The Capsule of the Fungal Pathogen *Cryptococcus neoformans* Paradoxically Inhibits Invasive Growth

Oscar Zaragoza[*,1], Manuel Cuenca-Estrella[1], Javier Regadera[2] and Juan Luis Rodríguez Tudela[1]

[1]*Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Carretera de Majadahonda-Pozuelo, Km 2, Majadahonda, 28220, Madrid, Spain*

[2]*Departamento de Morfología, Facultad de Medicina, Universidad Autónoma de Madrid, C/Arzobispo Morcillo, nº 2, Madrid 28029, Spain*

Abstract: Invasive growth in agar is an extended feature among many fungal species, and is correlated with morphological changes. In this paper we describe this phenomenon in the fungal pathogen *Cryptococcus neoformans*. This pathogen has a polysaccharide capsule around the cell body which is required for virulence. We found that acapsular mutants obtained by different methods invaded the agar in rich media, a phenomenon not observed in encapsulated strains. Agar invasion required the absence of capsule, since invasive growth was not present in *cap59* mutants reconstituted with the wild type gene. We compared multiple *C. neoformans* strains, including both *C. neoformans* and *C. gattii*, and confirmed that none of the encapsulated strains invaded the agar. In contrast, for other fungi (such as *Candida albicans*, *C. parapsilosis* and *C. krusei*), invasive growth in *C. neoformans* was not correlated with hyphae or pseudohyphae formation, and only yeast forms were observed in the agar. Melanisation of the cells did not affect the pattern of invasive growth. Our results demonstrate that the *C. neoformans* polysaccharide capsule inhibits invasive growth, a phenomenon that could have consequences for the dissemination of the microorganism during *in vivo* infection.

Keywords: *C. neoformans*, agar invasion, capsule, acapsular.

INTRODUCTION

Although the incidence of fungal infections in immunocompromised patients has significantly increased during the last years, the processes that result in disease remain poorly understood. One of the key processes that is required for systemic fungal infection is the dissemination of the microorganism from the site of initial infection to distant target organs. The main barriers that fungi have to cross to invade target organs are epithelia and endothelia. There have been several mechanisms described that allow the passage of fungal cells through these barriers, including the secretion of lytic enzymes that digest the surface of biological surfaces, endocytosis and transcytosis, phagocytosis and diapedeses inside the phagocytic cell and inclusion between endothelial cells (see seminal review in [1]).

In laboratory conditions it is difficult to reproduce tissue and organ invasion. One phenomenon that may provide an approximation of the capacity of pathogens to invade, and therefore, produce infection, is *in vitro* invasive growth. Invasive growth is the ability that some microorganisms have to penetrate into solid media and to grow included in this matrix. In laboratory conditions, invasive growth is easily observed in agar plates, by washing the plates and observing the amount of microorganism that remains included in the agar. Agar invasive growth is a common feature for many types of fungi, such as *Candida species, Saccharomyces cerevisiae, Exophiala* (*Wangellia),* and several plant pathogens [2-7]. In the case of the human pathogenic fungi, invasive growth suggests the ability of microorganism to invade and grow in host tissues, or cross biological barriers, such as epithelia, blood vessels or the blood-brain barrier. In addition, *in vitro* invasive growth constitutes a simple model to study the role of single elements during invasion. As an example, the contribution of melanin to hyphal penetration of *Exophiala* (*Wangellia)*spp into tissues has been studied *in vitro* using various supports [8].

Invasive growth has been correlated in many fungi with morphological changes, normally to the appearance of hypha or filamentous forms. In some plant fungal pathogens, invasion is also correlated with the formation of specialized filamentous forms, known as appresorium, which undergo biophysical changes that allow penetration in the plant leaves [9]. In the case of mammalian pathogenic fungi, invasive growth and morphogenesis have been primarily studied in *Saccharomyces cerevisiae* and *Candida albicans.* These yeasts can form pseudo- and true hypha, respectively, which are thought to be more invasive due to the higher capacity of penetration compared to the yeast form [10, 11]. In *S. cerevisiae*, filamentous growth occurs mainly during nitrogen and carbon source starvation and stress conditions, and it is thought that this form helps the cell to evade adverse conditions and to search for an environment richer in nutrients [4, 12]. Filamentous and invasive growth occur in both haploid in diploid strains, although there are some phenotypic differences between these two forms [13, 14]. Two main pathways are involved this type of growth: cAMP-dependent pathway

*Address correspondence to this author at the Servicio de Micología, CNM, ISCIII, Carretera de Majadahonda-Pozuelo, Km 2, Majadahonda, 28220, Madrid, Spain; Tel: (+34) 91 822 3661; Fax: (+34) 91 509 70 34;
E-mail: ozaragoza@isciii.es

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and MAP kinases (see reviews in [10, 15]). The interplay of these pathways during filamentous growth results in gene expression regulation. Many of these genes are involved in cell adhesion, such as flocculins, and in changes in cell shape. In *Candida albicans*, many pathways are involved in filamentous and invasive growth (see review in [16-21]). In the case of *C. albicans*, blastoconidias are normally found outside the epithelial and endothelial cells during tissue infection, and hyphae are the forms normally found inside the endothelial and epithelial cells [22, 23].

Cryptococcus neoformans is another human fungal pathogen whose incidence among immunocompromised patients has increased significantly in recent years [24]. *C. neoformans* is unique among fungal pathogen because it has a polysaccharide capsule that surrounds the cell body. The capsule is composed of three components: glucuronoxylomannan (GXM, 95%), galactoxylomannan (GalXM, 5%) and mannoproteins (<1%). During pathogen-host interactions, the *C. neoformans* capsular polysaccharide is abundantly released into tissues [25], and has been associated with a myriad of deleterious immunological effects [26-32]. In addition, the capsule has antiphagocytic properties [33-38], although it has been described that it can interact with phagocytic receptors [32, 39-42]. During infection, dissemination from the lung to other organs, especially the brain, where it produces meningitis, is associated with the death of the host. Several studies suggests that *C. neoformans* crosses the blood-brain barrier *via* trancytosis [43]. Dissemination in the host correlates with changes in capsule structure [44, 45]. It has been recently reported that it can produce biofilms *in vitro* [46], which suggests another mechanism by which adherence to solid matrixes promotes dissemination and disease.

