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REVIEW ARTICLE

Candida parapsilosis Secreted Lipase as an Important Virulence Factor

Renata Toth, Adel Toth, Csaba Vagvölgyi and Attila Gacser*

Department of Microbiology, University of Szeged, Szeged, Hungary

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Abstract: The prevalence of *Candida parapsilosis*, an opportunistic human pathogenic fungal species, is increasing at an alarming rate in the hospital environment. Patients at risk for *C. parapsilosis* infection include those with immunosuppression, such as individuals with cancer, AIDS, and low birth weight premature neonates as well as patients that had undergone abdominal surgery. Neonatal candidiasis caused by *C. parapsilosis* has been widely reported across the globe. Various reports have shown that, compared to other *Candida* species, certain *C. parapsilosis* clinical isolates were less susceptible to antifungals such as amphotericin B, fluconazole, and caspofungin. In addition, some studies have even reported multi-echinocandin or multi-azole resistant strains of *C. parapsilosis*. *C. parapsilosis* has several virulence factors that contribute to its capacity for host invasion and among these factors extracellular lipases have a major role in pathogenesis. In this review we have collected all the recent relevant studies that confirm the involvement of secreted lipases in *C. parapsilosis* pathogenesis, using both *in vitro* and *in vivo* models of infection. Of particular note, an available lipase deficient *C. parapsilosis* strain has been utilized to demonstrate that the lack of secreted lipases decreased virulence, reduced tissue damage, and was less able to survive within phagocytes or mice compared to the wild type. Since fungal secreted lipases have different characteristics than lipolytic enzymes present in humans, *C. parapsilosis* extracellular lipases may be potential targets for the development of novel antifungal drugs.

Keywords: *Candida parapsilosis*, extracellular lipase, pathogenicity, macrophages, dendritic cells, reconstituted human tissue, *in vivo* models.

INTRODUCTION

Despite of the wide range of available antifungal medicines, nosocomial fungal infections remain a major concern [1-3]. Recent studies detail that invasive candidiasis is the leading cause of mycosis-associated mortality worldwide [4-7]. Although the opportunistic pathogen *Candida albicans* is the most common species found in the hospital environment, numerous non-albicans *Candida* (NAC) species have been isolated from patients as a source of primary infection [8, 9]. More significantly, the frequency of invasive disease due to NAC species such as *C. parapsilosis* is rising alarmingly [10, 11]. One reason for the increased incidence of *C. parapsilosis* infections is that the species displays notable resistance to antifungals drugs [11, 12]. Several studies have reported *C. parapsilosis* clinical isolates with reduced susceptibility to different types of antifungals such as Amphotericin B, fluconazole, anidulafungin, and caspofungin when compared to other *Candida* species [13-15, 14c]. In addition, multi-echinocandin and multi-azole-resistant clinical strains have been also isolated from patients [14b]. Horizontal transmission is also a characteristic of this species [16]. Taken together, these factors suggest that, the number of resistant *C. parapsilosis* strains might further increase in the future.

Since the '90s, reports have frequently associated *C. parapsilosis* with nosocomial infections among children and newborns [17-21]. Several recent studies have further reported *C. parapsilosis* to be the predominant species causing neonatal infections at intensive care units (ICU), threatening especially low birth weight infants, with significant neonatal mortality [7, 22-24]. Additionally, diverse adult patients with diminished immunity are also at risk [25-28]. Further risk factors include the use of lipid rich parenteral nutrition and prolonged use of implanted devices, as *C. parapsilosis* readily forms and maintains stable biofilms on the surfaces of commonly used medical equipments [29].

