-V5-HRP antibodies. This was determined after the β -Galactosidase expression control protein was

detected using polyclonal anti-β-Galactosidase antibodies. A new anti-V5 antibody was then used in the immunoblotting experiments. This time the expressed protein was detected.

Initial antigenicity testing was done by immunoblotting with sera from patients with proven systemic candidiasis. Pep1 expression samples were used to detect antibodies against Pep1 in the infected patient's sera. A positive result would indicate that Pep1 is immunogenic and anti-Pep1 antibodies are present. The results of these initial experiments were, however, inconclusive. Antibodies were detected by both, the expression samples and the negative control (*E. coli* cell lysate with no vector), at the molecular weight that Pep1 would be observed. There were also multiple other proteins that reacted with the patient's antibodies used in the blotting experiment. It could be speculated that the patient possesses natural antibodies against the *E. coli* cell lysates used in these experiments. Unfortunately, there were proteins that reacted at the same molecular weight as the Pep1 peptides, therefore it cannot be determined if all, part, or none of the reaction at that particular molecular weight is the result of anti-Pep1 antibodies reacting with the expressed Pep1 protein.

According to previous software analysis, it was expected that both, Pep1 and Pep2 fusion proteins would be antigenic. This was confirmed when preliminary data, gathered by our collaborating group in Cleveland, showed that synthetic Pep1 and Pep2 fragments are indeed antigenic. They showed that these synthetic peptides were detected by antibodies in patients with systemic candidiasis when used in an ELISA (Unpublished Data). These results

established that patients with systemic candidiasis have antibodies that reacted against Pep1 and Pep2, and that the titers are significantly higher than those in healthy control sera.

Further testing needs to be done to evaluate Pep1 and Pep2 as specific diagnostic markers of systemic candidiasis. For instance, the expressed Pep1 and Pep2 proteins needs to be purified to eliminate other *E. coli* proteins to determine the antigenicity of the peptides. Once their expressed products have been purified and their antigenicity determined, the specificity of the peptides needs to be evaluated. Evaluation of the specificity for candidiasis could be accomplished by reacting purified Pep1 and Pep2 with sera from patients with other diseases including the mycoses. It would be expected that little or no reaction would be observed if the peptides are indeed highly specific for *C. albicans*. Testing should also be done on different patient populations with other candidal infections to evaluate the utility of the Plb1-specific assay. These should include patients who are afflicted with mucosal and cutaneous candidiasis, candidiasis as a result of a neutropenic state, candidiasis from non-neutropenic diseases such as HIV/AIDS, and the appropriate negative controls (sera from healthy people). Testing should also be done in order to determine if anti-fungal therapy affects the detection of anti-Plb1 antibodies or Plb1 antigen. This is of paramount importance since this assay could also be used to monitor therapy.

In summary, our study showed that the caPlb1 molecule could be studied at the molecular level to identify putative unique regions not present in other

fungal Plb1s. This study found that two previously identified putative unique regions, Pep1 and Pep2, can be PCR amplified, and cloned, with Pep1 being expressed. However, additional antigenicity experiments, with the purified Pep1 antigens, should be carried out to fully demonstrate their potential as molecular markers. Although our antigenicity experiments were not conclusive, we have developed a system that can be later used to test this approach in other pathogenic microorganisms.

BIBLIOGRAPHY

Ashman RB and JM Papadimitriou. 1995. Production and function of cytokines in natural and acquired immunity to *Candida albicans* infections. Microbiol. Rev. 59(4):646-672.

Ashman RB, JM Papdimitriou, AK Ott, and JR Warmington. 1990. Antigens and immune response in *Candida albicans* infections. Immunol. Cell. Biol. 68:1-13.

Baugn RE, M Demecs, LH Taber, and DM Musher. 1996. Epitope mapping of b-cell determinants on the 15-kilodalton lipoprotein of *Treponema pallidan* (Tpp15) with synthetic peptides. Inffect. Immun. 64(7):2457-2466.

Bennett JE. 1987. Rapid diagnosis of candidiasis and aspergillosis. Rev. Infect. Dis. 9(2):398-402.

Berg FT. 1846. Om tursk hos barn. LJ Hjerta. Stockholm.

Bougnoux M, C Hall, D Moisscenet D, *et al*. 1990. Comparison of antibody, antigen, and metabolite assays for hospitalized patients with disseminated or peripheral candidiasis. J. Clin. Microbiol. 28(5):905-909.

Buchheidt D, H Sklandny, C Baust, and R Hehlmann. 2000. Systemic infections with *Candida* spp. in immunocompromised patients with hematological malignancies: current serological and molecular diagnostic methods. Chemotherapy. 46:219-228.