In this manuscript we show that the polysaccharide capsule inhibits invasive growth in agar plates. Since the capsule is the main virulence factor, this fact might seem paradoxical, since it suggests that the absence of the capsule enhances tissue invasion, a process that in turn could influence the course of fungal dissemination.

MATERIAL AND METHODS

Strains and growth media. The *C. neoformans* strains used in this study are listed in Table **1**. Strains C536 and C538 were kindly provided by Dr. Kwon-Chung (NIH, Bethesda). Strain C536 is a disruptant mutant of the *CAP59* gene, and strain C538 is a reconstituted strain of the C536 mutant which contains the wild type *CAP59* gene under the control of the *GAL7* promoter. In addition, a total of 37 isolates from the Yeast Collection of the Reference Mycology Laboratory of the Spanish National Centre for Microbiology were tested for invasive growth. These included *C. neoformans, C. gattii*, *Candida albicans*, *Candida krusei* and *Candida parapsilosis*

The cells were regularly grown in Saboraud medium (Difco, Le Pont de Claix, France) at 30°C with moderate shaking (150 r.p.m.). When the cells were grown in solid medium, we prepared 2% agar plates containing 1% peptone and different carbon sources (glucose, galactose or ethanol) at a final 2% concentration. To induce melanization, the cells were grown in minimal media (30 mM KH_2PO_4, 10 mM $MgSO_4 \cdot 7H_2O$, 2.7 mM glucose, 30 μM thiamine, 80 nM biotin, 7.5 mM asparagine, 6.8 mM L-glutamine, 13.3 mM glycine,) containing L-DOPA (0.2 mM) for 7 days at 30°C. To obtain control non-melanized samples, the cells were grown in the same medium without L-DOPA. Then, invasive growth was studied in the same media containing 2% agar. To test the role of the cAMP dependent pathway, we prepared plates containing 10 mM cAMP (Roche) or several concentration of the specific cAMP-dependent protein kinase inhibitor, H89 (Sigma Aldrich, St. Louis, MO).

Invasive growth. Invasive growth was defined as the ability of fungal cells to penetrate into agar, and in consequence, retained in the solid medium after extensive washing of the Petri dish. To test for invasive growth *C. neoformans* cells from various strains were grown in Sabouraud medium at 30°C at 150 r.p.m. The cells were collected in the late exponential phase of growth, and washed twice with PBS. The cells were suspended in the same buffer at a cell density of 10^6 cells/mL, and serial dilutions of 10^5, 10^4 and 10^3 cells/mL were prepared. Four to eight μL were placed on plates, allowed to dry and incubated for different time periods at the temperature indicated in each experiment. After the incubation time, invasive growth was observed as follows. The plates were photographed using a Nikon D70 digital camera, and then, an abundant amount of water was added to the plates, and cells growing on the agar surface were discarded by rubbing the surface with a loop. This process was repeated at least three times, and we confirmed that the superficial yeast was removed by passing a finger wearing a glove. The residual water was eliminated by placing the plates in inverted position on bench filter paper for 10-20 minutes, and pictures of the yeast retained inside the agar were taken again. The pictures were analysed with Adobe Photoshop 7.0 for windows software (Adobe, San Jose, CA).

Microscopy. Digital images were taken with a Nikon Eclipse E-400 microscope connected to a Nikon Coolpix 995 digital camera, or with a Leica DMRD connected to a Leica DC200 digital camera with IM1000 software. The images were processed with Adobe Photoshop 7.0 for Windows (Adobe, San Jose, CA).

***C. neoformans* and *C. albicans* penetration into mouse lung**. Male CD1 mice (8-12 weeks old, Charles River, etc) were sacrificed and the lungs isolated. The lungs were placed in 6-well plates, each well containing 2 mL of feeding medium (RPMI (Sigma Aldrich, MO), 10% FCS, 2 mM L-glutamine, 1% non-essential amino acids (Sigma Aldrich, MO), 100 U/mL Penicillin and 100 mg/L Streptomycin) supplemented with 2% glucose and 1% peptone. An amount of 10^7 *C. neoformans* (B3501 or C536) or *C. albicans* (SC5314) cells were placed on the medium, and allowed to sediment on top of the tissue. The organs were incubated for 6 days. For histopathologic studies, the lungs were fixed *in toto* by immersion in neutral buffered formalin solution for 48 h, and serial transverse sections were made of the whole lung. These sections were again fixed in formalin for 12 h, followed embedding in paraffin. Finally, 5- micras- thick sections were obtained from the paraffin blocks and stained by hematoxylin and eosin (HE) as well as with peryodic acid Schiff (PAS) stain, for histological findings and fungal visualization.

Table 1. Strains Used in this Study

Specie	Strain	Capsule	Reference
C. neoformans	H99	YES	[65]
	24067	YES	ATCC (†)
	B3501	YES	[66]
	cap59	NO	[59]
	cap67	NO	[67]
	C536 (*CAP59:: URA5*)	NO	[68]
	C538 (C536 background, *GAL7p:: CAP59* reconstituted)	Only on galactose	[68]
	CL2132	YES	YC-NCM*
	CL3146	YES	YC-NCM*
	CL3691	YES	YC-NCM*
	CL4018	YES	YC-NCM*
	CL4839	YES	YC-NCM*
	CL4851	YES	YC-NCM*
	CL4866	YES	YC-NCM*
	CL4978	YES	YC-NCM*
	CL5539	YES	YC-NCM*
	CL5632	YES	YC-NCM*
	CL5707	YES	YC-NCM*
	CL5750	YES	YC-NCM*
	CL5801	YES	YC-NCM*
	1078	YES	CECT (#)
Candida albicans	6451	NO	ATCC (†)
	SC5314	NO	[69]
Candida parapsilosis	CL4509	NO	YC-NCM*
Candida krusei	6258	NO	ATCC (†)
Cryptococcus gattii	CL212	YES	YC-NCM*
	CL4998	YES	YC-NCM*
	CL4999	YES	YC-NCM*
	CL5000	YES	YC-NCM*
	CL5001	YES	YC-NCM*
	CL5002	YES	YC-NCM*
	CL5003	YES	YC-NCM*
	CL5004	YES	YC-NCM*
	CL5005	YES	YC-NCM*
	CL5006	YES	YC-NCM*
	CL5007	YES	YC-NCM*
	CL5008	YES	YC-NCM*
	CL5009	YES	YC-NCM*
	CL5010	YES	YC-NCM*
	CL5011	YES	YC-NCM*
	CL5012	YES	YC-NCM*
	CL5013	YES	YC-NCM*
	CL5014	YES	YC-NCM*
	CL5015	YES	YC-NCM*
	CL5016	YES	YC-NCM*

(†) American Type Culture Collection; (*) Yeast collection of the Spanish National Centre for Microbiology; # Colección Española de Cultivos Tipo.

