

Intracellular parasitism of macrophages by
Cryptococcus

by

Hansong Ma, BSc

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Abstract

The pathogenic fungi *Cryptococcus neoformans* and *Cryptococcus gattii* are two of the main causes of life-threatening meningoencephalitis in immunocompromised and immunocompetent individuals respectively. Following inhalation, cryptococci are engulfed by phagocytic cells in the lung and previous studies by our group and others have demonstrated that they are then able to survive inside these cells (especially macrophages), thus acting as intracellular parasites. This intracellular phase is thought to underlie the ability of the pathogens to remain latent for long periods of time within infected individuals. Here, we demonstrate that cryptococci can also manipulate host macrophages in order to mediate an exquisitely controlled 'escape' process. This expulsive process, which we have termed 'vomocytosis', can occur either into the extracellular milieu or, remarkably, into neighbouring host cells, thus resulting in direct cell-to-cell transmission ('lateral transfer'). After vomocytosis, both the host macrophages and the expelled cryptococci appear morphologically normal and continue to proliferate. Vomocytosis therefore represents an important mechanism by which pathogens are able to escape from phagocytic cells without triggering host cell death and thus inflammation. Moreover, direct cell-to-cell spread of cryptococci allows the pathogen to remain concealed from the immune system and protected from antifungal agents, thus achieving long-term latency.

This project has also provided a possible explanation for the molecular cause of a recent *C. gattii* outbreak on Vancouver Island, Canada. We found that isolates from the outbreak have dramatically increased their ability to replicate within macrophages in comparison with other *C. gattii* strains, despite the fact that they are genetically very similar to each other. We further demonstrate that such enhanced intracellular parasitism is directly linked to virulence in a murine model of cryptococcosis, suggesting that this ability might be the cause of the outbreak. Finally, application of high-density whole genome tiled arrays, confocal microscopy and mating assays reveal regulation of mitochondrial activity to be a major driver of virulence in this pathogen group. Taken together, these data indicate that a recent change in mitochondrial regulation within the *C. gattii* lineage has led to an increased intracellular proliferative capacity, resulting in the hypervirulent phenotype that underlies the outbreak. Such shifts in intracellular replication capacity may be a widespread phenomenon in other human pathogens and could potentially underpin disease epidemics caused by otherwise unrelated pathogens.

This thesis is dedicated to my dearest parents: **Fu Miaojuan & Ma Furong**

仅把此论文献给母亲傅淼娟和父亲马福荣

Acknowledgments

After terminating my first PhD within one year, I wondered whether to dedicate a further 3 plus years on another project. Now I look back and feel really lucky that I made the right decision. Not only did this PhD offer me the opportunity to work with some first-class researchers and vastly expand my knowledge on host-pathogen interaction, a topic that I will keep working on as a post-doc, but it also provided me with some treasured memories.

I would like to take this opportunity to express my appreciation to Dr Robin C May for giving me the chance to study for this PhD. As a supervisor, he was always there to help, listen and support me whatever the cost; I could not have wished for a kinder or more considerate supervisor. As a role model, his enthusiasm towards science has certainly encouraged me to march forward in the field of biology after this PhD.

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support: my PhD would have been very boring without you. Claudia- you were, are and will always be my best female friend 'on the 4th floor'. Thank you for sharing your scientific ideas and inspiring me in various ways. Pedro- thank you for the hours of entertainment and jokes. I am sure with your talent, you will speak fluent Chinese one day. Farhat- thank you for all kinds of birthday presents, the weekend chats and being there for my turmoil. I am also grateful to many of my friends, especially Junhong Liu, Luming Lou, Feifei Song, Fang Peng, Ralf Webber, Jikai Wen and Jing Zhang for their encouragements and invaluable time. Without them, my PhD time would have been far less colourful.

Finally and most importantly, I acknowledge my parents, who have worked very hard in order to send their daughter for higher education. I will always be in debt to their unconditional love.

List of Abbreviations

5'-Fc	flucytosine
5'-Fu	5-fluorouracil
AFLP	amplified fragment length polymorphism
AIDS	acquired immunity deficiency syndrome
BBB	blood-brain barrier
CFU	colony forming unit
CGH	comparative genome hybridisation
CNS	central nervous system
CR3	complement receptor 3
CSF	cerebrospinal fluid
Cy	cyanine
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
FBS	foetal bovine serum
FcR	fragment crystallisable receptor
GalXM	galactoxylomannan
GM-CSF	granulocyte macrophage-colony stimulating factor
GXM	glucuronoxylomannan
HBMEC	human brain microvascular endothelial cells
HI-FBS	heat inactivated-foetal bovine serum
IFNγ	interferon gamma
IgG	immunoglobulin G
IL	interleukin
IPR	intracellular proliferation ratio
L-DOPA	3, 4-dihydroxyphenylalanine
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
M-CSF	macrophage-colony stimulating factor
MLST	multi locus sequencing typing
mtDNA	mitochondrial genome
PFGE	pulsed field gel electrophoresis
NK	natural killer
NKT	natural killer T
PBS	phosphate buffered saline
HPBMC	human peripheral blood mononuclear cell
PCR	polymerase chain reaction
PI	phagocytosis index
PKA	protein kinase A
PMA	phorbol myristate acetate
RAPD	random amplification of polymorphic DNA

rcf	relative centrifugal force
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute (medium)
SDA	Sabouraud dextrose agar
SDS	sodium dodecyl sulphate
SFM	serum free medium
SREBP	sterol regulatory element binding protein
Th	T-helper
TLR	Toll like receptor
TNFα	tumour necrosis factor alpha
v/v	volume per volume
VIO	Vancouver Island outbreak
w/v	weight per volume
YPD	yeast peptone dextrose

Thesis overview

As human beings, we live in a world where novel infectious diseases are appearing and old infectious diseases are spreading. Disease emergence is a routine event in the evolutionary ecology of pathogens, during which pathogen populations can become more virulent or shift arrays of host species. Recently, the increasing incidence of *C. neoformans* infection as a result of the AIDS epidemic, the outbreak of a hypervirulent *C. gattii* strain in Vancouver Island (Canada) and the fact that mortality from cryptococcal disease remains high have stimulated intensive research into these organisms.

Cryptococcus has been found to live inside host cells for more than 40 years, but detailed studies on its intracellular behaviour only started about 10 years ago, mainly by several groups, including Arturo Casadevall, Stuart Levitz and others. To date, both *in vitro* and *in vivo* data have demonstrated that *Cryptococcus* belongs to the group of intracellular pathogens that can adapt inside various host cells including phagocytes like macrophages, dendritic cells, monocytes and neutrophils. Studies with macrophages have showed intracellular survival is not a consequence of active escape from the mature phagosome, but rather is associated with accumulation of intracellular capsular polysaccharides and possibly also the presence of other virulence factors, such as the synthesis of melanin-like pigment in the cell wall and secretion of proteinases and phospholipases. In this thesis, I review the recent advances in our understanding of *C. neoformans* and *C. gattii*, including intraspecific complexity, virulence factors, and key signalling pathways regulating virulence, along with the interaction between these pathogens and the host immune system. I then present my main findings on how *Cryptococcus* can manipulate macrophages in order to achieve efficient dissemination, and long term latency & persistence. Finally, I provide a short summary discussing the contribution of my work to the field and potential experiments can be carried out in future.

The thesis is written in a conventional way with one Introduction section (Chapter I), one Materials & Methods section (Chapter II) and four Result chapters (Chapter III-VI). For each Result chapter, the discussion follows immediately in order to better explain the data. A general discussion (Chapter VII) is given at the end to summarise all the work. A brief description of each Result Chapter is given below.

Chapter III focuses the phagocytosis of *Cryptococcus* by macrophages. This part of the work was carried out at the beginning of my PhD in order to optimise various parameters. It provides the basis for the later work discussed in chapter IV-VI.

Chapter IV describes two phenomena that occur to *Cryptococcus* following phagocytosis by macrophages: 'vomocytosis' and 'lateral transfer'. They are new observations which had never been reported with *Cryptococcus* at the time the work was carried out.

Chapter V discusses my work on intracellular proliferation capacity of various clinical and environmental strains. This part of the PhD revealed that strains from the Vancouver Island outbreak (VIO) showed enhanced intracellular proliferation in comparison to many other *C. gattii* strains and that such enhanced intracellular parasitism was linked to virulence in the murine model of cryptococcosis. Subsequent microarray and confocal microscopy studies demonstrated that the enhanced virulence might be due to regulation of cryptococcal mitochondria to form a tubular morphology inside the host cells.

Chapter VI depicts my attempts to verify the role of the mitochondrial genotype in virulence. To date, it seems that, although the presence of VIO mitochondrial genotype alone is not sufficient, it is required for virulence. Therefore, we propose that both mitochondrial genotype and the regulation of mitochondrial activity by nuclear-encoded proteins are important for the observed hypervirulence of the VIO strains. However, this part of project is still in its preliminary stage and needs more work to draw a solid conclusion.

Several sections of the thesis have been published: Chapter I was published as a book chapter in *Advanced in Applied Microbiology* (2009); Much of the work in Chapter IV was published back to back with Arturo Casadevall's group in *Current Biology* (2006) and *BMC Immunology* (2007); The work in Chapter V on intracellular proliferation was recently accepted by *Proceedings of the National Academy of Sciences* (2009).

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Chapter I: Introduction

1.1 *Cryptococcus*

The genus *Cryptococcus* contains 40 heterobasidiomycetous fungal species characterised as variously encapsulated budding yeasts, of which only *Cryptococcus neoformans* and *Cryptococcus gattii* are commonly considered as the causative agents of cryptococcosis (Casadevall and Perfect 1998). *C. neoformans* was first identified as a human pathogen in the 1890s (Buschke 1895; Busse 1894). It exists predominantly as a vegetative haploid form and is heterothallic with each cell existing as one of two distinct mating types: MAT_a or MAT_α. In response to nutrient limitation, cells of opposite mating type mate to form the filamentous teleomorph and produce basidiospores (Kwon-Chung 1975; Kwon-Chung 1976). Under the microscope, most clinical isolates of *C. neoformans* appear as encapsulated spherical yeasts in both tissue and culture (Mitchell and Perfect 1995). The capsule size varies according to the strain and culture conditions with most isolates having a medium-sized capsule resulting in a total diameter of 4-10µm (Figure 1). Poorly encapsulated strains have diameters of only 2-5µm whereas heavily encapsulated isolates can have a cell diameter of up to 80µm (Casadevall and Perfect 1998).

C. neoformans can cause human infections following inhalation of the small airborne propagule (believed to be either basidiospores or poorly encapsulated yeast cells) originating from certain environments such as soil and avian habitats (Casadevall and Perfect 1998; Velagapudi et al. 2009). Therefore, the lung is invariably the portal of entry and initial site of infection. However, *C. neoformans* not only has the ability to simply colonise the host's respiratory tract without causing disease (latency) in immunocompetent individuals (Garcia-Hermoso et al. 1999), but is also capable of disseminating to any organ of the human body, with a predilection for the central nervous system (CNS). The resulting meningoencephalitis represents the most severe form of the disease and is uniformly fatal if untreated (Casadevall and Perfect 1998).

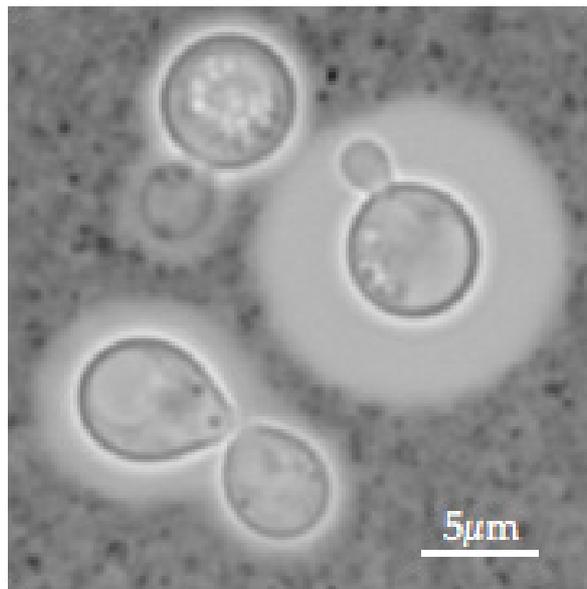


Figure 1: India ink stained capsule (with various sizes) around budding *C. neoformans* cells.

Conventional nomenclature classified *C. neoformans* into five serotypes (A, B, C, D and AD) and three varieties: *C. neoformans* var. *neoformans* (serotype D), *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *gattii* (serotype B & C) (Franzot et al. 1999; Kwon-Chung et al. 1982). Each serotype is characterised by a specific structure of glucuronoxylomannan (GXM), the main capsule component (Cherniak et al. 1995). In the last decade, a number of DNA genetic typing techniques have been used to genotype and study the epidemiology of *C. neoformans* species. These techniques, including electrophoretic karyotyping by pulsed field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), DNA hybridization studies, amplified fragment length polymorphism (AFLP), polymerase chain reaction (PCR) fingerprinting and multi locus sequence typing (MLST) (Boekhout et al. 2001; Boekhout et al. 1997; Brandt et al. 1995; Currie et al. 1994; Litvintseva et al. 2006; Meyer et al. 1999; Ruma et al. 1996; Varma and Kwon-Chung 1992), have resulted in the elevation of *C. neoformans* var. *gattii* to the species level, based on genetic variability and lack of evidence for genetic recombination between *C. neoformans* and *C. gattii* (Kwon-Chung et al. 2002). Moreover, *C. gattii* differs from *C. neoformans* in phenotypic characters, natural habitats, epidemiology, clinical manifestations of disease and response to antifungal treatments (Casadevall and Perfect 1998; Chen et al. 2000; Sorrell 2001; Speed and Dunt 1995). The *C. neoformans*-*C. gattii* species complex is further divided into 9 major molecular types or genotypes (Figure 2) (Bovers et al. 2008; Ngamskulrungrroj et al. 2009). *C. neoformans* var. *grubii* isolates correspond to molecular types VNI, VNII and VNB; *C. neoformans* var. *neoformans* corresponds to VNIV; and serotype AD isolates correspond to molecular type VNIII. *C. gattii* corresponds to four molecular types: VGI, VGII, VGIII and VGIV and a recent study by Bovers *et al* has proposed to treat these four molecular types as different taxa (varieties), just like var. *neoformans* and var. *grubii* (Bovers et al. 2008). Further morphological and mating studies are required in order to draw final conclusions (Ngamskulrungrroj et al. 2009).

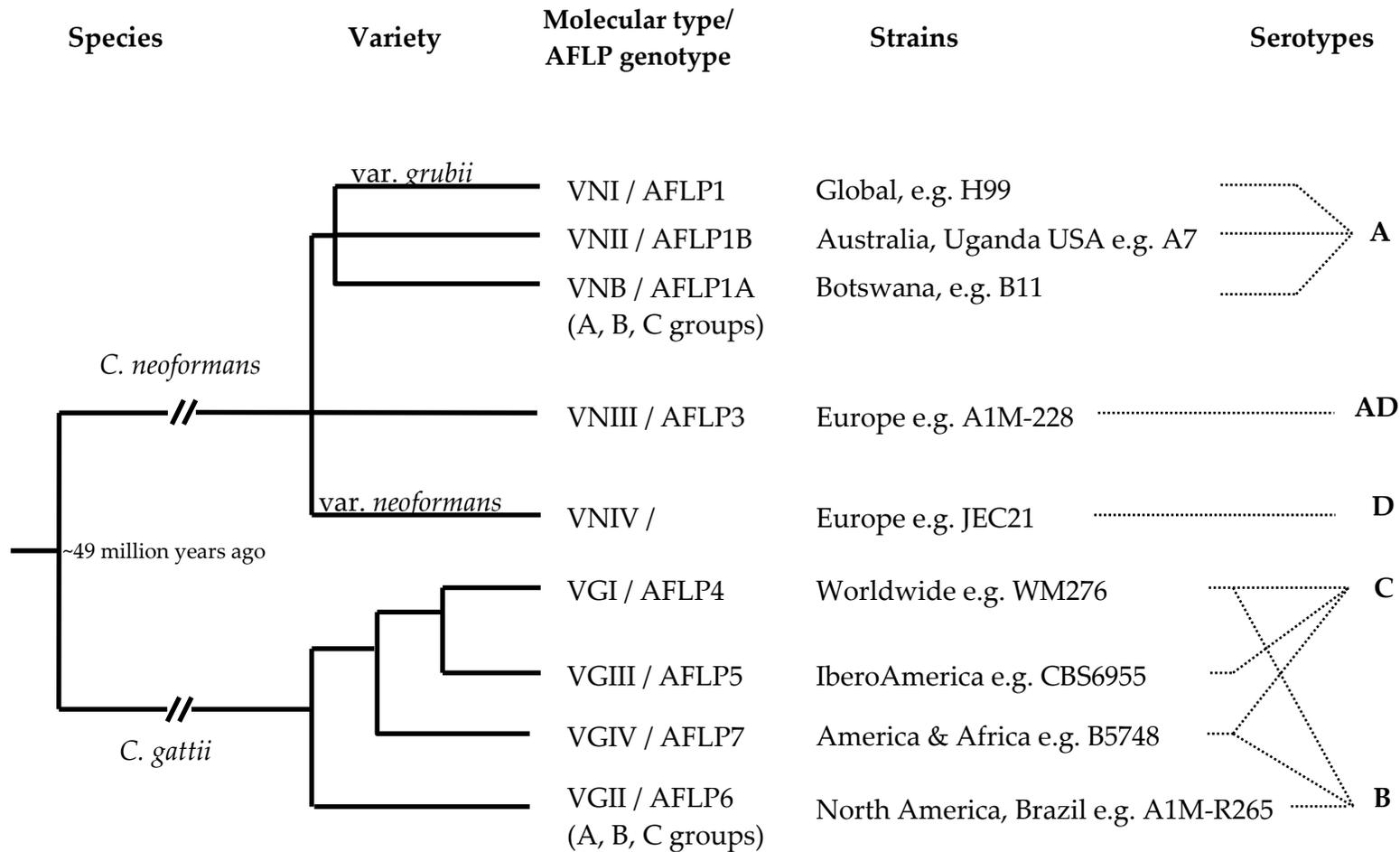


Figure 2: A schematic phylogeny of *C. neoformans*-*C. gattii* species complex. For *C. neoformans*, two monophyletic lineages, corresponding to variety *grubii* and *neoformans* consistently present along with the hybrid population. Serotypes correspond to genotypes. For *C. gattii*, four monophyletic lineages corresponding to the previously described genotypic groups are consistently found. Serotypes and genotype do not necessary correlate to each other. The molecular type VNIV and VGII forms the basal clade within the *C. neoformans* and *C. gattii* branch of the phylogenetic tree respectively.

1.1.1 *C. neoformans*

C. neoformans usually infects immunocompromised patients (although some exceptions have recently been reported, e.g., (Chen et al. 2008)). It can be found in the environment worldwide, and is commonly associated with pigeon guano or soil (Casadevall and Perfect 1998). Most *C. neoformans* isolates are either serotype A or serotype D. A and D serotypes diverged about 18 million years ago and have always been described as varieties, not as separate species (Fan et al. 1994; Xu et al. 2000b). Nevertheless, a recent proposal is that these two varieties should be described as different species (Bovers et al. 2008), because they have diverged to such an extent that normal mating is no longer possible (Sun and Xu 2007), and comparison of their genomes shows that there has not been any recent DNA exchange between these two varieties (Kavanaugh et al. 2006).

Serotype A is the predominant serotype of *C. neoformans* isolated from infected patients, responsible for 95% of all *C. neoformans* infections (Hull and Heitman 2002). It is subdivided into three molecular types: VNI (AFLP1), VNII (AFLP1B) and VNB (AFLP1A) according to MLST and AFLP analysis (Boekhout et al. 2001; Bovers et al. 2008; Litvintseva et al. 2006) (Figure 2). Such sub-classification is confirmed by recent comparative genome hybridisation (CGH) data (Hu et al. 2008). VNI is the most common molecule type, contributing 78% of *C. neoformans* isolates (Meyer et al. 1999). The VNB cluster can be further separated into three groups: VNB-A, VNB-B and VNB-C (Litvintseva et al. 2006). Initially, VNB strains were found only in Botswana (Litvintseva et al. 2006), but more recently they have also been recovered from Brazilian pigeon droppings and patients in Rwanda, Portugal and Brazil (Bovers et al. 2008).

Serotype D strains are found globally, but they are more prevalent in areas with temperate climates, such as Europe, where 30% of isolates are serotype D (Dromer et al. 1996). This restricted distribution may be due to the fact that serotype D strains are

more susceptible to high temperature than cells of serotype A (Martinez et al. 2001). The clinical manifestations of human infections caused by serotype A or D are similar, although differences in virulence potential in animal models have been reported (Barchiesi et al. 2005; Lin et al. 2008)

Serotype AD is the result of a fusion event between a serotype A strain and serotype D strain followed by impaired meiosis due to genomic incompatibilities (Boekhout et al. 2001; Cogliati et al. 2001; Lengeler et al. 2001; Xu et al. 2002). AD strains are therefore diploid (or aneuploid), containing two sets of chromosomes, and possessing two mating type alleles. Serotype AD strains are relatively common: a recent analysis of environmental and clinical populations of *C. neoformans* in North America revealed that approximately 7.5% of strains isolated from the environment are AD hybrids (Litvintseva et al. 2005a). Thus far, the majority of the globally isolated serotype AD strains originate in Africa (Litvintseva et al. 2007).

1.1.2 *C. gattii*

C. gattii was first described after being isolated from a leukemic patient in 1970 (Vanbreuseghem and Takashio 1970). It mainly infects individuals with no immunological defects, although AIDS-associated *C. gattii* infections have also been reported (Chen et al. 2008; Chen et al. 2000; Litvintseva et al. 2005b). It has been consistently isolated from decaying wood of several tree species, especially the red gum group of *Eucalyptus* trees (*Eucalyptus* ser. *Exsertae* Blakely) (Ellis and Pfeiffer 1992; Ellis and Pfeiffer 1990; Fortes et al. 2001; Krockenberger et al. 2002; Lazera et al. 2000). The geographic distribution of *C. gattii* was originally thought to be limited to tropical and subtropical regions of the world (Kwon-Chung and Bennett 1984). However, recent studies have revealed its worldwide distribution: VGI (AFLP4) strains were found to be the most widely distributed (Campbell et al. 2005; Chen et al. 2008; Meyer et al. 2003); Strains of the VGII (AFLP6) type are found in areas like Australia and America (Fraser et al. 2005; Kidd et al. 2005; Kidd et al. 2004; Meyer et al. 2003); The

VGIII (AFLP5) type predominates IberoAmerican countries (Meyer et al. 2003) and can also be found in India (Bartlett et al. 2007), whilst the VGIV (AFLP7) type, which has been associated with infections in HIV-positive patients (Bovers et al. 2006; Litvintseva et al. 2005b), is found in South Africa (Meyer et al. 2003) and Central America (Bartlett et al. 2007) etc.

Until recently, *C. gattii* has been under-studied because *C. gattii* infections comprise only 1% of cryptococcosis cases worldwide. Even in areas like Australia, where *C. gattii* is endemic, the rate of infection is 0.94 cases per million residents per year (Chen et al. 2000; Sorrell 2001). However, a recent outbreak of cryptococcosis caused by *C. gattii* has stimulated detailed investigation of this organism. This ongoing outbreak was first noted in 1999 on Vancouver Island, British Columbia, Canada. Between 2002 and 2006, the average annual cryptococcosis incidence rate was 6.5 cases/million in British Columbia and 27.9 cases/million on Vancouver Island (Control 2007). In addition to human infections, cryptococcal disease has been diagnosed in animals such as dogs, cats, horses and even porpoises. In fact, veterinary cases have been diagnosed two to three times more frequently than human cases (Lester et al. 2004). So far, the fungus has infected more than 176 individuals and spread from Vancouver Island to other regions of Canada and the Pacific Northwest (Byrnes et al. 2009a; Byrnes et al. 2009b; MacDougall et al. 2007; Upton et al. 2007).

Interestingly, the majority (~90%-95%) of cryptococcal isolates from the island have been found to belong to the VGII molecular type, with the rest being VGI (Kidd et al. 2004). These VGII isolates have been further separated into two discrete subtypes: a major form common in environmental and clinical isolates (VGIIA/AFLP6A, hypervirulent, e.g., A1M-R265), and a rare minor form presented by one clinical and several environmental samples (VGIIB/AFLP6B, with attenuated virulence, e.g., A1M-R272) (Fraser et al. 2005; Kidd et al. 2005; Kidd et al. 2004). So far, the VGIIA genotype has accounted for 78% of the examined veterinary cases and 87% of the human cases on Vancouver Island (Bartlett et al. 2007). Surprisingly, MLST (Kidd et al. 2005) and

gene genealogy analysis (Fraser et al. 2005) revealed that VGIIA and VGIIB strains found on Vancouver Island share similar or identical genotypes with isolates from other parts of world. For example, the VGIIA genotype was also shared by the NIH444 strain (from a patient in Seattle, 1971, which is considered as the potential origin of the VGIIA subtype), CBS7750 (from a *Eucalyptus* tree in San Francisco, 1992), and isolates from other parts of the North America (e.g., KB10455). The VGIIB genotype was also observed among environmental and clinical isolates from Australia (e.g., Ram005, NT-13), as well as a clinical isolate from Thailand (MC-S-115) (Fraser et al. 2005; Kidd et al. 2005). More recent studies confirmed the global distribution of the outbreak genotypes (Byrnes et al. 2009a; Byrnes et al. 2009b; Meyer et al. 2007). The wide distribution of Vancouver genotypes in other geographical areas makes it difficult to accurately determine a specific origin. Current hypotheses are that the species is either a long-term resident of British Columbia (ancient population), or represents a particularly virulent genotype that may be well-adapted to the local conditions and has been recently introduced to British Columbia. For instance, Fraser *et al* reported that the VGIIA and VGIIB strains from Vancouver Island shared 14 identical loci after examining 30 alleles, and hypothesised that VGIIA isolates might be the result of same-sex mating (α/α) between a VGIIB isolate and a second unknown VGII isolate in Australia, in transit or in the Pacific Northwest (Fraser et al. 2005). However, Meyer *et al* revealed that there were VGIIA and VGIIB isolates recovered as early as in 1986 in South America, suggesting that these genotypes may have been present for a long time in the Americas rather than being a result of a recent recombination event as suggested by Fraser *et al* (Meyer et al. 2007).

1.1.3 Other species

Besides *C. neoformans* and *C. gattii*, there are at least 38 other cryptococcal species found in a wide variety of environmental locations, such as Antarctica, the Himalayas and saline water (Casadevall and Perfect 1998). However, since most of them are not able to survive in mammalian tissue due to the relatively high body temperature and host

immune system, infection caused by these species is rare (Kordossis et al. 1998; Kraiden et al. 1991; Kunova and Krcmery 1999; Loison et al. 1996). Among those causing non-*neoformans* cryptococcosis, *C. laurentii* (20 cases) and *C. albidus* (18 cases) are responsible for most (80%) of such infections (Khawcharoenporn et al. 2007). The transmission, virulence factors and host immune response to these species resembles that of *C. neoformans* (Ikeda et al. 2000; McCurdy and Morrow 2003).

1.1.4 Genome sequencing project

The genomes of five cryptococcal strains (JEC21, B3501A, H99, WM276 and A1M-R265) have been sequenced (Hu et al. 2008). The JEC21 genome (sequenced at TIGR) is 20Mb in size, containing approximately 6572 genes (Loftus et al. 2005), 10% of which are unique to *C. neoformans* (Idnurm et al. 2005). The intron-rich genome encodes a transcriptome abundant in alternatively spliced (4.2% of transcriptome) and antisense messages (53 genes). The genome is also rich in transposons (~5%), many of which cluster at centromeric regions. The presence of these transposons results in genetic plasticity and may be responsible for karyotype instability and phenotypic variation (Loftus et al. 2005). The sequence difference between JEC21 and B3501A (another serotype D isolate, sequenced at Stanford University) is restricted to 50% of their genomes, which overall are 99.5% identical at the sequence level (Loftus et al. 2005).

As a basidiomycete fungus, *Cryptococcus* is evolutionarily distinct from ascomycete fungi such as *Saccharomyces cerevisiae*, the fission yeast *Schizosaccharomyces pombe*, and many common human fungal pathogens including *Candida albicans* and *Aspergillus fumigatus* (Hull and Heitman 2002). The completed *C. neoformans* and *C. gattii* genome sequences permit comparative genomics with fungi from other phyla, although a detailed comparison based on all five cryptococcal genomes has not yet been undertaken. In addition, the availability of these sequences has made the construction of tiling microarrays and CGH studies feasible. So far, CGH in combination with physical mapping and sequencing has been applied to study the genome variability

within *C. neoformans* species; and it potentially allows for detailed characterisation of the genome of emerging clinically significant strains (e.g., isolates from the VIO) in the future (Hu et al. 2008). These studies will provide important information on the mechanisms of genome microevolution in these pathogens.

1.2 Cryptococcosis

Following inhalation of the infectious particle, a primary pulmonary lymph-node complex is formed. In most cases, symptoms do not develop, indicating that most immunocompetent people either clear or control the infection before widespread symptomatic dissemination occurs (Casadevall and Perfect 1998). Yet frequently the yeast will reside in a dormant state, probably within the lymph-node complex (Baker 1976). Among patients with significant alterations of immunity, including those with prolonged corticosteroid administration, haematological malignancies or HIV infection, however, disseminated disease is often seen. *Cryptococcus* can cause localised infections in any organ involving the skin, eyes, myocardium, bones, joints, lungs, prostate gland, urinary tract or CNS (Perfect 1989). Dissemination may occur from a primary infection; acute infection was reported to occur when immunocompromised individuals are exposed to large numbers of cryptococcal cells (Nosanchuk et al. 2000b). However, there is increasing evidence indicating that dissemination is the result of reactivation of dormant disease. For instance, patients coming from tropical areas can be diagnosed with *C. gattii* cryptococcosis long after they have left their countries of origin (Dromer et al. 1992). Similarly, Garcia-Hermoso and colleagues analysed cryptococcal clinical isolates recovered from patients diagnosed with cryptococcosis in France but born in Africa. The RAPD profiles of these isolates were significantly different from that of those from 17 European patients, suggesting that *Cryptococcus* can be acquired long before the infection develops, as these patients had been living in France for

approximately 10 years and had not been in contact with an African environment for as long as 13 years (Garcia-Hermoso et al. 1999).

Cryptococcosis occurs in both animals and humans, but animal-to-human or human-to-human transmission has not been documented, other than rare examples of iatrogenic transmission (Lin and Heitman 2006) and a mother-to-child transmission (Sirinavin et al. 2004). The clinical presentation of cryptococcosis can be acute or chronic, and manifestation varies depending on stage of the disease. Typical symptoms associated with meningoencephalitis are significantly raised cerebrospinal fluid (CSF) opening pressure ($>25\text{cm H}_2\text{O}$) (occurs in more than 50% of patients with HIV-associated cryptococcal meningitis) (Graybill et al. 2000), resulting in headache, fever, altered mental status, visual loss, dementia or even coma (Casadevall and Perfect 1998). For pulmonary cryptococcosis, symptoms range from asymptomatic pulmonary nodules to acute respiratory distress syndrome (Casadevall and Perfect 1998; Saag et al. 2000). According to a recent study in 166 patients, symptoms including cough (58%), dyspnoea (46%), and fever (38%) are the most frequent manifestations of infection (Baddley et al. 2008). Both *C. neoformans* and *C. gattii* affect the lung and CNS, but the infections caused by the two species have important differences in epidemiology, clinical presentation, and therapeutic outcome (Kwon-Chung and Bennett 1984; Sorrell 2001). For instance, *C. gattii* appears to invade the brain parenchyma more commonly than *C. neoformans*, and in *C. gattii* infected patients, pulmonary infections and pulmonary mass-like lesions are more common (Mitchell and Perfect 1995; Speed and Dunt 1995).

Since 1981, infections due to *Cryptococcus* have been a major cause of morbidity and mortality in individuals with suppressed immune system as a consequence of the AIDS epidemic, as 5-10% of all individuals with CD4+ lymphopenia develop cryptococcosis (Steenbergen and Casadevall 2003). Nowadays, cryptococcosis ranks as one of the three common life-threatening opportunistic infections in people with AIDS worldwide (Levitz and Boekhout 2006). Even though the prevalence of cryptococcosis

in HIV-infected individuals has declined because of highly active antiretroviral therapy, it remains epidemic in Africa and Southeast Asia, where up to 30% of AIDS patients are affected (Bicanic and Harrison 2004; Idnurm et al. 2005). In fact, cryptococcosis has been recognised as an AIDS-defining illness in areas like Zimbabwe, where 91% of AIDS patients are infected (Mwaba et al. 2001). Although less common, cryptococcosis in HIV-negative patients also has a high mortality rate (Kiertiburanakul et al. 2006), particularly in areas such as northern Brazil, where *C. gattii* is endemic and accounts for 62.7% of all cryptococcosis cases (Nishikawa et al. 2003).

1.2.1 Antifungal therapy

Untreated cryptococcal meningitis is uniformly fatal, although survival can range from years to only a few weeks (Mwaba et al. 2001). There are several well-established antimicrobial reagents for treatment, and amphotericin B, a polyene introduced in the mid-1950s, was the first effective therapy developed. Amphotericin B binds to ergosterol in the fungal plasma membrane to cause increased permeability to protons and monovalent cations such as potassium (Brajtburg et al. 1990). It was also found to stimulate inflammatory cytokine production from innate immune cells through CD14 and Toll-like receptors (TLRs) (Sau et al. 2003). In many resource-poor areas where amphotericin B is not available, fluconazole, a triazole that inhibits fungal ergosterol synthesis is widely used (Jarvis and Harrison 2007). It has excellent absorption and CSF penetration and is widely available at low cost in generic form. However, the slow response to therapy with fluconazole means that it is better suited to long-term maintenance therapy than initial therapy (Bozzette et al. 1991; Powderly et al. 1992). Flucytosine (5-Fc) is another commonly used anticryptococcal drug. It is a synthetic antimycotic compound and was initially developed as an anticancer drug in the 1970s. It has no intrinsic antifungal capacity, but after it has been taken up by *Cryptococcus*, it is converted into 5-fluorouracil (5-Fu), a pyrimidine analogue that inhibits fungal RNA and DNA synthesis (Vermees *et al.*, 2000). 5-Fc is commonly prescribed in combination with amphotericin B, because such combination has been shown to have higher

efficiency compared to amphotericin B alone in both non-HIV-associated and HIV-associated infection (Bennett et al. 1979; Brouwer et al. 2004; van der Horst et al. 1997). The optimal current therapy is with amphotericin B 0.7-1mg/kg/day plus 5-Fc 100mg/kg/day for two weeks, followed by fluconazole 400mg/day for 8 weeks and 200mg/day thereafter (Bicanic and Harrison 2004).

The emergence of antifungal drug resistance has not been a major problem to date in areas like Australia and New Zealand (Chen et al. 2000). However, in sub-Saharan Africa, resistance can be very high. In Nairobi Kenya, 5-Fc resistance was observed in 21% of cryptococcal strains and only 23.8% of these strains were susceptible to fluconazole (65% susceptible in a dose-dependent manner and 11.2% resistant) (Bii et al. 2007). Differences in the antifungal susceptibilities of the two species of *Cryptococcus* have also been reported. Trilles *et al* found that *in vitro*, *C. gattii* was less susceptible to seven antifungal compounds as compared with *C. neoformans*, although both showed equal susceptibility to amphotericin B and 5-Fc (Trilles et al. 2004). However, a more recent study did not detect serotype susceptibility differences after measuring antifungal activity against 128 cryptococcal isolates (86 of *C. neoformans* and 42 of *C. gattii*) (Thompson et al. 2009). Isavuconazole, posaconazole, and voriconazole demonstrated excellent potency against each isolate and serotype, including isolates with reduced fluconazole susceptibilities. In future, more controlled experiments are required in order to compare different studies.

1.2.2 Immunotherapy

Immunotherapeutic strategies, mainly based on introducing antibodies and cytokines, have been developed to restore and boost host defence mechanisms to *Cryptococcus*. Antibodies against capsule and cell wall have been demonstrated to provide protection in animal models of cryptococcal infection (Casadevall et al. 1998; Dromer et al. 1987; Mukherjee et al. 1992; Rachini et al. 2007; Sanford and Stollar 1990). However, adjunctive use of antibody therapy in mice with established cryptococcal infection was

also reported to cause cardiovascular collapse and death in some strains due to the release of platelet-activating factor (Lendvai et al. 2000; Savoy et al. 1997). Nevertheless, a murine IgG1 (mAb 18B7) has reached phase I trial in patients recovering from HIV-associated cryptococcal meningitis (Larsen et al. 2005) and radioimmunotherapy (radiation was delivered by specific radio-labelled antibodies leading to antibody-specific killing of *Cryptococcus*) is under evaluation in the murine model (Bryan et al. 2009; Dadachova et al. 2004).

Several cytokines have been shown to augment the antifungal activity of effector cells against cryptococcal infection. In a murine cryptococcal infection, administration of IL-12 resulted in up to 10-fold decreases in the cryptococcal burden in the CNS. Significantly, the combination of fluconazole with IL-12 showed synergetic effects on reducing organism burden (Clemons et al. 1994). Similarly, the importance of interferon- γ (IFN γ) in the clearance of cryptococci, especially from the CSF, has been demonstrated by several groups (Kawakami et al. 1996; Siddiqui et al. 2005; Zhou et al. 2007). In addition, IFN γ can potentiate amphotericin B reduction of infection in the brain (Lutz et al. 2000). A recent phase II study to evaluate the safety and antifungal activity of adjuvant recombinant interferon (rIFN)- γ 1b in HIV patients with acute cryptococcal meningitis showed a trend towards improved mycological and clinical success without adverse effects on CD4 count or HIV viral load (Pappas et al. 2004). However, studies also showed IFN γ treatment can reduce the ability of human alveolar and monocyte-derived macrophages to eliminate intracellular cryptococci (Levitz and Farrell 1990; Reardon et al. 1996; Voelz et al. 2009). In future, more studies should be carried out to evaluate whether IFN γ treatment is an appropriate therapeutic approach.