Calderone R, S Suzuki, R Cannon *et al*. 2000. *Candida albicans*: adherence, signaling and virulence. Med. Mycol. 38(Suppl 1):125-137.

Casadevall A. 1995. Antibody immunity and invasive fungal infections. Infect. Immun. 63(11):4211-4218.

Chaffin WL, JL Lopez-Ribot, M Casanova, D Gozalbo, and JP Martinez. 1998. Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. Microbiol. Molec. Biol. Rev. 62(1):130-180.

Chapman SW, CR Daniell III. 1994. Cutaneous manifestations of fungal infections. Infect. Dis. Clin. N. Amer. 4:879-910.

Cole GT, AA Halawa, and EJ Anaisse. 1996. The role of the gastrointestinal tract in the hematogenous candidiasis: from the laboratory to the bedside. Clin. Infect. Dis. 22(Suppl 2):573-588.

Cutler JE. 1991. Putative virulence factors of *Candida albicans*. Ann. Rev. Microbiol. 45:187-218.

Davis D, JE Edwards, AP Mitchell, and AS Ibrahim. 2000. *Candida albicans RIM101* pH response pathway is required for host-pathogen interactions. Infect. Immun. 68(10):5953-5959.

De Bernardis F, G Santont, M Boccanera *et al*. 2000. Local anticandidal immune responses in a rat model of vaginal infection by and protection against *Candida albicans*. Infect. Immun. 68(6):3297-3304.

De Repemtigny L, F Aumont, K Bernard, and P Belhomeur. 2000. Characterization of binding *Candida albicans* to small intestinal mucin and its role in adherence to mucosal epithelial cells. Infect. Immun. 68(6):3172-3179.

Edmond MB, SE Wallace, DK McClish *et al*. 1999. Nosocomial bloodstream infections in United States hospitals: a three-year analysis. Clin. Infect. Dis. 29:239-249.

Espinal-Ingroff A. 1997. Clinical relevance of antifungal resistance. Infect. Dis. Clin. N. Amer. 11:929-944.

Fallow K, K Bausch, J Noonan, E Huguenel, and P Tamburni. 1997. Role of aspartic proteases in disseminated *Candida albicans* infections in mice. Infect. Immun. 65(2):551-556.

Fernandez M, EH Moylett, DE Noyola, and CJ Baker. 2000. Candidal meningitis in neonates: a 10 year review. Clin. Infect. Dis. 31:458-463.

Fidel PL, JA Vazquez, and JD Sobel. 1999. *Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *Candida albicans*. Clin. Microbiol. Rev. 12(1):80-96.

Galen. 130 - ca200. De remedies parabilibus I - III. In: Kuhn CG (ed) 1965. *Opera Omlia*. Geoge Olms, Hildersheim.

Ghannoum M. 2000. Potential role of phospholipases in virulence and fungal pathogenesis. Clin. Microbiol. Rev. 13(1):122-143.

Gottfredsson M, GM Cox, and JR Perfect. 1998. Molecular methods for epidemiological and diagnostic studies of fungal infections. Pathol. 30:405-418.

Greenfield RA. 1992. Host defense system interactions with *Candida*. J. Med. Vet. Mycol. 30:89-104.

Gutierrez J, C Maroto, G Piedrola *et al.* 1993. Circulating candidal antigens and antibodies; useful markers of candidemia. J. Clin. Microbiol. 31(9):2550-2552.

Hadley S and AW Karchnur. 1995. Fungal infections in solid organ transplant recipients. Infect. Dis. Clin. N. Amer. 9(4):1045-1074.

Harley WB and SJ Drummer. 1994. Diagnosis of disseminated candidiasis by detection of antigenaemia: a critical review. Infect. Dis. Clin. Practice. 3:168-172.

Hippocrates. ca 460-377 BC. *Epidemics, book 3.* Translated F Adams. 1939. Williams and Wilkens, Baltimore.

Ibrahim AS, F Mirbod, SG Faller *et al.* 1995. Evidence implicating phospholipase as a virulence factor of *Candida albicans*. Infect. Immun. 63(5):1993-1998.

Jong AY, MF Stins, S Huang, SHM Chen, and KS Kim. 2001. Transversal of *Candida albicans* across the human blood-brain barrier *in vitro*. Infect. Immun. 69(7):4536-4544.

Kalin M and B Petrini. 1996. Clinical and laboratory diagnosis of invasive candida infections in neutropenic patients. Med. Oncol. 13:223-231.