RESULTS

Acapsular Mutants Manifest Invasive Growth in Different Media

We observed that in plates containing both wild type and acapsular strains, only the acapsular mutant was able to invade the agar. This observation suggested us that the *C. neoformans* capsule interfered with agar invasive growth, a phenomenon well described in several fungal species. Since the cryptococcal capsule is one of the eukaryotic fungal virulence factors best characterized, we decided to study this phenomenon in more detail. As a first approach, we streaked both the wild type and acapsular strains on Sabouraud plates. We confirmed that after several days of growth, the acapsular strain invaded the agar meanwhile the encapsulated strain only grew on the surface of the agar (Fig. **1A**). To confirm that the process was due to penetration of the yeast into the solid media, we cut small pieces of the agar, and observed microscopically the fungal penetration. As shown in Fig. (**1B**), acapsular mutants penetrated into the agar, confirming the presence of invasive growth in this strain. Invasive growth was not affected by the agar concentration of the plates, since even at concentrations as low as 0.5%, the encapsulated strain did not invade the agar (result not shown).

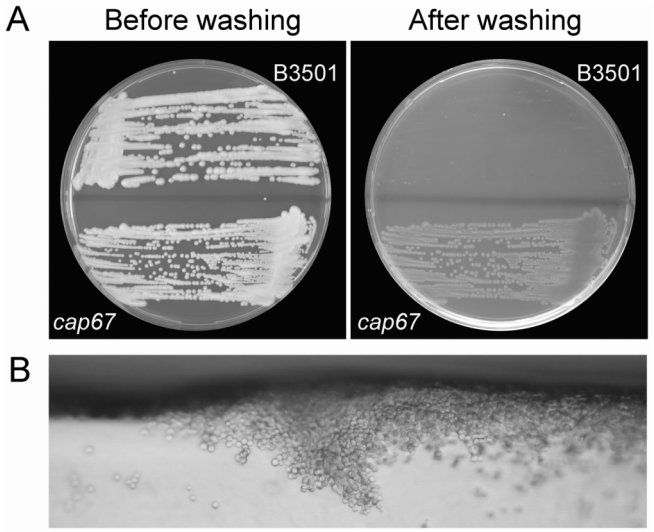

Fig. (1). Acapsular mutants show invasive growth. (**A**) Strains B3501 and *cap67* were streaked on Sabouraud plates, and invasive growth was tested after 5 days of growth at 30°C. (**B**) Transversal view of an agar cut with acapsular *C. neoformans cap67* strain grown as in A) and after washing of the plate.

We wanted to confirm that invasive growth was due to the lack of capsule and not to other mutations that could be accumulated in this acapsular strain. So we followed several approaches to confirm this hypothesis. First, we investigated the ability of several acapsular mutants from different sources and generated in different ways to invade the agar, and compared these strains with different wild type strains widely used in the field. As shown in Fig. (**2**), all the acapsular strains invaded the agar after three days of growth. None of the wild type strains showed this phenotype. We obtained the same result after 7 days of growth (result not shown). Since these mutants were isolated by different laboratories by different approaches, we believe that invasive growth is due to the lack of capsule and not secondary mutations.

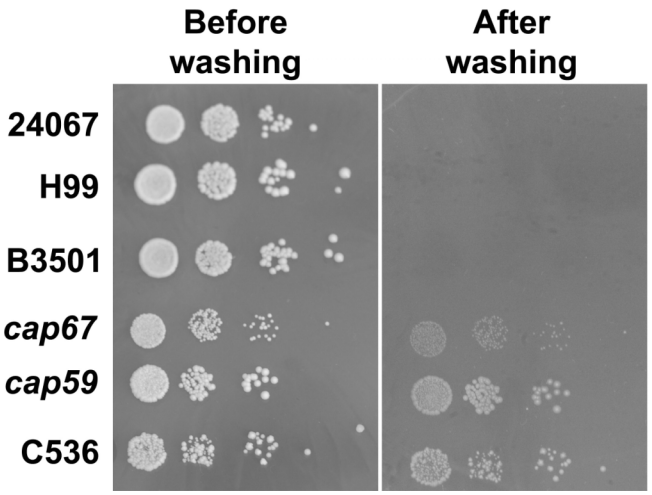

Fig. (2). Comparison of different wild type and acapsular mutant strains. Serial dilutions of encapsulated strains H99, B3501 and 24067, and acapsular mutants *cap67*, *cap59* and C356 (which carries a disruption of the *CAP59* gene) were prepared and 8 µL were spotted on Sabouraud plates. Invasive growth was tested after 7 days of growth at 30°C.

To further confirm the inhibitory role of the capsule in this type of growth, we used a reconstituted strain which accumulates capsule only in the presence of galactose (strain C538, see Table **1**). Using this strain and the isogenic acapsular mutant (C536), we observed that the reconstituted strain invaded the agar on glucose, but not on galactose plates (Fig. **3**). This phenotype correlated with the absence and presence of capsule in the reconstituted strain, respectively.