With the use of molecular biology, several genes and their encoded proteins have now been identified which may help elicit a protective immune response. One such group is the mannoproteins. Mannoproteins are a group of glycoproteins present in the capsule (discussed in detail in Chapter II). They are recognised by the mannose receptor and

presented to T cells by dendritic cells (Levitz and Specht 2006; Mansour et al. 2006). Recent *in vivo* and *in vitro* studies have reported that mannoproteins were the major T cell antigenic determinants from *C. neoformans* and both CBA/J and C57BL/6 mice benefited from immunisation with mannoproteins (Mansour et al. 2004; Specht et al. 2007). Another molecule with therapeutic potential is a synthetic oligodeoxynucleotide containing an unmethylated CpG motif (CpG-ODN). CpG-ODN is a TLR ligand, which was found to protect mice from infection with *C. neoformans* by altering the Th1-Th2 cytokine balance toward a Th1-biased immune response (Edwards et al. 2005; Miyagi et al. 2005). Combination of CpG-ODN with antifungal chemotherapy or with mannoproteins seems to provide a beneficial effect in a murine model of pulmonary and disseminated infection (Dan et al. 2008, Kinjo, 2007 #740), suggesting a rationale for vaccination strategies that combine mannosylated antigens with TLR ligands to achieve synergistic promotion of host defence against *C. neoformans* infection.

1.2.3 Outcomes

The mortality from cryptococcosis remains unacceptably high. The last US Mycoses Study Group treatment trial of HIV-associated cryptococcal meningitis showed the lowest mortality to date, which is still 9.4% at 10 weeks (Bicanic and Harrison 2004). In France, Dromer *et al* observed an overall mortality rate of 6.5% in the first two weeks and 11.5% over the next ten weeks (Dromer et al. 2007). In Southeast Asia, even in the context of amphotericin B based therapy, acute mortality has ranged from 22% to more than 40% (Brouwer et al. 2004; Imwidthaya and Pongvarin 2000). For instance, with amphotericin B plus 5-Fc, 34% of patients with *C. gattii* meningitis in Papua New Guinea died during their first admission, at a median of 8 days (Seaton et al. 1996). In African areas where amphotericin B is not available, results with fluconazole monotherapy at 200mg/day or fluconazole plus 5-Fc in combination showed 44% mortality at 8 weeks (Mayanja-Kizza et al. 1998; Mwaba et al. 2001). The main reasons for the ongoing high mortality of cryptococcal disease include the inadequacy of antifungal therapy, restricted access to some drugs in many areas and the problem of

raised CSF pressure (Antinori 2006; Bicanic and Harrison 2004; Jarvis and Harrison 2007; Perfect 2007).

1.3 Virulence factors

C. neoformans and *C. gattii* have a number of well-defined virulence factors, which strongly influence the degree of pathogenicity of individual isolates. A recent study by Rodrigues *et al* demonstrated that *C. neoformans* was able to secrete vesicles containing many of its virulence factors, including GXM, laccase, urease and phospholipase B (Rodrigues *et al.* 2008). The extracellular vesicles manifested various sizes and morphologies, including electron-lucid membrane bodies and electron-dense vesicles. During disseminated cryptococcosis, measurable levels of cryptococcal products are detected in the body fluid of patients (Gordon and Vedder 1966), suggesting that these 'virulence factor delivery bags' may represent an efficient and general way of delivering pathogenesis-related molecules to the extracellular environment by *C. neoformans* (Rodrigues *et al.* 2008). Below several well-characterised virulence factors are discussed in detail.

1.3.1 Capsule

The capsule is composed of 90-95% GXM and 5% galactoxylomannan (GalXM) (Rakesh *et al.* 2008). GXM is a large polymer with a repeating structure of α -1,3-mannose with β -D-xylopyranosyl, β -D-glucuronosyl and 6-O-acetyl branching. This structure determines the serotype of *C. neoformans* and *C. gattii*, because different capsule structures can be distinguished by antibodies. GalXM is an α -1,6 galactan that contains branches of β -1,3-galactose- α -1,4-mannose- α -1,3-mannose (Vaishnav *et al.* 1998). It has a much smaller mass than GXM: 1.01×10^5 g/mol versus $1.7-7.4 \times 10^6$ g/mol (McFadden *et al.* 2006). In addition to GXM and GalXM, several mannoproteins (<1%) such as MP-98

and MP-99 have been identified within the cryptococcal capsule (Huang et al. 2002; Levitz et al. 2001). To date, a total of 53 mannoproteins have been predicted by genomic databases (Levitz and Specht 2006). These mannoproteins share several structural features, including N-terminal signal sequences, serine/threonine (S/T)-rich C-terminal regions, and glycosylphosphatidylinositol anchor motifs. When mannosylated and glycosylated, they act as critical cryptococcal antigens responsible for stimulating T-cell responses by promoting dendritic cell maturation and activation (Mansour et al. 2004; Pietrella et al. 2005; Specht et al. 2007).

Many *C. neoformans* genes in capsular synthesis and formation have been identified. Chang *et al* cloned and sequenced four genes (*CAP10*, *CAP59*, *CAP60* and *CAP64*) responsible for capsule synthesis in serotype D isolates. These genes are all required for virulence in a murine model (Chang and Kwon-Chung 1994; Chang and Kwon-Chung 1998; Chang and Kwon-Chung 1999; Chang et al. 1996). *CAP59* encodes a transmembrane protein (Chang and Kwon-Chung 1994; Chang et al. 1995), which is involved in the process of GXM export (Garcia-Rivera et al. 2004). *CAP64* was used to complement an acapsular strain (602), resulting in capsule production and a fatal infection in mice (Chang et al. 1997; Chang et al. 1996). *CAP60* and *CAP10* encode proteins localised to the nuclear membrane and cytoplasm respectively (Chang and Kwon-Chung 1998; Chang and Kwon-Chung 1999). All four CAP genes have been shown to be essential in capsule synthesis, but the biochemical function of their products is ill defined. There are many other genes involved in, but not essential to, capsule formation. For instance *CAS1* and *CAS3* are involved in the acetylation of GXM (Janbon et al. 2001; Moyrand et al. 2004), whilst *UXS1* and *UGD1* along with *CAS31*, *CAS32*, *CAS33*, *CAS34* and *CAS35* are important for proper xylosylation of GXM (Bar-Peled et al. 2001; Moyrand et al. 2004). Genome analysis identified more than 30 new genes that are likely to be involved in capsule biosynthesis, including a family containing seven members of the capsule-associated (*CAP64*) gene and a second family of six capsule-associated (*CAP10*) genes (Loftus et al. 2005). Interestingly, some of the

genes in the capsule biosynthesis pathway are also required for the processes of sexual development and sporulation (e.g., *CAP10*, *CAP60*, *CAP64* & *CAP67*) (Botts et al. 2009).

The capsule is important for *C. neoformans* survival in its host, where it increases the fitness of *C. neoformans* by providing direct protection for the yeast. For instance, the capsule inhibits phagocytosis of *C. neoformans* by professional phagocytes in the absence of opsonins (Kozel and Gotschlich 1982) and resists phagosome digestion (Tucker and Casadevall 2002). Capsular material also acts directly against the host. In macrophages, *C. neoformans* releases polysaccharide from its capsule into vesicles around the phagosome and accumulation of these vesicles in the cytoplasm of the host cell results in macrophage dysfunction and lysis (Feldmesser et al. 2000; Tucker and Casadevall 2002). High levels of capsular polysaccharide antigens in the CSF can change the osmolarity of the CSF, thereby affecting its outflow and leading to increased intracranial pressure, headaches and visual disturbance (Denning et al. 1991). In addition, capsular material was reported to repress the migration of host phagocytes (e.g., neutrophils) (Dong and Murphy 1995; Dong and Murphy 1997; Ellerbroek et al. 2004), interfere with cytokine secretion (Retini et al. 1996; Villena et al. 2008), directly inhibit T-cell proliferation (Yauch et al. 2006), induce macrophage apoptosis mediated by Fas ligand (Villena et al. 2008) or nitric oxide generation (Chiapello et al. 2008), and delay maturation and activation of human dendritic cells (Lupo et al. 2008; Vecchiarelli et al. 1994).

The cryptococcal capsule size varies depending on the environmental conditions and seems to be tightly regulated. In nature, cryptococcal cells rarely display the large capsule seen in clinical isolates. The infectious particles, in order to be inhaled and penetrate the small airway, have to be smaller than 4µm in diameter with little or no capsule (Casadevall and Perfect 1998). However, during infection, the capsule is dynamically enlarged and the size varies depending on the affected organ. The lung and brain environment appears to act as an active inducer of capsule growth (Rivera et al. 1998). Capsule size can also be experimentally modulated by growing *C. neoformans*

in diluted Sabouraud broth in the presence of serum, or in a CO₂ rich atmosphere in Dulbecco's Modified Eagle media (DMEM) with low iron concentration (Vartivarian et al. 1993; Zaragoza and Casadevall 2004). These conditions are present in the host environment and may thus promote capsule production during infection.

Although the presence of capsule significantly contributes to the virulence of *C. neoformans*, it is not the only requirement. Many non-*neoformans* cryptococcal species possess a capsule, but are not pathogenic. Also, in one study acapsular *C. neoformans* was found to cause persistent infections in the brains of nude mice, but not in mice with defects only in innate immunity (Casadevall and Perfect 1998), suggesting that when mammalian immunity is sufficiently impaired, even non-capsular strains retain their virulence potential.

1.3.2 Melanin

The ability of *C. neoformans* to produce melanin (Figure 3) was discovered by Staib in the 1960s (Polacheck 1991). Melanin is a negatively charged, hydrophobic pigment of high molecular weight that is formed by the oxidative polymerisation of phenolic compounds (Casadevall et al. 2000). Melanin synthesis in *C. neoformans* is catalysed by laccase in the presence of certain o-diphenolic compounds, such as 3,4-dihydroxyphenylalanine (L-DOPA) (Williamson 1997). In the environment, melanin protects yeast from UV light, high temperatures, freezing and thawing (Rosas and Casadevall 1997; Wang and Casadevall 1994). In hosts, *C. neoformans* cells recovered from human brain tissue are melanised (Nosanchuk et al. 2000a) and gene disruption studies indicate that wild type melanin-producing *C. neoformans* are more virulent (Casadevall et al. 2000). Compared to non-melanised *C. neoformans* cells, melanised cells are less susceptible to oxidants (Emery et al. 1994), and killing by antifungal drugs (e.g., caspofungin and amphotericin B) (Doering 1999; van Duin et al. 2002). Since production of an oxidative burst after phagocytosis is an important mechanism by which immune effector cells mediate antimicrobial action, these results suggest that

melanin may enhance virulence by protecting fungal cells against immune attack. This is further supported by the observation that melanised cells were more resistant to phagocytosis and cell death caused by phagocytic effector cells (Huffnagle et al. 1995). It is important to point out that some non-*neoformans* cryptococci are able to form melanin as well, such as *Cryptococcus podzolicus* (Petter et al. 2001), although they are not pathogenic.

The importance of melanin production to the virulence has motivated studies to define components of this pathway. Two laccase genes: *LAC1* (Torres-Guererro and Edman 1994) and *LAC2* (Missall et al. 2005; Zhu and Williamson 2004) were identified as central enzymes in melanin biosynthesis. Other genes including *VPH1*, *CLC1*, *CCC2*, *ATX1* and *MBF1* have also been found to be essential (Erickson et al. 2001; Walton et al. 2005; Zhu and Williamson 2003), although in most cases the mode of action is not well characterised.



Figure 3: Non-melanin and melanin forming colonies of *C. gattii* serotype B on L-DOPA medium after 7 days at 25°C.

1.3.3 Ability to grow at physiological temperature

The ability to grow at physiological temperatures is essential for the virulence of *C. neoformans* and *C. gattii*. Some cryptococcal species also possess capsules and/or produce melanin (e.g., *C. podzolicus*), but only rarely are they capable of *in vitro* growth at 37°C, and thus none of them cause consistent infection in mammals (Perfect 2005). *C. neoformans* is enriched in bird guano, but birds do not become infected, probably

because *C. neoformans* does not live well at the avian body temperature of 40°C to 42°C (Mitchell and Perfect 1995). Therefore, this temperature restriction is an important determinant of *C. neoformans* pathogenicity.

Early studies identified over a dozen genes as being necessary for high-temperature growth. One such gene, *CNA1*, encodes the *C. neoformans* calcineurin. When *CNA1* was disrupted in H99, the resulting mutant strain was viable at 24°C but not at mammalian physiological temperature. Correspondingly, the mutant strain was avirulent in an immunocompromised rabbit model of cryptococcal meningitis (Odom et al. 1997). Therefore, a role for the regulation of growth at elevated temperatures by signalling cascades involving calcineurin has been proposed. Many cryptococcal genes are known to be regulated by temperature, although they are not necessarily required for high-temperature growth. A microarray transcriptional profiling of *C. neoformans* genes showing altered expression at 37°C versus 25°C described 49 genes induced at 37°C, including *MGA2*, which showed significantly higher expression during growth at 37°C, and was also important for normal growth at high temperature (Kraus et al. 2004). Similarly, a more recent study using an alternative approach called representational difference analysis has revealed 29 genes that are up-regulated at 37°C, with some overlaps with the genes identified by Kraus *et al* (Rosa et al. 2008). These newly-defined genes seem to have a variety of functions, ranging from stress signalling, cell wall assembly, membrane integrity and basic metabolism (Kraus et al. 2004; Rosa et al. 2008; Steen et al. 2002). Functional studies of genes identified in these work by targeted gene disruption followed by validation in animal models may contribute to a better understanding of their role in virulence and pathogen-host interactions.

1.3.4 Degradative enzymes

Proteinase: Both environmental and clinical isolates of *C. neoformans* have proteinase activity (Casadevall and Perfect 1998). They have been shown to degrade host proteins including collagen, elastin, fibrinogen, immunoglobulins and complement factors

(Chen et al. 1996). Tucker and Casadevall also proposed that replication of *C. neoformans* inside macrophages is accompanied by the production of enzymes including proteinases and phospholipases to damage the phagosomal membrane (Tucker and Casadevall 2002). Therefore, cryptococcal proteinases can cause tissue damage, provide nutrients to the pathogen and protection from the host.

Phospholipase: Phospholipases are a heterogeneous group of enzymes that are able to hydrolyse one or more ester linkages in glycerophospholipids. The action of phospholipases can result in the destabilisation of membranes, cell lysis and release of lipid second messengers (Ghannoum 2000; Santangelo et al. 1999). *C. neoformans* secretes a phospholipase enzyme that demonstrates phospholipase B (PLB), lysophospholipase hydrolase and lysophospholipase transacylase activities. As with proteinases, phospholipases contribute to the degradation of phagosomal membrane and thus cell lysis. There is a correlation between phospholipase expression and virulence in a dose-dependent manner among the strains used to infect mice (Chen et al. 1997; Ghannoum 2000). Disruption of *PLB1* gene led to reduced virulence *in vivo* and growth inhibition in macrophages (Cox et al. 2001). Phospholipase can also cleave dipalmitoyl phosphatidylcholine, one of the main components of lung surfactant, and thus assists fungal spread (Steenbergen and Casadevall 2003). Furthermore, recent studies demonstrated that phospholipase B of *C. neoformans* enhances adhesion of *C. neoformans* to a human lung epithelial cell line (Ganendren et al. 2006) and dissemination of cryptococcosis in a murine model (Santangelo et al. 2004).

Urease: Urease catalyses the hydrolysis of urea to ammonia and carbamate and is an important pathogenic factor for certain bacteria (Steenbergen and Casadevall 2003). The cryptococcal urease, Ure1, is an important virulence factor and mice infected with a ure1 mutant strain live longer than those infected with the wild type strain H99 (Cox et al. 2000). Although Ure1 is not required for growth in the brain, the dissemination patterns in the brain, spleen, and other organs after intravenous inoculation differed from the wild type strain, leading to the proposal that Ure1 is important for the CNS

invasion by enhancing yeast sequestration within microcapillary beds (such as within the brain) during haematogenous spread, thereby facilitating blood-to-brain transmission (Olszewski et al. 2004). A more recent study also defines cryptococcal urease as a pulmonary virulence factor that promotes immature dendritic cell accumulation and a potent, yet non-protective, Th2 immune response (Osterholzer et al. 2009).

1.3.5 Mating type

Most clinical and environmental cryptococcal isolates have been observed predominantly as vegetative haploid yeast. Like other basidiomycetes, traditional mating can occur in response to nutrition limitation, where opposite mating types (a and α) secrete peptide pheromones, which trigger cell to cell fusion to produce a filamentous dikaryon, resulting in a transient a/α diploid state that immediately undergoes meiosis and sporulation producing a and α haploid progeny (Kwon-Chung 1975; Kwon-Chung 1976) (Figure 4). *Cryptococcus*, although less often, can also undergo same-sex mating (monokaryotic fruiting), especially between two α cells to form stable α/α diploids and also α haploid progeny (Lin et al. 2005) (Figure 4). Mating without a partner of the opposite mating type might provide a survival advantage, particularly under harsh or changing conditions (Lin et al. 2007).

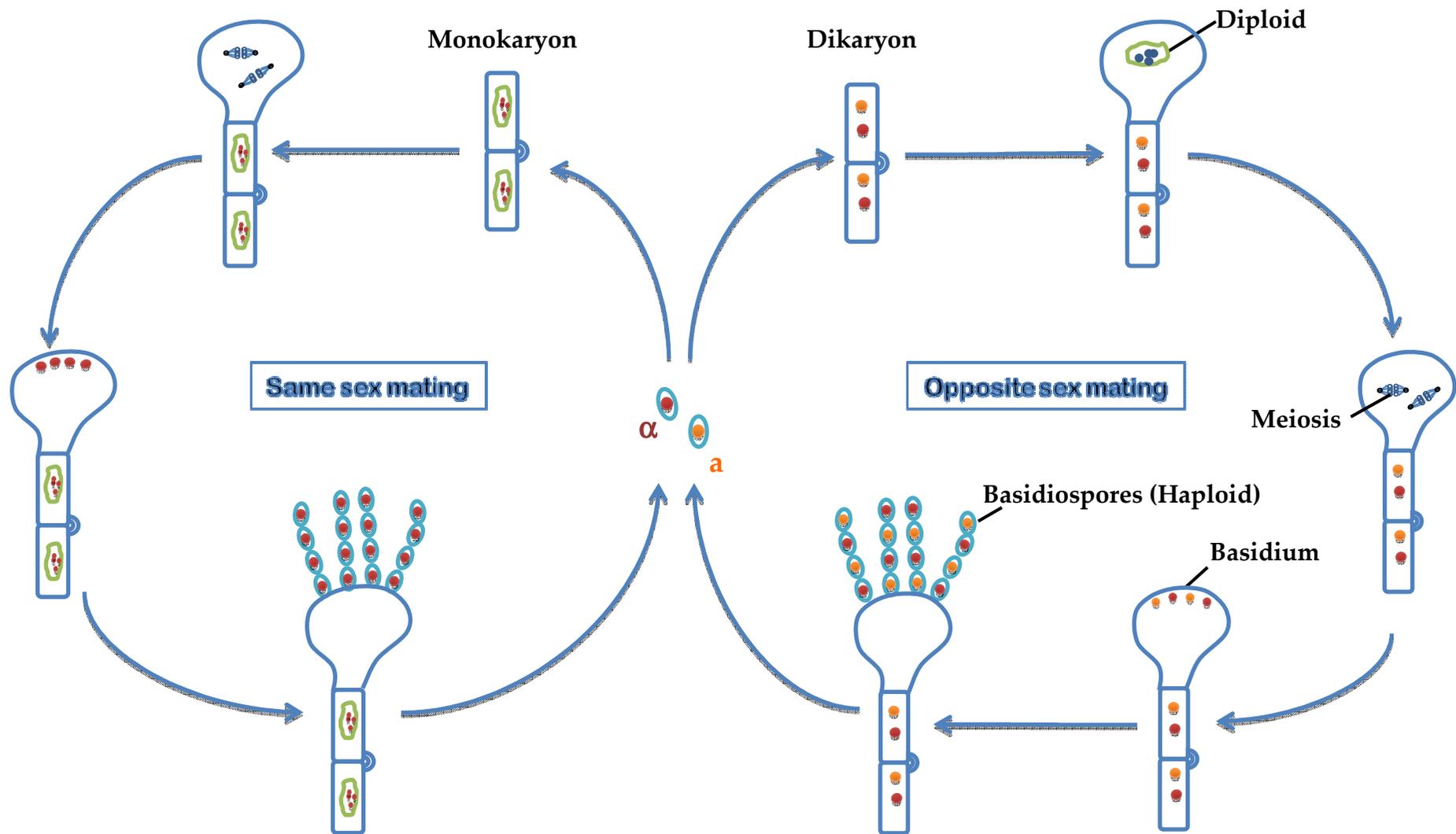


Figure 4: Mating between the opposite sex and the same sex. During opposite sex mating, there are five distinct stages: 1) Dikaryon formation, 2) nuclear fusion between two parental nuclei, 3) Meiosis to produce four meiotic products, 4) Basidiospores formation, and 5) Mitosis and basidiospore proliferation (sporulation). Spores will ultimately be released into the environment. During monokaryotic fruiting, diploidisation (nuclear fusion) occurs before the monokaryon formation, which is followed by meiosis and sporulation.

Several interesting observations implicate mating type as a virulence factor. Firstly, MAT α cells are much more prevalent than MATa cells. In a survey of natural and clinical isolates, the MAT α mating type was 40-fold more abundant in environmental isolates and 30-fold more abundant in clinical isolates than its MATa counterpart (Kwon-Chung and Bennett 1978). In addition, most of the Vancouver isolates are α mating type (Fraser et al. 2003). Secondly, when congenic α and a strains (JEC21) of serotype D (genetically identical except at the mating type locus) were studied in a murine model of cryptococcosis, the MAT α strain was found to be significantly more virulent than the MATa strain (Kwon-Chung et al. 1992). Congenic α and a cells in the serotype A H99 background show the same pathogenicity level in various mammalian models (Nielsen et al. 2003), but α cells have an enhanced predilection to penetrate the CNS during coinfection with a cells, which provides an explanation for the prevalence of α strains in clinical isolates (Nielsen et al. 2005).

The finding that MAT α cells are more prevalent and virulent than MATa cells has promoted molecular analysis of the MAT α mating type locus. Initially, an approximately 50kb region present only in MAT α strains was defined as the MAT α locus, and it contains many α -specific genes including *STE12 α* (Karos et al. 2000). However, the actual size of the MAT locus appears to be much larger than that. It is more than 100kb for both *C. neoformans* and *C. gattii*, containing more than 20 genes, including those involved in pheromone production and sensing, establishing cell type identity, components of a MAP kinase pathway and those do not seem to have a function in mating (Fraser and Heitman 2004; Lengeler et al. 2002) (Figure 5). There is still much to be learned about the linkage of sex and pathogenesis, especially at the genetic level.

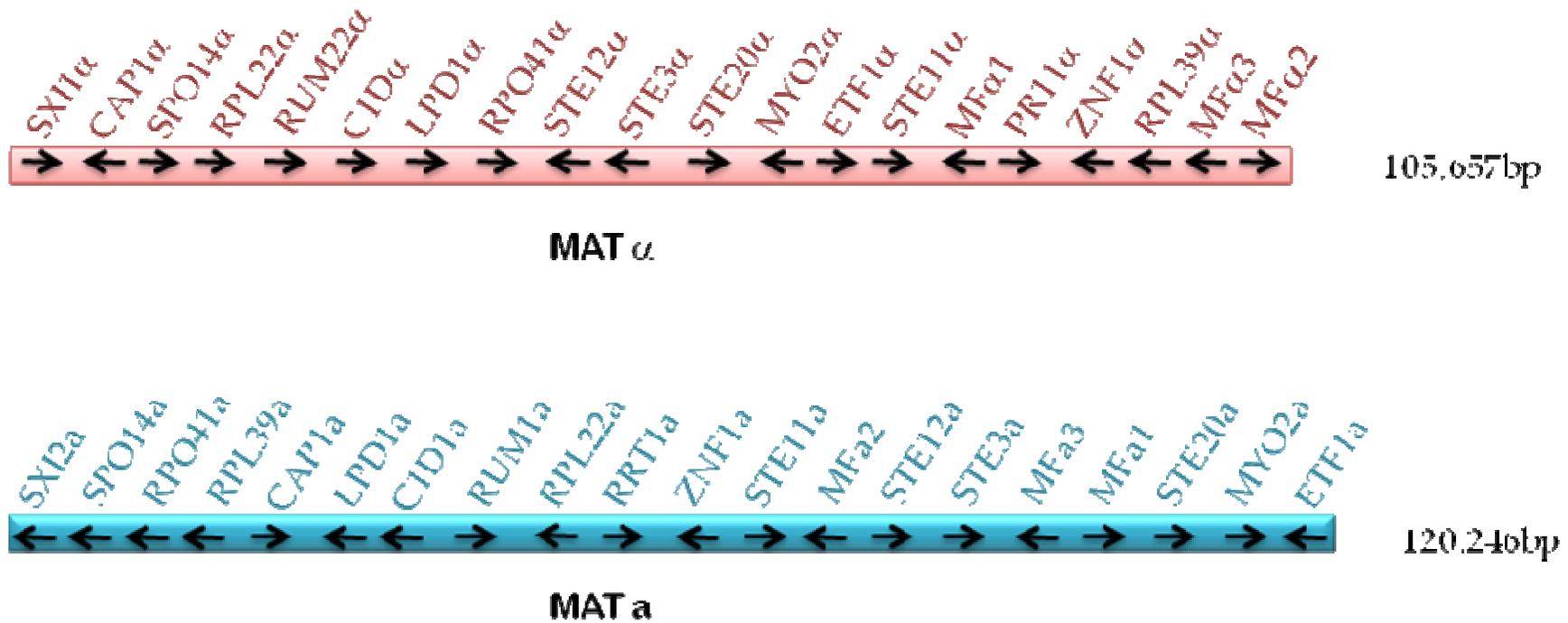


Figure 5: The mating type locus of *C. neoformans* (based on information from (Idnurm et al. 2005)). The locus (>100 kb for both mating type) is located on chromosome 4.

1.3.6 Phenotypic switching

Phenotypic switching has been observed in both prokaryotes and eukaryotes and involves stochastic switching between two or more alternative and heritable phenotypes. It occurs by spontaneous tuning in gene expression in order to escape recognition by the immune system and to adapt to a new host environment. Phenotypic switching is reversible and readily detectable in a fraction of the cell population (D'Souza and Heitman 2001b).

The first detection of phenotypic switching in *C. neoformans* was reported by Fries and Casadevall in 1998, in which they showed *C. neoformans*'s ability to undergo microevolution during chronic infection (Fries and Casadevall 1998). Subsequently, Fries *et al* showed that *C. neoformans* was able to undergo phenotypic switching *in vivo* during serial passage in mice (Fries et al. 2001). So far, phenotypic switching has been reported in serotype A, B and D strains (Guerrero et al. 2006; Jain et al. 2006) and always leads to changes in virulence by causing changes in capsule or cell wall morphology. For instance, a *C. gattii* strain was found to switch reversibly between two colony morphologies. Switching to mucoid colonies (with a thicker layer of capsule) was observed during pulmonary infection and resulted in enhanced intracellular survival due to a larger capsule. However, only smooth colonies (with a thin layer of capsule) could be grown from brain homogenates in infected mice, probably because the thin capsule permits better crossing of the blood-brain barrier (Jain et al. 2006). Phenotypic switching of *C. neoformans* was also shown to influence the outcome of the human immune response (Guerrero and Fries 2008). For example, the mucoid colony phenotype elicits a macrophage- and neutrophil-dominated immune response, while the smooth colony phenotype elicits a lymphocyte-dominated immune response (Pietrella et al. 2003). The ability of this organism to cause chronic infections even after prolonged antifungal therapy may be, in part, attributable to phenotypic switching (Guerrero et al. 2006).

1.3.7 The origin and maintenance of virulence factors

C. neoformans and *C. gattii* are environmental saprophytes, mainly found in soil and trees, so humans probably represent an inadvertent host species rather than a primary niche. There is much evidence supporting the hypothesis that cryptococcal virulence originated due to environmental selective pressure. Firstly, many environmental isolates of *C. neoformans* are virulent in animals, indicating that these virulence factors have been developed without previous interaction with host animals. Secondly, a broad range of animals are susceptible to these organisms and the hosts are not required for replication or viability of the pathogen (Casadevall et al. 2003). Thirdly, many virulence factors appear to have 'dual use' capacities that allow survival advantages in both animal hosts and in the environment. For instance, in bird excreta, the primary role of urease probably is to enable *C. neoformans* to convert urea to the usable nitrogen source ammonia (Levitz 2001). Decaying wood contains large amount of the aromatic polymer lignin, a substrate of laccases. Thus it has been hypothesised that cryptococcal laccase helps the organism establish an ecological niche in rotting wood (Lazera et al. 2000). The capsule can protect the fungus against dehydration and thus provide a survival advantage in conditions of low humidity (Aksenov et al. 1973). Melanised *C. neoformans* cells, as mentioned earlier, are more resistant to UV radiation, temperature extremes and heavy metals (Rosas and Casadevall 1997). In addition, phospholipase and protease can serve important nutritional roles (Chen et al. 1996). Hence these virulence factors are not solely developed for survival inside mammalian cells and hosts.

Finally, *C. neoformans* is a facultative intracellular parasite, surviving both inside and outside of phagocytes. Infection of macrophages and amoebae by *C. neoformans* was found to be very similar, and it has therefore been postulated that mammalian virulence factors in *C. neoformans* evolved as a defence mechanism against environmental predators (Malliaris et al. 2004; Steenbergen and Casadevall 2003; Steenbergen et al. 2001). The observation that *C. neoformans* can be ingested by living

amoebae was first reported by Bunting and colleagues nearly 30 years ago (Bunting et al. 1979). Subsequently, it was demonstrated that incubation of *C. neoformans* and the amoeba *Acanthamoeba castellanii* results in phagocytosis of yeast cells and intracellular proliferation in a phagocytic vacuole followed by killing of amoebae; a process that is identical to that seen to occur in mammalian macrophages infected with this pathogen (Steenbergen et al. 2001). Another amoeba, *Dictyostelium discoideum*, is also susceptible to infection with *C. neoformans* and the interactions are similar to those described previously for this fungus with macrophages. In addition, *C. neoformans* virulence was enhanced after growth in *D. discoideum*, and this enhancement correlated with increased capsule size and melanisation (Steenbergen et al. 2003). Both studies support the idea that pathogenicity of *C. neoformans* towards macrophages and vertebrate hosts may result from evolutionary pressure exerted by environmental predators. Similarly, Mylonakis *et al* have demonstrated that soil-dwelling nematodes may also exert strong selective pressure on cryptococcal species (Mylonakis et al. 2002). Whilst non-pathogenic cryptococcal species (*C. laurentii* and *C. kuetzingii*) are killed by the nematode *Caenorhabditis elegans*, wild type strains of *C. neoformans* are lethal to the worms. Furthermore, the interaction involves a number of genes that are also important during the host pathogen interaction in mammals, including *GPA1*, *PKA1*, *RAS1* and *PKR1* (Mylonakis et al. 2002). Virulence might also be maintained through infection of small rodents or other mammals that, after death, re-introduce virulent strains back to the environment (Idnurm et al. 2005).

In conclusion, it appears increasingly likely that many virulence factors in *C. neoformans* and *C. gattii* are 'ready made' (Casadevall et al. 2003) due to environmental selective pressure rather than 'specially made' in order to colonise mammalian hosts. There are many existing environmental reservoirs which are expected to affect the fitness of fungal cells in that environment and to provide selective pressures for virulence attributes leading to differences in fitness during mammalian infection.

1.4 Signalling pathways regulating pathogenicity

Six major signalling pathways have been demonstrated to modulate morphological differentiation, virulence and stress responses. They are the cAMP-PKA pathway, three MAP kinase pathways involving Cpk1, Hog1 and Mpk1, the Ras specific pathway and the Ca²⁺-calcineurin pathway. These pathways are also responsible for regulating differentiation and pathogenicity in other fungi and are largely structurally and functionally conserved in serotype A and D strains, although there are serotype-specific differences.

1.4.1 cAMP-PKA

There is conservation of function in cAMP signalling pathways in fungi since a large and diverse group of fungi (including *C. albicans* and *A. fumigatus*) employ similar signalling elements (Alspaugh et al. 1998; Liebmann et al. 2003; Rocha et al. 2001). In *C. neoformans*, cAMP signalling is triggered by environmental stimuli (mainly nutritional, such as starvation) through a G-protein-coupled receptor (e.g., Gpr4 (Xue et al. 2006)) and the G α protein called Gpa1 (Alspaugh et al. 1997). Gpa1 activates a conserved cAMP pathway through the enzyme adenylyl cyclase (Cac1), which generates cAMP and leads to activation of protein kinase A (PKA) by causing the release of the regulatory subunits (Pkr1) from the two catalytic units of PKA (Pka1 and Pka2) (Pukkila-Worley and Alspaugh 2004). In serotype A strains, Pka1 plays a major regulatory role, while in serotype D strains, Pka2 does so (Hicks et al. 2004).

The cAMP-PKA pathway regulates several important processes in *C. neoformans*, including capsule production, melanin formation and mating. A gpa1 mutant, cac1 mutant and pka1 mutant all display similar defects in mating, capsule and melanin production (Alspaugh et al. 1997; Alspaugh et al. 2002; D'Souza and Heitman 2001a). For instance, *C. neoformans* gpa1 mutant strains could not produce melanin, showed

markedly attenuated capsule production in response to the normal inducing condition of severe iron starvation, and were sterile. Correspondingly, in a rabbit model of cryptococcal meningitis, the mutant strain was markedly impaired in the ability to maintain the CNS infection compared to the isogenic wild type strain (Alspaugh et al. 1997). Disruption of *PKR1* suppresses the capsule and melanin defects of the *gpa1* mutant, causes cells to display an enlarged capsule phenotype, and results in hypervirulence (D'Souza and Heitman 2001a). In addition, a recent microarray study comparing the transcriptome of mutants (*pka1* and *pk1*) to a wild type strain revealed a novel relationship between cAMP signalling and the secretory pathway in *C. neoformans* (Hu et al. 2007). In the *pka1* and *pk1* mutants, transcriptional changes occur to many key components important for the secretory pathway, such as those responsible for translocation (Sec61 and Hsp70/Kar2), vesicle formation and fusion (Bet1, syntaxin), Golgi transport (α -1,6-mannosyltransferase), and vesicle delivery to the plasma membrane (e.g., Ypt3). This study along with the observation that *C. neoformans* secretes vesicles containing many of its well-defined virulence factors, suggests a model in which PKA regulates the expression of secretory pathway components to control the elaboration of virulence factors at the cell surface (Hu et al. 2007; Rodrigues et al. 2008).

1.4.2 MAP kinase pathway

The pheromone-activated MAP kinase pathway is another conserved pathway, in which the G protein β subunit (Gpb1) activates the transcriptional regulator Ste12 α , whose downstream targets include *STE20*, *STE11* and *STE7* (Lengeler et al. 2000). *gpb1* mutants are sterile, defective in haploid fruiting and exhibit a severe defect in cell fusion assays (Wang et al. 2000). Although studies disrupting the *STE12 α* found that in both serotype A and D, Ste12 α is absolutely required for monokaryotic fruiting, it seems only to augment virulence in serotype D: in serotype D strains, Ste12 α controls the expression of many virulence-associated genes, and disruption of *STE12 α* resulted in significant reduction in virulence in a mouse model (Chang et al. 2000), whereas the

STE12 α homolog is largely dispensable for virulence in a number of serotype A strains (Yue et al. 1999).

More recently, the Pbs2-Hog1 MAP kinase pathway has been shown to have a significant impact on virulence of serotype A and some serotype D strains (Bahn et al. 2005). The fungal Hog1 MAPK mediates responses to a plethora of environmental cues, including osmotic shock, UV irradiation, oxidative damage and high temperature. Intriguingly, Hog1 is regulated in an opposite fashion in a majority of *C. neoformans* strains (especially highly pathogenic isolates, e.g., H99), compared to some of the serotype D strains and other model yeasts. In *S. cerevisiae*, MAPK Hog1 is dephosphorylated in normal conditions and following osmotic shock, a two component system can activate MAPK kinase Pbs2 through activation of Ssk1, which subsequently phosphorylates the MAPK Hog1 (Bahn et al. 2006). Phosphorylated Hog1 then translocates to the nucleus where it activates expression of target genes (Hohmann 2002). A similar pathway has been observed for some *C. neoformans* serotype D strains, such as JEC21. However, in most *C. neoformans* strains, the Hog1 MAPK is constitutively phosphorylated by Pbs2 MAPK kinase under normal *in vitro* growth condition and, upon osmotic shock, Hog1 is rapidly activated by Hog1-dependent dephosphorylation (Bahn et al. 2005). It was proposed that phosphorylated Hog1 under normal conditions is mainly responsible for negatively regulating virulence factors, including capsule and melanin, and sexual development. In addition, phosphorylated Hog1 concentrates in the nucleus, where it can interact with other transcription factors resulting in cross-talk with signalling cascades that regulate virulence factor expression in *C. neoformans* (Bahn et al. 2006). For example, experimental data demonstrated that Hog1 negatively regulates melanin production by acting on PKA downstream targets for melanin synthesis, whilst Hog1 also negatively regulates capsule production by acting upstream of Gpa1 or PKA itself (Bahn et al. 2005). Under stress conditions, Hog1 is rapidly dephosphorylated, and presumably then induces stress defence genes in *C. neoformans*. It was reported that

fludioxonil treatment can activate the Hog pathway by rapid dephosphorylation of the Hog1 MAPK in the majority of *C. neoformans* strains (Kojima et al. 2006).

The Mpk1 MAP kinase pathway regulates cell-wall integrity and growth at high temperature. It is well studied in *S. cerevisiae*, and the function of Mpk1 in promoting growth at 37°C in *S. cerevisiae* is conserved in *C. neoformans*. In this pathway, upstream components such as membrane sensors that detect stresses to the cell wall (Gray et al. 1997; Verna et al. 1997) and the Rho1 GTPase are responsible for activating protein kinase C, which in turn activates the Mpk1/Slt2 MAPK cascade (Kamada et al. 1996; Lee et al. 1993). *C. neoformans* mutants lacking Mpk1 are attenuated for virulence in the mouse model of cryptococcosis (Kraus et al. 2003) and become more sensitive to antifungal drugs like fludioxonil (Kojima et al. 2006).