Kanoh H, S Nakashima, Y Zhao *et al.* 1998. Molecular cloning of a gene encoding phospholipase D from the pathogenic and dimorphic fungus *Candida albicans*. Biochim. Biophys. Acta. 1398:359-364.

Kwon Chung KJ and JE Bennett. 1992. *Medical Mycology.* Lea and Febiger, Philadelphia. 280-336.

Leidich SD, AS Ibrahim, Y Fue *et al.* 1998. Cloning and disruption of *caPLB1*, a phospholipase B gene involved in the pathogenicity of *Candida albicans*. J. Biological Chem. 273(40):26078-26086.

Lew MA. 1989. Diagnosis of systemic *Candida* infections. Ann. Rev. Med. 40:87-97.

Lipton SA, WF Hickey, and JH Morris. 1984. Candidal infections in the central nervous system. Am. J. Med. 76:101-108.

Long-Krug S, K Fischer, R Hysmith, and J Ravdin. Phospholipase A enzymes of *Entamoeba histolytica*: description and subcellular localization. J. Infect. Dis. 152:536-541.

Lui L, K Kang, M Takatara, KD Cooper, and MA Ghannoum. 2001. Hyphae and yeasts of *Candida albicans* differentially regulate interleukin-12 production by human blood monocytes: inhibitory role of *Candida albicans* germination. Infect. Immun. 69(7):4695-4697.

Lundstrom T and J Sobel. 2001. Nosocomial candiduria: a review. Clin. Infect. Dis. 32:1602-1607.

Maenza JR and WG Merz. 1997. Candidemia: epidemiology and laboratory detection. Infect. Dis. Clin. Practice. 6:83-88.

Marsden HS, K MacAulay, J Murry, and IW Smith. Indentification of an immunodominant sequential epitope in glycoprotein G of herpes simplex virus type 2 that is useful for serotype-specific diagnosis. J. Med. Virol. 56:79-84.

Martin MV. 1999. The use of fluconazole and intraconazole in the treatment of *Candida albicans* infections: a review. J. Antimicrob. Chemother. 44: 429-437.

Martinez JP, ML Gil, J Lopez-Ribot, and WL Chaffin. 1998. Serological response to cell wall mannoproteins and proteins of *Candida albicans*. Clin. Microbiol. Rev. 11(1):121-141.

Matthews RC. 1996. Comparative assessment of the detection of candidal antigens as a diagnostic tool. J. Med. Vet. Mycol. 34:1-10.

Matthews R, JP Burnie, and W Lee. 1991. The application of epitope mapping in the development of a new serological test for systemic candidosis. J. Immunol. Methods. 143:73-79.

McCullough MJ, BC Ross, and PC Reade. 1996. *Candida albicans*: a review of its history, taxonomy, virulence attributes, and methods of strain differentiation. Int. J. Oral Maxillofac. Surg. 25:136-144.

Mirbod F, M Banno, MA Ghannoum *et al*. 1995. Purification and characterization of lysophosphlolipase-transacylase (h-LPTA) from a highly virulent strain of *Candida albicans*. Biochim. Biophys. Acta. 1257:181-188.

Na B and C Song. 1999. Use of monoclonal antibody in diagnosis of candidiasis caused by *Candida albicans*: detection of circulating aspartyl proteinase antigen. Clin. Diag. Lab. Immunol. 6(6):924-929.

Naito M, N Ohara, S Matsumoto, and T Yamada. 1998. Immunological characterization of alpha antigen of *Mycobacterium kansasii*: b-cell epitope mapping. Scand. J. Immunol. 48:73-78.

Nawrot J, K Grzybek-Hryncewicz, J Zielska, A Czarny and J Podwinska. 2000. The stud of cell-mediated immune response in recurrent vulvovaginal candidiasis. FEMS Immunol. Med. Microlbiol. 29:89-94.

Narrow RE, M Huezenroeder, and PA Manning. 1996. Antigenic epitope mapping of the M24 protein of *Streptococcus pyogenes*: implications for serodiagnosis of rheumatic fever. FEMS Immunol. Med. Microlbiol. 16:267-271.

Noverr M, SM Phare, GB Toews, MJ Coffey and GB Huffnagle. 2001. Pathogenic yeasts *Cryptococcus neoformans* and *Candida albicans* produce immunomodulatory prostaglandins. Infec. Immun. 69(5):2957-29-63.

Obayashi T, M Yoshida, T Mori *et al.* 1995. Plasma 1-3-Beta-glucan measurements in diagnosis of invasive deep mycosis and fungal febrile episodes. Lancet. 345:17-20.

Odds FC. 1988. *Candida and Candidosis.* Bailliere Tindall W. B. Saunders. London.