Based on these results, we focused subsequent studies with the C536 strains, since this mutant contains a whole disruption of the *CAP59* gene, and reversion of the mutation are highly improbable. To investigate if invasive growth is correlated with the growth rate of the yeast and to try to simulate the complex nutritional situation that the yeast confronts during infection, we studied if the *cap59* disruptant strain (C536) presented invasive growth in different carbon sources and temperatures. We found that for the disruptant mutant (C536 strain), invasive growth occurred in all the conditions tested (30 and 37°C, and glucose, galactose and ethanol as carbon sources, result not shown).

Invasive Growth in Multiple *C. neoformans* Strains

We tested a total of 37 different *C. neoformans* isolates for invasive growth in agar. We included in the study *C. gattii* strains (serotype B and C), which differ in their capsule structure from *C. neoformans* var *grubii* or *var neoformans* strains (serotype A and D, respectively). The *C. neoformans* strains used differed also in the antifungal susceptibility [47]. We also studied other pathogenic yeast species that undergo morphological transitions (hyphae or pseudohyphae). In this experiment, we observed that encapsulated *C. neoformans* strains (*neoformans* and *grubii*) did not invade the agar, and only both acapsular strains presented this type of growth (C536 and C538, Fig. **4**). Among the *C. gattii* strains tested only one presented a residual invasive growth, and the rest behaved as non-invasive yeast. The three species of *Candida* tested (*albicans*, *parapsilosis* and *krusei*) were each able to invade the agar. When we examined the morphology of the

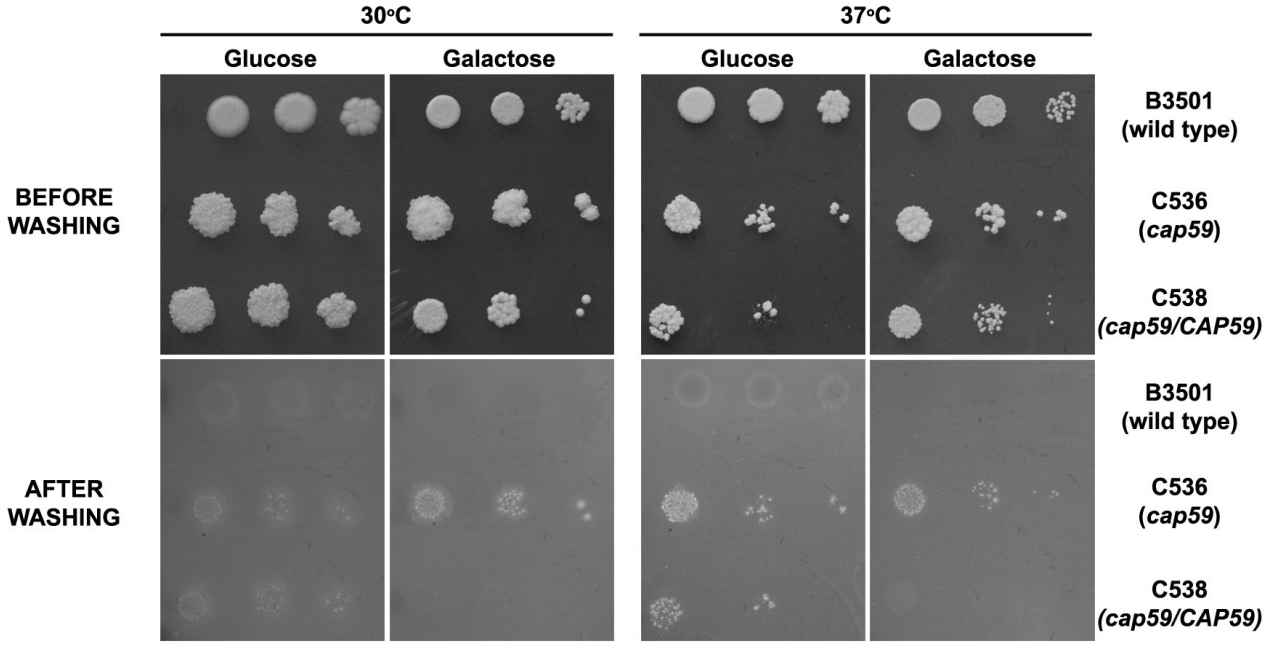

Fig. (3). Invasive growth of C536 y C538 strains on glucose and galactose. B3501 (wild type), C536 (*cap59* disruptant mutant) and C538 (*cap59*/*CAP59*) were grown in YNBglucose, and after 48 hours, serial dilutions were prepared, and spotted by duplicate on YNBGlucose and YNBGalactose plates. The plates were incubated at 30°C or 37°C, and invasive growth was observed after 7 days.

yeasts that were retained inside the agar, we observed that only *C. neoformans* exhibited exclusively yeast forms (Fig. **5**), while the other species presented true hyphae (*C. albicans* and *C. krusei*) or pseudohyphae (*C. parapsilosis*, inset in Fig. (**5**)).

Effect of Melanisation on Invasive Growth

C. neoformans accumulates melanin at the cell wall in the presence of diphenolic compounds [48, 49] and is a virulence factor also produced during infection [50]. We argued that the presence of melanin could affect some cell wall properties required for invasive growth. Hence, we decided to investigate if melanisation affects invasive growth in both encapsulated and acapsular mutants. We induced melanisation by growing the cells in the presence of L-DOPA, and tested invasive growth in minimal media agar plates with and without L-DOPA. We used as control cells that were grown in minimal media without L-DOPA (non-melanized cells). As shown in Fig. (**6**), acapsular melanized cells invaded the agar in a similar way than non-melanized cells.