1.4.3 Ras pathway & the Ca²⁺-calcineurin pathway

The Ras-Cdc24 pathway and Ca²⁺-calcineurin pathway independently control *C. neoformans* growth at high temperature. *C. neoformans* ras1 mutant strains are viable, but they are unable to grow at 37°C, and are thus less virulent in rabbit and murine models of cryptococcosis (Waugh et al. 2002), a phenotype associated with a failure of actin polarisation at elevated temperature (Waugh et al. 2002). Similarly, calcineurin mutant strains are found to be viable, but do not survive *in vitro* conditions that mimic the host environment and are no longer pathogenic in a murine model of cryptococcal meningitis (Odom et al. 1997).

Ras also plays a dual role to activate a MAP kinase cascade and to regulate cAMP production in *C. neoformans*. Initial experiments defining the Ras pathway in a serotype A strain indicated that Ras1 mediates MAP kinase, cAMP and Ras-specific signal transduction pathways (Alspaugh et al. 2000). By northern blot analysis, Ras1 was demonstrated to play a major role in the transcriptional regulation of genes in the pheromone response pathway. It also controls pheromone-independent signalling

mechanisms which are essential for filamentation, development and pathogenicity (Waugh et al. 2003). Ras2 is expressed at a very low level compared to Ras1, and a ras2 mutant showed no differences in vegetative growth rate, differentiation or virulence factor expression, nor was it attenuated in the murine inhalational model of cryptococcosis. However, when over-expressed, Ras2 was able to restore mating and high temperature growth of a ras1 mutant, indicating Ras1 and Ras2 may share overlapping functions (Waugh et al. 2002).

The calcineurin pathway, in addition to its importance for growth at high temperature, is also essential for cell integrity, monokaryotic fruiting and mating (Cruz et al. 2001; Fox et al. 2001; Kraus et al. 2003; Kraus et al. 2005; Odom et al. 1997). In this pathway, both calcineurin A and B subunits were found to be essential for virulence (Fox et al. 2001), by binding to calcineurin binding protein 1 (Gorlach et al. 2000) and activating as-yet unidentified downstream transcription factors.

1.5 *Cryptococcus* and the host immune response

Exposure to *C. neoformans* is thought to be common, but in a normal host the infection is usually self-limiting. In contrast, in immunocompromised individuals, the infection is not restricted to the primary site of infection, but frequently disseminates to the CNS. This suggests that phagocytes *in vivo* are able to dispose of *C. neoformans* effectively (or at least maintain the pathogen in a latent stage), only when T-cell defences are intact. This probably involves activation of macrophages by Th1 type cytokines (including TNF α , IFN γ , IL-2 and IL-12) and granuloma formation to contain replicating organisms. In other words, phagocytes are 'temporary protectors' until the acquired immune response is established. This part of the introduction will focus on the interaction between phagocytic effector cells and *C. neoformans* in the absence and presence of a secondary immune response.

1.5.1 Immunocompromised host

In immunocompromised individuals, the innate immune response is the major barrier to cryptococcal infection. Although many studies have identified several innate factors such as serum, complement factors and saliva that discourage infections (Baum and Artis 1961; Baum and Artis 1963; Hendry and Bakerspigel 1969; Igel and Bolande 1966; Nassar et al. 1995; Szilagyi et al. 1966), the outcome of the infection is largely dependent on the interaction between the pathogen and phagocytic effector cells (Shao et al. 2005).

1.5.1.1 Neutrophils

An *in vivo* study on cryptococcal infection in mice by Feldmesser *et al* (2000) noted that macrophages and neutrophils are the only inflammatory cells in contact with *C. neoformans* in the lung. Many *in vitro* studies also demonstrated that neutrophils could phagocytose and kill *C. neoformans* (Chaturvedi et al. 1996; Kozel et al. 1984; Mambula et al. 2000; Miller and Mitchell 1991). However, *in vivo*, neutrophils were only found to occasionally ingest *C. neoformans* for the first few days after infection, indicating that they predominate only in the early stage of an experimental infection (Feldmesser et al. 2000). Furthermore, neutrophil-depleted mice infected with *Cryptococcus* had significantly higher levels of IL-4/IL-10 (Th2 cytokines) and IL-12/TNF α (Th1 cytokines), and they lived longer than wild type mice, suggesting neutrophil depletion is protective against *C. neoformans* pulmonary infection. The enhanced survival observed in neutrophil-depleted mice could be a result of a more effective killing of the pathogen triggered by IL-12 and TNF α , and reduced damage to the host moderated by IL-4 and IL-10 (Mednick et al. 2003). Therefore, neutrophils probably do not contribute significantly to direct killing of invading *C. neoformans*, but rather play an important role in balancing the Th1/Th2 cytokine profile in the late stage of infection.

1.5.1.2 Dendritic cells

Recent studies also show that dendritic cells phagocytose *C. neoformans* both *in vitro* (Kelly et al. 2005; Syme et al. 2002) and *in vivo* (Wozniak et al. 2006). Intracellular yeast cells then enter the endolysosomal pathway of dendritic cells and can be killed by lysosomal components (Wozniak and Levitz 2008). Dendritic cells are antigen-presenting cells that act as sentinels in the peripheral tissues, constantly sampling the antigens in their environment. During cryptococcal infection, dendritic cells are thought to be more important in the initial presentation of antigens to the naive T cells to induce an adaptive immune response. Indeed, they induce a stronger T-cell response to *C. neoformans* than alveolar macrophages or monocyte-derived macrophages (Mansour et al. 2006; Syme et al. 2002). Several major antigens (e.g., mannoproteins) known to drive T cell responses to *C. neoformans* were also found to be mainly presented by dendritic cells (Levitz and Specht 2006).

1.5.1.3 Macrophages

Macrophages, also involved in antigen presentation and cytokine production (Casadevall and Perfect 1998), have long been regarded as the phagocyte that initially encounters inhaled *C. neoformans* and act as the primary phagocytic cell at all times of infection in both murine and rat models of infection (Bolanos and Mitchell 1989; Feldmesser et al. 1998; Feldmesser et al. 2000; Goldman et al. 2000; Levitz 1994). Phagocytosis of *C. neoformans* by macrophages can be mediated by receptors such as the mannose receptor, β -glucan receptor, antibody receptors and complement receptors. Phagocytosis via the latter two receptors is efficient (Casadevall and Perfect 1998). Depending on the environment they adapt to, *C. neoformans* cells can actively 'choose' to avoid being phagocytosed to a certain extent by regulating their antiphagocytic factors. For example, *C. neoformans* was found to switch reversibly between two colony morphologies which were associated with changes in capsule (Jain et al. 2006). Capsule is a major anti-phagocytic factor in the absence of opsonins (Kozel and Gotschlich 1982; Kozel and Mastroianni 1976). It inhibits phagocytosis partly by

lessening presentation of phagocytic ligands to alveolar macrophages (Vecchiarelli et al. 1994). In addition, encapsulated *C. neoformans* have a more negatively charged surface than acapsular cells, which causes electrostatic repulsion between the cryptococci and negatively charged phagocytic cells and thus reduces cell-cell interaction (Nosanchuk and Casadevall 1997). However, in the presence of opsonins including antibody and complement components (*in vivo*), the antiphagocytic property of the capsule is usually diminished (Feldmesser et al. 2000). App1 (antiphagocytic protein 1) is another factor found to regulate phagocytosis. It was first identified as a regulator of complement-mediated phagocytosis and inhibits phagocytosis through a specific and novel mechanism without affecting other cryptococcal anti-phagocytosis factors, such as capsule and melanin (Luberto et al. 2003). Without App1, *C. neoformans* is more likely to be ingested by macrophages. Interestingly, the app1 mutant is less virulent than the wild type strain in A/J, CBA/J and C57BL/J mouse models, which are immunocompetent, whereas in a T-cell and natural killer (NK) cell deficient mouse model, the app1 mutant exacerbated the infection as compared with the infection caused by a wild type strain. These data suggest that when the cellular immune response is impaired, phagocytosis aids the spread of *C. neoformans* infection as the pathogen can be transported in a more efficient manner by macrophages from organ to organ (Del Poeta 2004; Luberto et al. 2003). Therefore, modulation of the expression of antiphagocytic factors by *C. neoformans* may play a key role in the outcome of infection.

Following particle internalisation by macrophages, the resulting intracellular vacuole (known as the phagosome) is subsequently fused with lysosomes to form the phagolysosome. This process is called phagosome maturation and the newly formed phagolysosome possesses a number of complementary degradative properties including a very low pH, hydrolytic enzymes for particle digestion, bactericidal peptides, and the ability to generate toxic oxidative compounds (Vieira et al. 2002). Usually the phagolysosome is very efficient at digesting encased microorganisms. However, for *C. neoformans*, three outcomes have been observed after phagocytosis: 1) The yeast is killed by the macrophage (Brummer and Stevens 1994; Casadevall and

Perfect 1998); 2) the yeast remains latent inside the macrophage (Alvarez and Casadevall 2007; Del Poeta 2004; Ma et al. 2007); and 3) the yeast grows within the phagosome, eventually causing macrophage lysis (Feldmesser et al. 2001a; Feldmesser et al. 2000; Tucker and Casadevall 2002) (Figure 6). Currently it is unclear as to what decides the outcome of the intracellular yeast, but it is generally established that, *in vitro*, macrophages activated with Th1 cytokines are more efficient at eradicating intracellular cryptococci than those activated with Th2 cytokines (Brodie et al. 1994; Chen et al. 1994; Kawakami et al. 1995; Mody et al. 1991; Weinberg et al. 1987). In addition, the fate of intracellular cryptococci varies with strain (Ma et al. 2009; Zaragoza et al. 2007); and other local environmental stimuli (e.g., oxygen concentration) (Voelz et al. 2009, unpublished data).

In the absence of a T-cell mediated immune response, intracellular survival and proliferation of *Cryptococcus* is very common. This intracellular behaviour is important for pathogenicity, because it provides a basis for dissemination and latency: intracellular cryptococci are carried by infected macrophages to different parts of the body without being exposed to any extracellular hazards, such as complement components or antifungal agents present in the blood. This so-called 'Trojan Horse' mechanism of dissemination (Figure 7) (Chretien et al. 2002; Santangelo et al. 2004) is supported by the observation that *C. neoformans* was found almost exclusively in macrophages in chronic and latent infection (Feldmesser et al. 2000; Goldman et al. 2000) and macrophage depletion often leads to reduced clinical manifestations and fungal burden in many organs studied (Charlier et al. 2009; Shao et al. 2005).

Intracellular parasitism of macrophages by *C. neoformans* was reported in the early 1970s, when most ingested *C. neoformans* were found to be resistant to intracellular killing by either peritoneal exudate cells from Lewis rats or monocyte-derived macrophages (Diamond and Bennett 1973; Mitchell and Friedman 1972). Unlike many other intracellular pathogens which persist within the phagosome by either affecting phagolysosome maturation (e.g., *Legionella pneumophila*) (Nguyen and Pieters 2005) or

by escaping from the phagosome and then proliferating in the host cytosol (e.g., *Listeria monocytogenes*) (Cossart et al. 2003), *C. neoformans* has been demonstrated to persist inside apparently normal mature phagosomes in human monocyte-derived macrophage (Levitz et al. 1999) (Table 1). The pH of *C. neoformans*-containing phagosomes was similar to that observed following uptake of dead fungi over 24h, and these phagosomes also co-localised with LAMP-1, a highly glycosylated protein found in endosomal and lysosomal compartments that is commonly used as a late mature phagosome marker, indicating that *C. neoformans* does not interfere with phagosome-lysosome fusion. In fact, *C. neoformans* grows more rapidly in acidic media than in neutral or alkaline media and appears to be able to resist the action of the macrophage lysosomal enzymes, which function optimally at acid pH (Levitz et al. 1999). *In vivo*, intracellular persistence was associated with replication and residence in a membrane bound phagosome (Feldmesser et al. 2001a; Feldmesser et al. 2000). Recent electron microscopy studies by Tucker and Casadevall revealed that intracellular residence by *C. neoformans* is accompanied by the accumulation of polysaccharide-containing vesicles, which originated from the phagosome, followed by macrophage dysfunction and lysis (Tucker and Casadevall 2002) (Figure 6). Many virulence factors required for cryptococcal intracellular survival have already been identified, including capsule and melanin synthesis proteins, proteinases and phospholipases, an alternative oxidase (Aox1) (Akhter et al. 2003), inositol phosphosphingolipid- phospholipase C1 (Isc1) (Shea et al. 2006), Skn7 (Coenjaerts et al. 2006), and vacuole protein Vps41 (Liu et al. 2006), most of which contribute to defence against exogenous oxidative stress. However, the detailed intracellular survival mechanism needs further investigation.

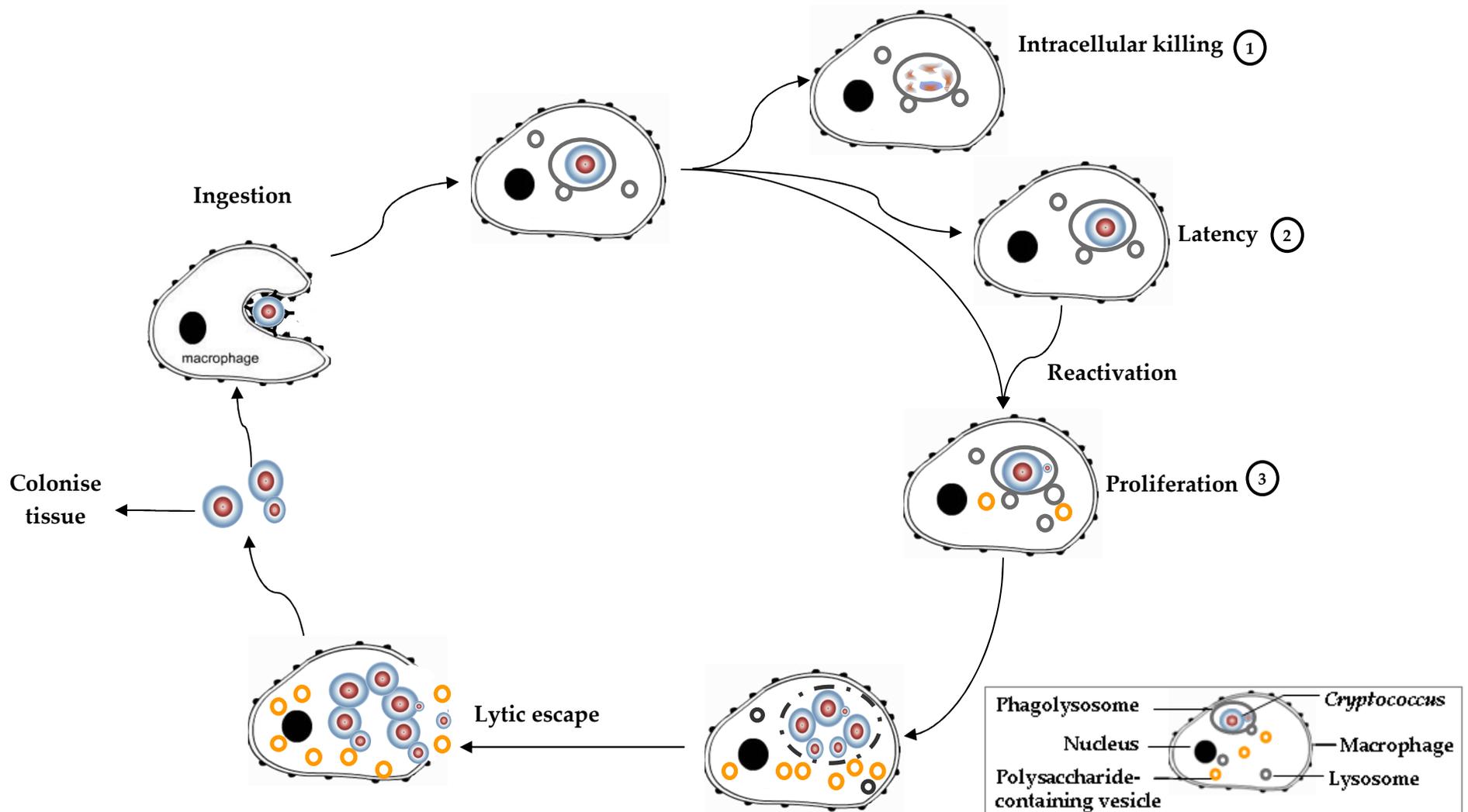


Figure 6: Macrophage parasitism by *C. neoformans*. Following phagocytosis, the internalised cryptococci can be killed by macrophages (1), or they remain latent (2). When the host becomes immunocompromised, some of the cryptococci or latent population can reactivate and proliferate intracellularly (3), followed by the lytic burst of the host cells and release of the intracellular yeast cells into the extracellular environment. The released yeast cells can then infect more macrophages or establish their extracellular dominance.

Intravenous Delivery

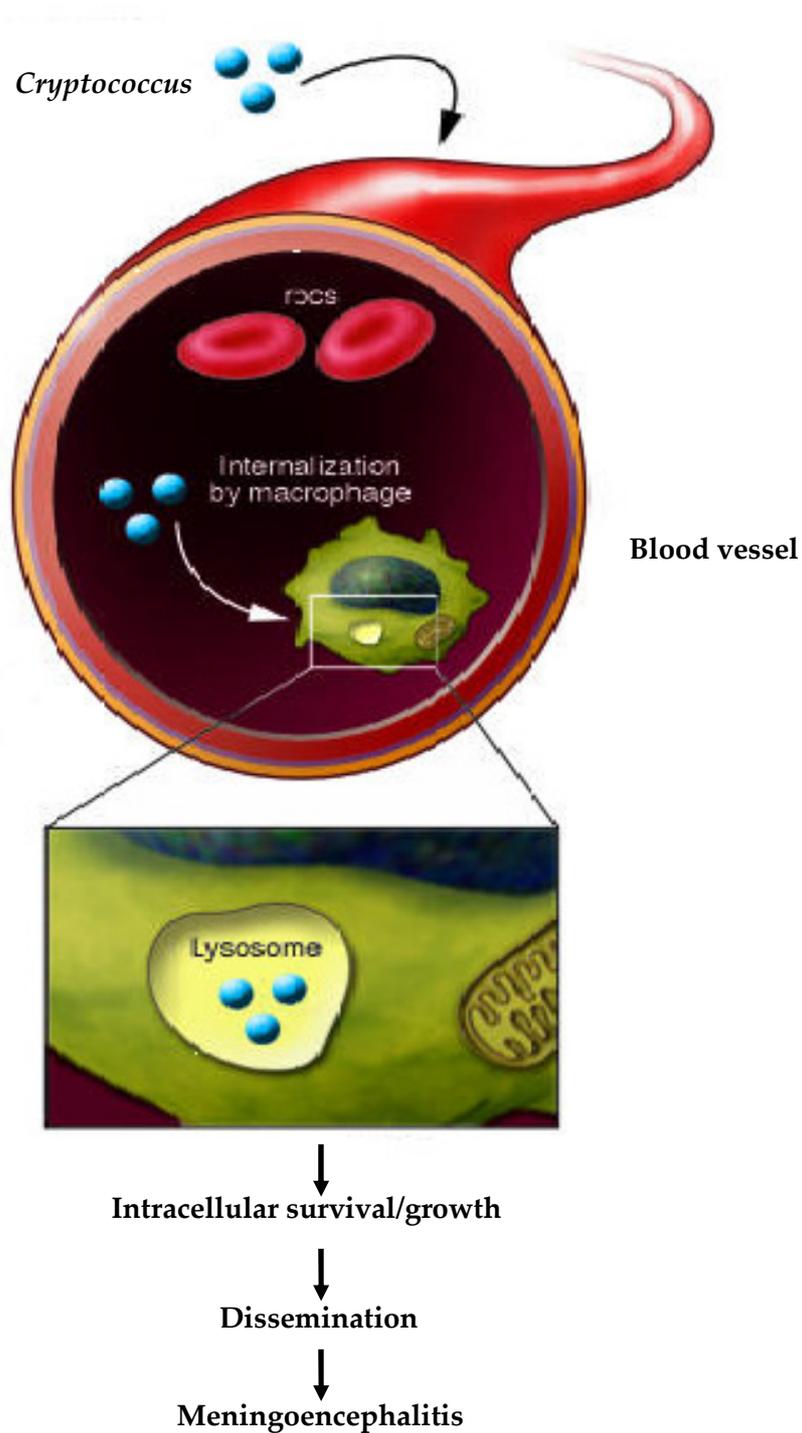


Figure 7: A schematic illustration of Trojan Horse mechanism, during which intracellular cryptococcal cells are delivered by macrophages to various tissues via the host circulatory system (modified from (Mitchell 2006)).

Table 1: A list of strategies used by selected pathogens to achieve intracellular survival and growth (Adapted from (Feldmesser et al. 2001b)).

Microorganism	Escape from phagosome	Inhibition of phagosome maturation	Destruction of mature phagolysosome membrane
<i>Listeria monocytogenes</i>	Yes	No	No
<i>Legionella pneumophila</i>	No	Yes	No
<i>Chlamydia psittaci</i>	No	Yes	No
<i>Mycobacterium tuberculosis</i>	No	Yes	No
<i>Histoplasma capsulatum</i>	No	Yes	No
<i>Leishmania donovani</i>	No	Yes	No
<i>Toxoplasma gondii</i>	No	Yes	No
<i>Cryptococcus neoformans</i>	No	No	Yes

1.5.2 Immunocompetent host

The host defence against *C. neoformans* is critically regulated by cell-mediated immunity (Lim and Murphy 1980), especially T lymphocytes, which play a central role in eradicating this infection (Hill and Harmsen 1991; Huffnagle et al. 1991; Mody et al. 1990). The mechanisms by which the lymphocytes facilitate elimination of cryptococci have not yet been elucidated. It is generally thought that lymphocyte clearance of *C. neoformans* acts indirectly through production of cytokines to enhance clearance of the organism by natural effector cells, particularly macrophages (Brodie et al. 1994; Chen et al. 1994; Kawakami et al. 2000; Kawakami et al. 1995; Lindell et al. 2005; Weinberg et al. 1987; Zhang et al. 1997).

Exposure to various pathogens can stimulate at least two patterns of cytokine production, mainly by CD4⁺ T cells: Th1 and Th2. For *C. neoformans*, the balance between Th1 and Th2 cytokines markedly influences the outcome of infection: the predominant synthesis of Th1 cytokines over Th2 protects mice from infection, whereas infection is exacerbated under Th2 dominant conditions (Hoag et al. 1995; Huffnagle 1996; Koguchi and Kawakami 2002; Snelgrove et al. 2006). Mice depleted of Th1 type cytokines are highly susceptible to cryptococcal infection (Huffnagle 1996; Kawakami et al. 1996), while the infection is less severe in mice lacking Th2 cytokines relative to control mice (Blackstock and Murphy 2004; Decken et al. 1998). A recent study shows the observed poor prognosis of a Th2 cytokine profile might be due to inhibition of anti-cryptococcal macrophage functions, such as phagocytosis and intracellular clearance by Th2 cytokines (Voelz et al. 2009).

For cryptococcal infection, the Th1/Th2 balance is maintained mainly by phagocytic effector cells (e.g., dendritic cells and neutrophils as discussed earlier) (Mednick et al. 2003, Wozniak et al. 2006) and some primary lymphocytes (e.g., natural killer T (NKT) cells and $\gamma\delta$ antigen receptor-bearing T cells) (Kawakami 2004; Nanno et al. 2007; Zhang et al. 1997). A remarkable feature of NKT cells is the abundant production of

IFN γ and IL-4 upon stimulation via their antigen receptors. After cryptococcal infection, NKT cells were found to be recruited to the lung, and trigger a Th1-mediated, but not Th2-mediated, immune response (Kawakami et al. 2001). In contrast, $\gamma\delta$ T cells play a down-modulatory role in the development of Th1 responses and host resistance against *C. neoformans* (Uezu et al. 2004). Therefore, $\gamma\delta$ T cells may act to keep the balance of Th1-Th2 responses in a proper manner by suppressing the exaggerated Th1 response caused by NKT cells (Kawakami 2004). The contrasting roles of NKT and $\gamma\delta$ T cells, and the fact the neutrophil depletion can enhance both Th1 and Th2 cytokines (Mednick et al. 2003), suggest that these innate immune response are not only important for induction of proper host defence but also to balance the level of defence.

1.5.2.1 Antifungal effect of activated macrophages

When a T-cell mediated immune response is present, the majority of the intracellular cryptococci are eradicated. Properly activated macrophages have a variety of microbicidal mechanisms that are potentially active against *C. neoformans*, including both oxidative and nonoxidative mechanisms and granuloma formation. The oxidative microbicidal mechanism involves the generation of reactive oxygen- and nitrogen-derived intermediates (ROI and RNI). ROI, such as superoxide anions, hydroxyl radicals, and hydrogen peroxide are generated as a result of the incomplete reduction of oxygen during respiratory metabolism (Turrens and Boveris 1980). Nessa *et al* showed that *C. neoformans* induced a markedly higher increase of oxidative metabolism in macrophages than did inert silica particles in an *in vivo* rabbit model of infection (Nessa et al. 1997a). Such an increase was also observed with rat alveolar macrophages and *Candida* and *Aspergillus* species in *in vitro* studies (Nessa et al. 1997b; Nessa et al. 1997c), indicating that production of ROI is a general mechanism of intracellular killing employed by macrophages. Cryptococcal strains lacking proteins (e.g., Aox1 and Skn7) that protect against reactive oxygen species inside macrophages, show reduced intracellular survival of *C. neoformans* and thus reduced virulence in animal models of infection (Akhter et al. 2003; Coenjaerts et al. 2006). RNI, produced by several

mammalian cells, are also powerful antimicrobial molecules against intracellular *C. neoformans*. Nitric oxide, one of the key RNI molecules, is produced by macrophages through the action of inducible nitric oxide synthase on L-arginine (Tripathi et al. 2007) and acts to suppress cryptococcal growth (Tohyama et al. 1996). In addition, NK cells promote anticryptococcal activity of macrophages through enhancing nitric oxide activity (Kawakami et al. 2000). Resistance to oxygen- and nitrogen-derived oxidants has been found to be a major factor in determining the outcome of infection with *C. neoformans* (Xie et al. 1997), implying the importance of ROI and RNI in intracellular killing by macrophages.

In the presence of intact T cell function, macrophages also often form a histiocytic ring around *C. neoformans* cells and may fuse to form giant multinucleated cells in order to engulf heavily encapsulated yeast. This is called granuloma formation and has been demonstrated to be the most effective host response to localise the infection and prevent dissemination (Casadevall and Perfect 1998; Hill 1992). Furthermore, resolution of infection, when it occurs, almost always follows granuloma formation. For instance, intratracheal infection of rats with *C. neoformans* was found to elicit a strong granulomatous response and resulted in minimal or no dissemination (Goldman et al. 1994; Goldman et al. 1996; Kobayashi et al. 2001). Granulomatous inflammation is more likely to be reported in non-HIV-associated cryptococcosis (Lee et al. 1996; Mohanty et al. 2003; Shibuya et al. 2005), and there is evidence that a strong granulomatous response is dependent on intact T cell function (Clemons et al. 1996; Hill 1992), indicating a mechanism by which abnormalities of cell-mediated immunity can translate into poor inflammatory responses.

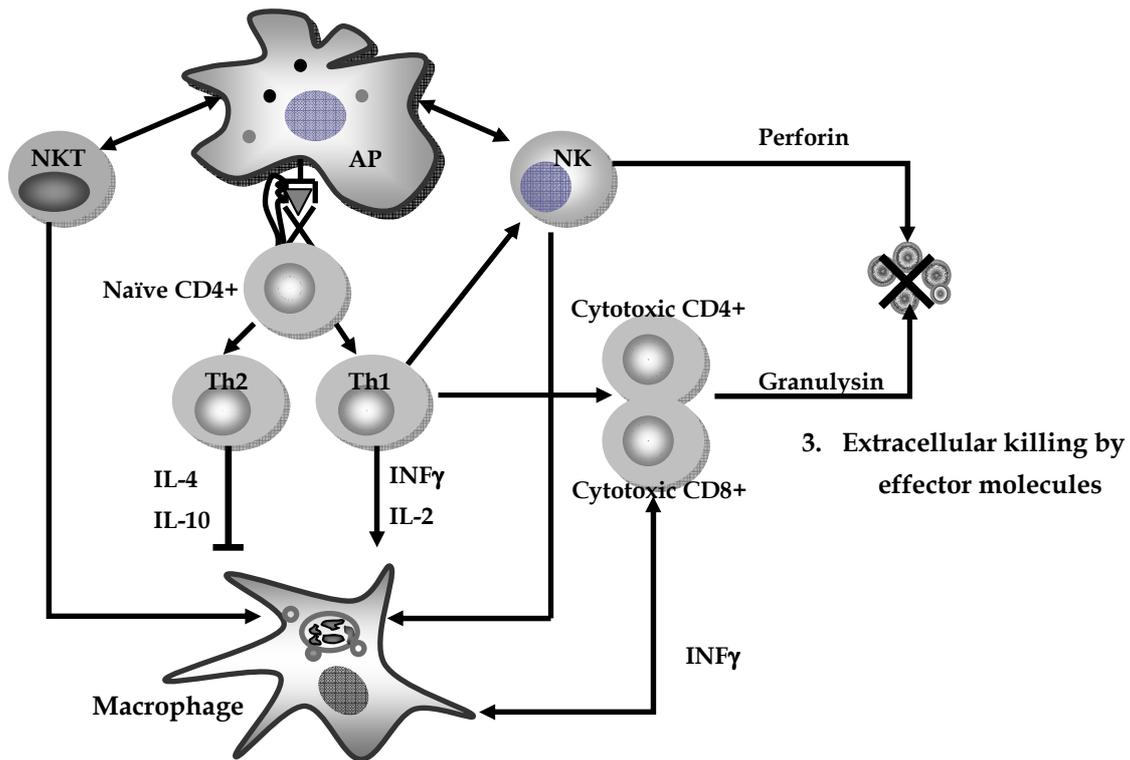
The fungistatic activity of macrophages can be enhanced by the presence of antibody; antibody against capsular GXM promoted nitric oxide production in macrophages (Rivera et al. 2002), and antibody-treated mice have a more intense granulomatous response than control mice (Casadevall and Perfect 1998). Nevertheless, a small number of cryptococci are able to survive and remain latent inside macrophages in

immunocompetent individuals, despite the presence of Th1 cytokines and antibody. This latency is probably due to the presence of cryptococcal anti-ROI/RNI factors and virulence factors (such as capsule, melanin, Aox1, Sod1, Ccp1, Isc1 and Skn7 (Akhter et al. 2003; Alvarez and Casadevall 2007; Coenjaerts et al. 2006; Cox et al. 2003; Giles et al. 2005; Liu et al. 2006; Ma et al. 2007; Missall et al. 2004; Zaragoza et al. 2008)), rapid changes in virulence mediated by phenotypic switching and Th2-polarised responses later in infection to avoid tissue damage caused by the early Th1 response (Kawakami 2004; Mednick et al. 2003). This latent population is then able to trigger cryptococcosis later on in life when the host immune system becomes compromised (Garcia-Hermoso et al. 1999).

1.5.2.2 Direct antifungal effects of T lymphocytes

Much evidence suggests that NK cells and T lymphocytes function as both regulators (by secreting cytokines, e.g., CD4+ T helper cell) and effectors (cytotoxic cells) during the immune response against *C. neoformans*. Hence, direct inhibition of cryptococcal cells by these host cells may be another important means of host defence against *C. neoformans*. Early studies by Levitz *et al* demonstrated the competence of freshly isolated human CD4+, CD8+ lymphocytes, and CD16/56+ NK cells (but not B cells) to directly bind and inhibit the growth of *C. neoformans* in the absence of MHC restriction (Levitz and Dupont 1993; Levitz et al. 1994). These findings are in agreement with several previous studies (Hidore et al. 1991; Murphy et al. 1991; Murphy et al. 1993; Nabavi and Murphy 1985). Recent studies have improved our understanding of the underlying detailed mechanisms. These studies found that direct anticryptococcal activities of CD4+ and CD8+ cytotoxic cells are dependent on the expression of granulysin after activation by CD4+ T helper cell (or IL-2/IL-15, which can substitute T cell helper) (Ma et al. 2002; Zheng et al. 2007), whereas NK cells used perforin instead (Ma et al. 2004) (Figure 8). Granulysin, a novel host defence protein, is able to increase membrane permeability of bacteria and fungi, and thus trigger osmotic lysis (Ernst et al. 2000). Granulysin expression in CD4+ cytotoxic T cells is controlled by PI3K and

STAT5 signalling pathways through promoting IL-2R β induction (Zheng et al. 2008). CD4⁺ cytotoxic T cells from HIV patients fail to induce granulysin expression due to defective PI3K and STAT5 pathways, resulting in inefficient killing (and growth inhibition) of *C. neoformans* (Zheng et al. 2007). Similarly, CD8⁺ T cells express granulysin in the presence of IL-15 and CD4⁺ T cells, and the up-regulation of granulysin correlated with the acquisition of anticryptococcal activity (Ma et al. 2002). Perforin, stored in secretory vesicles (granules) of T lymphocytes and NK cells, is another pore-forming effector molecule that acts by inserting into the target cell's plasma membrane, triggering lysis (Voskoboinik et al. 2006). Perforin-mediated anticryptococcal killing is accompanied by activation of PI3K-ERK1/2 signalling pathway (Wiseman et al. 2007). It is essential for NK cells, although both granulysin and perforin are constitutively expressed by this cell type (Ma et al. 2004; Marr et al. 2009)



- Intracellular killing by macrophages (oxidative and non-oxidative)
- Granuloma formation



Figure 8: Killing of *C. neoformans* by the immune response in immunocompetent individuals. Killing can occur either intracellularly when macrophages are activated by Th1 type cytokines or extracellularly by effector molecules secreted by cytotoxic T lymphocytes (CD4+ and CD8+) and NK cells. Many other cells from the immune system contribute to the elimination of cryptococci directly or indirectly by triggering and balancing Th1 type cytokine release.

1.5.3 Conclusion

C. neoformans is a facultative intracellular pathogen, capable of living both outside and inside cells. The current model of cryptococcal infection is based on five steps: internalisation, dormancy, reactivation, proliferation and dissemination (Figure 6). In the initiation stage, *C. neoformans* interacts with and is engulfed by lung phagocytes (mainly macrophages). Normally, in an immunocompetent individual, a T-cell mediated immune response (driven especially by CD4⁺ cells) develops. This leads to activation of macrophages via cytokine release and granuloma formation, resulting in either destruction of the intracellular fungus or containment in a latent state. Direct antifungal activity of lymphocytes also improves the host defence (Figure 8). Subsequently, when the individual becomes immunocompromised, *C. neoformans* can start proliferating inside the macrophage, followed by macrophage lysis and release of *C. neoformans*. The released organism can then enter other phagocytes, causing dissemination and increased proliferation. Long-term growth inside macrophages and host often leads to enlargement of the capsule, which probably sequesters available complement proteins. The unopsonised organisms are poorly recognised by phagocytes and thus establish extracellular dominance. During prolonged infections, the yeast population can undergo microevolution, which results in both phenotypic and genotypic changes in order to be better adapted to local organs or environments (Lortholary et al. 1999). The identification of genes and factors that contribute to either extra or intracellular proliferation of this pathogen may lead to the development of novel prevention and treatment strategies for cryptococcosis.

1.6 Current understanding on how *Cryptococcus* crosses the blood-brain barrier (BBB)

Cryptococcal meningoencephalitis develops as a result of haematogenous dissemination of inhaled *Cryptococcus* from the lung to the brain. In order to penetrate into the brain, the yeast must cross the endothelium of the BBB, which is composed of brain microvascular endothelial cells connected by tight junctions between the cells (Rubin and Staddon 1999).

Although the mechanisms of entry into the CNS for the majority of meningoencephalitis-causing microorganisms are not clear, three potential models have been described. Pathogens may cross the BBB paracellularly (e.g., *Trypanosoma* species) (Grab et al. 2004), transcellularly (e.g., *Streptococcus pneumoniae*) (Ring et al. 1998), and by means of infected immune cells (Trojan Horse mechanism, e.g., HIV) (Dallasta et al. 1999; Erlander 1995). In the case of *Cryptococcus*, several lines of evidence support the hypothesis that the yeast crosses the BBB transcellularly. In 1995, an *in vitro* study showed that *C. neoformans* (especially acapsular strains) were able to adhere to and then be internalised by endothelial cells, subsequently causing damage to the host cell. Furthermore, they found that internalisation required the presence of a heat-labile serum factor, which could be one of the components of the classical complement pathway (Ibrahim et al. 1995). Chretien and colleagues then reported that, *in vivo*, *Cryptococcus* was phagocytosed by endothelial cells of the leptomeningeal capillaries (Chretien et al. 2002). Subsequently, *C. neoformans* was found to alter the cytoskeletal morphology of human brain microvascular endothelial cell (HBMEC) through the ROCK-LIMK-cofilin pathway, and cross the HBMEC layer transcellularly without affecting the monolayer integrity (Chang et al. 2004; Chen et al. 2003). Importantly, the virulence factor Skn7 has been demonstrated to co-regulate the adaptive strategy of *Cryptococcus*, allowing intraphagocytic survival by conferring resistance to phagolysosomal killing in endothelial cells (Coenjaerts et al. 2006).

However, compared to *C. albicans*, the efficiency of adhesion and invasion is low (Chang et al. 2004; Chen et al. 2003; Jong et al. 2001).

Concomitantly, several studies also demonstrated that phagocytes might act as a means of allowing *Cryptococcus* to invade the brain. For example, microscopy of the leptomeninges of a mouse with severe meningoencephalitis showed cryptococci internalised both within mononuclear cells circulating within meningeal capillaries and within host cells touching the outer membrane of the capillaries (Chretien et al. 2002). In a murine model of cryptococcosis, a reproducible threefold increase in the fungal burden measured in the brain 24 hours after inoculation with bone marrow derived macrophage-associated yeasts were observed compared to the burden after inoculation with free yeasts, providing another piece of evidence for the Trojan Horse mechanism (Charlier et al. 2009).

Furthermore, although direct transfer of *C. neoformans* from infected phagocytes to endothelial cells has not been demonstrated, such events have been observed between two macrophages (Alvarez and Casadevall 2007; Ma et al. 2007). When travelling throughout the host circulatory and lymphatic system, macrophage cells interact intimately with one another and with other cell types through transient contacts. It is possible that internalised *C. neoformans* may use such transient contact in order to cross the BBB by direct cell-to-cell spread from adherent infected macrophages to microvascular endothelial cells. In fact, spreading from macrophages to other cell types during dissemination has been demonstrated for other pathogens *in vitro*. For instance, *L. monocytogenes* can infect neurons by cell-to-cell spread from adherent macrophages, a more efficient process than direct invasion of neurons (Dramsi et al. 1998). More importantly, cell-to-cell spread of bacteria from adherent infected phagocytes to endothelial cells of the CNS has also been reported (Drevets and Leenen 2000) and it will clearly be of great interest to investigate whether a similar process may occur during cryptococcosis.