Microbial secreted hydrolytic enzymes are known to contribute to the process of fungal pathogenesis. Such enzymes include extracellular proteinases, phospholipases and lipases [30-32]. The role of fungal secreted lipases is pleiotrop as they modulate the immune response in various ways [33]. Both cellular and humoral host responses are affected and they also contribute to host tissue damage, allowing fungal cells to invade the host [33-35]. Based on a large number of recent studies, *C. parapsilosis* is considered an emerging

*Address correspondence to this author at the Department of Microbiology, University of Szeged, Közép fasor 52,H-7626 Szeged, Hungary;
Tel: +36/62544849; Fax: +36/62544823; E-mail: gacsera@gmail.com

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pathogen, and since its clinical relevance is clear, attention should be increased towards this NAC species.

A number of reports and previous reviews have already demonstrated the role of *C. parapsilosis* in nosocomial infections [23, 24, 29, 36]. In this review, we aim to provide a description of a critically important effector protein of *C. parapsilosis* that has been proven to promote invasion, including altering host immune responses to the benefit of the fungus. We will detail how fungal secreted lipases influence the virulence of *C. parapsilosis* and thus provide evidence that fungal lipases should be considered a target for developing antifungals in the future.

FUNCTION OF FUNGAL SECRETED LIPASES

Fungal secreted lipases can have a variety of functions, due to their ability to catalyze a wide range of reactions such as the hydrolysis or synthesis of triacylglycerols [37]. Many microbial extracellular lipases are used for industrial purposes as biocatalysts and *Candida* species are frequently used to produce hydrolytic enzymes for industrial use [38, 39]. For example, *C. antartica* and *C. rugosa* secreted lipases are the most frequently used biocatalysts that are involved in detergent industry, biosensor modulation, and even biocatalytic resolution of pharmaceuticals such as antibiotics, virucids, probiotics and non-steroid anti-inflammatory drugs (NSAIDs) [38, 40, 41]. Thus, fungal secreted lipases are considered beneficial for biotechnological applications.

However, the role of secreted lipase as a potential virulence factor on the level of a pathogenic microorganism is also quite evident [31, 42]. In addition to the key roles of lipases in nutrient acquisition, they might also augment adhesion to host tissues, lyse competitive microflora, and modulate host inflammatory reactions, which can also result in further tissue damage [29, 43]. Therefore, fungal secreted lipases contribute to host invasion in diverse ways.

C. albicans is known to have 10 lipase encoding genes, one (*LIP1*) characterized by Fu *et al.* [44] and nine (*LIP2-LIP10*) by Hube *et al*. [31]. Further examinations of *C. albicans* lipases revealed that individual genes are regulated differently during an infection and gene expression of each ORF was mainly dependent upon the stage of infection [45]. Interestingly, the different genes had variable contributions to virulence. The role of *LIP5* and *LIP8* in *C. albicans* virulence was highlighted as their expression levels remained the highest when examining isolates recovered from systemically infected mice or from human oral specimens [45]. Furthermore, the generation of a *C. albicans* strain lacking *LIP8* led to experiments demonstrating that this null mutant was less virulent *in vivo* compared to wild-type [46]. These reports suggest that differential regulation of lipase gene expression has helped *C. albicans* to adapt to the human host.

Lipid acquisition from the host has already been reported in *Candida* species. Both *C. albicans* and *C. parapsilosis* assimilate exogenic arachidonic acid in order to produce human prostaglandin-like molecules (PGE_2 and PGD_2) [47, 48]. Fungal prostaglandins were shown to modulate host immune responses via a variety of mechanisms to promote host colonization [49]. Furthermore, *C. albicans* morphogenesis is also influenced by PGE_2 [49]. Secreted lipases contribute to the release of lipid intermediers during host invasion, and therefore also indirectly influence fungal virulence.

CpFIT2 (Fat storage-Inducing Transmembrane protein 2) is involved in fungal lipid droplet (LD) formation. According to Nguyen *et al*. *FIT2* has a role in triacil glycerol (TAG) synthesis and may be required for the balance between lipid species via LD formation. It is notable, that defect of *CpFIT2* leads to reduced LD formation, altered fatty acid composition, growth disabilities and increased susceptibility to oxidative stressor. Furthermore, *FIT2* deletion also resulted in reduced virulence in murine infection models, further supporting the role of fungal lipids in pathogenicity [50].