Invasion of lung tissue. We wanted to reproduce the results obtained *in vitro* in a more physiological ex vivo model. For this purpose, we isolated lungs and liver tissue from mice, and incubated them in the presence of encapsulated and acapsular strains. We also included *Candida albicans*, as a control of a microorganism with high invasion ability. We observed that the liver was not well preserved in these conditions (data not shown), so we focussed our studies in lung tissues. We observed that the *C. neoformans* growth was highly compromised in the lung incubation conditions. After the incubation and subsequent histological analysis, *C. albicans* (which demonstrated an optimal growth rate) showed high invasion in these conditions, proving the feasibility of the method. In the case of C. *neoformans*, we observed the presence of microorganism inside the lung tissue incubated with the acapsular strains. *C. neoformans* were located into the alveolar cavities at peripherical subpleural areas of the pulmonary parenchyma. The alveolar wall showed a moderate oedema of the interstitial tissue, without inflammatory cells infiltrates. No other relevant histopathological lesions were found (Fig. **7**). We have to highlight that these areas were not very abundant, but we believe that this is related to the poor growth of the *C. neoformans* strains in these conditions. These areas were not found in the encapsulated strain (Fig. **7**). On contrary, pulmonary tissue incubated by *C. albicans* determined an intense colonization of both pleural membrane and alveolar lumen, associated with irreversible alveolar wall destruction (Fig. **7**). In conclusion, these experiments suggest that the phenomenon observed *in vitro* could be occurring also during *C. neoformans* infection in mammalian models.

DISCUSSION

We have demonstrated that the fungal pathogen *Cryptococcus neoformans* manifests agar invasive growth in laboratory conditions without undergoing any morphological change. Only a few reports have shown invasive growth in *C. neoformans*, and all of them used limited nitrogen media [51, 52], which are conditions known to induce invasive growth in other organisms in *S. cerevisiae*. In our conditions, there is no nutrient limitation, so all the phenomena observed occur in the absence of environmental inducing signals. The effect was only observed with acapsular strains. Invasive growth has been largely studied in other fungi, including pathogenic and non-pathogenic microorganisms. In the case of pathogenic microorganisms, it has been specially characterized in *Saccharomyces cerevisiae* and *Candida albicans*. In this pathogen, tissue invasion is a key process in the pathogenesis and it has been associated with hyphae formation. It is believed that the ability to form hyphae provides

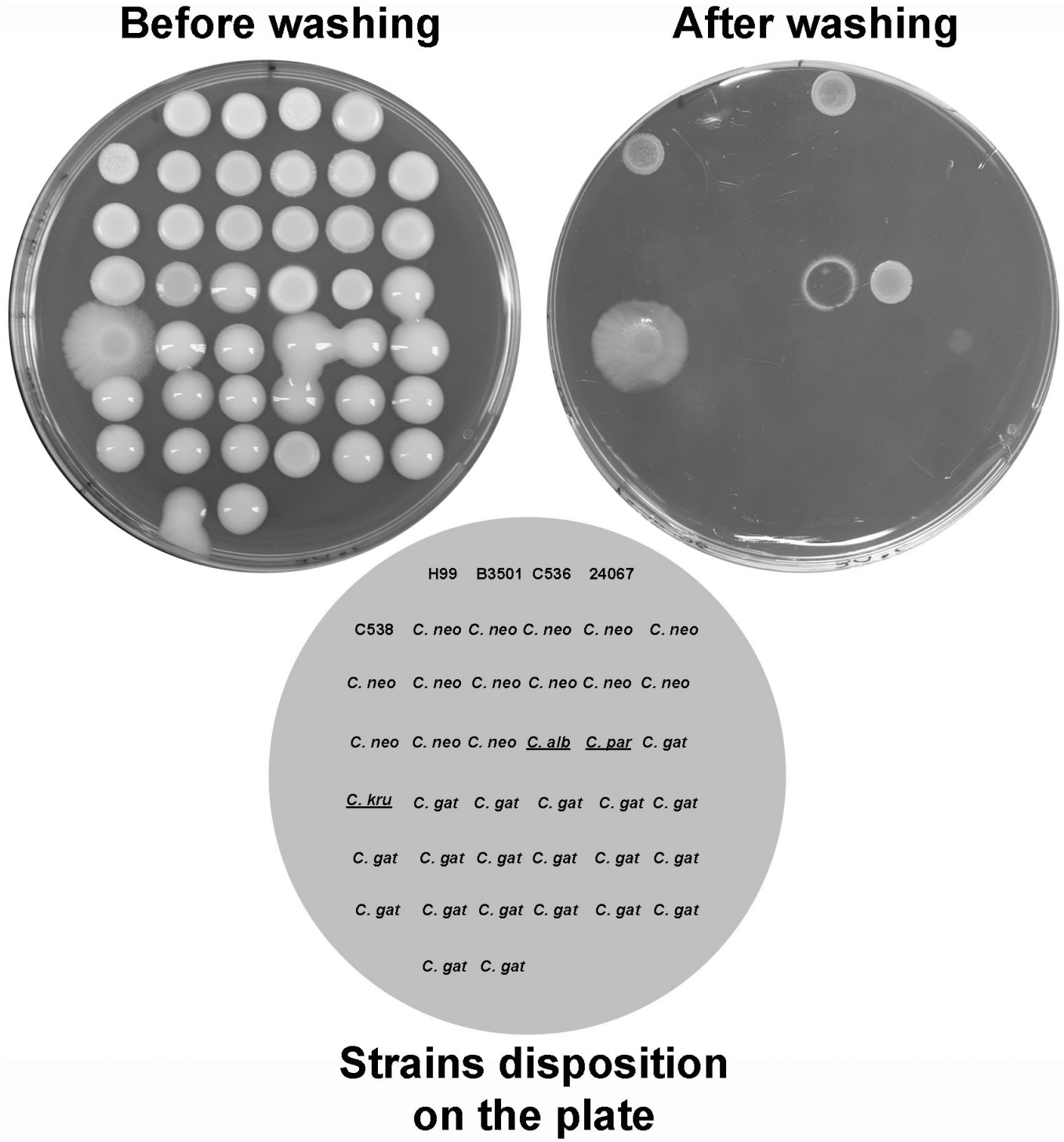

Fig. (4). Invasive growth of different pathogenic yeast isolates. Several *C. neoformans* isolates (encapsulated and acapsular, and from varieties *neoformans* and gattii), *Candida albicans*, *Candida parasilopsis* and *Candida krusei* were grown in liquid sabouraud media, and after 36 hours of growth at 30oC, 3 μL from each strain were placed on Sabouraud agar plates. Invasive growth was tested after 7 days of growth at 30°C. The diagram at the bottom shows the disposition of all the strains spotted (C.neo, *Cryptococcus neoformans*; C.gat, *Cryptococcus gattii*; C.alb, *Candida albicans*; C.par, *Candida parasilopsis*; C.kru, *Candida krusei*). Candida species are underlined.