Since cryptococcosis is very common in HIV-infected patients, it is not implausible to suspect that the presence of HIV may enhance cryptococcal entry into the CNS. Numerous studies have demonstrated that HIV is able to cause damage to the endothelial cell layer and thus facilitate the entry of other microorganisms into the CNS (Dallasta et al. 1999; Ricardo-Dukelow et al. 2007; Toborek et al. 2005). The interaction between HIV and *C. neoformans* has not been well studied, but a recent study reported an interesting interplay between the yeast and the HIV-1 protein gp41. Jong *et al* demonstrated that the binding of *C. neoformans* to HBMEC could be enhanced by HIV-1 gp41 *in vitro* and also in a murine model. Therefore, they speculated that HIV-1 gp41 may play a role as a trans-predilection factor for *C. neoformans* invasion, thus resulting in a deteriorating meningoencephalitis in HIV-infected patients (Jong et al. 2007).

In summary, there are three possible ways by which *Cryptococcus* can cross the BBB and enter the CNS (Figure 9). They are: 1) direct transcellular crossing, during which free cryptococci are internalised by endothelial cells and exit through the abluminal surface of the cells; 2) Trojan Horse mechanism, which proposes that cryptococci are engulfed by phagocytic cells at an early stage of infection and then trafficked by these host cells into the CNS; and 3) direct transfer from infected phagocytes into endothelial cells followed by exit at the abluminal surface of the cells. Moreover, the presence of HIV-1 may facilitate *Cryptococcus* to cross the blood-brain barrier by destroying the integrity of the blood-brain barrier and/or by acting as a trans-predilection factor.

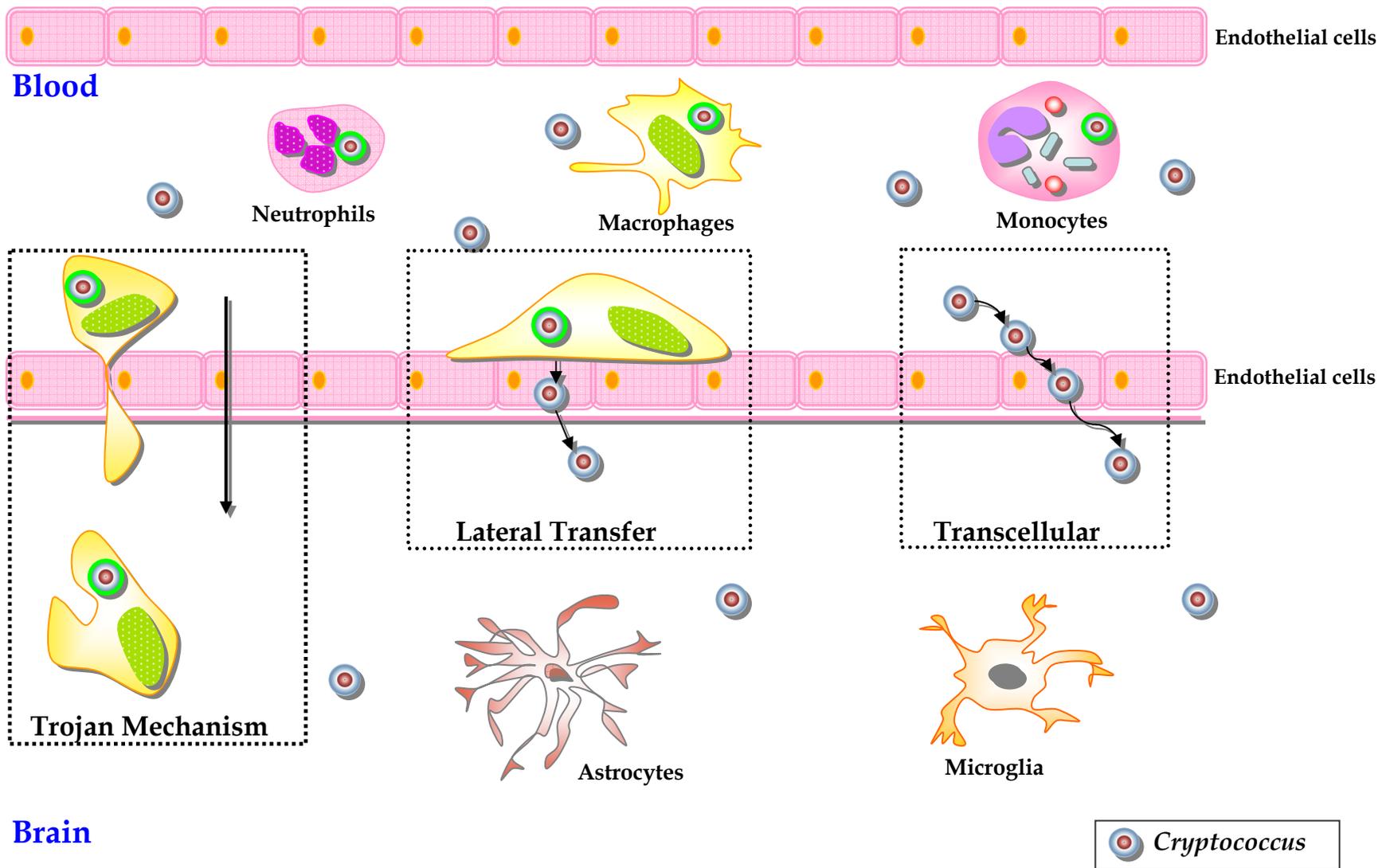


Figure 9: Possible routes for cryptococci to cross the BBB: 1) Trojan mechanism; 2) Lateral transfer; and 3) Transcellular cross.

1.7 Project outline

The AIDS pandemic has resulted in a new wave of research on *C. neoformans* over the last 30 years, and our understanding of the biology of this pathogen has improved dramatically. As a result of technical improvements, such as the development of transformation techniques (electroporation (Chang et al. 1996; Edman and Kwon-Chung 1990), biolistic transformation (Alspaugh et al. 1997; Odom et al. 1997; Toffaletti et al. 1993), and *Agrobacterium*-mediated transformation (McClelland et al. 2005)) and the recent completion of several whole-genome sequences, many genes responsible for virulence in *C. neoformans* during infection have been identified and the mutants have been verified in robust animal models. Its clinical significance and well-defined virulence factors, along with advanced genome-wide analysis tools, have made *C. neoformans* an organism of choice for the study of fungal pathogenesis in general. To date, the clinical management of cryptococcosis is possible, but the morbidity and mortality remain high. A critical challenge will be to develop novel treatments based upon advances in genomics, proteomics and metabolomics. This requires a better understanding of host-pathogen interplay.

C. neoformans and *C. gattii* can live and replicate inside phagocytes, but the long term latency and intracellular survival mechanism have not been investigated in detail. This project is aimed to understand the molecular basis of phagocyte parasitism during cryptococcosis. Since macrophages are the primary phagocytic cell at all time of infection, we have used J774 mouse macrophage-like cells as the main cell line to elucidate intracellular survival and replication of *Cryptococcus*. J774 was derived from a tumour in a female BALB/c mouse and has been shown to possess characteristics typical of macrophages, such as the expression of both Fc and complement receptors (Ralph and Nakoinz 1975). In recent years, J774 has been used extensively to study intracellular survival of pathogenic bacteria including *Mycobacterium avium* and *Francisella tularensis* (Hostetter et al. 2003; Telepnev et al. 2003). The similarities

between J774 and human peripheral blood monocytes have also been demonstrated (Alvarez et al. 2009), supporting the suitability of mouse cells for the study of intracellular pathogenesis mechanisms. The first part of the project asked whether different phagocytosis routes lead to different outcomes for intracellular cryptococci. During this part of work, we discovered and characterised a novel escape mechanism used by *Cryptococcus*. The second part of the project aimed to reveal how *Cryptococcus* achieves intracellular replication. By combining strain screening together with microarray analysis and mating assays, we identified factors promoting the intracellular proliferation of *Cryptococcus*. We hope that in the future, this improved understanding of cryptococcal virulence will aid in development of potential anti-fungal compounds.

Chapter II: Materials and Methods

All the chemicals and solutions are purchased from Sigma-Aldrich unless otherwise stated.

2.1 Yeast culture techniques

2.1.1 Strains and growth conditions

All cryptococcal and yeast strains used in this project are listed in Appendix I. Cryptococcal strains and *S. cerevisiae* were grown overnight in antibiotic-containing yeast peptone dextrose (YPD) medium (2% D-glucose, 1% peptone and 1% yeast extract) with moderate shaking (240 rpm) at 25°C. Antibiotics were added to the media at the following concentrations: ampicillin 50µg/ml or kanamycin 25µg/ml. For experiments using *S. pombe* wild type 972, the strain was grown in YES medium (0.5% yeast extract, 3% glucose, 0.1g/L amino acid supplement containing adenine, uracil, leucine, histidine, lysine and arginine) overnight with moderate shaking (240 rpm) at 25°C. Before use, the overnight yeast cells were collected by centrifugation at 2000 rpm for 2min, washed twice with PBS (pH=7.4) and resuspended in PBS.

2.1.2 Freezing and defrosting cryptococcal strains

To freeze down the strains, 1ml of 50% glycerol was added to each cryogenic vial and sterilised by autoclaving. 200µl of cryptococcal culture grown in YPD for 24h at 25°C were added to a glycerol vial and vortexed to make the glycerol suspension homogeneous. The vial was stored at -80°C immediately. When isolates were needed, a small piece of frozen culture was chipped and inoculated onto YPD plates. The vial was immediately returned to the -80°C freezer to prevent further thawing. Individual isolate was re-plated onto a new YPD plate every month.

2.1.3 Micromanipulation of meiotic basidiospores and segregation analysis

Mating reactions of the desired *C. gattii* strains were established by co-culturing the opposite mating-type cells on V8 plates (pH=5). Matings were conducted at room temperature in the dark for 3–4 weeks until robust filamentation and sporulation were observed by microscopy. Each basidium fruiting structure harbours a single diploid nucleus, which undergoes a single round of meiosis, and then repeated rounds of mitosis give rise to chains of >10 basidiospores (Figure 4). The filaments and the

basidiospores on the edges of a mating patch were removed on an agar plug taken with a glass Pasteur pipette and transferred to an YPD plate. The basidiospores were randomly selected with a 25µm microneedle and arranged in a grid on the surface of the YPD plate. Subsequently, random spore analysis was conducted. Isolated basidiospores were incubated at 30°C for 2–3 days to allow the spores to germinate, and the resulting yeast colonies were subcultured to fresh YPD medium. Each yeast colony was further purified by spreading the culture on to YPD plates for single colony selection.

2.2 Cell culture techniques

2.2.1 Defrosting cell lines

The cells were thawed in a 37°C water bath immediately after taking from liquid nitrogen. The cells were then centrifuged at 1500 rpm for 5min in 9ml of warm complete media to remove dimethyl sulfoxide (DMSO). The cell pellets were then resuspended in 1ml complete media before transferring to flask with adequate amount of complete media. The dead cells were removed the next day and healthy cells were left to grow until they were ready to split.

2.2.2 Passaging cell lines

J774.16 cells (A murine macrophage-like cell line derived from a reticulum sarcoma) were grown in complete media, containing DMEM supplemented with 10% (v/v) heat-inactivated foetal bovine serum (HI-FBS), 1% penicillin (100u/ml) /streptomycin (100µg/ml) and 2mM L-glutamine. Cells were kept in a humidified incubator at 37°C with 5.0% CO₂. When cells reached 80%-90% confluence, they were split in the following manner. Old media were replaced with fresh media and cell scrapers were used to dislodge cells from the bottom of the flask. The cells were counted using a haemocytometer and then seeded at the appropriate density into a new flask. The cell line was used between 3-30 passages.

Cos-7 cells (African green monkey kidney fibroblast-like cell line) were incubated in DMEM supplemented with 10% (v/v) HI-FBS, 1% penicillin (100u/ml) /streptomycin (100µg/ml). Cells were grown in a humidified incubator at 37°C with 5.0% CO₂. When cells reached 80-90% confluence, they were split using trypsin-EDTA at 1:4 ratio.

2.2.3 Freezing cell lines

When cells reached about 80% confluency, J774 cells were covered with 5ml fresh complete media and scraped from the flask. While Cos-7 cells were washed once with 5ml PBS followed by trypsin/EDTA treatment. The dislodged cells were then mixed with 5-10ml complete media and centrifuged at 1500 rpm for 5min. After that, cell pellets were suspended in 1ml DMEM containing 10% DMSO and 20% HI-FBS. Subsequently, cells were transferred to -20°C freezers and then -80°C freezers and finally to liquid nitrogen for long-term storage.

2.2.4 Transfection of Cos-7

Cos-7 cells were transfected using Lipofectamine reagent (Invitrogen). The day before transfection, cells were split onto a 35mm plate to achieve 50%-70% confluency next day. Before transfection, the cells were washed once with serum-free DMEM (SFM) and covered with 1750µl SFM. After that, 10µl SFM was mixed with 3µl Lipofectamine reagent to form Lipofectamine-SFM, and the same time, 15µl SFM was mixed with 0.7µg plasmid DNA to form DNA-SFM. Subsequently, Lipofectamine-SFM was added to DNA-SFM dropwise. The new mixture was incubated at room temperature for 15min to allow DNA-Lipofectamine complex to form. Finally, 200µl of SFM was mixed with DNA-Lipofectamine complex, which was added to Cos-7 cells dropwise. The cells were incubated in lipofectamine containing SFM for six hours and then changed to complete DMEM. Expression was checked 24h after transfection.

2.2.5 Generation and activation of human blood-derived macrophages (HPBMC)

HPBMC were prepared as described previously (Fazal et al. 1995). Briefly, the cells were resuspended at 6×10^6 cells/ml in RPMI-1640 medium containing 2% FBS, 2mM L-glutamine and 1% penicillin (100u/ml) /streptomycin (100 μ g/ml). The HPBMC were then seeded into T175 tissue culture flasks. Non-adherent cells were removed by extensive washes with PBS and adherent monocytes incubated overnight in RPMI media supplemented with 10% FBS and GM-CSF (100iu/ml). Following overnight incubation adherent cells were removed from flasks by incubation on ice in pre-chilled PBS and cultured for 1-2 days in RPMI + 10% FBS supplemented with GM-CSF (100iu/ml). The cells were adjusted to 1×10^6 cells/ml and aliquoted into 3cm plastic petri dishes or 24-well plates. The cell media were replenished on days 3 and 5 yielding adherent, confluent macrophage cultures at Day 7. When required the macrophages were activated with lipopolysaccharide (LPS) (1 μ g/ml) and human IFN γ (1000u/ml), which were added to the culture dishes 24h prior to infection with *Cryptococcus*.

2.2.6 Drug treatment of macrophages

Drugs used to treat macrophage include concanamycin A, chloroquine, cytochalasin D and nocodazole. They were used at the following concentrations: concanamycin A 100nM to block the acidification of phagosome; chloroquine 10 μ M to increase the pH of the phagosome; cytochalasin D 2.5 μ M, 4 μ M and 10 μ M to block actin polymerisation; and nocodazole 2.5 μ g/ml to depolymerise microtubules. Chloroquine was added to J774 one hour prior the onset of phagocytosis assay, while concanamycin A, cytochalasin D, and nocodazole were added to J774 after two-hour phagocytosis assay because they affected uptake of *Cryptococcus* by J774. All these drugs were present thoroughly the intracellular proliferation measurement assay and timelapse filming.

2.3 Phagocytosis assay

J774 cells or HPBMC (4×10^5 cells/ml) were plated into a 35cm tissue-culture plate or 1×10^5 cells/ml in 24-well dishes 16-24h before the assay. Shortly before use, cells were incubated for one hour in SFM containing 150ng/ml phorbol myristate acetate (PMA).

Similarly, the primary macrophage cells (which have been activated with LPS and IFN γ) were incubated for one hour in SFM. Meanwhile, cryptococcal cells were washed three times with PBS and counted in a haemocytometer. For experiments with opsonised *Cryptococcus*, both live and heat-killed fungal cells were incubated with 10 μ g/ml of the monoclonal antibody 18B7 or 20% human or guinea pig sera at 37°C for one hour. For some strains, the antibody titration is optimised (between 1 μ g/ml to 10 μ g/ml) in order to reduce the number of attached extracellular yeast cells after phagocytosis. For non-opsonised cryptococci, *S. cerevisiae* and latex beads, opsonins were replaced with water. To commence the assay, the medium containing PMA was removed and replaced by normal SFM containing yeast cells at a ratio of yeast cell and macrophage from 10:1 to 1:1 depending on the experiment. The phagocytosis was allowed to proceed for two hours at 37°C in 5.0% CO₂ atmosphere. Non-internalised yeasts were then removed by 4-5 successive washes with pre-warmed SFM or PBS. After that, the cells were fixed for further analysis, or timelapse imaging, or intracellular proliferation assay.

2.4 Intracellular proliferation assay

A proliferation assay was developed to monitor intracellular proliferation of individual strains for a 64-hour period following phagocytosis. For this assay, J774 macrophages, HPBMC or primary human alveolar macrophages (provided by David Lammas, University of Birmingham) were exposed to cryptococci opsonised with 18B7 antibody for two hours as described above. Each well was washed with PBS 4-5 times to remove as many extracellular yeast cells as possible and 1ml of fresh SFM was added. For time point T₀, the 1ml SFM was discarded and 200 μ l of sterile dH₂O was added into wells to lyse macrophage cells. After 30min, the released intracellular yeast cells were collected. Another 200 μ l dH₂O was added to each well to collect the remaining yeast cells. The intracellular yeast was then mixed with Trypan Blue at a 1:1 ratio and the live yeast cells were counted. For the subsequent five time points T₁₆, T₂₄, T₄₀, T₄₈ and T₆₄, both extracellular and intracellular cryptococci were collected and independently counted by haemocytometer in the same manner. For each strain tested, each time course was

repeated at least three times on different occasions and using different batches of macrophages. Intracellular proliferation ratio was calculated by dividing the maximum intracellular yeast number (which is T_{24} for most of the strains) by the initial intracellular yeast number at T_0 . Compared to conventional colony forming unit counting method, this assay is more sensitive in detecting the clustered yeast population or yeast cells undergoing budding.

2.5 Measurement of cryptococcal growth rate in YPD and macrophage lysates

In order to demonstrate that the high intracellular proliferation capacity of VIO strains was not due to a shorter generation time or better utilisation of nutrient sources available in the host cell, a selection of *C. gattii* strains with different IPR values was chosen to measure their growth rates in YPD at 25°C (shaking) and at 37°C (non-shaking with 5.0% CO₂), or in the presence of macrophage lysates (at 37°C, non-shaking with 5.0% CO₂) for 48h. To prepare the macrophage lysate, 5x10⁶ J774 macrophages were collected and lysed in 2 ml ice-cold H₂O for 10min on ice. The lysate was then vigorously vortexed for 1 min and mixed with 48 ml of SFM. Subsequently, 10⁵ cryptococcal cells of each strain were mixed with 1ml SFM-lysate mixture and incubated at 37°C with 5.0% CO₂ for 48h. The number of yeast cell was counted using haemocytometer every 24h. All the experiments were repeated 3 times with different macrophage cultures.

2.6 Microscopy

2.6.1 Light microscopy

2.6.1.1 Calculation of phagocytosis index

Coverslips (13mm) were either treated with 20ml nitric acid for 10min followed by 2-3 times 100% ethanol washes or autoclaved before being plated in 24-well dishes. Cells

were seeded in 24-well dishes at 1×10^5 cells/ml to carry out phagocytosis assay as described earlier. Afterwards, cells were washed twice with PBS and fixed by incubating in 4% PFA for 20min at 4°C. Following fixation, cells were washed three times with PBS and two times with water before being mounted in mowiol mounting media on slides. Phagocytosis Index (PI) was calculated as the percentage of the macrophages with internalised *C. neoformans*. For each PI calculation, more than 500 cells were counted.

2.6.1.2 Mitochondrial staining

Yeast cells, grown overnight in YPD medium at 25°C at 240 rpm, or grown at 37°C in DMEM in a 5.0% CO₂ incubator without shaking for 24h, or isolated from macrophages 24h after infection, were harvested, washed with PBS twice and resuspended in PBS containing the MitoTracker Red CMXRos (Invitrogen) strain at a final concentration of 40nM. Cells were incubated for 30min at 37°C. After staining, cells were washed three times and resuspended in PBS. For each condition, more than 60 yeast cells for each tested strains were randomly chosen and analysed in blind. For quantifying different mitochondrial morphologies, images were collected on a Nikon Eclipse E300 microscope using 60x oil immersion 1.40NA plan apo objective lens. Both fluorescence images and bright-field images were collected simultaneously. Images were captured with identical settings on a Hamamatsu Orca C4745-12NRB with a 0.5x camera lens using Openlab (version 5.5.0; Improvision, Coventry, UK). All Images were processed identically in Photoshop CS2 and mitochondrial morphologies were analysed and counted blind. For confocal microscopy, images were collected on a Nikon Eclipse E600 confocal microscope with BioRad Radiance 2000 MP laser scanning system using a 100x oil immersion WD 0.20 objective lens. Images were processed using ZeissSharp2000 software (version 6.0).

2.6.2 Timelapse microscopy

Timelapse live imaging was carried out for all the strains in order to calculate phagocytosis rate and verify IPR values calculated using the proliferation assay

described above. Cells were maintained at 37°C and 5.0% CO₂ using a temperature-controlled chamber (Okolab) and imaged on a Nikon Eclipse TE2000-U microscope with 20x phase contrast objective and 1.5x optivar. The timelapse movies were made using the software NIS-Elements AR 3.0, capturing one frame every 90s for 24h on a Nikon Digital Sight DS-Qi1Mc camera. The vomocytosis rates (which equals to the number of vomocytosis occurred divided by the number of macrophages with intracellular *Cryptococcus*) and IPR values for various strains were scored manually by eye. All the experiments were repeated three or more times on different days using independent cultures of J774 macrophage and *Cryptococcus*.

2.7 Microarray study

2.7.1 RNA isolation from intracellular cryptococci after 24h

Phagocytosis assay was carried out as mentioned earlier. After 24h, extracellular cryptococci were removed with pre-warmed PBS washes and macrophages containing intracellular cryptococci were lysed with 10ml ice-cold ddH₂O for 30min before being scraped from T75 tissue flasks. The whole mixture was then centrifuged at 1500 rpm at 4°C for 5min and the resulted pellet was washed twice with ice-cold ddH₂O. Subsequently, the pellet was resuspended in 10ml of ddH₂O supplemented with 0.05% sodium lauryl sulphate (SDS) for 5min to remove most of the macrophage RNA, because at this SDS concentration, the macrophages were lysed while cryptococci remained intact and viable (Fan et al. 2005). The remaining yeast cells were washed three times with ice-cold ddH₂O by centrifugation and resuspension. The final pellet was then used for RNA isolation, or it was frozen at -80°C until RNA preparation.

Yeast RNA was isolated using Micro-to-Midi Total RNA Purification System (Invitrogen). Basically, 3-9x10⁷ yeast cells were suspended in 500µl of freshly prepared RNA lysis solution containing 5µl of 2-mercaptoethanol. The suspension was added dropwise onto the crushed dry ice and the mixture was ground until the dry ice had evaporated. The liquid was then transferred to a 4ml tube and homogenised at 6400 rcf

for 2x15s. After homogenisation, the homogenate was centrifuged at 12,000 rcf for 2min and the supernatant was mixed with 500µl of 70% ethanol, added to the RNA Spin Cartridge and centrifuged at 12,000 rcf for 15s at 25°C. Subsequently, 700µl of Wash Buffer I was added to the cartridge and spin down at 12,000 rcf for 15s at 25°C, which was followed by washing the cartridge with 500µl of Wash Buffer II twice and spin down at 12,000 rcf for 15s at 25°C. Finally the cartridge membrane was dried by centrifugation at 12,000 rcf for 1min, and RNA was collected by adding 30-50µl of RNase-free water to the cartridge and centrifuged at 12,000 rcf for 2min at 25°C. The RNA was then quantified with Nanodrop-1000 and stored on ice for immediate use or -80°C for later use.

2.7.2 Microarray experiment

An Agilent printed whole genome tiling array with 242,003 probes generated against two genomes of *C. gattii*, A1M-R265 (Broad Institute) and WM276 (Jim Kronstad, personal communication), was designed by Oxford Gene Technology (OGT). Average interprobe distance was 140-nucleotide long, evenly distributed across both DNA strands. After round one of design, any gaps of over 1.5kb (due to repeat regions, etc) were then filled using suboptimal probes (700 probes in total) in the same manner. There are 20,212 probes (8.4%) that map onto both genomes. Using this design, 24 *C. gattii* RNA samples were used to determine differences in their gene expression 24h after internalisation by macrophages. For the array experiment, RNA samples from each strain were labelled with Cy3, whilst the control, consisting of a pooled sample containing equal quantities of RNA samples from all 24 strains (reference) was labelled with Cy5.

The microarray experiment was performed by OGT. Briefly, the standard Agilent protocol for labelling, hybridisation and washing (Two-Colour Microarray-Based Gene Expression Analysis, Version 5.5, February 2007) was followed. The starting concentration of total RNA from each sample used was 2µg. For the control samples, 2µg of each test sample was pooled together and then aliquoted into 24 sets of 2µg samples. Once the appropriate concentration of sample had been prepared, the

samples were reverse-transcribed for two hours at 40°C to produce cDNA. Following denaturation of the enzyme (15min, 65°C), the samples were then transcribed to produce cRNA (two hours, 40°C). During this transcription step, amplification of the sample occurs, as well as the incorporation of the fluorescent Cy dye molecules. Following purification of the cRNA (30µl), the concentration and dye incorporation of each sample was checked. The concentration that was required of each sample for hybridisation was 750ng. The samples were then made up to 190µl, using nuclease-free H₂O. To this, 11µl 10x blocking agent and 2.2µl 25x fragmentation buffer was added and incubated at 60°C, for 30min, to fragment the RNA. Then 55µl of 2x gene expression hybridisation buffer was added to each sample. The samples were then hybridised to the microarray as follows. The microarray slide was hybridised at 65°C, for 17h. Once hybridised, the slides were washed and scanned according to the protocol outline in Agilent's standard protocol for washing and scanning gene expression microarrays. Briefly, the microarray slides were disassembled in Gene Expression wash 1, and then washed in Gene Expression wash 1 for 1min. Next, the microarray slides were transferred to Gene Expression wash 2 and washed for 1min. The microarray slides were then carefully removed from the wash buffer and immediately scanned, at 5µm resolution, at 100% PMT (photo-multiplier tube). The microarray slides were then feature extracted using Agilent Feature Extraction software 9.5.3.1.

2.7.3 Data analysis

Data were background subtracted using Bayesian method, normalised using Loess normalisation (Stekel 2003) and analysed using the statistical package R (<http://www.r-project.org/>) based on linear regression against IPR values. Data were corrected for false-discovery rate (Benjamini and Hochberg 1995) before candidates (from the A1M-R265 genome) showing q-values smaller than 0.086 were BLASTed against both *C. neoformans* and *C. gattii* databases. All hits were also retained for statistical analysis of probe distribution. The main R scripts used for the data analysis can be found in Appendix IV.

2.8 PCR

2.8.1 DNA isolation from *Cryptococcus*

Yeast cells isolated from inside the macrophages or from overnight culture were centrifuged and washed with PBS twice before mixed with 750µl of lysis buffer (0.5% w/v SDS, 0.5% w/v Sarkosyl in TE buffer) and homogenised. The cells were then centrifuged at 13,000 rcf for 2min and mixed with 750µl of phenol-chloroform solution (1:1, pH=8.0) by strong vortex. Subsequently, the crude extract was spun down at 17,000 rcf for 15min at 4°C. The top aqueous layer which contains the DNA was transferred to another tube and mixed with 750µl of 100% ethanol and 100µl of 3M ice cold sodium acetate. The samples were kept at -20°C for one hour in order to enhance DNA precipitation. After that, the genomic DNA was spun down at 17,000 rcf for 15min at 4°C and dissolved in 30µl of pre-heated TE buffer. The concentration of genomic DNA was measured by Nanodrop-1000.

2.8.2 PCR for profiling mitochondrial genotype

Seven pairs of primers were designed to profile the mitochondrial genotype of *C. gattii* isolates. The primers are listed in Table 2A (primer pair 6 and 7 are from published work (Bovers et al. 2009)). Only primer pair 1 (ATP-1) was able to distinguish VGII mitochondrial genotype from the VGIII mitochondrial genotype. It was therefore chosen to analyse the mitochondrial genome of the progeny generated by crossing VGII and VGIII isolates. The PCR reactions were carried out with required DNA template, dNTPs and Taq polymerase with the appropriate buffers provided. Cycles for these PCR reactions are shown in Table 2B.

2.8.3 Gel electrophoresis

DNA was typically resolved on a 1% (w/v) TBE agarose gel containing 0.2µg/ml ethidium bromide. Before loading, DNA was mixed with approximately 0.2 volumes of 5x DNA loading dye. DNA was run as standard at 60V for approximately 40-60min

along with 0.25 μ g 1kb ladder, or 0.2 μ g 100bp ladder for size and quantification. DNA fragments were visualised under UV light using Geneflow Syngene Bio Imaging.

2.8.4 Real time PCR

Real time PCR using the Sybr Green method was performed on the genomic DNA generated from newly isolated intracellular cryptococci to check the copy number of mtDNA in comparison to a nuclear region. For the reaction, four strains (ENV152, A1M-R271, CBS7750 and CBS8684) were chosen. Every reaction in the experiment was done in triplicate. The details of the candidates and primers are listed in Table 2C.

2.9 Mouse survival assay

The mouse survival experiments were performed by Edward Sionov and Rama Falk from Itzhack Polacheck's lab at the Department of Clinical Microbiology and Infectious Diseases, Hadassah-Hebrew University Medical Center, Jerusalem, Israel. Briefly, yeast inocula (10^6 yeast cells per mouse from a 48h culture of cryptococci on Sabouraud dextrose agar (SDA) at 30°C) were injected into the tail veins of male albino BALB/c mice (weight, 20-23g) by administration of a single bolus injection of a 0.2ml suspension in PBS. The yeast concentration was determined by counting with a haemocytometer. The viable count was measured as the number of CFU on SDA plates after 24-48h of incubation at 30°C. With these inocula, systemic infections are regularly produced in mice, and they cause total killing within 5 to 50 days. For each experiment 10 mice were used, maintained in separate cages. The number of surviving animals in each group was recorded daily over a period of 45 days using a blinded experimental design such that animal handlers were unaware of the corresponding IPR value for the strains used. All procedures, care and treatment of mice were in accordance with the principles of humane treatment outlined by the Guide for the Care and Use of Laboratory Animals of the Hebrew University, and were approved by the Committee for Ethical Conduct in the Care and Use of Laboratory Animals (approval number OPRR-A01-5011).

Table 2: A) The list of primers used for profiling mitochondrial genotype; B) PCR cycles used for primers listed in A; C) Primers used to quantify mtDNA copy number in four *C. gattii* strains.

A)

Region	Primer 5'	Primer 3'
1 ATP6-1	ACTTGCGGCTGAATGATAAAAATCTAA	GTGGAGATGTAATAAAGTGTGTCATG
2 Cytochrome C	TACTGTAGGTAATGTGAATAGCCGA	AGATATAACCATCAGTAGCCCGAGT
3 Intergenic-1	TAAGGACGAGCGGAATCGA	TAACCTCGCTGACGATGATTGC
4 Intergenic-2	TGGGGAACACCAGGATACT	TGCGTAAGGGGAGTTGAGTT
5 Intergenic-3	TTCGTCTTGCTGGTCGACTT	TGGGAGTTGTTGATCGTTG
6 ATP6-2	ATTACATCTCCACTAGAACAATTC	AGTTCAATGGCATCCTTGATATAG
7 mtlrRNA	GACCCTATGCAGCTTCTACTG	TTATCCCTAGCGTAACTTTTATC

B)

Temperature (°C)	Duration	Number of cycles
94	2min	1
94	25s	30
54-58	30s	30
72	1min	30
72	7min	1
15	5min	1

C)

Candidate loci	Primer 5'	Primer 3'
Nuclear locus (GADPH)	GGTCGAATTGTTCTCAGG	ACCGTCCTTAACCTCGAC
Mitochondrial locus	TTCGTCTTGCTGGTCGACTT	TGGGAGTTGTTGATCGTTG

2.10 Phenotypic analysis of *Cryptococcus*

2.10.1 Proteinase assay

Proteinase activity was measured as described in (Braga et al. 1998). To make plates, 900ml casein medium (pH=7.0) (7.5g casein, 5.0g glucose and 20.0g agar) was prepared and autoclaved. Meanwhile, yeast nitrogen base 10x medium was prepared by dissolving 6.7g yeast nitrogen base in 100ml of dH₂O and autoclaved by filtering through a 0.22µm filter. The 100ml filter-sterilised yeast nitrogen base 10x medium was then added to the casein medium when the casein medium has cooled down to ~55°C. Once the plates were made, 2µl of overnight yeast culture was inoculated in the middle of the plates for 7 days at 37°C. The diameter of the colony and the zone around the colony were measured and Pz value was calculated by dividing the zone-diameter with the colony diameter. Each measurement was repeated three times.

2.10.2 Phospholipase assay

The method used to measure phospholipase activity was adapted from (Vidotto et al. 1996). In order to make plates, 900ml of Sabourand dextrose medium (pH=7.0) (20.00g SDA, 58.44g NaCl and 0.74g CaCl₂·2H₂O) was prepared and autoclaved before supplemented with 100ml sterile Bacto™ egg yolk enrichment 50%. Once the plates were made, 2µl of overnight yeast culture was inoculated in the middle of the plates for 7 days at 37°C. Subsequently, the diameter of the colony and the zone around the colony were measured. Pz value was calculated by dividing the zone-diameter with the colony diameter. Each measurement was repeated three times.

2.10.3 Melanin production measurement

To make plates for melanin production, 900ml dopamine medium (pH=7.0) was prepared (3.00g glucose monohydrate, 1.20g MgSO₄, 3.50g KH₂PO₄, 0.98g glycine and 20.00g agar) and autoclaved before being supplemented with 100ml sterile L-DOPA solution (0.20g L-DOPA, filtered through 0.22µm filter) and 1.0ml vitamin B1 solution (0.25g thiamine in 250ml dH₂O and sterilise through a 0.22µm filter). Subsequently, 2µl

of overnight yeast culture was inoculated in the middle of the plates for 10 days at 37°C. The melanin production was recorded every day as a score from 1 to 5. Each measurement was repeated three times. For the statistical analysis, melanin production was calculated as follows:

Maximum score/the number of days required to achieve the maximum score

2.10.4 Capsule size measurement

Yeasts were grown overnight, shaking at 240 rpm in capsule medium (1% peptone, 1% glucose, 1.5% Ox-bile) at 37°C. On day two, 2µl of yeast culture was mixed with 2µl of Indian ink and examined under a microscope. For each sample, 60 yeast cells were chosen randomly measured for capsule size using ImageJ. The capsule index is calculated by the formula below:

Capsule index = the actual cell size/ the total cell size (including the capsule).

2.10.5 Statistical test

Unless otherwise stated, statistical tests were performed by linear regression to test whether any of the tested phenotype was correlated with IPR values.

**Chapter III: Phagocytosis of *Cryptococcus* by J774
macrophages**

The fate of pathogens phagocytosed by macrophages depends on phagosome acidification and fusion with different intracellular vesicles (Bouvier et al. 1994). As phagosome-lysosome fusion is modulated by the mode of particle recognition by the phagocyte, phagosomes formed via the various receptor-ligand pairs may have divergent fates. For example, FcR-derived, but not CR3-derived phagosomes containing *Salmonella typhimurium* can fuse with secretory granules in neutrophils (Joiner et al. 1989). Similarly, phagosomes containing IgG-opsonised mycobacteria, but not those opsonised with complement components, can fuse with lysosomes (Vieira et al. 2002). Another example is *Toxoplasma gondii*. Normally, after entering phagocytic and nonphagocytic cells, live *T. gondii* inhibits phagosomal fusion with preexisting secondary lysosomes and thus the acidification process. Since *T. gondii* is highly susceptible to low pH conditions, blocking phagosome fusion and acidification is thought to be important for its intracellular survival. Interestingly, in macrophages or CHO cells transfected with FcR, the blocks in lysosome fusion and acidification can be overcome by opsonising live parasites with specific antibody before cell entry. This effect was not due to the antibody interfering with some critical parasite surface component and often results in efficient parasite killing even in the absence of an oxidative burst (Anderson et al. 1976; Joiner et al. 1990; Sibley et al. 1985). Therefore, not all phagosomes fuse equally for the same pathogen.

For intracellular *C. neoformans*, three outcomes have been observed following phagocytosis by macrophages (being killed, staying latent or proliferating intracellularly) as discussed in Chapter I. We were therefore interested to test whether phagocytosis mediated via different surface receptors can lead to different outcomes for intracellular yeast. It is possible that yeast phagocytosed entirely through FcR are prone to be killed, while those phagocytosed via mannose receptors (non-opsonised) are more likely to undergo proliferation, in a similar manner to that was observed with *T. gondii*. In order to address this question, we carried out phagocytosis assay on non-opsonised, complement-opsonised and antibody-opsonised cryptococci respectively. Phagocytosis index (PI) and intracellular proliferation of *C. neoformans* were measured for each route. We use proliferation rate as an indicator of intracellular

processing since it was difficult to tell the difference between latency and death of intracellular yeast cells within 10h of timelapse filming whereas proliferation was relatively easy to spot.

3.1 The presence of opsonins enhances phagocytosis

In order to obtain PI, yeast cells were opsonised with fresh human or rodent serum, Fc monoclonal antibody 18B7 (which recognises the capsule, provided by Arturo Casadevall) or left unopsonised before the phagocytosis assay. We found that the efficiency of antibody-mediated phagocytosis was the highest for all of the strains. In addition, there was an inverse correlation between the capsule volume and the efficacy of non-opsoninic and complement-mediated phagocytosis, but not for antibody-mediated phagocytosis (JEC21, NIH-B4131 and CBS919) (Table 3). This result is consistent with previous findings that capsule inhibits phagocytosis in the absence of opsonins (Kozel and Gotschlich 1982; Nosanchuk and Casadevall 1997; Vecchiarelli et al. 1994).