C. parapsilosis is considered to be less pathogenic than *C. albicans*, although its relevance is increasing in the hospital environment and it even outranks *C. albicans* in some health care centers as a cause of invasive candidiasis [29]. *C. parapsilosis* has only two indentified secreted lipase coding genes [29]. The presence of only two secreted lipase encoding genes facilitated the construction of a secreted lipase-null strain, a double knock-out strain, which has proven to be a valuable tool for studying the role of extracellular lipases during host-pathogen interactions.

IDENTIFICATION OF *C. PARAPSILOSIS* SECRETED LIPASES

Fu *et al*. published the first report that suggested the presence of secreted lipases in *C. parapsilosis* in 1997 [44]. Five years later, Neugnot *et al.* identified two lipase encoding ORFs in this species [51]. Amino acid sequence comparison revealed that these identified proteins might belong to the same *Candida* lipase gene family that was described by Hube *et al*. in *C. albicans*, thus they might be also involved in fungal pathogenesis. The newly found *C. parapsilosis* lipase encoding ORFs showed the highest homology with *C. albicans LIP1*. Accordingly, the ORFs were named *CpLIP1* and *CpLIP2*. Expression analysis of the two *C. parapsilosis* lipase genes in *S. cerevisiae* and later in *P. pastoris* revealed that only *CpLIP2* coded for a functional protein [51, 52]. *C. parapsilosis LIP1* and *LIP2* are encoded by two neighboring ORF regions with an approximate size of 1300 bp for each [51]. Both lipases consist of a deduced 465 amino acid sequence with two CUG codons present in *CpLIP1* but none in *CpLIP2*. As *C. parapsilosis* is a member of the CUG-clade (serine instead of leucine translation), this might be an explanation why the enzyme encoded by *CpLIP1* is not functional when expressed in *S. cerevisiae* [51]. *CpLIP2* was further described with a molecular mass of 60 kDa and was shown to contain the Gly-X-Ser-X-Gly consensus motif that is highly conserved and commonly found in the serine catalytic core of lipolytic enzymes [51]. The lipase from *C. parapsilosis* catalyses the alcoholysis of various esters. According to Briand el al., this lipase showed the highest activity when unsaturated fatty acids with a cis-delta 9 double bond or the ester bond of long-chain fatty acids were present in the medium [53]. During the investigation of biochemical properties, it was also shown that the catalytic activity was also dependent upon the temperature of the reactant medium [53].

After whole genome sequencing, two additional *LIP*-like genes were suggested that may be present in the *C. parapsilosis* genome, however, these remained uninvestigated [29]. Expression studies of *CpLIP* genes using other yeasts highly contributed to our knowledge on *C. parapsilosis* lipase secretion, but did not address their role in virulence. Although there were advances in *C. parapsilosis* genetic manipulation [12, 54] , it was not until 2007 that an innovative method for targeted gene deletion became available, and *LIP1 - LIP2* were one of the first genes to be deleted from the diploid genome of *C. parapsilosis,* using a dominant selection marker carrier flipper cassette [34]. The available null mutant strain (*Cp ΔΔlip1-ΔΔlip2*) provided us a novel tool for studying *C. parapsilosis* lipases and facilitated numerous subsequent virulence related investigations. Early studies demonstrated the role of *CpLIP1-LIP2* in viability in lipid rich environment, biofilm formation, and adherence to host surfaces as well as to immune response modulation [34].