the yeast a way to cross biological barriers and penetrate into target organs [1]. In the case of the brewers´ yeast *Saccharomyces cerevisiae*, invasive growth correlates also with pseudohyphae formation, and in this case, it is believed to be a mechanism to look for more favourable nutritional environments [10]. In the case of acapsular *Cryptococcus neoformans* strains, we found that invasive growth in rich media does not correlate with any morphological change. This is consistent with the fact that no filamentous growth has been described in *Cryptococcus neoformans*, except during mat-

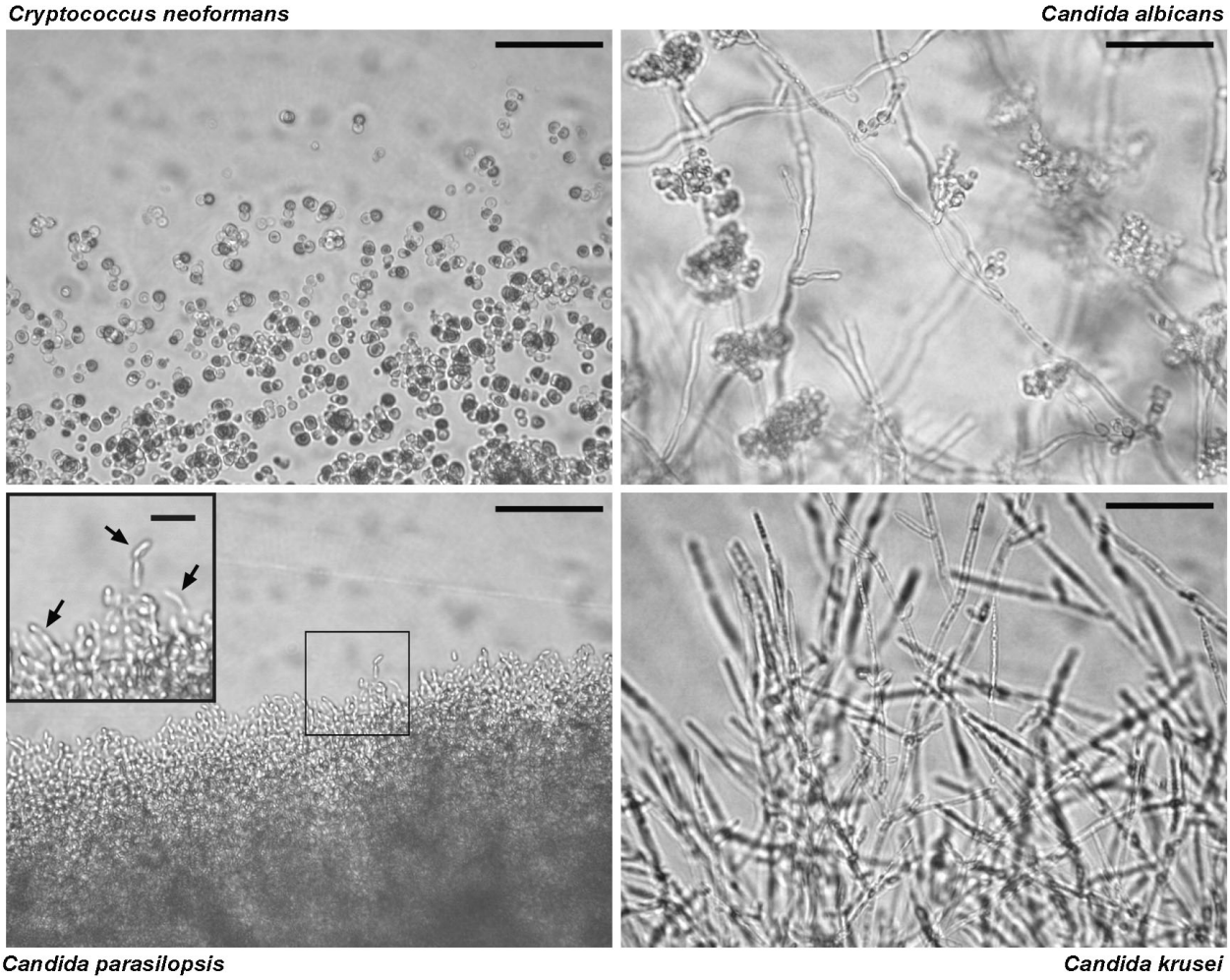

Fig. (5). Morphology of different fungi after invasive growth. Morphology of acapsular C536 *C. neoformans* strains, *C. albicans*, *C. parasilopsis* and *C. krusei* was observed by microscopy. Scale bar, 50 microns. Inset in the lower left panel shows a detail a pseudohyphae from C. parasilopsis. Scale bar inside this inset, 10 microns.

ing and monokaryotic fruiting (absent in our conditions). We do not have an explanation for the invasive growth in the absence of capsule and without any requirement for filamentous growth. However, acapsular mutants show a high adherence phenotype, and these mutants present a flocculent-like phenotype (Fig. **8**). We believe that this property is due to a peculiar cell wall structure that facilitates the adherence and penetration into the agar, resulting in invasive growth. It is worthy to mention that in *Candida albicans*, hyphae formation correlates also with higher adhesion of the filamentous form, which produces also visible clumps. We think that this phenomenon is important for invasion, since cell aggregation most probably reflects a higher adherence of the cells, not only to other cells, but also to different matrixes, such as the agar. Interestingly, it has been recently shown that *C. neoformans* can form biofilms on polystirene substrates, and this adherence is higher in encapsulated strains. The presence of the capsule confers different physical properties to the fungal cells, such as zeta potential and cell charge [53]. We believe that all these results indicate that adhesion of *C. neoformans* cells depends on the characteristics of the substrate (composition, charge, etc), being the capsule in some cases required for the binding of the cell to the solid support. This is agreement with the finding that under stress nutritional conditions, a low degree of invasive growth found in encapsulated strains [52]. During *in vitro* growth on rich media plates, the presence of the capsule does not allow penetration in the agar. This suggests a mechanism of invasion in acapsular mutants, such that a higher cell adherence to the agar allows a strong binding to the surface. It is worthy to mention that the acapsular mutants used in this study (Cap59) lack the production of the major component of the capsule, glucuronoxylomannan (GXM), but can still synthesize the other polysaccharide that is found in a lower proportion (around 5% of the capsule), which is galactoxylomannan (GalXM) [54, 55]. The biochemical function of Cap59p is not known, but it has been involved in the GXM secretory pathway [56]. In *cap59* mutants, GalXM is secreted to the growth medium, but the question of whether it remains attached to the cell wall has not been definitively answered yet. However, even if GalXM remains attached to the cell wall in *cap59* mutants, it does not form a physical structure that could be defined as a capsule since it is not visible by India Ink staining. However, it cannot be discarded that the secreted GalXM in acapsular mutants is producing some effects in the host. Concerning invasion, it seems that the amount of GalXM produced by acapsular mutants is not enough to inhibit invasive growth.