Fluorescence labelling was used to investigate the localisation of opsonins on the capsule after opsonisation. In strains with small capsule size (NIH-B4131), both 18B7 and C3 localised to the outer capsule edge since the cell wall and outer edge of the capsule were very close to each other. In contrast, in strains with moderate (JEC21) or large capsules (CBS919), C3 was not able to reach the cell wall and thus most of deposition occurred at the budding site of the yeast cells where the capsule was absent. This leads to poor opsonisation of complement components and explains why complement-mediated phagocytosis has a relatively low PI. Interestingly, we found that *uxs1*Δ strain (NE178) was less resistant to complement-mediated phagocytosis, whereas the *cas1*Δ strain (NE150) was more resistant (Table 3, both of the PIs are significantly different to the PI of JEC21). NE178 is a strain that lacks xylose in its GXM and is avirulent in mouse (Kozel et al. 2003). NE150 strain lacks the *CAS1* gene, which encodes a membrane protein necessary for the O-acetylation of capsular

polysaccharide, and it is hypervirulent in mouse (Janbon et al. 2001). The molecular mechanisms causing differences in virulence are not yet known. It is likely that the ability to resist phagocytosis may have a great impact on the virulence of the strains.

3.2 The fate of intracellular cryptococci is independent of phagocytosis route

In order to quantify intracellular proliferation capacity, real time timelapse imaging of macrophage cells was carried out. For all the movies, macrophage cells were fed with cryptococcal cells for two hours and macrophage cells with internalised cryptococcal cells were filmed in the next 10-16 hours. The timelapse movie showed that intracellular proliferation occurred at a similar rate following the uptake of yeast via all phagocytosis routes (Table 4), suggesting that although the presence of opsonins enhances phagocytosis, cryptococcal proliferation rate inside J774 cells is independent of the phagocytosis route. Interestingly, intracellular proliferation was significantly reduced in J774 cells treated with concanamycin A (Table 4). Early studies have shown that *C. neoformans* grow much faster inside the acidic phagosome as compared with the neutral extracellular environment (Feldmesser et al. 2001b). Since concanamycin A is an inhibitor of the V-type ATPase, known to reduce phagosome maturation by block acidification of the phagolysosome (Drose and Altendorf 1997), it is therefore not surprising to observe decrease in intracellular proliferation of *C. neoformans*. Based on these two observations, we conclude that the fate of intracellular *Cryptococcus* is not determined by the entry route, but rather is altered by additional signals inside the phagosome. Alternatively, it is the organism surface (e.g., the presence of capsule) that decides its intracellular fate, as has been found in *Salmonella montevideo* (Pilszczek et al. 2005). Since antibody opsonised *Cryptococcus* often yields the highest intracellular yeast number and phagocytosis route is unlikely to affect intracellular fate of this pathogen, we use antibody opsonised cryptococci for most of the experiments later on.

Table 3: The PI of six yeast strains and three phagocytosis routes. Data were generated by counting the number of macrophages with internalised *Cryptococcus* out of total 1000 cells in each case. / indicates the PI was not studied for these strains.

Strains	Non-opsonised	Serum opsonised	Fc antibody opsonised
JEC21	2.67%±0.24%	3.54%±0.68%	20.08%±2.72%
CBS919(big capsule)	<1%	<1%	15.9%±3.44%
NIH-B4131 (cap67)	39.45%±5.2%	47.60%±4.8%	/
NE178 (usx1 mutant)	/	9.25%±0.73%	/
NE150 (cas1 mutant)	/	1.96%±0.53%	/
<i>S. cerevisiae</i> (AH109)	63.78%±5.9%	/	/

Table 4: Percentage of macrophages which contain intracellular particles that underwent proliferation. There is no significant difference in term of intracellular proliferation rate among cryptococci phagocytosed by different routes ($P=0.895$, ANOVA, $n=3$). However, JEC21 engulfed by concanamycin A-treated macrophages showed much lower intracellular proliferation capacity ($P<0.001$, t-test). *S. cerevisiae* was used as a control to verify the system.

Particles	Macrophages with intracellular particles	Intracellular particles undergoing proliferation
mAb18B7 opsonised JEC21	134	63 (47.0%)
Complement opsonised JEC21	27	17 (63.0%)
Non-opsonised JEC21	45	28 (62.2%)
<i>S. cerevisiae</i> (AH109)	28	0 (0%)
JEC21+mAb18B7+concanamycin A	68	5 (7.35%)*

Chapter IV: Vomocytosis & Lateral transfer

Ma, H., Croudace, J. E., Lammas, D. A., May, R. C. (2007), Direct cell-to-cell spread of a pathogenic yeast, *BMC Immunology* 8: 15

Ma, H., Croudace, J. E., Lammas, D. A., May, R. C. (2006), Expulsion of live pathogenic yeast by macrophages. *Current Biology* 16: 2156-60

4.1 Vomocytosis: a novel way to exit

Some bacteria and fungi have evolved strategies to survive within a phagocyte after uptake, but most of them must eventually kill the host cell if they are to escape and infect other tissues (Cossart and Sansonetti 2004; Nguyen and Pieters 2005). Lysis of *Cryptococcus*-infected macrophages is a general phenomenon that has been observed both *in vivo* (Feldmesser et al. 2000) and *in vitro* (Feldmesser et al. 2001a; Tucker and Casadevall 2002). As for most pathogens, it is assumed that *Cryptococcus* can only be released by lysing its host cells. However, during the investigation of cryptococcal intracellular proliferation, we made an observation that the fungal cell is able to escape from within macrophages without killing the host cell, by a novel mechanism that we have termed ‘reverse phagocytosis’ or ‘vomocytosis’. We observed vomocytosis in both cultured J774 cells and primary human macrophage cells (both alveolar and blood-monocyte derived macrophages) (Movie 1^I and Movie 2^{II}).

^I **Movie 1: Quicktime movie showing an example of vomocytosis in J774 murine macrophages.** J774 macrophages were exposed to *C. neoformans* JEC21 (opsonised with monoclonal antibody 18B7) for two hours. Extracellular yeast cells were then washed away before the dish was placed on an inverted microscope for imaging. The movie shows timelapse images (one frame per minute) over the course of almost 10h (time shown in bottom left). After almost two hours of recording, the intracellular pathogen starts to produce a visible (phase bright) capsule (first seen at 1:59:58). This capsule grows dramatically over the next four hours until, between 6:38:01 and 6:40:01, the yeast cell is vomocytosed. Thereafter, both the macrophage and yeast cell remain in the field of view and appear morphologically normal. Indeed, the small daughter cell, present on the pathogen whilst it is intracellular, continues to grow after vomocytosis.

^{II} **Movie 2: Quicktime movie showing an example of vomocytosis in human primary macrophages.** Macrophages were exposed to *C. neoformans* JEC21 (opsonised with fresh human serum) for two hours. Extracellular yeast cells were then washed away before the dish was placed on an inverted microscope for imaging. The movie shows timelapse images (one frame per minute) over the course of 10h (time shown in bottom left). Note that the primary macrophages are more dynamic than the J774 cells, which results in the intracellular *C. neoformans* cell moving up and down in the focal plane. Approximately three hours after recording commences, the intracellular yeast cell starts to bud, producing a distinct daughter cell by 04:54:57. At 05:26:27 the original mother cell is suddenly expelled, leaving the daughter cell within the phagosome. The expelled *C. neoformans* cell remains attached to the macrophage surface for several hours, until it drifts off towards the end of the movie.

It appears to occur when a mature phagosome containing one or more *C. neoformans* cells fuses with the plasma membrane, releasing the yeast cell(s) into the extracellular medium (Figure 10). Neither the host macrophage nor the *C. neoformans* cell is killed by this event and both appear morphologically normal for the duration of the experiment thereafter (several hours). Indeed in several cases we were clearly able to observe cryptococcal cells that had been vomocytosed subsequently undergoing replication, demonstrating that the yeast cells were alive when expelled by the macrophage (Movie 1 and Figure 10). The expelled yeast cells can either be phagocytosed by other macrophages, or proliferate extracellularly, or simply propagate.

Vomocytosis can lead to expulsion of all the intracellular yeast cells simultaneously (known as complete phagosomal extrusion). Complete phagosomal extrusion requires fusion of cryptococcal-containing phagosomes, which often leads to the formation of a giant compartment (Alvarez and Casadevall 2006). However, complete phagosomal extrusion is just a special type of vomocytosis, as vomocytosis can also lead to release of only one or some of the intracellular yeast particles. During our study, we have observed numerous examples of vomocytosis occurring to only one cryptococci-containing phagosome when there was more than one such phagosome present in the same macrophage. It was also common to see when there were several cryptococci in one phagosome, but only one of them underwent vomocytosis. Sometimes, the internalised *Cryptococcus* first underwent budding, and then only the mother cell in the same phagosome was expelled, leaving the daughter cell behind (Movie 2). This type of vomocytosis is rather intriguing as one would expect the fusion between phagosome membrane and plasma membrane will release all the particles within that phagosome. Possibly, the membrane fusion was shortly followed by membrane fission, so some of the yeast cells did not have chance to escape.

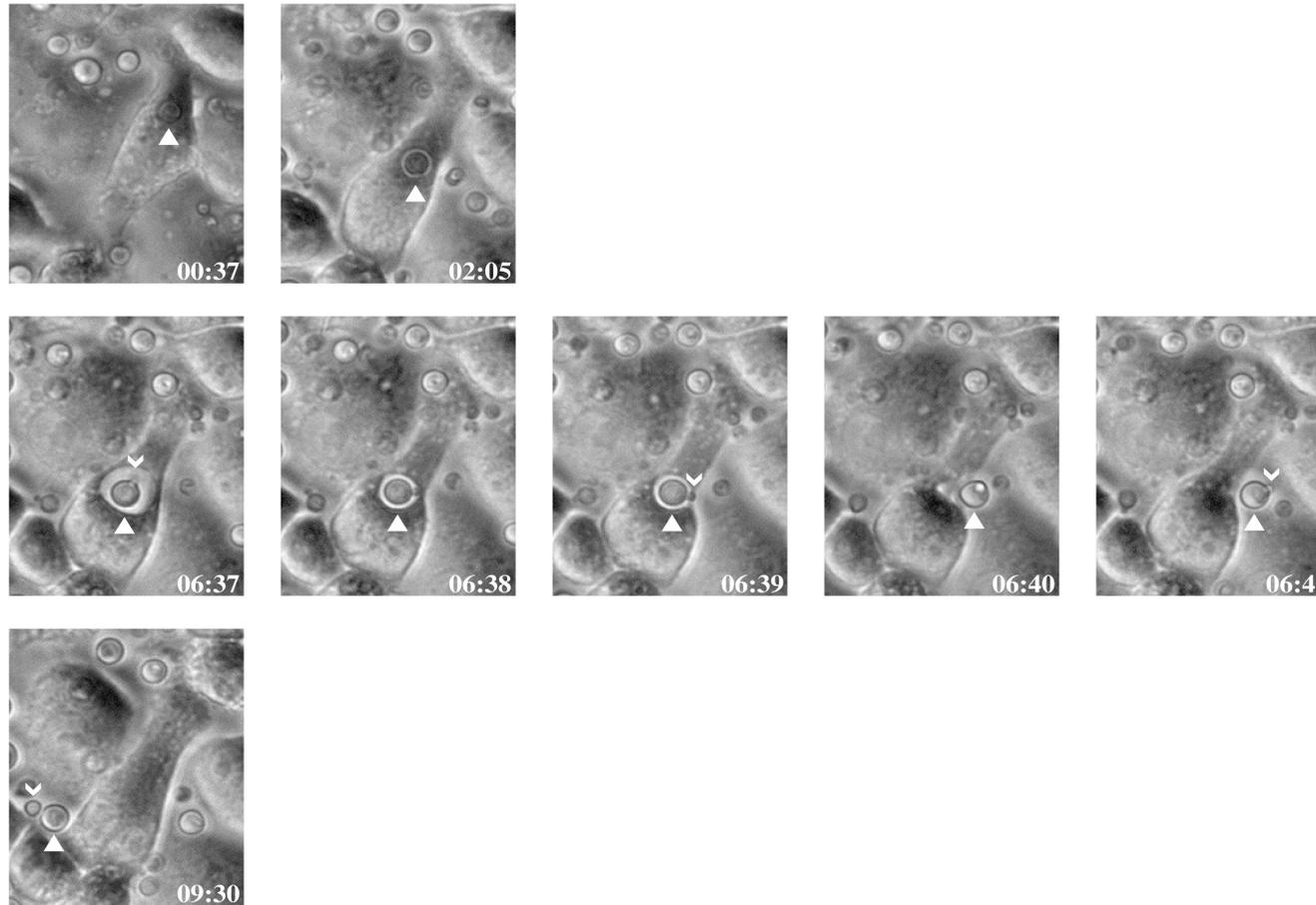


Figure 10: Vomocytosis in J774 cells. The internalised *C. neoformans* (large arrow) is initially contained within a 'tight' (phase-dark) phagosome. Two hours later the phagosome begins to swell – the yeast cell is surrounded by a phase-bright region between the cell wall and the phagosome membrane, which is believed to contain secreted capsular polysaccharide. By six hours into the time series, the phagosome has grown dramatically; note that the *C. neoformans* cell is proliferating and a small daughter bud can be clearly seen (small arrow). 400min after the onset of filming, the *C. neoformans*-containing phagosome suddenly fuses with the plasma membrane. The phase-bright material is released and, 2min later, the yeast cell is clearly outside of the host macrophage. Importantly, both the macrophage and the yeast cell are still alive three hours later, by which time the *C. neoformans* daughter cell (small arrow) has clearly grown in size.

4.1.1 Vomocytosis rate

Vomocytosis appears to occur independently of the initial route of phagocytic uptake, since both antibody/serum opsonised and non-opsonised yeast cells were capable of being expelled (Table 5). However, we never observed vomocytosis of dead (heat-killed) *C. neoformans* cells nor of inert latex beads (2 different sizes), suggesting that vomocytosis requires the presence of a live organism within the phagosome (Table 5).

Based on our observations with cultured J774 cells, for most of the strains, vomocytosis is a relatively rare event compared to intracellular proliferation, as the majority of phagocytosed *C. neoformans* either proliferates or remains latent within the host macrophage (Table 5). This probably explains why previous studies have not reported it. However, we are likely to underestimate the true frequency of reverse phagocytosis, as events that occur outside of the filming time (10-16 hours) will remain undetected in our experimental setup. Interestingly, the rate of vomocytosis in human primary macrophages is significantly ($P < 0.001$, t-test) higher than that in J774 cells (Table 5) (also reported in (Voelz et al. 2009)), although the time-distribution (Figure 11) is the same for both cell types ($P > 0.2$, t-test).

Vomocytosis has been observed with both *C. neoformans* and *C. gattii*. However, the rate varies from 0% to up to 45% for different strains (Table 6). Such strain-to-strain differences were also reported in (Alvarez and Casadevall 2006), implying the specificity of *Cryptococcus*-macrophage interaction. It is worth noting that vomocytosis also occurred to acapsular mutants including CBS7931 & NIH-B4131, which lacks GXM in their capsule. Hence, GXM is not essential to trigger vomocytosis.

Table 5: Vomocytosis rate recorded for five particle types in J774 cells and for primary human macrophages over 10 hours of timelapse filming. Each value represents the total number of recorded events from three or more independent timelapse experiments. mAb: monoclonal antibody; MØ: macrophage

Host cell	Target particle	Number of MØ observed	MØ with internalised particle	Vomocytosis rate
J774	mAb(18B7) opsonised JEC21	603	134 (22.2%)	13 (9.7%)
J774	Non-opsonised JEC21	903	45 (5.0%)	3 (6.7%)
J774	Latex beads (3µm)	336	157 (46.7%)	0
J774	Latex beads (12µm)	299	97 (32.4%)	0
J774	Heat-killed JEC21	277	80 (28.8%)	0
Primary	Serum opsonised JEC21	661	177 (26.8%)	47 (26.6%)

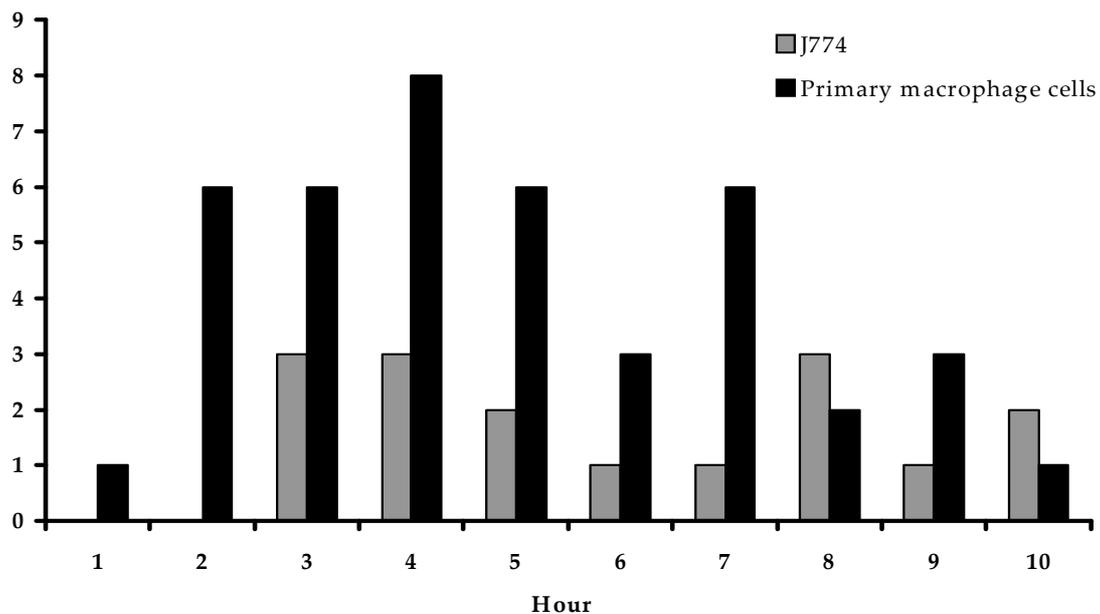


Figure 11: The distribution of vomocytosis events over the course of the timelapse recordings. Data are pooled from all timelapse recordings (15 independent experiments) of J774 macrophages (11 experiments, 1506 cells, 179 with internalised JEC21) and human primary macrophages (4 experiments, 661 cells, 177 with internalised JEC21). Although the rate of vomocytosis is higher in primary macrophages than in J774 cells, the distribution of vomocytosis events over the 10h timelapse recording does not differ between these cell types ($P > 0.2$, two-tailed Student's T-Test).

Table 6: Vomocytosis rate for various mAb (18B7)-opsonised cryptococcal strains after engulfed by J774 cells. *Vomocytosis rates were measured when macrophage culture was supplemented with HEPE buffer during the timelapse movie in the absence of CO₂ control. MØ: macrophage

Strain name	Genotype (AFLP)	Number of MØ observed	MØ with intracellular yeast	Number of vomocytosis	Vomocytosis rate (%)
A1M-R271	6	487	50	0	0
ENV131	6	789	73	4	0.055
CBS10089	6	490	53	8	0.151
CBS10090	6	562	22	1	0.045
CBS10485	6	943	57	7	0.123
A1M-F2866	6	680	50	1	0.020
A1M-F3016	6	732	33	1	0.030
A1M-R265	6	699	94	5	0.053
CBS7750	6	872	39	3	0.077
ENV152	6	1329	226	14	0.062
RB14	6	391	72	3	0.042
RB50	6	432	25	1	0.040
CBS6956	6	544	19	2	0.105
CBS8684	6	429	66	10	0.152
A1M-R376	6	361	50	0	0
A1M-R406	6	783	106	9	0.085
ICB184	6	481	49	6	0.122
A1M-R272	6	1004	114	13	0.114
WM728*	5	114	17	2	0.118
CBS1930	6	833	45	4	0.089
LA362	6	652	44	1	0.023
CBS7229	4	983	77	2	0.026
WM276	4	733	48	2	0.042
56A	4	1142	39	5	0.128
CBS5467*	2	155	41	1	0.025
JEC20*	2	260	54	6	0.111
B3501*	2	192	25	9	0.360
JEC21*	2	603	134	13	0.097
H99*	1	267	98	24	0.245
125.91*	1	186	45	8	0.178
WM714*	1	585	141	18	0.128
P152*	1	189	37	11	0.297
ATCC90112*	1	456	85	37	0.435
CBS8336*	1	390	144	24	0.167
CBS996T*	1	176	65	16	0.246
MT105*	1	115	46	0	0

4.1.2 Vomocytosis does not require phagosome maturation or presence of host actin, but is dependent on host microtubule

Previous studies have demonstrated that *C. neoformans* is well adapted to survive within the acidic environment of the phagosome and, unlike many other pathogens, it does not inhibit phagolysosomal fusion (Levitz et al. 1999). We therefore wondered whether vomocytosis occurs only after maturation of the phagosome. By blocking phagosome maturation using both 100nM concanamycin A (a V-ATPase inhibitor that inhibits acidification of phagosome) (Drose and Altendorf 1997) and 10µM chloroquine (a weak base that accumulates within the phagosome by ion trapping) (Levitz et al. 1997), we found that vomocytosis was not blocked in the presence of either drug (Table 7A), suggesting that this process does not require phagosome maturation, although Alvarez *et al* showed that phagosome-lysosome fusion still occurred prior to vomocytosis (Alvarez and Casadevall 2006). Intriguingly, the rate of vomocytosis appears to be enhanced by the presence of chloroquine (Table 7A). This effect may explain the earlier finding of Levitz and colleagues that chloroquine suppresses intracellular growth of *C. neoformans* to a level that cannot be explained simply by its ability to inhibit phagosome acidification (Levitz et al. 1997).

The molecular mechanism that drives cryptococcal expulsion remains to be elucidated. Given the speed with which expulsion occurs, we considered a possible role for the macrophage cytoskeleton in providing the required force. To test this, we exposed J774 macrophages to 2.5µg/ml nocodazole, a drug that depolymerises microtubules (Samson et al. 1979). Such a treatment totally abolished vomocytosis (e.g., from 24.5% to 0 for H99) (Table 7B), suggesting that microtubules are essential for this process. Microtubule is known to be essential for vesicle transportation in many cells (Schroer and Sheetz 1991). Since vomocytosis is likely to be a consequence of phagosome and plasma membrane fusion, it is not surprising that disruption of microtubule depolymerisation led to the suppression of vomocytosis. We also treated J774 cells with cytochalasin D to block barbed-end growth of actin filaments (Urbanik and Ware 1989). Cryptococcal expulsion was still observed even at concentrations of up to 10µM

of cytochalasin D (Movie 3^{III} and Movie 4^{IV}), suggesting that actin-filament polymerisation is not required for this process. In fact, Alvarez *et al* reported that cytochalasin D treatment increased vomocytosis event, suggesting a restraining role of actin polymerisation in vomocytosis (Alvarez and Casadevall 2006). Actin polymerisation has been reported to both inhibit and facilitate exocytosis in various systems (Becker and Hart 1999; Bi et al. 1997; Muallem et al. 1995; Poucell-Hatton et al. 1997; Segawa and Yamashina 1989). As a negative regulator, sub-plasmalemmal actin can act as a physical barrier against exocytosis. For instance, increased actin polymerisation that occurs predominantly in the submembranous area is a key contributor to the osmotic inhibition of exocytosis in neutrophils (Rizoli et al. 2000). Based on our and others' observations, actin polymerisation is more likely to play a negative role in the case of vomocytosis.

^{III} **Movie 3: Quicktime movie showing cryptococcal expulsion after treatment with 4 μ M cytochalasin D.** Macrophages were exposed to *C. neoformans* JEC21 (opsonised with monoclonal antibody 18B7) for two hours. Extracellular yeast cells were then washed away, and the medium was replaced with serum-free medium containing 4 μ M cytochalasin D before the dish was placed on an inverted microscope for imaging. The movie shows time-lapse images (one frame per 90s) over the course of 10h (time shown in bottom left). At this low dose of cytochalasin D, the cells are partially retracted (note the "spiky" appearance), but morphology and behaviour is otherwise unaffected. Expulsion of *Cryptococcus* cells (at 03:13) occurs in a manner very similar to that in untreated cells (compare with Movie 1), although it is rather less dramatic, probably because of rounding of the host cell.

^{IV} **Movie 4: Quicktime movie showing cryptococcal expulsion after treatment with 10 μ M cytochalasin D.** Macrophages were exposed to *C. neoformans* JEC21 (opsonised with monoclonal antibody 18B7) for two hours. Extracellular yeast cells were then washed away, and the medium was replaced with serum-free medium containing 10 μ M cytochalasin D before the dish was placed on an inverted microscope for imaging. The movie shows time-lapse images (one frame per 90s) over the course of 10h (time shown in bottom left). At this dose of cytochalasin D, the macrophages are highly rounded and retracted, and substantial cell death starts to occur after approximately six hours of filming. Although it is difficult to score expulsion in these rounded cells with confidence, it appears that they remain capable of cryptococcal expulsion. One reasonably clear example is highlighted: The macrophage appears to expel a yeast cell (at 0:40), which initially remains attached to the cell surface, but by 05:19, the *Cryptococcus* cell is clearly extracellular and floats away.

Table 7: A) Vomocytosis rate of mAb (18B7)-opsonised JEC21 in J774 cells following treatment to block phagosome maturation. B) Vomocytosis rate of mAb (18B7)-opsonised H99 in J774 with and without the nocodazole treatment. Each value represents the total number of recorded events from three or more independent 10h timelapse experiments. MØ: macrophage

A)

Cell type/treatment	Number of MØ observed	MØ with internalised JEC21	Occurrences of vomocytosis
J774 untreated (data from Table 5)	603	134 (22.2%)	13 (9.7%)
J774 with concanamycin A (100nM)	449	90 (20.0%)	3 (3.3%)
J774 with chloroquine (10µM)	408	68 (16.7%)	14 (20.6%)

B)

Cell type/treatment	Number of MØ observed	MØ with internalised H99	Occurrences of vomocytosis
J774 untreated	267	98 (36.7%)	24 (24.5%)
J774 with nocodazole (2.5µg/ml)	285	82 (29.8%)	0

4.1.3 Is vomocytosis *Cryptococcus*-specific?

Although we did not observe vomocytosis in J774 with internalised *S. cerevisiae* (AH109) or *S. pombe* (972), currently, we still do not know whether it is unique to *Cryptococcus* or a more widespread cellular phenomenon. Recently, a similar process termed the 'extrusion pathway', has been reported in *Chlamydia*, an obligate intracellular pathogen causing sexually transmitted infections in humans, which is the leading cause of infectious blindness worldwide (Ryan and Ray 2003). The extrusion pathway involves a 'packaged release' mechanism, during which only a portion of the inclusion was released through a membranous protrusion, followed by the separation of the inclusion into compartments and tethering of the inclusion to the host cell membrane, leaving both the host cell and the residual inclusion intact (Hybiske and Stephens 2007) (Figure 12A). The extrusion pathway is not exactly the same as vomocytosis, because the process requires actin polymerisation and does not involve fusion between phagosome and plasma membranes (Hybiske and Stephens 2007). Another interesting non-lytic exit pathway was reported with *Orientia tsutsugamushi*, during which each single bacterium slowly extrudes from the cell membrane and buds from the host cell, leaving the host cell intact (Urakami et al. 1983; Yang et al. 2008) (Figure 12C). The released bacterium is enveloped with both the vacuole and plasma membranes, and can be subsequently phagocytosed by new host cells, after which both the vacuole and plasma membranes disintegrate by an unknown mechanism (Hybiske and Stephens 2008; Kadosaka and Kimura 2003). *O. tsutsugamushi*, a member of the scrub group of *Rickettsia*, causes tsutsugamushi disease or scrub typhus, which is a mite-borne acute febrile illness widely distributed through eastern and southern Asia, northern Australia and eastern Russia (Traub and Wisseman 1974; Watt and Parola 2003). To date, the detailed mechanism on budding remains poorly understood. Nevertheless, it morphologically resembles *Chlamydia* extrusion: the released bacterium is surrounded by both vacuole and plasma membrane (Figure 12). Cryptococcal expulsion is thus unique in representing a non-destructive mechanism by which pathogens can remerge from infected host cells.

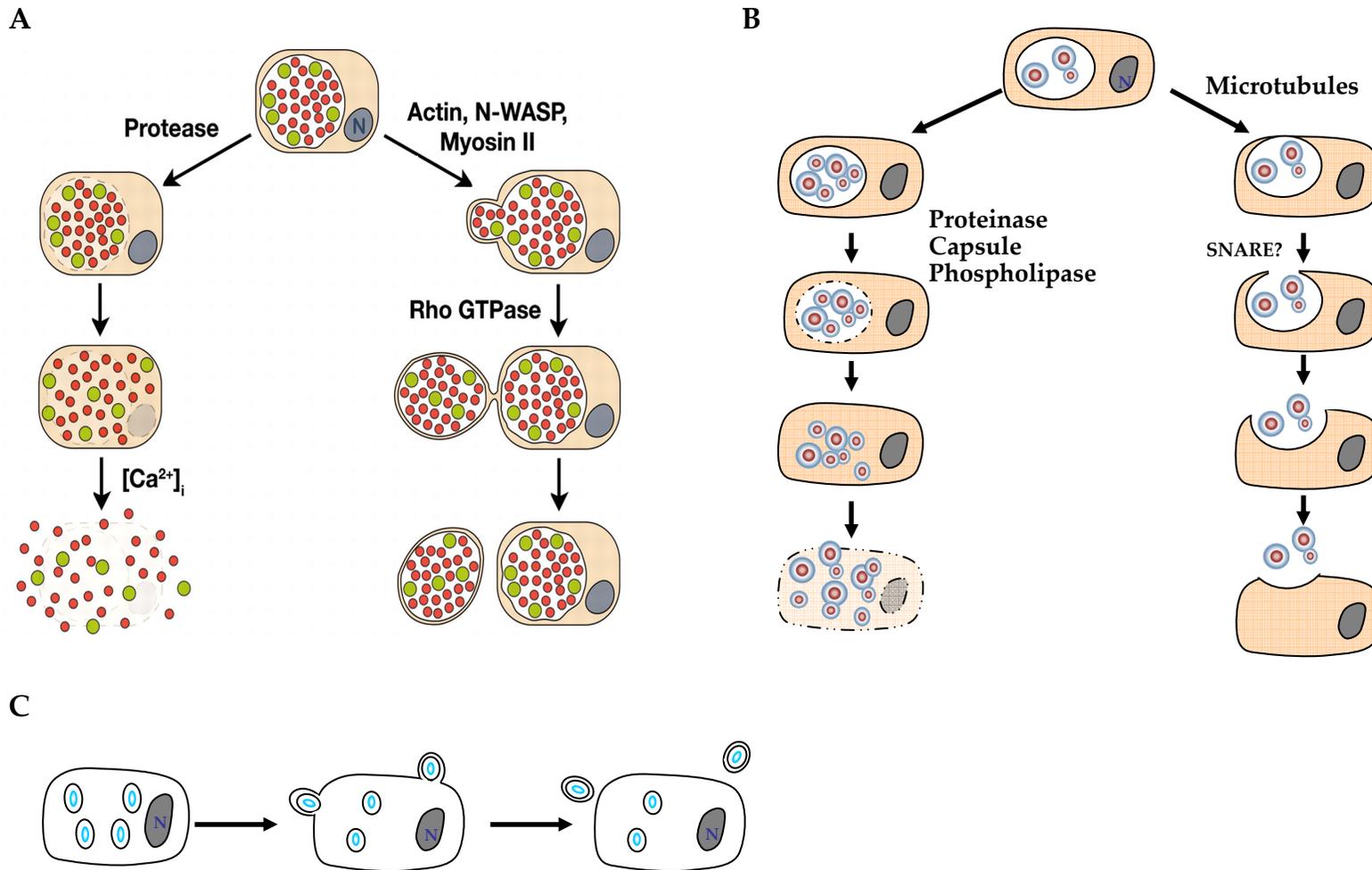


Figure 12: Non-lytic exit mechanism of A) *Chlamydia* (taken from (Hybiske and Stephens 2007)); B) *Cryptococcus*; and C) *O. tsutsugamushi*.

It is worth noting that the phagocytic amoeba *D. discoideum* has been observed to expel the remains of digested yeast cells in a process that is morphologically similar to cryptococcal expulsion (Clarke et al. 2002). The fact that we did not observe the expulsion of heat-killed *C. neoformans* or of inert latex beads (Table 5A) would argue against this process being a general mechanism for the expulsion of indigestible particles, although it is possible that such particles are expelled with a lower efficiency (and thus not detectable by our experimental setup). In addition, some retroviruses appear to be expelled from their host cell in vesicles termed “viral exosomes” (Pelchen-Matthews et al. 2004), and poliovirus is known to escape host cells non-lytically via fusion between virus-induced double membrane vesicles and the plasma membrane in a host microtubule-dependent manner (Dales et al. 1965; Taylor et al. 2009). Although these vesicles are one to two orders of magnitude smaller than the “expulsive phagosomes” observed in our system, it is possible that expulsion of *Cryptococcus* occurs by a related mechanism and thus represents a previously unrecognised form of exocytosis. Nonetheless, to the best of our knowledge, this is the first report of such an expulsive process in a vertebrate phagocyte and the first direct demonstration that live pathogens can be expelled from phagocytic cells.

4.1.4 Significance of vomocytosis

The existence of an alternative escape pathway for *Cryptococcus* apart from lytic release, coupled with the high incidence of cryptococcal expulsion in primary macrophage cells, has significant implications in cryptococcal infection, as it represents a mechanism by which *Cryptococcus* may be trafficked between tissues without triggering the localised inflammation that would occur if the host phagocyte was lysed (Fadok 1999). In addition, it might be one of the main means for cryptococcal strains that are not very capable of proliferating inside macrophages, to leave host cells and establish their extracellular dominance. Our study with a fibroblast cell line (Cos-7) showed that expulsion does not occur to Cos-7 cells transfected to express Fc receptor, although *Cryptococcus* still replicated intracellularly (Figure 13), indicating this process is host cell specific. Nonetheless, vomocytosis may still occur in cells other than macrophages,

making this a plausible mechanism by which *Cryptococcus* may be released into the CNS after being phagocytosed by endothelial cells of the BBB (Chang et al. 2004).

Vomocytosis was also reported by a second group at the same time (Alvarez and Casadevall 2006), who focused more on complete phagosomal extrusion. Later on, they proposed that although the expulsion event is independent of the initial route of the phagocytic uptake (Ma et al. 2006), the outcome of the complete phagosomal extrusion was affected by the mode of opsonisation (Alvarez et al. 2008). Extrusion of antibody-opsonised *C. gattii* and *C. neoformans* resulted in the release of a clump of yeast cells that remained attached to one another and continue to replicate extracellularly as a biofilm. In contrast, complement-opsonised *C. neoformans* cells were released from macrophages dispersed as individual cells, which then continued to divide in the extracellular milieu as single cells. Therefore, the biofilm-like microcolony exit strategy of *C. neoformans* and *C. gattii* following antibody opsonisation reduced fungal cell dispersion, suggesting that antibody agglutination effects persist even inside the phagosome to attach nascent daughter cells together and may thus contribute to antibody-mediated protection (Alvarez and Casadevall 2007; Alvarez et al. 2008).

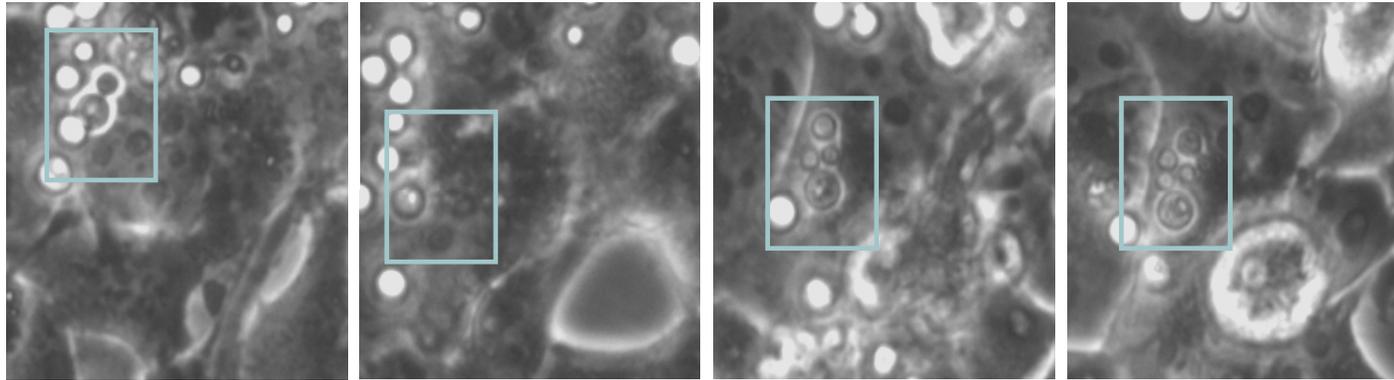


Figure 13: Representative timelapse images showing intracellular proliferation of H99 internalised by Cos-7 cells transfected to express Fc receptors. After monitoring more than 20 Cos-7 cells with internalised H99, no vomocytosis was observed (compared to 24.5% in J774 cells), but intracellular proliferation still occurs.

4.2 Lateral transfer: a novel way to disseminate

We also observed direct transfer of cryptococci between macrophages in both cultured J774 cells and PHMBC. During this process cryptococcal cells contained within the phagosome of an infected macrophage are passed directly to a neighbouring (uninfected) macrophage (Figure 14 and Movie 5^v). Compared to vomocytosis and intracellular proliferation, lateral transfer is a rare event (Table 8). In primary macrophages, we witnessed four events after monitoring 177 cells with internalised cryptococci. In immortalised J774 macrophages, the rate is even lower, since we observed only two events in more than 500 infected cells. However, again, we are likely to underestimate the rate of lateral transfer, since we only monitor infected cells for 10-16 h after phagocytosis.

Like vomocytosis, lateral transfer is independent of the initial route of uptake: both serum-opsonised and antibody-opsonised *C. neoformans* are able to undergo direct cell-to-cell transfer. We have also observed lateral transfer in both *C. neoformans* and *C. gattii* strains (including JEC21 (Serotype D), 125.91 (Serotype A); and CBS8684 (serotype B)), suggesting that it is not serotype dependent. Lateral transfer appears to be a very rapid process; transfer always completes within 10min of the onset of cell membrane fusion between the donor and recipient macrophage cells (Figure 14). Interestingly, three out of four lateral transfer events observed in primary macrophages were followed by yeast expulsion, suggesting that the cryptococcal phagosome may be in a special, highly fusogenic state.