IN VITRO HOST EFFECTOR CELL - *C. PARAPSILOSIS* INTERACTIONS

Using the previously mentioned lipase deficient strain, several studies confirmed the role of *C. parapsilosis* lipases in virulence (Fig. **1**) [33, 34, 55, 56]. Initial studies focused on the interactions of *C. parapsilosis* with murine macrophages. The lipase deficient *Cp ΔΔlip1-ΔΔlip2* yeast cells induced higher phagocytic and killing efficiency by murine macrophages *in vitro* when compared to the lipase secreting wild type cells (Fig. **2**) [34]. Subsequent studies with human peripheral blood mononuclear cell – derived macrophages (PBMC-DMs) [33] and dendritic cells (DCs) [55] reported similar outcomes. Overall, the *Cp ΔΔlip1-ΔΔlip2* strain appeared to be less virulent, as both types of human primary cells were able to kill mutant cells in a higher ratio compared to wild type cells. Intracellular survival of *C. parapsilosis* cells following phagocytosis has been recently reported [35]. In contrast with DCs, no difference was detected in the phagocytic efficiency of fungal cells by PBMC-DMs, although early phago-lysosome fusion was detected in both cases [33, 55]. These results led to the conclusion that fungal lipase secretion delays phagosome maturation, thus is considered to be one of the factors that contribute to fungal survival inside host cells.

In addition to their roles as professional phagocytes and professional antigen presenting cells, PBMC-DMs and DCs release certain types of effector molecules or cytokines to modulate immune responses. These cytokines include interleukin – 1 (IL-1), interleukin – 6 (IL-6) and tumor necrosis factor alpha (TNFα) as major inducers of inflammatory reactions; IL-8 as a neutrophil chemotactic factor and other chemokines to recruit leukocytes to the infection site [57, 58]. Cyclooxygenase 2 (COX-2) is essential for the synthesis of the biologically active inflammation inducer prostaglandin E2 [48]. Toth A. *et al*. and Nagy and Filkor *et al*. reported that both DCs and PBMC-DMs released IL-1, IL-6 and TNFα pro-inflammatory cytokines at a markedly increased level in the presence *Cp ΔΔlip1-ΔΔlip2* cells compared to that occurring in the presence of wild type yeast cells and an elevated level of IL-8 was detected after DCs were challenged with *Cp ΔΔlip1-ΔΔlip2* compared to wild type cells [55]. Similarly, higher levels of IL-8 and COX-2 expression were observed when PBMC-DMs were treated with the lipase deficient mutant in comparison with wild type strain. Interestingly, the anti-inflammatory response of PBMC-DM was also modulated, as an elevated IL-10 level was found after exposure to *Cp ΔΔlip1-ΔΔlip2* compared to that occurring after challenge with wild type yeast [33, 55].

In summary, the lack of the secreted lipase led to a stronger inflammatory response, shown by the increase in the pro-inflammatory cytokine and chemokine expression levels, as well as by more efficient killing and phagocytosis of *Cp ΔΔlip1-ΔΔlip2* cells by professional phagocytes. These reports suggest that *C. parapsilosis* secreted lipases suppress both the cellular and humoral immune responses and thus have a protective role against host responses.

TISSUE MODELS FOR *C. PARAPSILOSIS* EXTRACELLULAR LIPASES

In the macrophage - *C. parapsilosis* model, host cell damage by fungal cells was not affected by the presence of secreted lipases [33]. However, this did not mean that the extracellular lipases could not impact more complex tissues and the utilization of reconstituted human tissue (RHT) models allowed for the first assessment of *C. parapsilosis* lipase on virulence. Although RHTs had been used to model cutaneous, oral and vaginal *C. albicans* disease [59-61], they had not been applied to the study of *C. parapsilosis* to assess the histopathological effect of microbial secreted hydrolytic enzymes such as fungal extracellular lipases [34, 62]. In lipid rich environment fungi use secreted lipases to digest lipids in order to supply basic nutrient needs [29]. Oral epithelial RHT challenged with *C. parapsilosis* wild type cells develop severe histopathological changes such as atrophy, edema, and clefting in all tissue layers, along with higher rates of apoptosis [34]. In contrast, *Cp ΔΔlip1-ΔΔlip2* induced minimal injury, as the infected tissue was histologically similar to the uninfected control and their ability to attach to the epithelial surface was significantly decreased compared to wild type [34]. Using lactate-dehydrogenase (LDH) release as a determinant of RHT damage, the wild type *C. parapsilosis* yeasts caused significantly reduced cellular damage compared to the lipase mutant cells [34]. Additionally, inhibition of lipase by Ebelactone B, quinine or acetylsalicylic acid abrogated RHT infection and damage caused by *C. parapsilosis* [62, 63]. These findings further supported the involvement of *C. parapsilosis* extracellular lipases in tissue adhesion and destructive disease.