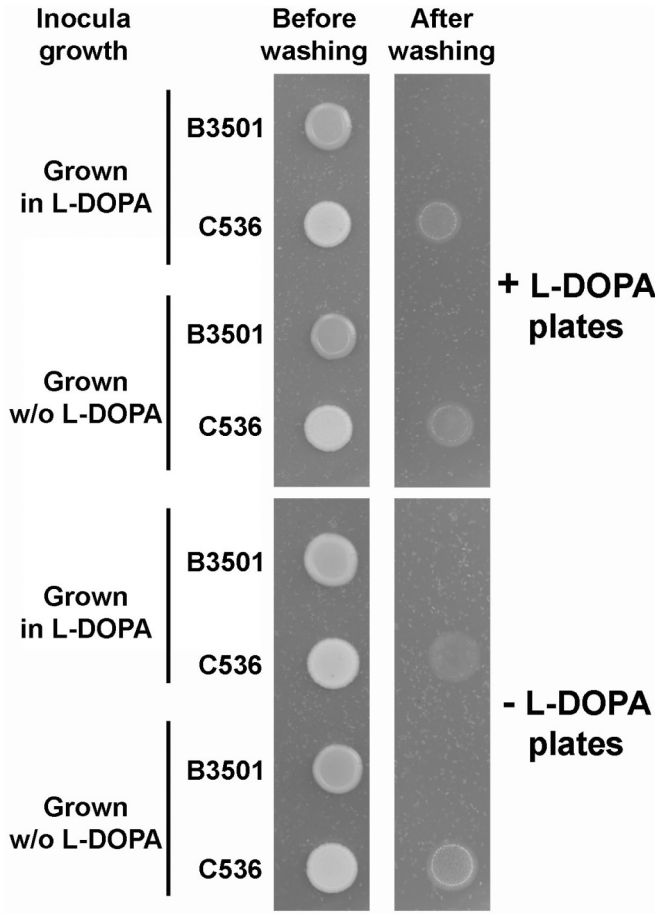

Fig. (6). Effect of melanization on invasive growth. B3501 (wild type) and C536 (*CAP59* disruptant) strains were grown in minimal media in the presence or absence of L-DOPA (left part of the figure). The cells (melanized and non-melanized cells) were spotted on agar plates containing the same media with or without L-DOPA (see right column), and invasive growth was tested after 7 days of growth at 30°C.

In *Saccharomyces cerevisiae*, invasive growth was first described in diploid strains [4], and although haploid strains also present this growth, there are some differences, which are mainly related to the environmental signals that induce invasive growth [10]. *C. neoformans* is haploid in laboratory conditions, which suggests that the sexual state is not a factor that influences in invasive growth. In addition, no stress or nutritional limitation is required for invasive growth in acapsular *C. neoformans*, which resembles the situation in other fungal pathogens, such as *C. albicans*. This indicates that invasive growth in *C. neoformans* in the absence of capsule is an intrinsic property, rather than an environmental response.

In other fungi, cAMP pathway is an important pathway involved in morphogenesis and invasive growth [15, 57]. We tried to modulate the cAMP pathway to investigate if it was involved in our phenotype by adding exogenous cAMP or blocking the pathway with the specific inhibitor H89. Although we did not observe any effect of the cAMP (result not shown), we are aware that in our approach, modulation of the cAMP pathway might not be complete. For example, we have not used *pde2* mutants (which lack the cAMP