^v **Movie 5: Lateral transfer of JEC21 from an infected to an uninfected human primary macrophage.** The infected (donor) cell is highlighted in the first frame. The donor cell moves underneath the recipient cell and, at 1:25, the recipient macrophage contacts the cryptococcal phagosome. About 160min after the onset of filming, membrane fusion starts to occur at the contact point of the two cells and initiates lateral transfer of the yeast from the donor cell to the recipient cell. The whole process takes only seven minutes. Upon completion, the cryptococcal cell is entirely in the recipient macrophage and the donor macrophage moves away.

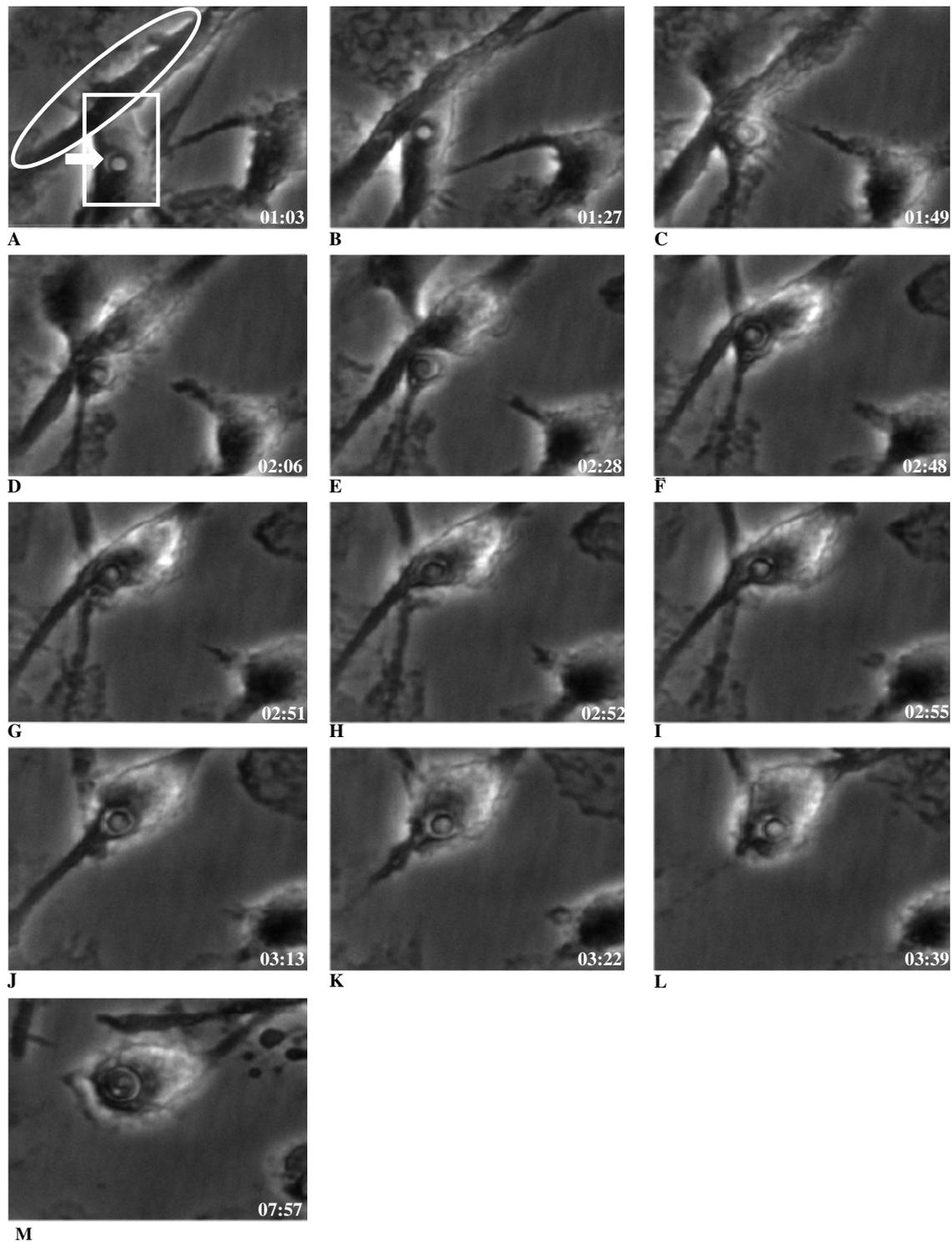


Figure 14: Lateral transfer of JEC21 (white arrow) from infected (donor, rectangle) to uninfected (recipient, oval) human primary cells. (A to E) The donor cell moves underneath the recipient cell and, after 85min, the recipient macrophage contacts the cryptococcal phagosome. (F to I) About 160min after the onset of filming, membrane fusion starts to occur at the contact point of the two cells and initiates lateral transfer of the yeast from the donor cell to the recipient cell. The whole process takes only 7min. (J to M) Upon completion, the cryptococcal cell is entirely in the recipient macrophage and the donor macrophage moves away.

Table 8: The rate of expulsion and lateral transfer events recorded for JEC21 in PHMBC.

	Primary human macrophages
Total macrophage observed	661
Macrophage with internalised yeast	177 (26.8%)
Occurrence of vomocytosis	47 (26.6%)
Occurrence of lateral transfer	4 (2.3%)

4.2.1 Significance of lateral transfer

Direct cell-to-cell spread has been described for some pathogenic bacteria, such as *Listeria*, *Rickettsia* and *Shigella*, and many viruses (e.g., poxvirus). Most of them undergo direct cell-to-cell spread via polarised actin polymerisation, which generates force to propel them through the cytoplasm of the infected cell and into uninfected neighbouring cells (Carlsson and Brown 2006; Johnson and Huber 2002; Tilney and Portnoy 1989). In contrast, it does not appear that a similar process occurs during the lateral transfer of *Cryptococcus*. Rather, we suggest that lateral transfer is related to vomocytosis, since both phenomena appear to be driven by rapid membrane fusion. Interestingly, the intracellular cryptococci shared by both macrophages were found to be surrounded by a high concentration of phalloidin-labeled actin from both macrophages (Alvarez and Casadevall 2007). However, since actin polymerisation is an extremely dynamic process, we do not know whether the observed actin ring was formed before or after the lateral transfer has been initiated. Therefore, at this stage, it is difficult to predict the role of actin polymerisation here.

Despite the low rate of lateral transfer observed *in vitro*, we hypothesise that this process may have significant clinical implications since it allows *C. neoformans* to remain intracellular, thus avoiding immune recognition. Furthermore, it allows the pathogen to move from weak to healthy phagocytes, thus ensuring intracellular persistence of the pathogen even if the host cell starts to die. Finally, infected macrophage cells may travel widely throughout the host circulatory and lymphatic systems, where they interact intimately with one another and with other cell types

through transient contacts (Johnson and Huber 2002). We speculate that internalised cryptococci may use such transient contact in order to cross the BBB by direct cell-to-cell spread from adherent infected macrophages to microvascular endothelial cells (Chang et al. 2004). In fact, spreading from macrophages to other cell types during dissemination has been demonstrated for other pathogens *in vitro*. For instance, the Gram-positive bacterium *L. monocytogenes* can infect neurons by cell-to-cell spread from adherent macrophages, a more efficient process than direct invasion of neurons (Dramsı et al. 1998). Intriguingly, cell-to-cell spread of bacteria from adherent infected phagocytes to endothelial cells of the CNS has also been reported (Drevets and Leenen 2000). In conclusion, lateral transfer of *Cryptococcus* is likely to be an important step regulating phagocyte-facilitated latency and dissemination.

4.3 Discussion

Persistence is a key concept in *Cryptococcus* pathogenesis and indicates the ability of *Cryptococcus* to avoid elimination within the host (even in the presence of antifungal reagents) and thus cause chronic infections. Unfortunately, evidence for exactly how this occurs at the cellular level is not sufficient. The discovery of vomocytosis and lateral transfer provides a possible framework for how persistence may be achieved by *Cryptococcus* residing within host phagocytic cells for an extended time. A better understanding of both processes can be of considerable importance in developing new therapeutic strategies against cryptococcosis. For instance, what is the mechanism of vomocytosis? What triggers vomocytosis? Can any environmental stimuli like CO₂ concentration and cytokine prolifera alter the frequency of vomocytosis? A more detailed discussion on future experiments to address the above questions is provided in Chapter VII.

Chapter V: Intracellular Proliferation

Ma, H., Hagen, F., Stekel, D., Johnston, S. D., Sionov, E., Falk, R., Polacheck, I., Boekhout, T., May, R.C. (2009) The fatal fungal outbreak on Vancouver Island is characterized by enhanced intracellular parasitism driven by mitochondrial regulation, PNAS 106: 12980-12985

5.1 Development of a novel method to monitor intracellular proliferation for various cryptococcal strains

In order to compare the ability of cryptococcal strains to replicate inside macrophages, I developed a method to quantify intracellular proliferation of cryptococci over 64 hours without performing lengthy timelapse imaging. The method involves lysing macrophages and counting live intracellular yeast particles using a haemocytometer at fixed time points (0, 16, 24, 40, 48 and 64h post-phagocytosis, See chapter 2.4 for details) after phagocytosis. Compared to timelapse imaging and conventional colony forming unit (CFU) counting methods, this system allows multiple strains to be analysed over a large time scale and is more sensitive in detecting the clustered yeast population or yeast cells undergoing budding. Since the phagocytosis route does not affect the intracellular processing of *C. neoformans* (demonstrated in chapter III), antibody-opsonised cryptococci were used for the whole experiment to maximise the initial intracellular yeast number. However, this method is not ideal for strains with low phagocytosis index (e.g., four *C. gattii* strains listed in Appendix I), as the intracellular yeast number for these strains remained low at all timepoints, and thus the number was very vulnerable to random variations.

As the presence of any extracellular yeast cells would be counted as a part of intracellular population, it was important to remove as many extracellular yeast particles as possible at each time point. Initially, flucytosine (5-Fc) was used to kill extracellular cryptococci. 5-Fc has no intrinsic antifungal capacity, but after it has been taken up by *Cryptococcus*, it is converted into 5-fluorouracil (5-Fu), which is a pyrimidine analogue that inhibits fungal RNA and DNA synthesis. The reason we chose this specific antifungal compound is because mammalian cells do not have receptors for 5-Fc and thus only the extracellular yeast will be affected. Unfortunately, after 5-Fc treatment, we found both extracellular and intracellular growth of *Cryptococcus* was affected. That may be due to the fact that 5-Fu, converted by extracellular yeast, was released from these dead extracellular yeast particles and then taken up by macrophages. Therefore, both macrophages and intracellular cryptococci

became sick. This was confirmed by real-time timelapse imaging. We therefore modified the protocol to include an extensive PBS wash step (instead of 5-Fc treatment) every time we collect intracellular population. This had the benefit of allowing us to simultaneously monitor both intracellular and extracellular growth.

We found that for most of the strains tested, the intracellular and extracellular yeast numbers changed in a fixed pattern. Commonly, intracellular yeast numbers increased to a certain time point (usually T_{16} or T_{24} , depends on the strains) and then started declining, whereas extracellular yeast number kept increasing from T_0 to T_{64} . The decline in intracellular number was caused mainly by excessive intracellular proliferation, which led to lysis of macrophages and thus release of intracellular particles into the extracellular environment. These released yeast cells were then counted as a part of the extracellular population. It is possible that for certain strains, significant vomocytosis may contribute to the decline in intracellular number, although we do not believe this to be a major factor. Figure 15 represents a typical growth curve for both intracellular and extracellular yeast. In this figure, value A and B stand for the maximum intracellular yeast number and initially internalised yeast number respectively. In order to measure how fast each strain proliferates intracellularly, we introduced a new parameter termed IPR (short for Intracellular Proliferation Ratio). IPR is calculated by dividing value A by value B (Figure 15) to represent the relative proliferation ratio. When IPR value is smaller than one, it usually suggests that the intracellular proliferation rate is overtaken by intracellular killing rate. So far, 77 natural isolates have been studied (7 serotype A strains; 12 serotype D strains, 47 serotype B strains, 5 serotype C strains and 4 hybrids) (Appendix I). Strains were chosen to ensure representation of all serotypes and both environmental and clinical isolates. Each analysis was repeated three times with different batches of J774 cells on different days.

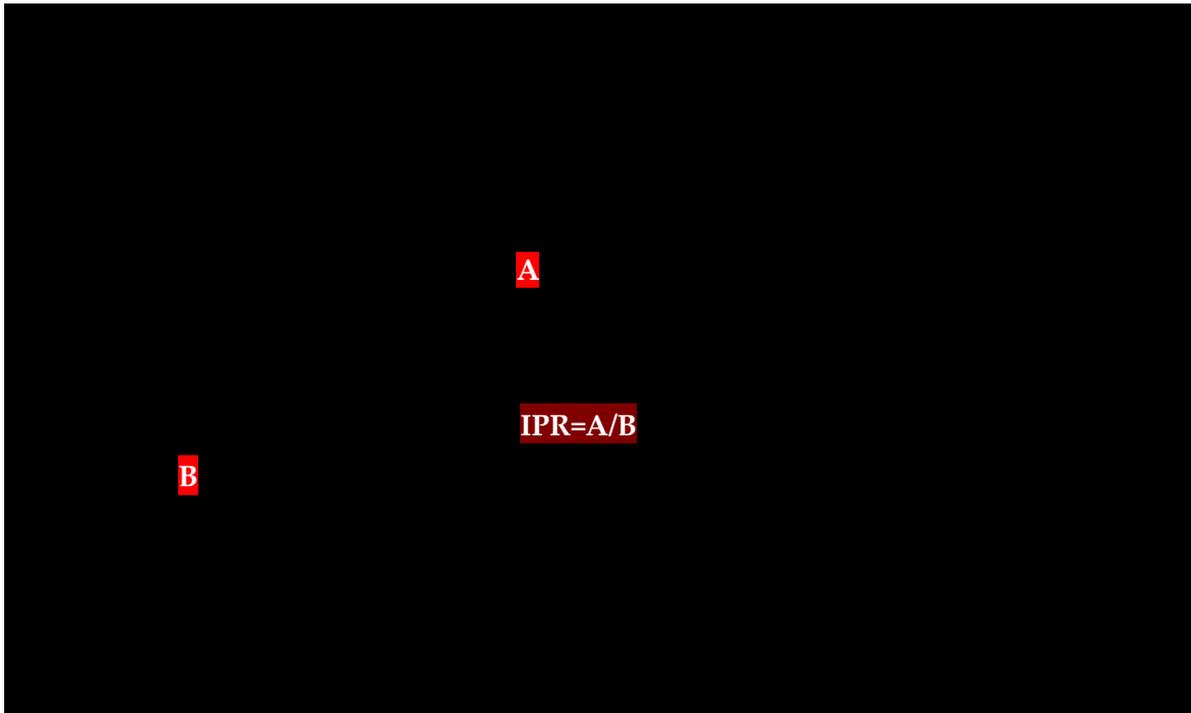


Figure 15: An example growth curve for intracellular and extracellular cryptococci after phagocytosis assay. For this specific example, intracellular yeast number reaches the maximum at T_{24} after phagocytosis. A: the maximum number of intracellular cryptococci; B: the number of initially internalised yeast particle; IPR equals to value A divided by value B. The error bars stand for the standard deviation of three or more repeats.

Furthermore, IPR values for 24 *C. gattii* strains (used for the microarray study later on) were also verified by real time timelapse imaging (Figure 16, Movies 6-8^{VI}). For each strain, more than three 16h-timelapse movies (with different culture of macrophages and yeast on different days) were made and analysed. In most of the case, IPR values agreed with the timelapse data, except for two strains (WM276 & CBS10089), which have low IPR values but moderate proliferation rates when checked with timelapse imaging. This is because with both strains there were a substantial number of extracellular yeast cells attaching to the macrophage surface at T_0 , which were extremely difficult to wash off and thus counted as intracellular population. Inevitably, the initial intracellular yeast numbers were over-estimated, which led to the underestimation of the IPR values.

Although the whole IPR assay was conducted with J774 cells, we also tested some strains with HPBMC and primary human alveolar macrophages (Table 9). The IPR values with primary macrophages (although generally lower) correlate well with the ones found in J774 cells, demonstrating the reliability of J774 as a cell line for this study (Figure 17).

^{VI} **Movie 6-8: Representative Quicktime movies showing the difference in intracellular proliferation rate (IPR) between 1) CBS8684 (IPR=0.90, Movie 6), 2) CBS6956 (IPR=1.35, Movie 7) and 3) A1M-F2932 (IPR=2.98, Movie 8).** J774 cells were exposed to individual strains (opsonised with 18B7 antibody) for two hours. Extracellular yeast cells were washed away before the macrophages were placed on an inverted microscope for imaging. The movies show time-lapse images (one frame every 90s) over the course of 18h. Within 18h, the good proliferator A1M-F2932 can produce more than 10 daughter cells, whereas the poor proliferator CBS8684 hardly replicates.

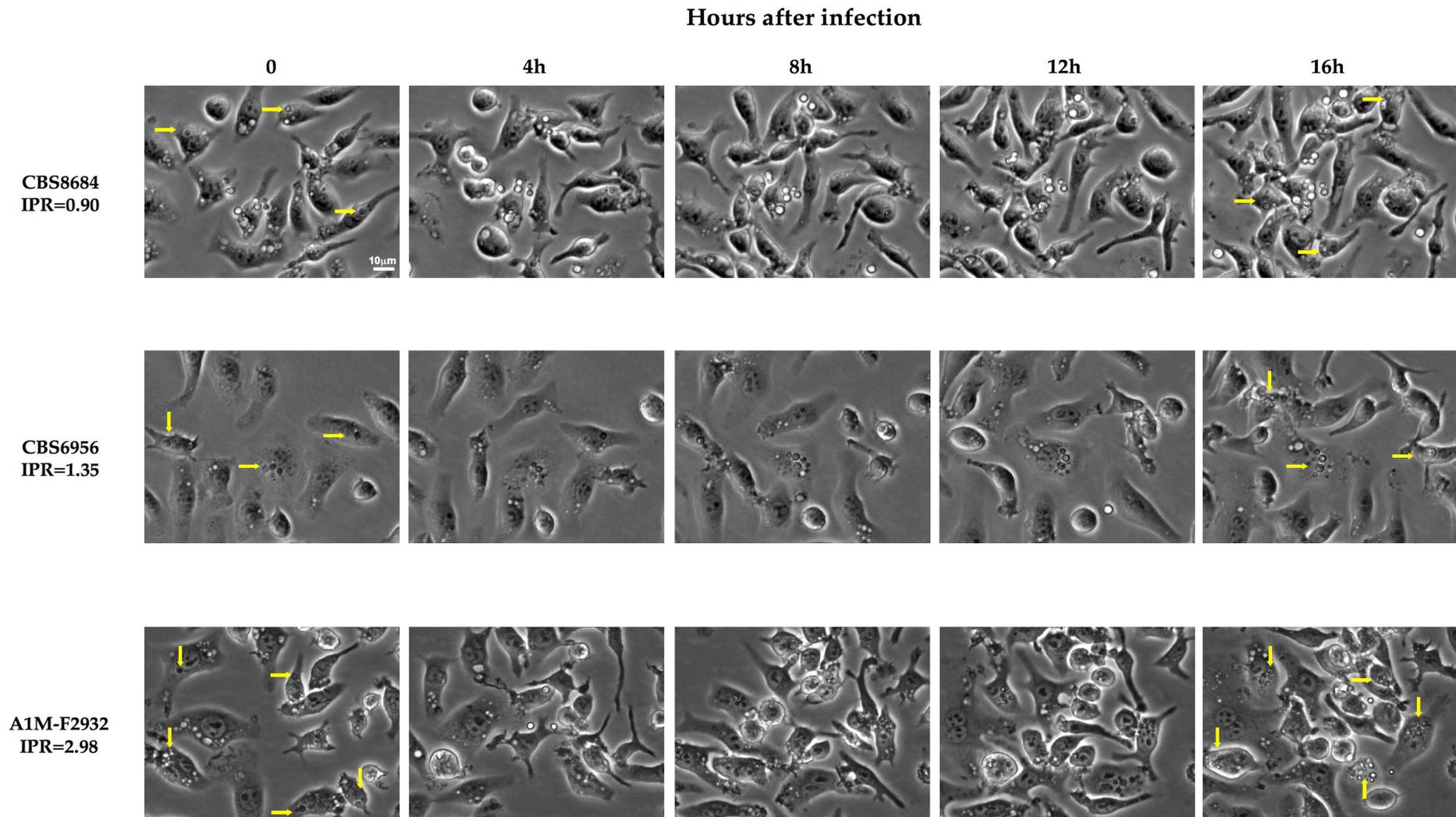


Figure 16: Representative frameshots showing the difference in IPR values between a) CBS8684 (IPR=0.90), b) CBS6956 (IPR=1.35), and c) A1M-F2932 (IPR=2.98). The panels show selected images at four hour intervals (4h, 8h, 12h and 18h after phagocytosis), derived from the Movie 6-8. Intracellular cryptococci (yellow arrows) can be seen to replicate rapidly in the good proliferator A1M-F2932, but less rapidly in CBS6956 and not at all in CBS8684.

Table 9: IPR values with HPBMC and primary human alveolar macrophages in comparison to mouse J774 macrophages. The IPR values obtained with HPBMC and alveolar macrophages are generally lower than those seen in J774 macrophages, which may be a result of the higher cryptococcal expulsion rates observed in primary cells, as reported in chapter IV.

Strains	IPR in J774	IPR in PHBMC	IPR in alveolar macrophages
CBS7750	0.93±0.24	0.72±0.33	1.25±0.46
ENV152	2.28±0.16	1.64±0.29	2.01±0.29
A1M-F2932	2.98±0.40	1.88±0.68	1.73±0.45
A1M-R271	2.04±0.48	1.59±0.37	1.42±0.28
CBS1930	1.14±0.37	0.77±0.34	0.63±0.30
CBS8684	0.90±0.37	0.42±0.52	0.37±0.17
CBS10090	1.71±0.32	1.61±0.44	/
CBS7229	0.56±0.42	0.44±0.27	/

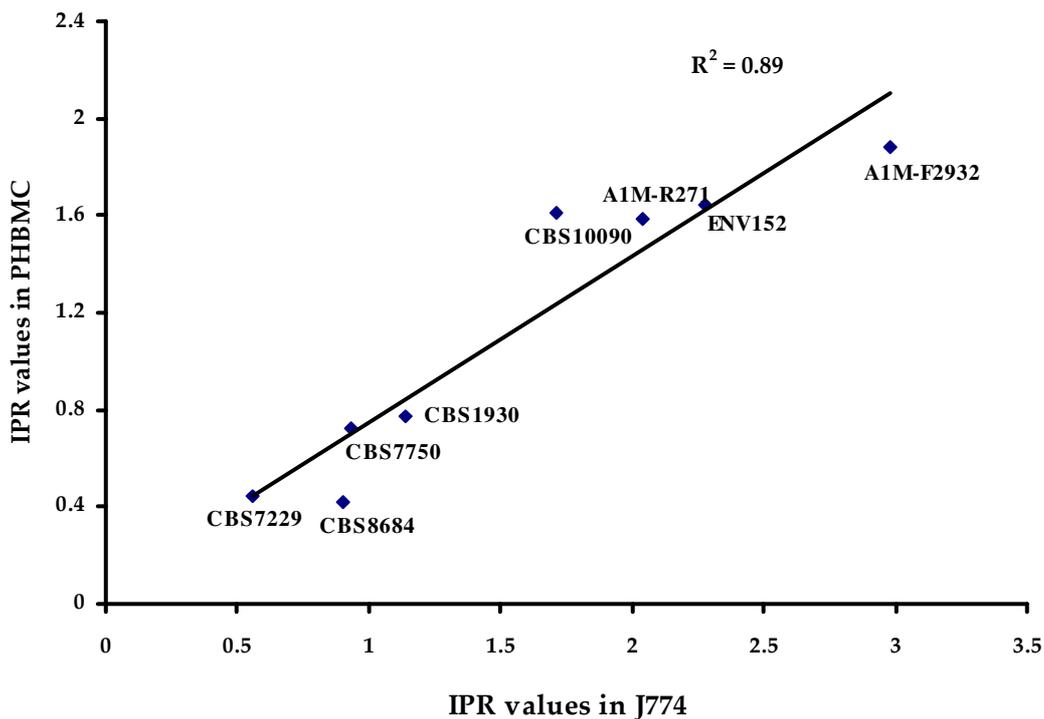


Figure 17: IPR values of eight *C. gattii* strains in HPBMC correlate significantly with those observed in J774 ($P=0.00044$, Spearman's test, $n=8$).

5.2 IPR predicts virulence of cryptococcal strains in murine models of cryptococcosis

Several early studies have highlighted the hypothesis that the presence of macrophages may exacerbate cryptococcal infection in mice because phagocytosis by macrophages can increase the dissemination capacity of *Cryptococcus*. For instance, alveolar macrophage depletion was associated with amelioration of disease in three murine strains as measured by lung fungal burden (Shao et al. 2005), and decreased the dissemination of a glucosylceramide-deficient mutant of *C. neoformans* in immunodeficient mice (Kechichian et al. 2007). We therefore went on to test whether high intracellular proliferation capacity is linked to the virulence. To do this, IPR values were compared with both published (Fraser et al. 2005) and newly-generated mouse median survival times (ST₅₀) (provided by Itzhack Polacheck's lab, Hadassah University, Israel). Remarkably, IPR and murine ST₅₀ are highly significantly correlated (Figure 18, $P=0.00017$, linear regression): a correlation that holds true for both *C. neoformans* and *C. gattii* and is independent of the mouse model used (BALB/c and A/Jrc strains). In other words, the ability to survive and proliferate inside a host macrophage contributes significantly to cryptococcal virulence in the murine model and presumably also in infected humans, as suggested by the human primary macrophage data (Table 9). Therefore, IPR values were used as a parameter to indicate virulence later on.

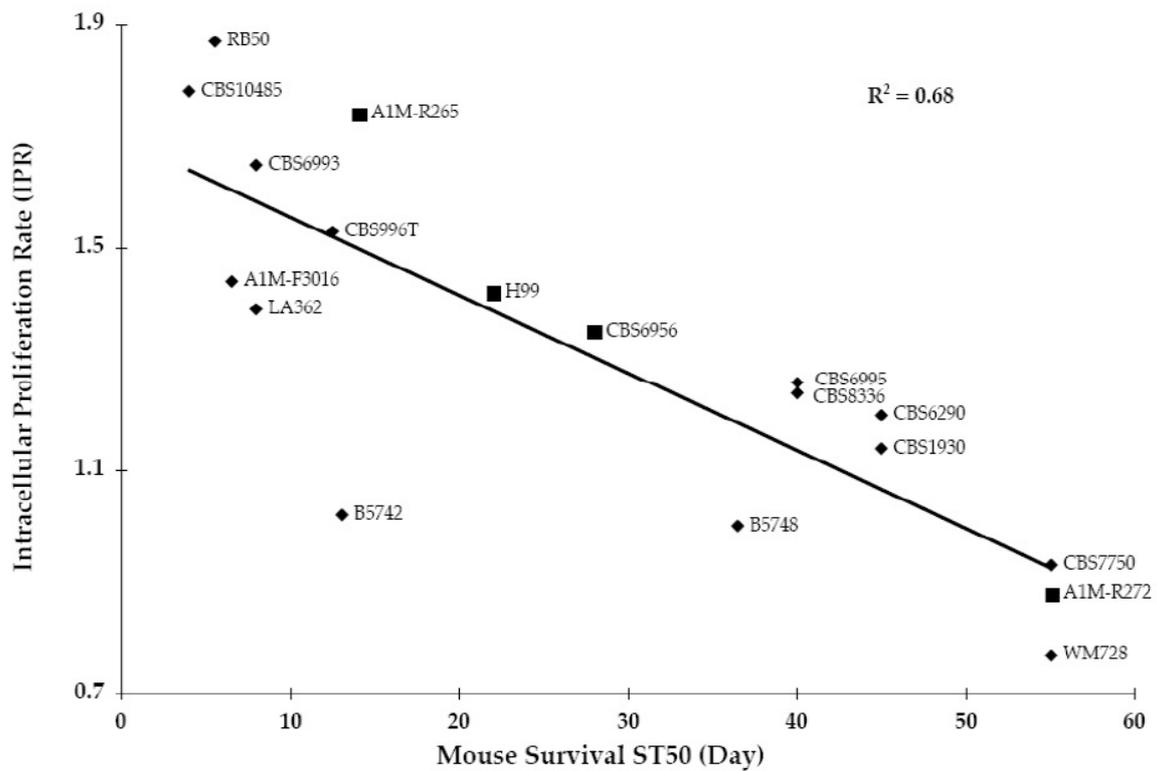


Figure 18: A significant correlation between mouse survival data (both previously published (Fraser et al. 2005) [■] and newly-generated [◆]) and IPR values. Mice survived longer when infected with strains with low IPR values as compared with animals infected with high IPR strains ($P=0.00017$, linear regression, $n=18$). For the unpublished mouse survival assays, experiments were conducted with BALB/c mice as described in the materials and methods section. Published mouse survival data are taken from (Fraser et al. 2005), where A/Jcr mice were used. For strains that did not cause mortality of 50% or more within the 45-day timeframe of the experiment, we arbitrarily assigned an ST_{50} of 55 days.

5.3 Strains from Vancouver Island outbreak show enhanced intracellular proliferation capacity

From appendix I, it is clear that IPR values vary from strain to strain. Generally speaking, the majority of *C. neoformans* strains show a great ability to proliferate intracellularly and the variation within this species is small: all the strains tested so far have IPR values larger than one. This is not surprising as *C. neoformans* isolates often only infect immunocompromised individuals and together with the prevalence of cryptococcosis caused by this species, one would expect it to be good at resisting the innate immune attack of host when the adaptive immune response is absent or compromised.

In contrast, *C. gattii* strains vary dramatically in their intracellular proliferation ability, especially within the VGII/AFLP6 subgroup (Appendix I). Some of AFLP6 strains have very high IPR values, similar to those observed with *C. neoformans*; while some AFLP6 isolates have an IPR value smaller than one, just like most of isolates belonging to the other *C. gattii* genotypes. Strikingly, all of the AFLP6A isolates from the Vancouver Island outbreak (VIO) exhibit much higher IPR values compared to other *C. gattii* strains, including AFLP6A strains isolated from other areas of the world (Figure 19). This is of particular interest because, as discussed in Chapter I, there is a high similarity (>88%) between the VIO strains and randomly chosen *C. gattii* strains from Australia and the US (Kidd et al. 2004). For instance, the Seattle strain NIH444, which was considered as the potential origin of the AFLP6A subtype, has an identical MLST profile to many isolates from Vancouver Island (e.g., A1M-R265), shores close to Vancouver Island (e.g., A1M-F3016) and other parts of North America (e.g., CBS7750) (Fraser et al. 2005; Kidd et al. 2005). However, it has a higher IPR value compared to CBS7750, but a lower IPR value compared to A1M-R265 and A1M-F3016, indicating that it cannot exploit the intracellular macrophage niche as successfully as the VIO isolates. The big variation in IPR values suggests that the IPR-value phenotype has evolved rapidly for AFLP6A isolates. Since we have linked IPR values to virulence in the murine model of cryptococcosis (Figure 18), the observed difference in IPR values

provides a possible explanation for why “ancestral” AFLP6A strains share the VIO genotype and yet do not lead to disease outbreaks.

Besides VIO strains, three Brazilian strains (LMM261, LMM265 and LMM645) tested in this project were also found to be capable of surviving inside macrophages (Figure 19). Currently, there are two theories on the origin of VIO strains: ‘newly made’ via same-sex mating, and ‘already made’ and migrated from South America (see Chapter I for detail). As these randomly examined Brazilian strains are as virulent as VIO isolates, it seems possible that the migration theory is more likely. Another interesting piece of data comes from a recent study on the mitochondrial genome (mtDNA) of AFLP6 strains. Xu *et al* found that mtDNA of three MATa strains (LA499, LA567 and LA584, all from Colombia) share identical mitochondrial alleles at five loci with each other and with strain A1M-R265. In contrast, none of the three Australian AFLP6 strains examined have the same mitochondrial genotype (Xu et al. 2009). Since early studies have shown LA499, LA567, LA584 and A1M-R265 share identical alleles at two of the three nuclear loci which are not associated with mating type locus (Kidd et al. 2005) and no MATa strains of the AFLP6 group have been found in North America, it is likely that the VIO lineage is a result of opposite-sex mating between strains in South America which then spread to North America (Xu et al. 2009). However, we still cannot eliminate the possibility of same-sex mating because only a small number of yeast isolates were tested in our study and in Xu *et al*'s study.

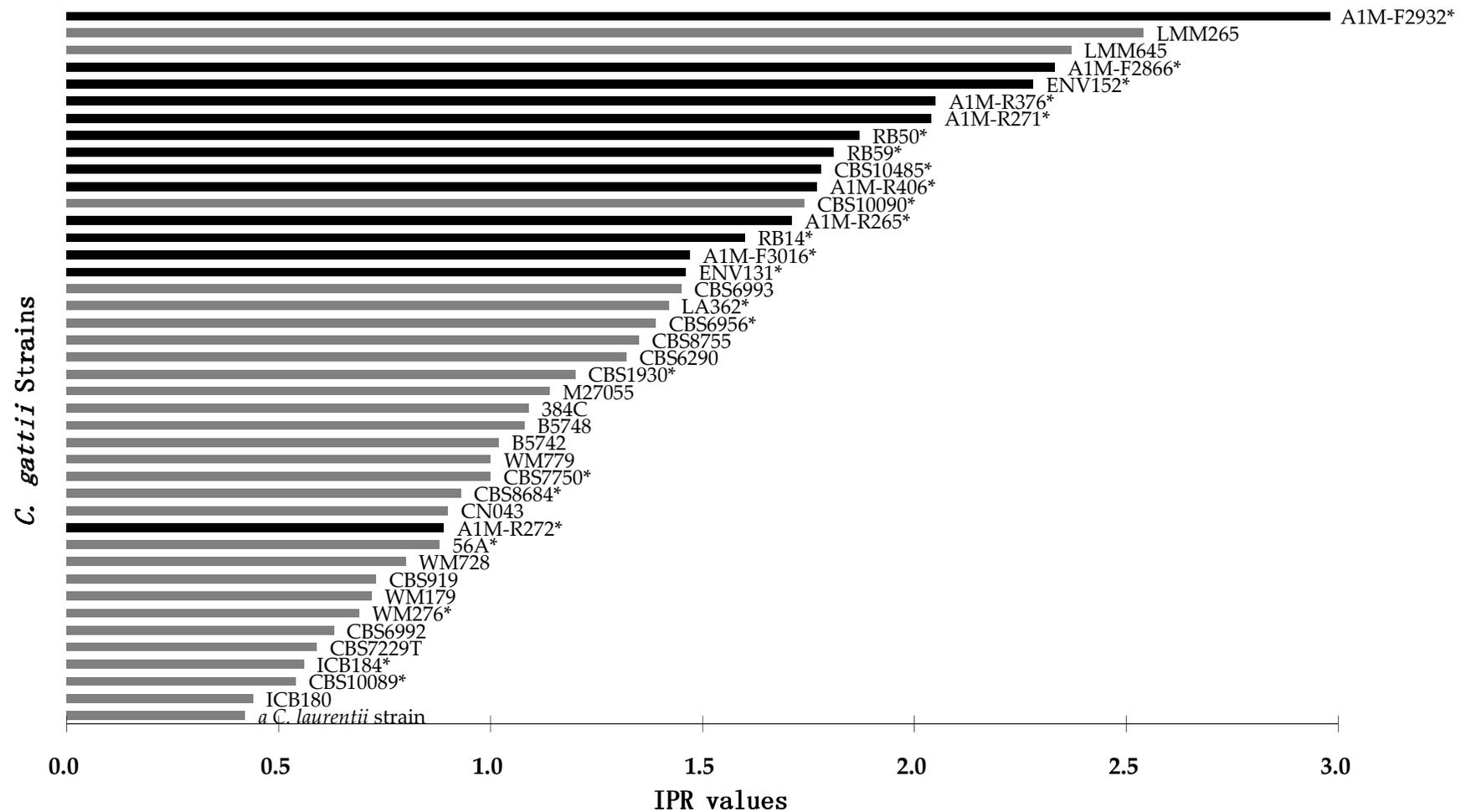


Figure 19: Significant inter-strain variation in IPR occurs within the *C. gattii* species (n=42). Black bars represent VIO isolates, which proliferate far better than other *C. gattii* strains (grey bars). The only exception was A1M-R272, which belongs to the minor (AFLP6B) group of the outbreak and has previously been shown to be less virulent than other VIO strains (Fraser et al. 2005). An asterisk (*) denotes strains (whose IPR values were verified by timelapse imaging) used for the microarray study.

5.4 Molecular cause of hypervirulence associated with VIO strains

5.4.1 The hypervirulence is not directly linked to any known virulence factors

To study the cause of hypervirulence within the VIO isolates, we undertook a high-throughput analysis of well-characterised virulence traits at 37°C (capsule size, melanin production, phospholipase activity, proteinase activity and other enzymatic activities) in 39 *C. gattii* strains representing both VIO and non-VIO isolates. However, there was no consistent difference between VIO and non-VIO isolates belonging to this genotype in any of the traits tested (Table 10), suggesting that the virulence of VIO strains does not simply result from over-expression of these individual cryptococcal pathogenicity factors. Such a finding is consistent with recent data suggesting that many cryptococcal virulence genes/factors remain to be discovered (Liu et al. 2008), and hypervirulence is likely to due to the combinational effect of these factors.

Table 10: Phenotypic analysis of 39 *C. gattii* stains with known IPRs. A) Activity of 19 enzymes measured by the API enzymatic assay; B) Proteinase and phospholipase activities, capsule and melanin productions were measured as described in Chapter 2.10. Linear regression analysis revealed that neither proteinase activity, phospholipase activity or capsule production showed a direct correlation with IPR. Melanin production correlated with IPR values when comparing all *C. gattii* strains, but this correlation was lost when only AFLP6 strains were studied. These data indicate that differential melanisation is an important virulence factor within the *C. gattii* species, but cannot explain the hypervirulence phenotype of the VIO isolates.