Ogawa H, RC Summerbell, KV Clemons *et al.* 1998. Dermatophytes and host defense in cutaneous mycoses. Med. Mycol. 36(Suppl 1):166-173.

Perez-Martin J, JA Uria, and AD Johnson. 1999. Phenotypic switching in *Candida albicans* is controlled by a *SIR2* gene. EMBOJ. 18(9):2580-2592.

Pfaller MA. 1996. Nosocomial candidiasis: emerging species, reservoirs, and modes of transmission. Clin. Infect. Dis. 22(Suppl 2):S85-94.

Phan QT, PH Belanger, and SG Filler. 2000. Role of hyphal formation in interactions of *Candida albicans* with endothelial cells. Infect. Immun. 68(6):3485-3490.

Reichart PA, LP Samaranayke, and HP Philipsen. 2000. Pathology and clinical correlates in oral candidiasis and its variants: a review. Oral Diseases. 6:85-91.

Reiss E, K Tanaka, G Bruker *et al.* 1998. Molecular diagnosis and epidemiology of fungal infections. Med. Mycol. 36(Suppl 1): 249-257.

Rex JH, TJ Walsh, JD Sobel *et al.* 2000. Practice guidelines for the treatment of candidiasis. Clin. Infect. Dis. 30:662-678.

Richardson M and M Ellis. 2000. Symposium on invasive fungal infections: clinical and laboratory diagnosis. Hospital Med. 61(9):610-614.

Rippon JW. 1998. Candidiasis and the pathogenic yeasts. *Medical Mycology*. 3rd edn. WB Saunders, Philadelphia. 532-581.

Rosa C, S Osborne, F Goretto *et al*. 1995. Epitope mapping of the NS4 and NS5 gene products of the hepatitis C virus and the use of chimeric NS4-NS5 synthetic peptides for serodiagnosis. J. Virological Meth. 55:219-232.

Rosen von Rosenstein N. 1771. Underrattelse om barms sjukdomer och deras botemedal. Wenneberg and Nordstrom, Stockholm.

Saffer LD and S Schwartzman. 1991. A soluble phospholipase of *Toxoplasma gondii* associated with host cell penetration. J. Protozool. 38:454-460.

Sanblas G, LR Travasso, BC Fries *et al*. 2000. Fungal morphogenesis and virulence. Med. Mycol. 38(Suppl 1):79-86.

Segal E and D Elad. 1998. *Candida* species and *Blastoschizomyces capitus*. *Topley and Wilson's Microbiology and Microbial Infections vol. 4 Medical Mycology*. Arnold, New York. 423-460.

Sendid B, M Tarbouret, JL Parot *et al*. 1999. New enzyme immunoassays for sensitive detection of circulating *Candida albicans* mannan and antimannan antibodies: useful combined test for diagnosis of systemic candidiasis. J. Clin. Microbiol. 37(5):1510-1517.

Sobel JD and J Vasquez. 1990. Candidemia and systemic candidiasis. Semin. Respir. Infect. 5:123-137.

Soll DR. 1992. High frequency switching in *Candida albicans*. CMR. 5(2):183-203.

Steffan P, JA Vasquez, D Boikov *et al*. 1997. Identification if *Candida* species by randomly amplified polymorphic DNA fingerprinting on colony lysates. J. Clin. Microbiol. 35(8):2031-2039.

Streyer L. 1995. *Biochemistry*. 4th edn. WH Freeman and Company. New York. 689-690.

Sugiyama Y, F Nakashima, F Mirbod *et al*. 1999. Molecular cloning of a second phospholipase B gene from *Candida albicans*. Med. Mycol. 37:61-67.

Thomas I. 1993. Superficial and deep candidosis. Inter. J. Dermatol. 11:778-783.

Titball RW. 1993. Bacterial Phospholipases. Clin. Microbiol. Rev. 57:347-366.

Underwood M. 1784. A treatise on the disease of children. P 43. J Matthews, London.

Verwiej PE, D Poulan, T Obayashi *et al*. 1998. Current trends in the detection of antigenaemia, metabolites, and cell wall markers for diagnosing and therapeutic monitoring of fungal infections. Med. Mycol. 36(Suppl 1):146-155.

Vincent JL, E Anassie, H Bruining *et al*. 1998. Epidemiology, diagnosis, and treatment of systemic *Candida* infections in surgical patients under intensive care. Intensive Care Med. 24:206-216.

Wahyuningsih R, H Freisleben, H Sonntag, and P Schnitzler. 2000. Sinple and rapid detection of *Candida albicans* DNA in serum by PCR for diagnosis of invasive candidiasis. J. Clin. Microbiol. 38(8):3016-3021.