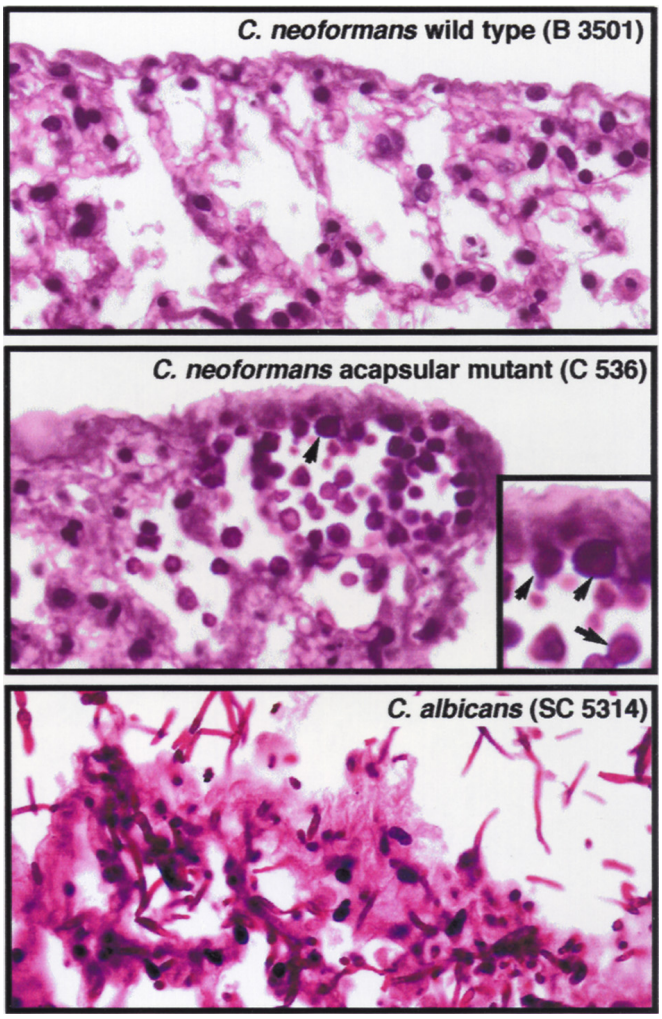

Fig. (7). *C. neoformans* invasion of lung tissues. Lung tissues were excised from mice and co-incubated with encapsulated (B3501) and acapsular (C536) *C. neoformans* strains and with *C. albicans* (SC5314) as described in Material and Methods. After paraffin inclusion and performing 5 microns sections, microorganisms were detected by PAS staining. Arrows denote the presence of microorganisms inside the lung tissue. Scale bar in upper panel denotes 10 microns, and applies to all the panels.

degrading enzyme phosphodiesterase 2) when we added exogenous cAMP. In the case of the yeast *S. cerevisiae*, exogenous cAMP produces many effects when using pde2 mutants, but not wild type cells. In the case of the cAMP-dependent protein kinase inhibitor H89, we found that it significantly reduces the growth rate of the yeast, which might also interfere with the results.

We were able to reproduce our findings using a model of invasion in mouse lung tissue. In this model, we observed that *C. albicans* presents a more aggressive invasive phenotype than *C. neoformans* strains, which correlates also with a higher tissue damage. We believe that this is related to the higher growth rate capacity of *C. albicans* in the experimental conditions, as well as to the hyphae formation, which allows a higher penetration in the tissue and the consequent alveolar wall destruction. In the case of *C. neoformans*, the growth rate in the tissue culture conditions was significantly compromised, which reduces the possibility of finding inva-

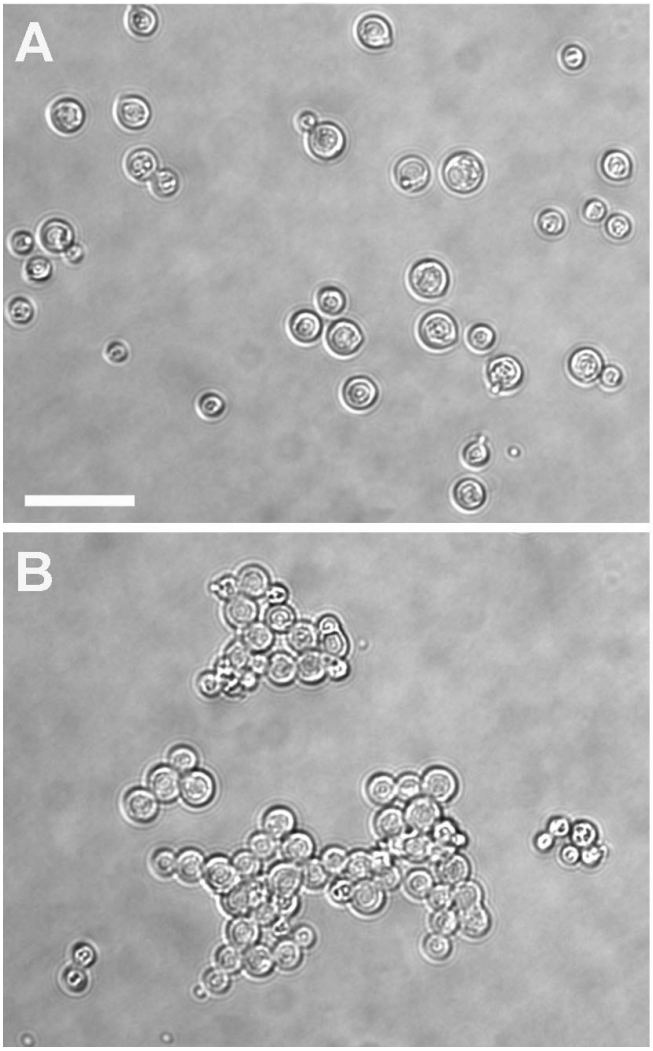

Fig. (8). Micrographs of encapsulated and acapsular *C. neoformans strains*. B3501 (**A**) and C356 (*cap59* mutant, (**B**) strains were grown in liquid Sabouraud media, and pictures showing the morphology of the yeast cells were taken. Scale bar, 20 microns.

sion areas. Despite this experimental inconvenience, invasion was observed in acapsular, but now in encapsulated strains. This finding suggests that the results found could occur *in vivo*, and raises the intriguing question of whether the capsule has a functional role *in vivo* modulating invasive growth. Although we cannot discard that during infection encapsulated strains undergo some degree of invasive growth as observed *in vitro* during ammonium limited conditions [52], we have proven that the phenomenon is much more enhanced in acapsular strains. The finding that the capsular polysaccharide inhibits invasive growth is paradoxical, since the capsule is the main virulence factor of this microorganism, and absence of the capsule significantly reduces the virulence of the microorganism [58, 59]. Our data show that in *C. neoformans* invasive growth is not correlated with virulence. We believe that the higher adherence of acapsular mutants difficults the dissemination through the organism, and supports the idea that the capsule is required for this process, through transcytosis mechanism and specific capsule-receptor interactions [43]. In this context, macrophages and other phagocytic cells have been proposed to serve as "trojan horses" for the fungal cells that in this way could disseminate through the organism, since encapsulated *C. neoformans* can survive and replicate inside macrophages [60, 61]. The role of the capsule during intracellular parasitism is still unknown. Although some articles suggests that encapsulated cells are more susceptible to killing by macrophages [62], others indicate that acapsular mutants are more susceptible to killing by phagocytic cells [63, 64], which suggests that in the absence of capsule, dissemination within phagocytic cells is compromised.

In summary, we have shown that the main cryptococcal virulence factors inhibits invasive growth, and suggests that in *C. neoformans, in vitro* invasive growth does not correlate with the outcome of the disease. Although this fact seems paradoxical, gives new insights about the dissemination mechanism of this pathogen during infection.

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