IN VIVO INFECTION MODELS TO STUDY *C. PARAPSILOSIS* LIPASE FUNCTION

In order to study systemic immune responses and inflammation triggered by pathogenic fungi, numerous *in vivo* infection models are in use. Even though, newly emerging non-conventional *in vivo* models of infection are becoming popular, mammalian models are still of central importance [64, 65]. Although *Galleria mellonella* has been described as a model to study *Candida* species virulence, including *C. parapsilosis* [65, 66], mice remain the most suitable and widely accepted model of candidiasis; however, occasionally, certain types of rats are also used. A common method to investigate non-lethal disease is to perform fungal burden

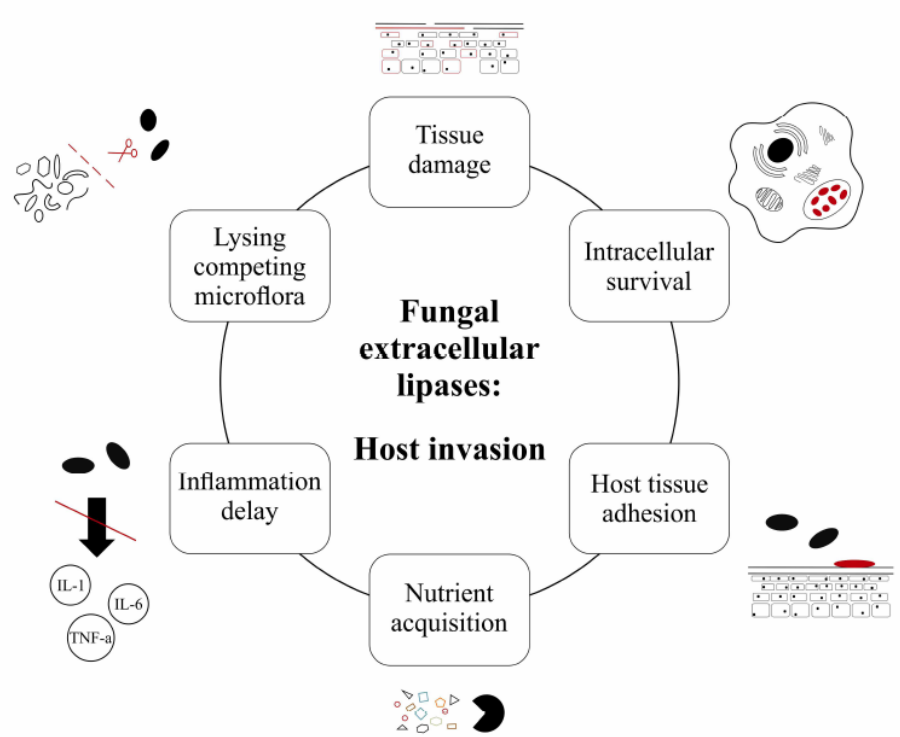

Fig. (1). The role of fungal extracellular lipases in host invasion. The image represents the putative role of *C. parapsilosis* secreted lipases during pathogenesis. Fungal secreted lipases directly contribute to nutrient acquisition and host cell lysis. Microbial extracellular lipases may also be responsible for directly lysing competitive microflora. Besides their direct roles in virulence they might influence fungal adhesion properties; delay inflammatory processes due to the release of chemical mediators from damaged cells; and contribute to intracellular survival as a result of phago-lysosome fusion inhibition.