A)

Strain	AFLP genotype	Negative control	Alkaline phosphatase	Esterase (C4)	Esterase lipase (C8)	Lipase (C14)	Leucine arylamidase	Valine arylamidase	Cystine arylamidase	Trypsin	alpha-chymotrypsin	Acid phosphatase	Naphthol-AS-BI-phosphohydrolase	alpha-galactosidase	beta-galactosidase	beta-glucuronidase	alpha-glucosidase	beta-glucosidase	N-acetyl-beta-glucosaminidase	alpha-mannosidase	alpha-fucosidase
56A	4	0	0	1	1	0	1	0	0	0	0	2	1	0	0	0	0	1	0	0	0
CBS6290	4	0	0	2	2	0	1	0	0	0	0	2	1	0	0	0	0	1	0	0	0
CBS6992	4	0	1	2	2	0	2	0	0	0	0	3	3	0	0	0	1	2	0	0	0
CBS7229 ^T	4	0	0	2	1	0	2	0	0	0	0	4	2	0	0	1	0	1	0	0	0
CBS919 ^T	4	0	0	1	1	0	2	0	0	0	0	3	2	0	0	2	0	0	0	0	0
WM179	4	0	0	2	2	0	2	0	0	0	0	4	3	0	0	1	0	1	0	0	0
WM276	4	0	1	2	2	0	2	0	0	0	0	4	2	0	0	1	1	2	0	0	0
384C	5	0	0	2	2	0	2	0	0	0	0	3	1	0	0	1	2	2	0	0	0
CBS6993	5	0	0	1	2	0	1	0	0	0	0	2	1	0	0	1	2	1	0	0	0
CBS8755	5	0	0	2	2	0	0	1	0	0	0	4	2	0	0	0	2	2	0	0	0

CN043	5	0	0	2	2	0	2	0	0	0	0	2	1	0	0	1	1	1	0	0	0
WM728	5	0	0	2	2	0	2	0	0	0	0	3	1	0	1	0	1	2	0	0	0
A1M-F2866	6	0	0	2	2	0	2	0	0	0	0	4	2	0	0	1	1	1	0	0	0
A1M-F2932	6	0	1	2	2	0	2	0	1	0	0	3	2	0	0	1	2	1	0	0	0
A1M-F3016	6	0	0	2	2	0	2	0	0	0	0	3	2	0	1	2	1	2	0	0	0
A1M-R265	6	0	1	2	2	1	2	1	0	0	0	4	2	0	2	1	1	2	0	0	0
A1M-R271	6	0	0	2	2	1	2	0	0	0	0	3	3	0	0	1	1	1	0	0	0
A1M-R272	6	0	2	2	2	0	3	0	1	0	0	3	2	0	1	2	1	2	0	0	0
A1M-R376	6	0	1	2	2	1	2	0	0	0	0	3	2	0	0	0	2	1	0	0	0
A1M-R406	6	0	1	2	2	0	2	0	0	0	0	3	2	0	0	1	2	2	0	0	0
CBS10089	6	0	0	2	2	0	2	0	0	0	0	3	1	0	0	3	1	1	0	0	0
CBS10090	6	0	1	2	2	0	2	0	1	0	0	3	2	0	0	2	1	2	0	0	0
CBS10485	6	0	1	2	2	0	2	0	0	0	0	3	1	0	0	1	1	1	0	0	0
CBS1930	6	0	1	2	2	0	3	0	1	0	0	3	1	0	0	1	2	2	0	0	0
CBS6956	6	0	0	2	2	0	1	0	0	0	0	3	1	0	0	0	1	1	0	0	0
CBS7750	6	0	0	2	2	0	1	0	0	0	0	3	1	0	0	0	2	1	0	0	0
CBS8684	6	0	1	2	2	1	2	0	0	0	0	3	2	0	0	3	2	2	0	0	0
ENV131	6	0	1	2	2	0	2	0	0	0	0	4	3	0	0	1	2	2	0	0	0
ENV152	6	0	0	2	2	0	2	0	1	0	0	4	2	0	0	1	1	1	0	0	0
ICB180	6	0	1	2	2	0	2	0	0	0	0	3	2	1	0	0	1	0	0	0	0
ICB184	6	0	1	2	2	0	3	0	0	0	0	3	2	0	0	3	1	1	0	0	0
LA362	6	0	0	1	2	0	2	0	0	0	0	4	2	0	0	1	1	1	0	0	0
RB14	6	0	1	2	2	0	2	0	1	0	0	4	2	0	0	1	1	1	0	0	0
RB50	6	0	0	2	2	0	2	0	0	0	0	4	2	0	0	0	1	2	0	0	0
RB59	6	0	0	2	2	0	2	1	0	0	0	5	2	0	0	1	2	2	0	0	0
B5742	7	0	0	2	1	0	1	0	0	0	0	2	1	0	0	0	1	1	0	0	0
B5748	7	0	0	2	1	0	1	0	0	0	0	2	1	0	0	0	1	0	0	0	0
CBS10101	7	0	0	1	1	0	2	0	0	0	0	3	2	0	0	1	1	2	0	0	0
M27055	7	0	0	2	2	0	2	0	0	0	0	4	1	0	0	1	0	2	0	0	0

B)

Strain name	AFLP Genotype	Proteinase	Phospholipase	Capsule index	Melanin										
					Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	
56A	4	0.197±0.010	1.000±0.000	0.591±0.073	0	0	0	0	0	0	0	0	1	1	1
CBS6290	4	0.202±0.022	0.792±0.039	0.531±0.062	0	0	0	0	0	0	1	1	1	2	2
CBS6992	4	0.152±0.018	0.957±0.067	0.804±0.090	0	0	0	0	0	0	0	0	0	0	1
CBS7229 ^T	4	0.190±0.019	0.751±0.055	0.663±0.081	0	0	0	0	0	0	0	0	1	2	3
CBS919 ^T	4	0.162±0.010	0.783±0.031	0.607±0.062	0	1	1	1	2	1	1	1	1	2	2
WM179	4	0.239±0.018	0.826±0.191	0.611±0.138	0	0	0	0	1	1	1	1	1	2	3
WM276	4	0.229±0.053	0.749±0.103	0.715±0.109	0	0	0	0	0	0	0	0	0	0	0
384C	5	0.194±0.027	0.810±0.074	0.719±0.102	0	0	0	0	0	0	0	0	0	1	2
CBS6993	5	0.201±0.053	0.733±0.086	0.746±0.094	0	0	0	0	0	0	0	0	1	2	2
CBS8755	5	0.192±0.009	0.954±0.075	0.826±0.101	0	0	0	0	0	0	0	0	0	0	0
CN043	5	0.180±0.013	0.746±0.065	0.776±0.113	0	0	0	0	0	0	0	0	0	0	0
WM728	5	0.229±0.018	1.000±0.000	0.780±0.113	0	0	0	0	0	0	0	1	2	4	5
A1M-F2866	6	0.210±0.009	0.706±0.035	0.644±0.071	0	2	2	3	4	4	5	5	5	5	5
A1M-F2932	6	0.235±0.031	0.768±0.072	0.596±0.091	0	2	2	3	4	4	5	5	5	5	5
A1M-F3016	6	0.200±0.040	0.770±0.035	0.631±0.062	0	1	1	2	3	5	5	5	5	5	5
A1M-R265	6	0.187±0.022	0.739±0.031	0.665±0.080	0	1	2	2	5	5	5	5	5	5	5
A1M-R271	6	0.188±0.015	0.690±0.066	0.659±0.081	0	2	3	4	4	5	5	5	5	5	5
A1M-R272	6	0.176±0.012	0.681±0.028	0.588±0.069	0	2	3	3	4	4	4	5	5	5	5
A1M-R376	6	0.186±0.021	0.702±0.097	0.667±0.057	0	3	3	3	4	4	5	5	5	5	5
A1M-R406	6	0.217±0.023	0.751±0.082	0.698±0.071	0	4	4	4	4	4	5	5	5	5	5
CBS10089	6	0.215±0.025	0.634±0.106	0.607±0.108	0	1	2	4	4	5	5	5	5	5	5
CBS10090	6	0.207±0.016	0.707±0.077	0.644±0.094	0	1	1	4	4	5	5	5	5	5	5
CBS10485	6	0.195±0.014	0.754±0.034	0.586±0.071	0	2	3	3	4	4	4	4	4	4	4

CBS1930	6	0.226±0.020	0.939±0.094	0.585±0.048	0	1	1	1	2	2	2	2	3	3
CBS6956	6	0.204±0.047	0.753±0.079	0.584±0.065	0	2	3	5	5	5	5	5	5	5
CBS7750	6	0.190±0.008	1.000±0.000	0.654±0.082	0	1	3	3	3	4	5	5	5	5
CBS8684	6	0.288±0.026	1.000±0.000	0.621±0.101	0	0	1	1	1	1	1	1	1	1
ENV131	6	0.179±0.013	0.689±0.124	0.607±0.102	0	1	1	3	4	4	4	5	5	5
ENV152	6	0.167±0.031	0.894±0.088	0.627±0.048	0	2	2	3	4	4	4	4	4	4
ICB180	6	0.211±0.031	0.719±0.032	0.595±0.074	0	2	2	2	3	3	4	5	5	5
ICB184	6	0.189±0.012	0.728±0.101	0.615±0.064	0	1	2	2	3	4	5	5	5	5
LA362	6	0.207±0.030	0.672±0.119	0.632±0.063	0	2	2	3	4	4	4	4	4	4
RB14	6	0.196±0.019	0.676±0.028	0.642±0.122	0	1	1	2	3	4	5	5	5	5
RB50	6	0.224±0.044	0.660±0.174	0.619±0.113	0	1	1	4	4	4	4	4	4	4
RB59	6	0.204±0.023	0.630±0.157	0.674±0.063	0	2	3	4	4	5	5	5	5	5
B5742	7	0.256±0.017	1.000±0.000	0.514±0.053	0	0	0	0	0	0	0	1	2	2
B5748	7	0.207±0.038	0.898±0.080	0.617±0.076	0	0	0	0	0	2	3	5	5	5
CBS10101	7	0.284±0.064	0.763±0.062	0.966±0.028	0	0	0	0	0	0	1	2	4	5
M27055	7	0.208±0.018	0.739±0.076	0.602±0.117	0	0	0	0	0	1	1	2	3	3
<i>P</i> value within <i>C. gattii</i> strains		0.985	0.085	0.657	0.000893***									
<i>P</i> value within AFLP6 strains		0.954	0.577	0.057	0.1872									

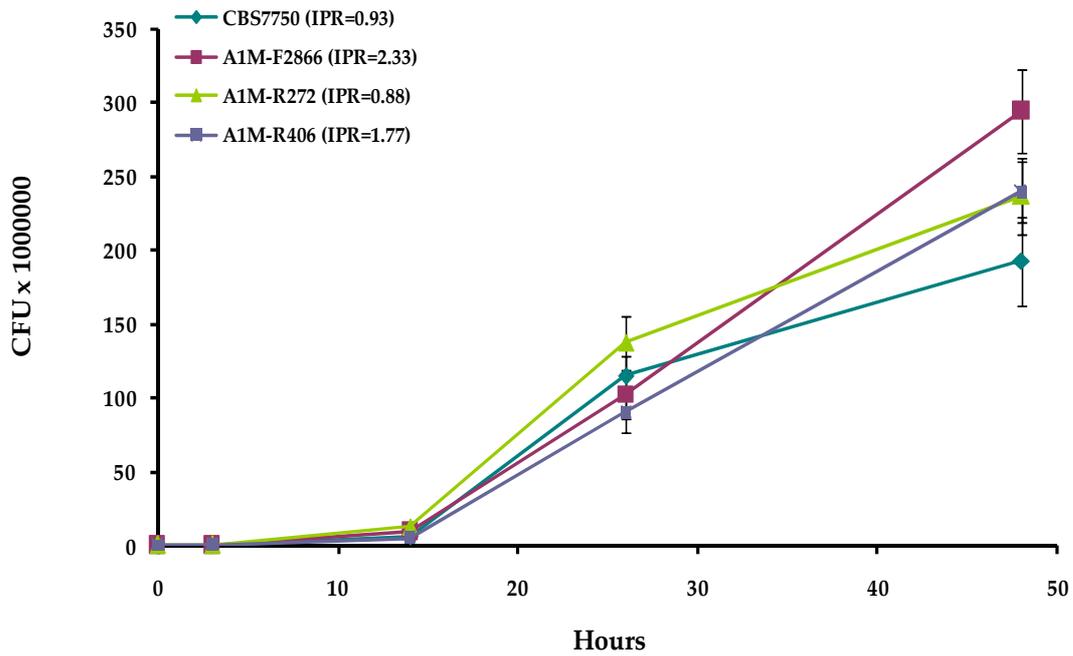
5.4.2 VIO hypervirulence is not due to shorter generation time or better utilisation of macrophage nutrition

The growth rate of selected cryptococcal strains were measured over 48h in YPD (at both 25°C (shaking) and 37°C (non-shaking with 5.0% CO₂)), and in the presence of macrophage lysate (at 37°C) in order to test whether the observed high IPR values are due to shorter generation time or better utilisation of intracellular nutrition by VIO strains. From Figure 20 and Table 11, it is clear that the growth rates for various isolates in YPD or in macrophage lysate do not correlate with IPR values. Strains that differ significantly in intracellular proliferation capacity showed similar growth rates in YPD and macrophage lysate (e.g., A1M-F2932 & CBS7750 in Table 11, and A1M-F2866 & A1M-R272 in Figure 20). We therefore conclude that the enhanced intracellular proliferation is not because VIO strains have a shorter generation time or can utilise intracellular macrophage nutrients in a better way.

Table 11: CFU counting of eleven *C. gattii* VGII strains in macrophage lysates at 37°C over 48 hours.

Strains	Initial CFU (x1000)	CFU 24h in macrophage lysate (x1000)	CFU 48h in macrophage lysate (x1000)	IPR
ENV152	100	1660±280	3060±140	2.28
A1M-F2932	100	1220±270	1760±420	2.98
CBS7750	100	1040±110	1760±350	0.93
A1M-R271	100	1030±200	1610±220	2.04
CBS8684	100	990±130	1930±310	0.90
CBS1930	100	1470±200	2430±270	1.14
A1M-R265	100	1550±80	2520±150	1.74
A1M-F2866	100	1580±80	1970±450	2.33
CBS6956	100	890±270	1600±290	1.35
RB14	100	1010±120	1340±280	1.60
ICB184	100	1240±240	1360±130	0.54

A)



B)

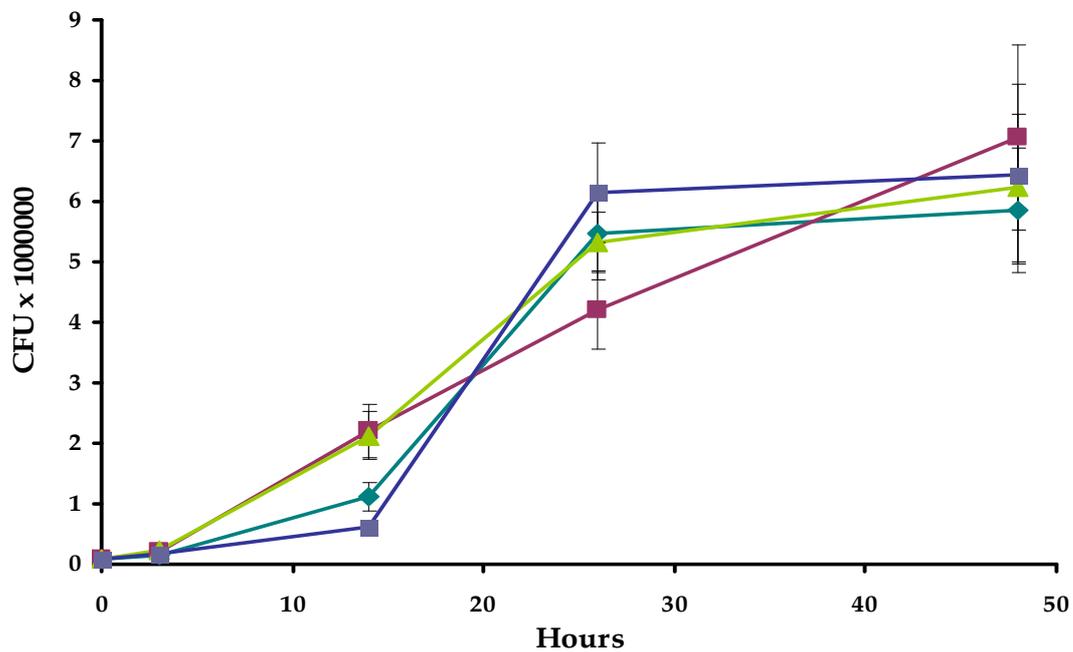


Figure 20: Growth rates of four *C. gattii* strains in YPD: A) at 25°C (shaking) and; B) at 37°C with 5.0% CO₂ (non-shaking). Among these four isolates, two have high IPR and other two have low IPR values. Nevertheless, they grow at a very similar rate in YPD at both temperatures, so VIO and non-VIO strains do not differ significantly in terms of generation time.

5.4.3 Microarray study: Identification of genes associated with enhanced intracellular parasitism

In order to investigate the molecular basis of enhanced intracellular proliferation (and thus hypervirulence) in the VIO isolates, we used custom-designed *C. gattii* whole-genome tiling microarrays to conduct transcriptional profiling of 24 *C. gattii* strains (21 AFLP6 and 3 AFLP4 isolates, listed in Figure 19) recovered from within J774 macrophages. These strains are genetically very similar but show a wide range of IPR values (Appendix I and Figure 19). RNA samples from each strain were isolated from intracellular cryptococcal cells 24h after infection and competitively hybridised against a pooled sample containing equal quantities of RNA from all 24 strains (See chapter II for details on array design and experimental details). At this timepoint, the intracellular number reaches the peak for the majority of strains. Linear regression identified 1367 target loci in the genome of A1M-R265 strains whose expression showed a significant correlation with IPR values. 224 of these have predicted function annotations, most of which can be categorised into one of five groups: carbohydrate metabolism, stress response, vesicle/vacuole fusion and transport, protein degradation and synthesis, and nucleotide metabolism (Appendix II). Interestingly, although our phenotypic analysis demonstrated that no single known virulence factor was responsible for the hypervirulence of the VIO isolates, several of these genes (e.g., *PLB1*, *CRG1*, capsule related genes, genes on the mating type locus, components of signalling pathways that are known to regulate virulence) showed a significant expression correlation with IPR values, suggesting that they may synergistically contribute to virulence within the AFLP6 lineage.

Due to poor annotation of the *C. gattii* genome, BLAST searching did not identify the function of most of the candidate genes we identified (Appendix II). We therefore mapped the genomic distribution of all the microarray hits by localising them on the 28 supercontigs of A1M-R265 genome. The distribution was homogenous across all supercontigs, with the remarkable exception of supercontig 25, corresponding to the mitochondrial genome (mtDNA), which was ten-fold over-represented (Figure 21, $P=10^{-24}$, Chi-square test). Initially, we suspected that the observed over-representation

of mitochondrial sequences could be due to an increase in mtDNA copy number, as a recent study showed that presence of reactive oxygen species is able to regulate mtDNA copy number in isolated yeast mitochondria by triggering recombination-mediated replication (Hori et al. 2009). However, this is not the case here because quantitative PCR demonstrates that AFLP6 strains with different IPR values nonetheless have similar mtDNA/genomic DNA ratios (Table 12). In addition, mtDNA copy number does not change significantly following intracellular replication, eliminating this as a possible explanation for the overrepresentation of mitochondrial hits (Table 12). Furthermore, the expression of many nuclear-encoded proteins that function in mitochondria is also up-regulated in the VIO strains (e.g., respiratory genes and mitochondrial proteins as listed in Appendix II), which is particularly relevant given early *in vivo* transcriptional profiling showing the high expression of several respiratory genes by this yeast at the site of a central nervous system infection (Steen et al. 2003; Toffaletti et al. 2003). Taken together, we propose that mitochondrial function might be critical for the virulence of this lineage.

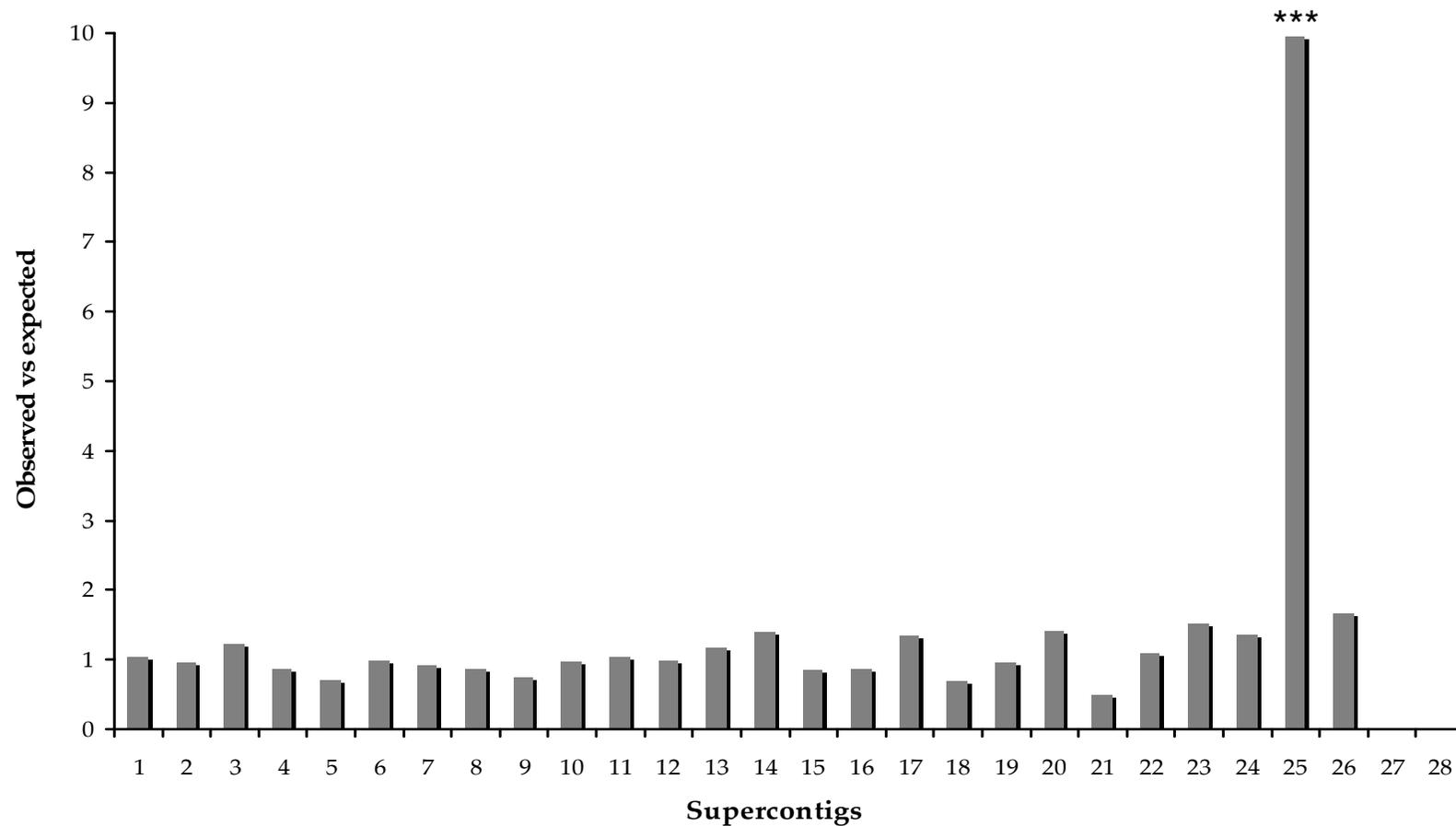


Figure 21: A1M-R265 Supercontig 25 is highly over-represented in the transcriptional analysis ($P=10^{-24}$, Chi-square test). 1367 probes whose expression showed significant correlation with IPR values were mapped onto the 28 supercontigs of the A1M-R265 genome and compared to the expected hit rate (assuming a random probe distribution) using the *Chi*-square test. Supercontig 25 is ten-fold over-represented.

Table 12: Real-time PCR to quantify mtDNA copy number per cell in *C. gattii*. Cryptococcal mtDNA copy number (ranging from 400-1600 copies per cell) does not vary among different AFLP6 strains (2 VIO isolates and 2 other AFLP6 strains) or change following intracellular replication within host macrophages.

Strains	RT-cycle difference between mitochondrial and nuclear loci	
	Intracellular yeast cells	Yeast cells grown in YPD at 25°C
ENV152	9.75±1.01	9.77±0.61
A1M-R271	8.74±0.38	10.68±1.04
CBS7750	9.44±0.19	10.14±1.59
CBS8684	10.71±0.63	10.44±0.53

5.4.4 Mitochondrial morphology changes in VIO strains following phagocytosis

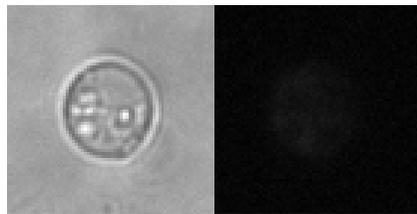
Given the over-representation of mitochondrial genes in the VIO strains, we analysed the morphology of cryptococcal mitochondria both before and after phagocytosis. Surprisingly, we observed a striking difference in mitochondrial morphology between the VIO and non-VIO strains following intracellular parasitism. During *in vitro* growth at 25°C (in YPD shaking, normal growth condition) or 37°C (in DMEM + 5.0% CO₂ in the absence of macrophages), more than 95% of cryptococcal cells have mitochondria with morphologies that we termed either “diffuse” or “globular” (Figure 22A), regardless of the strain tested. However, after growth inside J774 macrophages for a period of time, VIO strains developed a tubular mitochondrial morphology (Figure 22) that was rarely exhibited by non-VIO isolates ($P < 0.0001$, chi-square test for VIO versus non-VIO strains). Remarkably the percentage of cryptococcal cells exhibiting tubular mitochondria shows a strong, linear correlation with IPR values (Table 13 & Figure 23, $P = 0.00021$, linear regression), a relationship that raises the possibility of accurately predicting the virulence of novel cryptococcal genotypes based on a simple, one-step observation of mitochondrial morphology.

Mitochondrial tubular morphology is generally thought to result from mitochondrial fusion, a phenomenon that allows mitochondria within a cell to cooperate with each other (Chen et al. 2003) and protects cells from the detrimental effect of mtDNA mutations by allowing functional complementation of mtDNA gene products (Chan

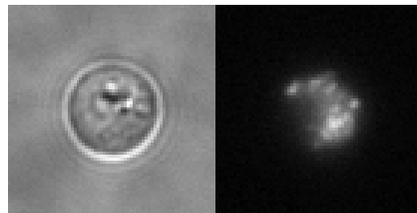
2006; Sato et al. 2006). Moreover, mitochondrial fusion has been found to protect cells from cell death (Karbowski et al. 2004; Sugioka et al. 2004). It therefore appears likely that the altered mitochondrial gene expression and morphology seen in the VIO strains is a protective response that facilitates rapid intracellular growth and thus enhanced virulence.

In yeast and mammals, several factors including Drp1/Dnm1 and Mfn/Fzo1 are known to regulate mitochondrial morphology by controlling membrane fission or fusion (Okamoto and Shaw 2005). Interestingly, we find that *FZO1* is up-regulated in the VIO strains (the gene appeared four times in the top 5000 candidates). Fzo (*Fussy onion* gene), isolated from a screen for genes involved in *Drosophila* spermatogenesis, is the first player identified in mitochondrial fusion (Hales and Fuller 1997). In mammals and yeast, it is known as mitofusin and Fzo1p respectively (Hermann et al. 1998; Santel and Fuller 2001). The protein contains a GTPase domain (exposed to the cytoplasm) at the N-terminus and a bipartite transmembrane domain (which spans the mitochondrial outer membrane twice) near the C-terminus (Fritz et al. 2001; Rojo et al. 2002). In *S. cerevisiae*, the *fzo1Δ* mutant is highly fragmented due to ongoing mitochondrial fission (Hermann et al. 1998; Rapaport et al. 1998) and over-expression of Fzo1p alters the fusion/fission protein ratio and thus inhibits cell apoptosis (Sugioka et al. 2004). Therefore, higher amounts of Fzo1p in VIO strains could be responsible for the tubular formation of mitochondria and also lead to a higher fusion/fission protein ratio, which is essential to increase the resistance of mitochondria and cells to apoptotic stimulation. However, a recent study by systematic screening of *C. elegans* mitochondrial proteins demonstrated that most fundamental mitochondrial functions, including metabolism and oxidative phosphorylation, are also necessary for the maintenance of mitochondrial tubular networks: of 719 genes predicted to code for most of the mitochondrial proteins, knockdown of >80% of them caused abnormal mitochondrial morphology, especially fragmentation (Ichishita et al. 2008). Hence the tubular morphology observed in this scenario could be a result of up-regulation of many candidate genes listed in Appendix II.

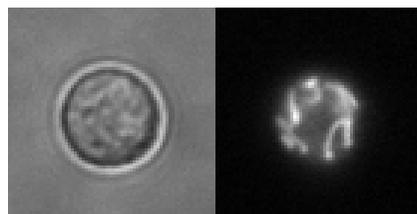
A)



Diffuse



Globular



Tubular

B)

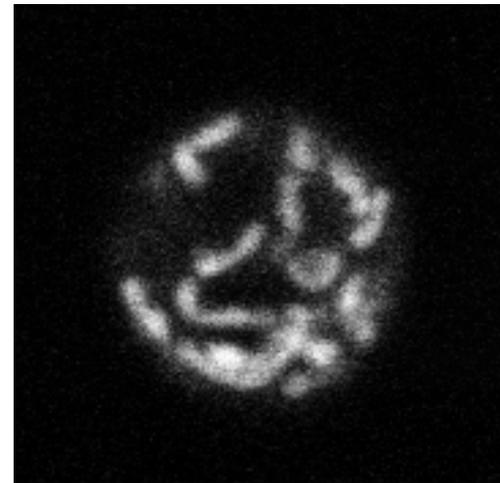


Figure 22: A) Representative images of the three different mitochondrial morphologies observed (diffuse, globular and tubular); B) A three dimensional confocal projection showing the tubular mitochondrial morphology of a VIO strain. *C. gattii* strain ENV152 (IPR=2.28), isolated 24h after growing within J774 macrophages and labeled with MitoTracker.

Table 13: The percentage of intracellular yeast cells with tubular mitochondria in six VGII strains (3 VIO and 3 non-VIO isolates). For each strain, a random selection of cryptococcal cells were scored blindly for the three different mitochondrial morphologies. Mitochondria with a tubular morphology were found only rarely in strains with low IPR values (non-VIO strains) or in strains (both VIO and non-VIO) that had been grown extracellularly.

Strains	Diffuse	Globular	Tubular	Total	Percentage of tubular	IPR values
CBS8684	15	46	0	61	0%	0.90
CBS7750	9	58	4	71	6%	0.93
CBS1930	10	44	6	60	10%	1.14
A1M-R271	7	32	24	63	38%	2.04
ENV152	11	28	39	78	50%	2.25
A1M-F2932	14	21	54	89	61%	2.98

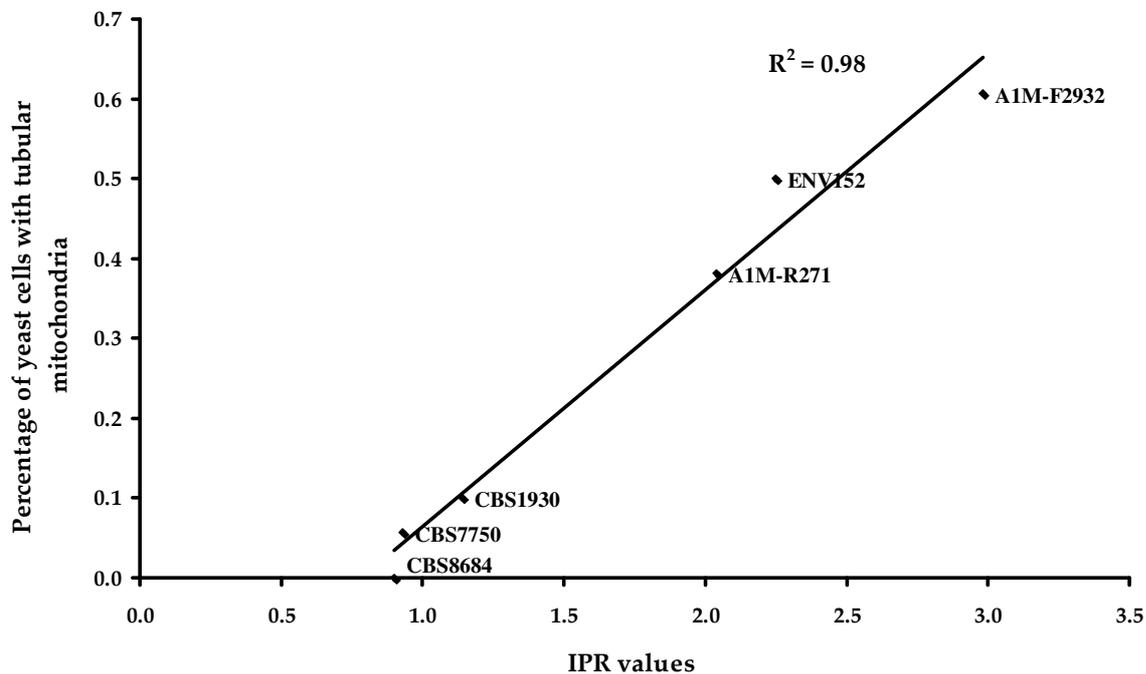


Figure 23: The percentage of intracellular yeast exhibiting a tubular mitochondrial morphology correlates significantly with IPR values ($P=0.00021$, linear regression, $n=6$).

5.5 Discussion

This study demonstrates a link between the intracellular parasitism of phagocytes and the virulence of a facultative intracellular pathogen in a murine model of infection. Moreover, we propose that a recent change in mitochondrial regulation within the *C. gattii* lineage has led to an increased intracellular proliferative capacity, resulting in the hypervirulent phenotype that underlies the VIO. This change leads to a quantitative linear relationship between intracellular proliferation ratio, mitochondrial gene expression, mitochondrial morphology and virulence.

The mitochondrion, as an essential organelle, has been linked to various cellular activities, such as intermediary metabolism and respiration, cell signalling, iron metabolism, apoptosis and aging (Cortopassi and Wong 1999; Green and Reed 1998). However, its role in modulating virulence of pathogens is unclear. Indeed such a role has been reported only once before, in *Heterobasidion annosum*, which, like *Cryptococcus*, is also a basidiomycete pathogen but of plants rather than animals (Olson and Stenlid 2001). Nevertheless, mitochondria can represent a source of rapid evolution of virulence in emerging pathogens because the mutation rate for the mtDNA genome is much higher than the nuclear genome due to a) the high amount of reactive oxygen species within mitochondria that can severely damage DNA and, b) the lack of replication error repair mechanisms within mitochondria (Denver et al. 2000; Haag-Liautard et al. 2008; Lynch et al. 2008). Within the *Cryptococcus* genus, mitochondrial genomes show conserved gene synteny but very different sizes (e.g., 34.7kb for *C. gattii*, 32kb for *C. neoformans* var. *neoformans*, and 24kb for *C. neoformans* var. *grubii*) (Litter et al. 2005). Moreover, a significantly higher within-lineage divergence for the mitochondrial genes than those for the nuclear genes within both the VGI and VGII lineages has been observed, indicating that they are under intense selection (Xu et al. 2009). This is further supported by the recent evidence of mitochondrial recombination in *C. gattii* (Bovers et al. 2009; Xu et al. 2009). For more information on the mitochondrial genome of *Cryptococcus*, see Chapter VI for detail.

Mitochondria are dynamic organelles that frequently divide and fuse with each other and it is thought that such behaviours are coordinated with their metabolic function (Chan 2006; Okamoto and Shaw 2005). Early studies on *Cryptococcus* have demonstrated the importance of mitochondria in responding to hypoxic conditions and oxidative stress (Ingavale et al. 2008; Narasipura et al. 2005). We therefore propose that after being engulfed by macrophages, the VIO strains are able to promote mitochondrial fusion to form long tubular mitochondria in order to more efficiently repair mtDNA damage caused by the oxidative stress and hypoxic conditions within the macrophage phagosome. Mitochondrially-regulated intracellular replication capacity may be a widespread phenomenon in other eukaryotic pathogens and hence an improved appreciation of this process is likely to have significant implications for our understanding of disease epidemics caused by a range of otherwise unrelated pathogens.

5.6 Extra work

After the above study, I further studied 8 VGIIC strains (kindly provided by the Heitman lab) for their virulence by measuring IPR values. VGIIC strains were only identified recently by Byrnes *et al* (Byrnes et al. 2009b). MLST analysis of 16 loci demonstrated that VGIIC strains have 7 novel alleles that are not seen in any of the other VGII genotypes. These isolates were recovered from both humans and animal in Oregon, but have never been found on Vancouver Island, in mainland British Columbia, or in Washington. It was speculated that they may have come from outside the region or may be recombined isolates (Byrnes et al. 2009b). The identification of the VGIIC genotype suggests the possibility of the emergence of a new VGII molecular type. Therefore, we were interested in studying their virulence. The IPR assay revealed that most of the VGIIC strains were as capable of exploring the intracellular niche as VIO strains with one exception (EJB52, Table 14). Mouse survival data also showed that VGIIC isolates were highly virulent (data from ongoing experiments, personal communication with Edmond Byrnes), further validating the IPR assay for predicting

virulence of cryptococcal strains in the mouse model of infection. It will be interesting to study more isolates of these subgroup and also test whether their mitochondria show the tubular morphology after phagocytosis, just like those of VIO strains. The IPR assay and mouse survival study suggest that the VGIIC population has the potential to cause another outbreak. In future, the surveillance of this subgroup should be increased for prevention purposes.

Table 14: IPR values of eight VGIIC strains in comparison to VIO strains.