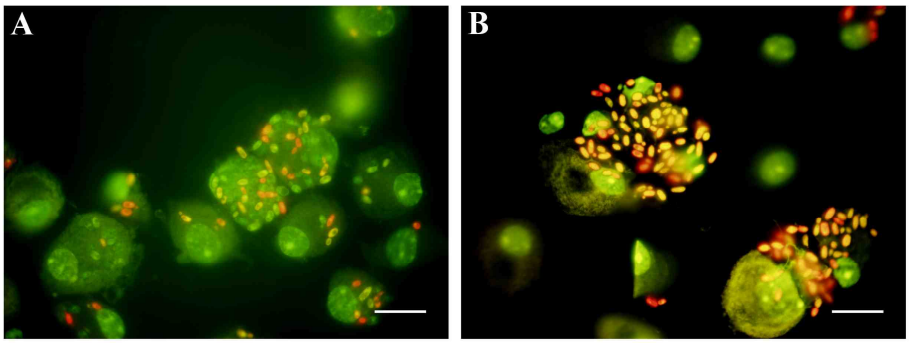

Fig. (2). Phagocytosis of *C. parapsilosis* cells. Representative image shows the phagocytosis of *C. parapsilosis* wild type (A) and lipase null mutant cells (B) by human peripheral blood mononuclear cell – derived macrophages. Acridine orange and crystal violet were used for staining. Orange / red fluorescence indicates cell death, while live cells emit green fluorescence. Scale bar: 20µm.

assessments of organs by means of colony forming unit (CFU) counting of plated organ homogenates. Using a intraperitoneal murine infection model, it has been demonstrated that *Cp ΔΔlip1-ΔΔlip2* cells were able to accumulate less in the kidney, liver and spleen of BALB/c mices 2 days post infection [34], but these mutant cells were eradicated within 4 days after the infection, which was significantly faster than clearance of wild type yeast [34]. As *C. parapsilosis* is one of the leading species associated with neonatal invasive infections, efforts have been applied to developing a suitable model to mimic newborn-related candidiasis [67-69]. Using newborn Sprague–Dawley rats, Trofa *et al.* demonstrated that premature neonates were highly susceptible to candidal infections. Moreover, this rat pup model proved to be effective for detecting the effects of the presence or absence of extracellular lipases. In their experiments, they infected newborn rats intravenously (IV), intragastrically (IG) and intraperitoneally (IP) either with the lipase secretion deficient strain of *C. parapsilosis*, a *LIP8* deficient strain of *C. albicans* or their corresponding wild type strains. Notably, both *CpΔΔlip1-ΔΔlip2* and *CaΔΔlip8* displayed significantly lower virulence compared to wild type strains as indicated by reduced organ fungal burdens. These findings of *in vivo* studies further demonstrate the crucial role of fungal secreted lipases in *C. parapsilosis* pathogenicity.

CONCLUSIONS

C. parapsilosis is an opportunistic human pathogenic species that is commonly associated with nosocomial fungal diseases, and this species continues to emerge in incidence and importance. Fundamental molecular investigations coupled with *in vitro* and *in vivo* studies have made major strides in elucidating several of the factors that facilitate the pathogenesis of *C. parapsilosis*. One of the best described virulence factors is the secreted lipases of *C. parapsilosis*, and the availability of lipase deletion strains has facilitated the study of *C. parapsilosis* virulence properties. There are now numerous reports that clearly detail the importance of *C. parapsilosis* extracellular lipases in pathogenesis as their presence alters cellular and humoral immune responses, delays inflammatory reactions, contributes to host tissue adhesion and promotes intracellular survival. In this review, we described the key role of secreted lipases in *C. parapsilosis* virulence, as another potential factor of this species that can be targeted by a novel class of antifungals of future medicine.

LIST OF ABBREVIATIONS

CpΔΔlip1-ΔΔlip2	=	*C. parapsilosis LIP1-LIP2* secreted lipase homozygous deletion strain;
PBMC-DM	=	peripheral blood mononuclear cell – derived macrophages;
DC	=	dendritic cell;
RHT	=	reconstituted human tissue.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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