Strains	Mating-Serotype	IPR values	Origin
A6M-R38	α -VGIIC	2.02±0.19	Oregon, United States
EJB12	α -VGIIC	1.53±0.07	Oregon, United State
EJB14	α -VGIIC	2.00±0.11	Oregon, United State
EJB15	α -VGIIC	1.72±0.23	Oregon, United State
EJB18	α -VGIIC	1.73±0.35	Oregon, United State
EJB52	α -VGIIC	0.97±0.22	Oregon, United State
EJB55	α -VGIIC	1.39±0.14	Oregon, United State
EJB74	α -VGIIC	1.60±0.06	Oregon, United State
Average VIO VGIIA	α-VGIIA	1.92	Vancouver Island, Canada

Chapter VI: Mitochondrial genotype and virulence

6.1 Introduction

6.1.1 Inheritance of mitochondria in *Cryptococcus*

The inheritance of mtDNA has been examined in many fungal species and diverse patterns have been observed. In both *S. cerevisiae* and *S. pombe*, mtDNA inheritance is biparental and the initial zygotes are heteroplasmic (Birky 1995; Gillham 1994). This is expected because zygotes in these yeast cells are the products of simple fusions between cells of different mating types. For *C. neoformans*, the zygotes are formed in a similar way to that of the budding and fission yeasts, but the mtDNA examined so far shows a largely mating-type dependent uniparental inheritance: the offspring predominantly receive their mitochondria from the MAT α parent, though a low level of leakage was also observed, where biparental inheritance and mitochondrial recombination occurs (Toffaletti et al. 2004; Xu 2005; Yan et al. 2007b). Early studies demonstrated that in *C. neoformans*, among 570 progeny examined from six independent crosses, no progeny were heteroplasmic or contained recombinant mtDNA (Xu et al. 2000a). Results from analysis of natural hybrids have been consistent with laboratory crosses (Xu 2002; Xu et al. 2002; Yan and Xu 2003).

Uniparental inheritance of mtDNA has been observed in many filamentous fungi such as *Neurospora*, *Aspergillus*, *Podospora* species, *Agaricus bisporus* and *Armillaria bulbosa*. Most of these cases of uniparental inheritance can be explained by the significantly biased cytoplasm inputs of the mating partners, or due to migration of only nuclei but not mitochondria during mating (Belcour 1975; Birky 1995; Lee and Taylor 1993; Mannella et al. 1979; Mason and Turner 1975). To date, the uniparental mitochondrial inheritance in *C. neoformans* has been demonstrated to be coordinately controlled by *SXI1 α* (located within the MAT α mating type locus) and *SXI2 α* (located within the MAT α mating type locus) (Figure 5) (Yan et al. 2007a; Yan and Xu 2003). However, the exact mechanism remains unknown and there are two hypotheses. The first hypothesis proposes that during sexual mating, mitochondria and cytoplasm from the two mating partners were not mixed equally to form a homogeneous cytoplasm in the fused cell and that hyphal formation occurred preferentially on the MAT α parent side, away

from the MAT α . Therefore, it is possible that during zygote formation, only the MAT α nucleus migrates through the conjugation tube into the MAT α cell, while mitochondria from the MAT α parent are left behind (Xu 2005). It is also possible that new daughter cells bud from the MAT α parent side of the zygote, away from the site of initial conjugation (Yan and Xu 2003). This hypothesis is supported by the microscopic observations showing the nucleus of the MAT α cell migrates into the conjugation tube whilst the recipient MAT α cell generates a hypha (McClelland et al. 2004). The second hypothesis proposes that uniparental inheritance is due to the selective elimination of the mtDNA type from MAT α after mating, similar to the current favoured mechanism to explain uniparental mitochondrial inheritance in the alga *Chlamydomonas reinhardtii* (Gillham 1994). When stable diploid yeast cells were synthesized directly from two mating partners without going through the filamentous stage, no mtDNA from the MAT α parent was found, suggesting a possible selective elimination mechanism for mitochondrial inheritance in *C. neoformans* (Yan and Xu 2003).

Although mitochondrial inheritance is mainly uniparental for *C. neoformans*, the patterns of mitochondrial inheritance in *C. gattii* are unknown. A recent study revealed that a high percentage of VGI isolates (more than 65%) possessed a recombined mitochondrial genome, suggesting that recombination occurs frequently in nature (Bovers et al. 2009). In addition, Yan *et al* demonstrated that both elevated temperature (up to 33°C) and UV irradiation can increase the leakage of the MAT α mitochondrial genome and result in biparental mitochondrial inheritance and recombination between strains with functional Sxi1 α and Sxi2 α in *C. neoformans* (Yan et al. 2007b). As *C. gattii* is predominantly found in tropical and subtropical regions, they will very likely to experience high temperature environments and great UV exposure. Therefore, biparental inheritance is expected to be more common in nature for *C. gattii*.

6.1.2 Mitochondrial genome of *Cryptococcus*

It is known that mtDNA of different yeast species can be highly variable in terms of both size and organisation. For example, within the *Saccharomyces* genus, the mtDNA of *S. cerevisiae* is 3.5 times bigger (86kB) (Foury et al. 1998) than that of *S. castellii* (26kB)

(Groth et al. 2000; Langkjaer et al. 2003). Considerable size differences have been observed even within the same species: a 9kb difference was found between two laboratory strains of *S. cerevisiae* (85kb & 76kb respectively) (de Zamaroczy and Bernardi 1985). For *C. neoformans*, two *C. neoformans* var. *neoformans* strains (IFM5844 & JEC21) and two *C. neoformans* var. *grubii* strains (IFO410 & H99) have been studied in details for their mtDNA structures. It was found that the order of coding genes in the two varieties are the same, but *C. neoformans* var. *neoformans* have much larger mtDNA (e.g., 32.6kb for IFM5844 and 33.2kb for JEC21) in comparison to that of *C. neoformans* var. *grubii* (e.g., 24.1kb for IFO410 and 24.9kb for H99) (Figure 24) (Litter et al. 2005; Toffaletti et al. 2004). The length variability is attributed to the presence and/or absence of optional intronic opening reading frames and also to the length of intergenic regions. For example, five introns were found in the *COX1* gene of IFM5844 and JEC21 strain, but no introns in IFO410 and H99. Similarly, IFO5844 and JEC21 have 2 introns in the *COB* gene, whereas IFO410 and H99 have only one (Litter et al. 2005; Toffaletti et al. 2004). Nevertheless, mtDNAs within varieties are highly similar in sequence: they displayed >99% similarities in the examined coding and non-coding regions (Litter et al. 2005). In comparison with other yeast species, the mtDNAs of *C. neoformans* are relatively compact and have short intergenic regions. This has been suggested to explain why all the mtDNAs have the same gene synteny since the variability in gene order might be connected with the presence of long intergenic regions (Litter et al. 2005).

For *C. gattii*, no detailed studies on mtDNA genome structure have been done, as the mtDNA has not been annotated for A1M-R265 (VGII) and WM276 (VGI) strains. We therefore used BLAST Open Reading Frame Software to predict the coding regions of A1M-R265 mtDNA. It seems that the mtDNA of this particular *C. gattii* strain has the same order of coding genes as that of both *C. neoformans* varieties, but it is intron-rich and resembles more var. *neoformans* in terms of size (Figure 24C). Simple alignment of A1M-R265 and WM276 mtDNA sequences (This was carried out under the guidance of Wenjun Li at Duke University) revealed most of the variations were found in gene-

coding regions whereas intergenic regions are very well conserved (Figure 24E), suggesting they are under intensive selection.

Recently, there have been two population genetic studies on the mitochondrial genome in *C. gattii* species. The first study revealed that the VGI genotype alone has five different mitochondrial genotypes based on the presence of ATP6 and MtLrRNA alleles, which did not form a monophyletic lineage (Bovers et al. 2009). The second study, by sequencing five mitochondrial DNA fragments of more than 50 VGI and VGII isolates, detected a significantly greater mtDNA divergence within VGI than that within VGII (Xu et al. 2009). Since studies on the nuclear genome showed VGII is basal to VGI and also VGIII & VGIV (Figure 2), the lower sequence diversity within VGII than those within VGI may reflect a recent bottleneck event, assuming the sampling is sufficient in this study (Xu et al. 2009). More importantly, both studies identified signatures of hybridisation between VGI and VGII lineages. For instance, about 65% VGI isolates studied possess mitochondrial genomes that consist completely of VGII sequence or that contain a combination of VGI and VGII sequences (Bovers et al. 2009). This is probably due to mating between VGI and VGII isolates, where mitochondrial inheritance is not uniparental, just as observed with *C. neoformans* (Toffaletti et al. 2004; Xu et al. 2000a), or mitochondrial recombination occurred (Bovers et al. 2009). Furthermore, Xu *et al* (2009) also found evidence for recombination in the mitochondrial genome within the VGII lineage. The recombination can be a consequence of biparental inheritance of mitochondrial genome in *C. gattii* or same-sex mating between strains of the MAT α mating type (Xu et al. 2009).

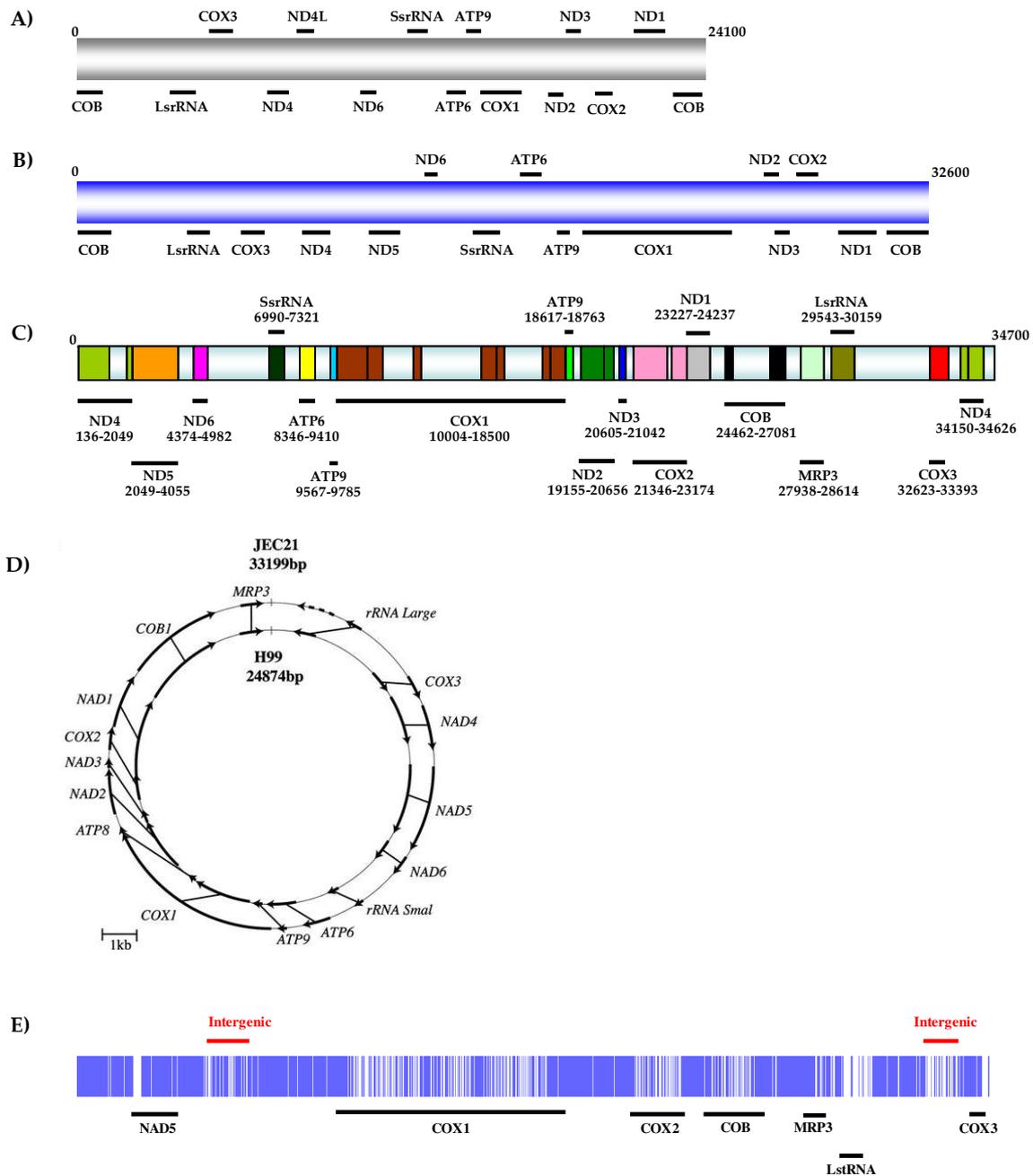


Figure 24: mtDNA structure of *Cryptococcus*: all the mtDNAs show a conserved gene synteny but have different sizes. A) IFM5844 (var. *neoformans*) and B) IFO410 (var. *grubii*). These two mtDNA structures were drawn to scale based on information from (Litter et al. 2005); C) mtDNA structure of A1M-265 (*C. gattii*, VGIIA). Sections with light blue colour are either introns or intergenic spaces. The open reading frames of A1M-R265 were predicted using Open Reading Frame Finder at NCBI at the following site: <http://www.ncbi.nlm.nih.gov/gorf/gorf.html>, in combination with alignment to the H99 mtDNA (<http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=AY101381>); D) Circular mtDNA structure of JEC21 (var. *neoformans*) and H99 (var. *grubii*) (taken from (Toffaletti et al. 2004)); E) Simple alignment of A1M-R265 and WM276 mtDNA using ClusterW. Before alignment, two repeat regions in both mtDNAs were removed (region one: 2434 nucleotides in COX1 gene; region two: 963 nucleotide at the end of the supercontig). Sections with white colour stand for the variations between two mtDNA sequences.

6.2 Verification of the role of mitochondrial genotype in intracellular proliferation and virulence for *C. gattii*

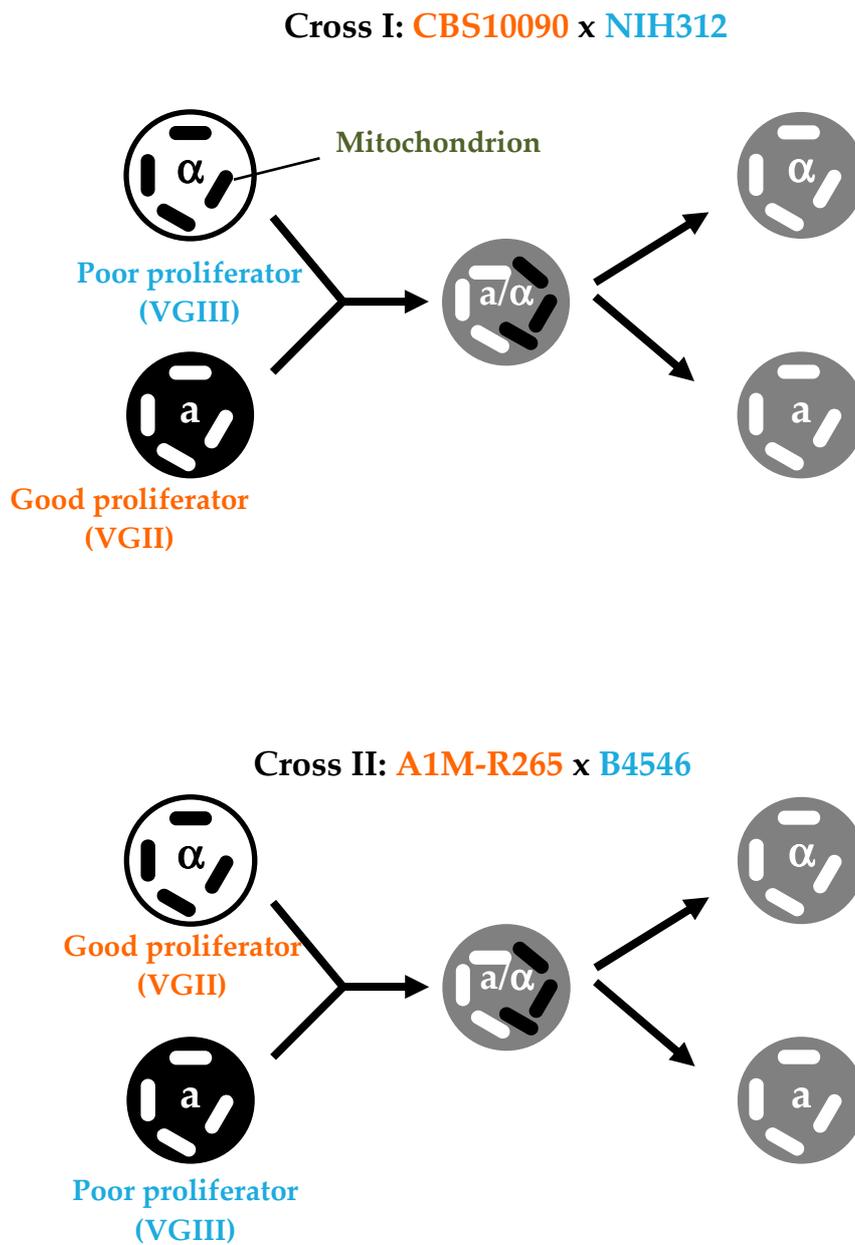
The mitochondrion has been linked to fitness in the past, as the organelle plays a key role in energy production and response to stress. Therefore, possessing different mitochondrial genotypes may lead to different fitness in an organism. Indeed, in *S. cerevisiae*, when mitochondria of wine yeasts were transferred to a laboratory strain, the latter showed increased viability and increased tolerance towards ethanol and high temperature (Jimenez and Benitez 1988). Furthermore, a perfect correlation between the mitochondrial type acquired by the *H. annosum* isolates and their virulence was reported, where hybrids with mitochondria of different origin have different virulence (Olson and Stenlid 2001).

For *C. neoformans*, a study conducted by creating stable AD hybrids to place serotype A and D mitochondria under different nuclear-genomic influences showed that either mitochondrial genotype was sufficient to support the full virulence of the H99 virulence composite for growth in the CNS of immunocompromised rabbits, suggesting that the mitochondrial genome is unlikely to have a significant influence on the differences between serotypes in their virulence composite in *C. neoformans* (Toffaletti et al. 2004). However, mitochondrial function and its genetic regulation may still be important to the virulence of *C. neoformans* and *C. gattii*, because many of the mitochondrial genes are encoded by the nucleus. These nuclear-encoded proteins are synthesised in the cytoplasm and then imported into mitochondria. They interact with mitochondrially encoded proteins (e.g., in the electron transport system), control mitochondrial biogenesis, regulate mtDNA copy number, influence mtDNA stability and alter mitochondrial morphology in a sophisticated manner (Cannino et al. 2007; Okamoto and Shaw 2005; Osiewacz and Kimpel 1999; Poyton and McEwen 1996). In addition, there is direct evidence showing that mitochondria might be involved in regulating virulence of *Cryptococcus*. Global *in vivo* transcriptional profiling of *C. neoformans* cells at the site of a CNS infection showed that the several respiratory genes were highly expressed by this yeast (Steen et al. 2003); There were significant

differences between mitochondrial gene regulation between the serotype A and D strains (e.g., *COX1*) (Toffaletti et al. 2003); Several other studies on *Cryptococcus* have demonstrated the importance of mitochondria in responding to hypoxic conditions and oxidative stress (Ingavale et al. 2008; Narasipura et al. 2005) etc. We were therefore very interested in verifying the role of mitochondrial genotype in the hypervirulence of VIO strains.

The ideal experiment to test the mitochondrial contribution towards intracellular proliferation in *C. gattii* would be to replace mitochondria of a poor proliferator with those from VIO strains or vice versa to see whether that alters the intracellular proliferation capacity of individual strains. However, such an experiment is practically difficult, as unlike *S. cerevisiae*, which can produce enough ATP by glycolysis, a pathway occurring in the cytoplasm that is independent of functional mitochondria (Osiewacz and Kimpel 1999), the presence of mitochondria seems to be essential to cryptococcal viability. Attempts to make *petite* (respiratory) mutants in strain H99 by standard methods using ethidium bromide or random mutagenesis with signature tags or to disrupt certain specific genes encoding proteins in the respiratory chain, such as *COX15*, have not been successful (Toffaletti et al. 2004). Nevertheless, the uniparental mitochondrial inheritance in *C. neoformans* means that it is possible to generate F1 progeny that only contain mitochondria from their good or poor proliferator parent and thus test the effect of mitochondrial genotype independently of nuclear genotype. If the presence of VIO mtDNA is sufficient to boost intracellular proliferation, progeny possessing the VIO mitochondrial genotype should all have high IPR values. Following this idea, we tried to cross two strains with very different IPR values followed by phenotyping the progeny. Disappointingly, after various attempts, we and the others were unable to mate MATa-VGII (a-VGII) with MAT α -VGII (α -VGII) strains in the lab, despite the fact the VGII strains are more fertile than the other *C. gattii* isolates (Fraser et al. 2003; Halliday and Carter 2003; Ngamskulrungrroj et al. 2008). Instead, a-VGII/ α -VGII strains (good proliferators) were chosen to mate with α -VGIII/a-VGIII strains (poor proliferators) to generate viable progeny. Although the progeny contain some of the VGIII nuclear genome, such crosses may still provide clues as to the importance of

mitochondrial genotype in *C. gattii*. In collaboration with the Heitman lab (Duke University, USA), two crosses were constructed on plates with V8 mating media and basidiospores were isolated by microdissection to generate recombinant F1 lines (See Figure 25 for experimental design). Cross I, constructed between CBS10090 (α -VGII, IPR=1.71) and NIH312 (α -VGIII, IPR=0.98), generated 16 progeny. Cross II, conducted between A1M-R265 (α -VGII, IPR=1.74) and B4546 (α -VGIII, IPR=0.87), produced 18 progeny. We then studied the IPR values of these 34 progeny. The whole experiment was based on one assumption, which is that in these two crosses, mitochondrial inheritance is uniparental: all the progeny only receive mitochondria from one of the parents.



	Parent MATa	IPR	Parent MATα	IPR	Number of progeny
Cross I	CBS10090 (VGII)	1.71	NIH312 (VGIII)	0.98	16
Cross II	B4546 (VGIII)	0.87	A1M-R265 (VGII)	1.74	18

Figure 25: A schematic illustration of the experimental design for two crosses.

6.2.1 All the progeny show the same mtDNA profile as their MATa parents

In order to check whether uniparental inheritance occurred during these two crosses, PCR of mtDNA was performed to profile the mitochondrial genotype. Seven primer pairs were designed and tested, but only one (named ATP-1, see Materials and Methods for details on sequence) was able to distinguish the VGII from VGIII mitochondrial genotype (Figure 26A): mtDNA from VGII isolates produced a much shorter PCR product (227bp) in comparison to the one from VGIII strains (>500bp), suggesting either a deletion in the ATP6 region occurred in the VGII strain or an insertion in the ATP6 region occurred to VGIII strains during evolution. Using the ATP-1 primer pair, we demonstrated that all the 16 progeny from cross I had the same mitochondrial genome profile as their MATa parent (CBS10090) (Figure 26B). Identical results were obtained for the progeny generated from cross II (data not shown, the PCR for these progeny were performed by Edmond Byrnes and Yoni Lewit at Duke University), suggesting that in these two crosses, the mitochondria were likely to be inherited uniparentally from MATa parents. Therefore, these progeny were used for the IPR study.

We note that the described approach to identify mitochondrial genome is not sufficient to demonstrate that the progeny have the exactly the same mtDNA as their MATa parents. It is possible that DNA recombination occurred between two mitochondrial genomes after fusion of MATa and MAT α cells and the region amplified by ATP-1 primers happened not to be recombined. In future, full sequence of the mtDNA of all the progeny should be carried out in order to ensure the complete inheritance of mitochondrial genome from MATa parents.

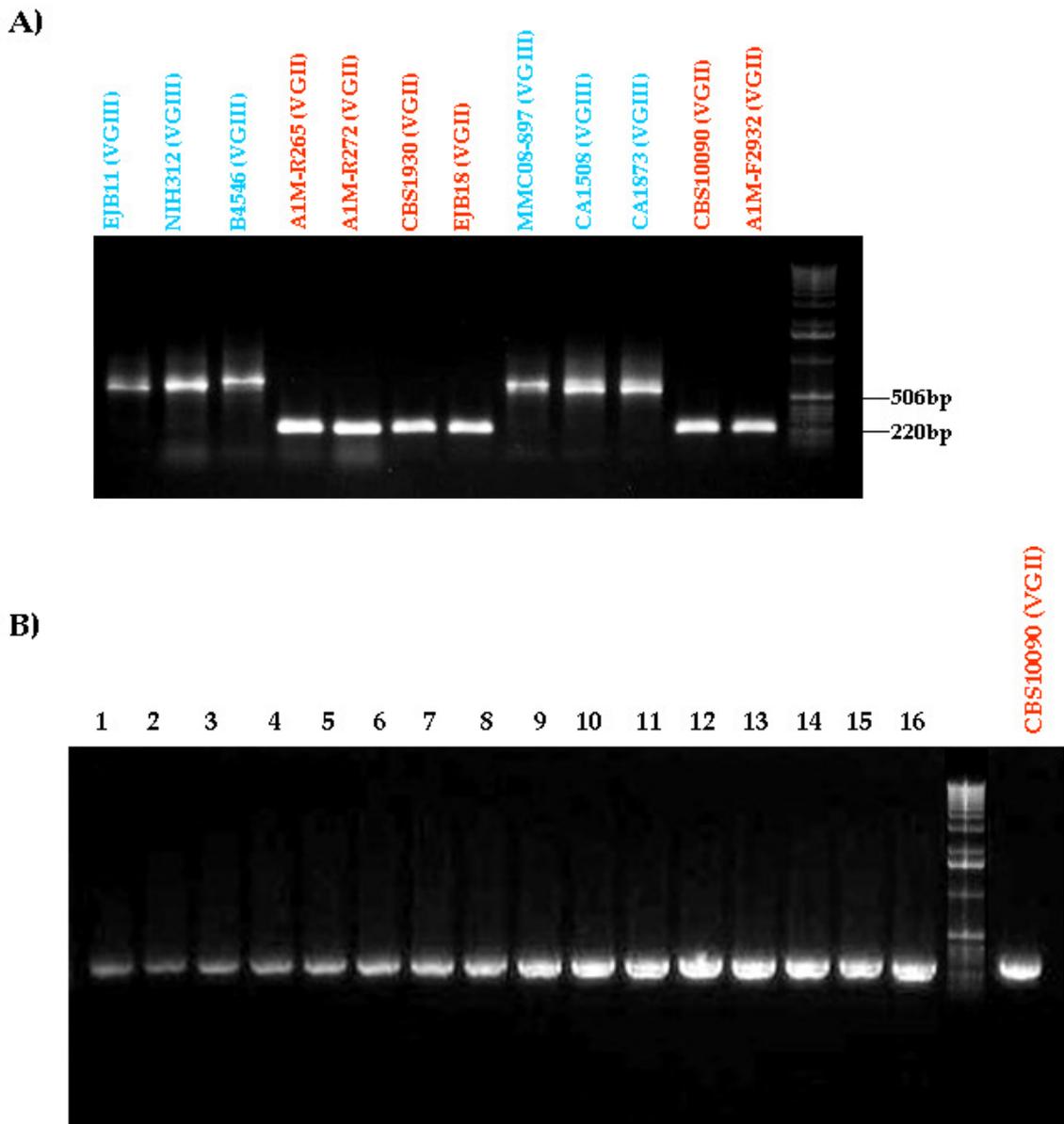
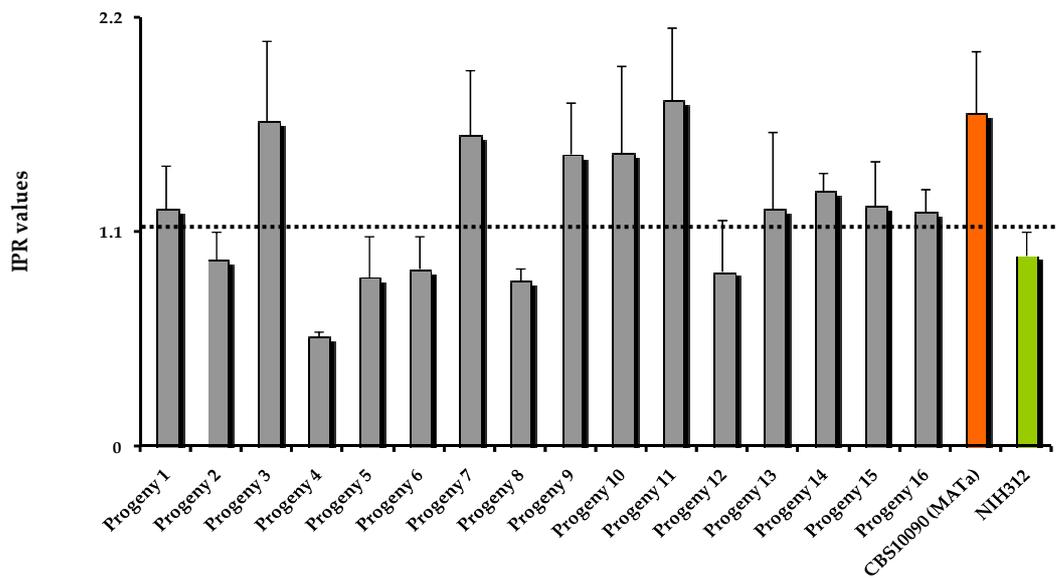


Figure 26: A) The analysis of PCR products of mtDNA of 12 strains (six VGII and six VGIII) using primer set ATP-1. mtDNA from VGII isolates produced much shorter PCR products in comparison to those of VGIII strains; B) PCR products of 16 progeny generated from cross I. mtDNA of all the progeny showed the same length as their VGIIA parent (CBS10090). The DNA ladder used in this experiment is the 1kb ladder from the Invitrogen.

6.2.2 The presence of VIO mitochondrial genotype is required but not sufficient for the hypervirulence of VIO strains

For progeny generated from crossing I, one would expect that all are good proliferators if the presence of the mitochondrial genotype from a good proliferator is sufficient to obtain high IPR values. However, this was not the case. The 16 progeny showed various IPR values, ranging from 0.57 to 1.78 (Appendix I & Figure 27A). Since the only difference between the progeny and CBS10090 is the content of the nuclear genome (due to recombination between the nuclear genome of the two parents during mating), the wide range of IPR values for the progeny suggests that possessing the “right” mitochondrial genotype does not guarantee the strains to be more virulent. The same study on progeny from cross II, however, showed that although mitochondrial genotype alone is not sufficient, it is absolutely required for virulence because none of the progeny are good proliferators when the right mitochondrial genotype is absent. All 18 progeny have a similar IPR values to their poor proliferator parent (Appendix I & Figure 27B). Taken together, these data imply that the presence of the ‘right’ mitochondrial genotype in the ‘right’ nuclear background is essential for the hypervirulence of VIO strains. Together with the recent demonstration regarding the importance of mitochondrial function in regulating survival of *Cryptococcus* (Ingavale *et al.* 2008), it is likely that mitochondrial activities (regulated by nuclear encoded proteins) determine the outcome of cryptococcal infection by protecting the pathogen from oxidative stress and hypoxic conditions in macrophage phagosomes and thus enhancing its survival.

A)



B)

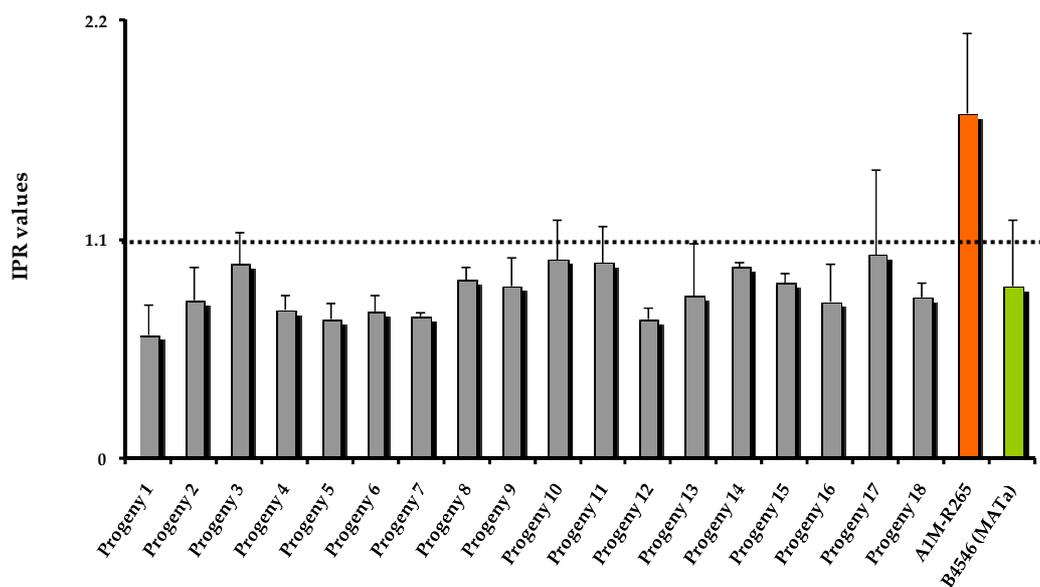


Figure 27: IPR values of the progeny and their parents of the two crosses. A) The progeny from cross I (CBS10090 × NIH312) show heterogeneous IPR values; B) The progeny from cross II (A1M-R265 × B4546) all have low IPR values. The good proliferator parents are represented with red bars and the poor proliferator parent with green bars. The IPR values represent the mean of three or more repeats.

At this point, it is important to point out that the above data from the two crosses are not sufficient to draw a solid conclusion about the role of mitochondrial genotype in virulence for several reasons. Firstly, as discussed earlier, we cannot rule out the possibility of mitochondrial recombination based on PCR amplifying of a single region of mtDNA. According to the study by Bovers *et al* (2009), mitochondrial recombination is likely to occur at high frequency in nature between VGI and VGII isolates (Bovers *et al.* 2009). Therefore, it is necessary to sequence the mtDNA of all the progeny and compare the sequences to those of their MATa parent in order to eliminate the possibility of recombination. Secondly, although *Cryptococcus* is able to undergo inter-genotype mating, this often result in a loss of viability in the basidiospores (<5%) and the generation of many diploid and even aneuploid progeny (Lengeler *et al.* 2001). Indeed, seven of the progeny from cross I were diploid and one might be aneuploid (Table 15, the FACS experiments were performed by Edmond Byrnes and Yoni Lewit at Duke University), indicating meiosis between the two genotypes was impaired because of their genomic divergence. This leads to the concern that progeny from such crosses may be generally less fit. Therefore, it will be necessary to study the normal growth rate of these progeny, especially ones from cross II, to make sure that the observed low IPR values are not due to longer generation time or growth deficiency at 37°C. Thirdly, although the MLST study (performed by Edmond Byrnes and Yoni Lewit at Duke University, data not shown) confirmed nuclear genome recombination between VGII and VGIII in cross I, we did not study the level of nuclear recombination for the two crosses in details. Bovers *et al* proposed that mating between different *C. gattii* genotypic groups may not result in the formation of a hybrid nuclear genome, although transfer of mitochondria and mitochondrial recombination still occur (Bovers *et al.* 2009). Hence, it is possible that the 18 progeny from cross II inherited both nuclear and mitochondrial genomes from MATa parent, which is a poor proliferator and that is the main reason for them to act poorly inside macrophages rather than not having the right mitochondrial genotype. Studying the nuclear background of the progeny is important for another reason. Communication between mitochondria and nucleus is bi-directional: nuclear gene expression can be influenced by signals coming from mitochondria and vice versa (e.g., as shown in (Epstein *et al.* 2001)). It has been

demonstrated that the mitochondrial genotype can influence nuclear gene expression in yeast (Parikh et al. 1987). If the mitochondrial genotype is not compatible to the nuclear genotype, their communication is likely to be blocked, which may lead to growth deficiency in the progeny, especially when dealing with stresses. In the future, more detailed study on nuclear genome recombination should be carried out. Finally, it is possible that mitochondrially-regulated virulence trait is restricted to the VGII strains. As mentioned earlier, the VGII population has much lower within-lineage divergence in both the nuclear and mitochondrial genome in comparison with the VGI population, although VGII is considered to be basal for the *C. gattii* species. If a recent bottleneck event occurred within the VGII population, the whole population might be selected to be generally fitter. Given that VIO VGIIA is a clonal population, it is possible that the trait was evolved just before its divergence from the other VGII populations. In future, the mitochondrial morphology of other *C. gattii* genotypes should be studied to test the above hypothesis.

Table 15: The ploidy of 16 progeny from cross I. These progeny were studied by FACS analysis. *Progeny 9 seems to contain both haploid and diploid populations despite numerous purifications of population by colony picking, suggesting it might be genetically unstable.

Progeny	Ploidy
1	Diploid
2	Haploid
3	Haploid
4	Diploid
5	Haploid
6	Haploid
7	Diploid
8	Diploid
9*	Haploid? Aneuploid? Diploid?
10	Diploid
11	Diploid
12	Haploid
13	Diploid
14	Haploid
15	Haploid
16	Diploid

Chapter VII: General discussion & Future work

In this thesis, I have demonstrated a complex interaction between *Cryptococcus* and macrophages as illustrated in Figure 28. It seems that *Cryptococcus* is able to use the host macrophage as a resource centre for replication (intracellular proliferation) as well as a vehicle for efficient dissemination (vomocytosis and lateral transfer), and that the pathogen might have developed unique mechanisms to control the balance between latency and dissemination in response to changes in the environment.

7.1 Vomocytosis and lateral transfer

The exit of intracellular pathogens from host cells is an important step in the infectious cycle because of its connection with dissemination and transmission. The route and timing of exit may also determine whether the pathogen avoids or engages immune responses. Early studies with *Cryptococcus* have assumed the cellular exit to occur by lysis, mainly due to the mechanical burden that is put on infected cells. The discovery of vomocytosis and lateral transfer demonstrates this pathogen's ability to escape in a more directed and organised way without killing of the host cell or the yeast itself. These two processes may represent the outcome of evolutionary pressure on intracellular cryptococci to be able to transmit between cells with minimal damage to the host cell and thus minimal immune response. Therefore, the pathogen can spread and infect new cells without altering the host response to its presence.

Currently, the detailed mechanism of vomocytosis remains to be explored. We believe that the initiating signals for *Cryptococcus* release are more likely to come from the yeast rather than the host cell, as the phenomenon was not observed with heat-killed *Cryptococcus*, latex beads or other yeast species tested so far. One possibility is that *Cryptococcus* secretes molecules into the phagosome that can trigger vomocytosis, as the pathogen has been demonstrated to secrete vesicles containing various virulence factors (Rodrigues et al. 2008). However, it is unlikely that *Cryptococcus* provides all the components required for vomocytosis, as vomocytosis resembles exocytosis, which is a

process regulated by a large number of protein complexes responsible for membrane fusion (Sollner 2003). Therefore, we suspect the intracellular yeast cells may be able to hijack the host exocytosis machinery, for example by targeting the SNARE complex (known as the minimal machinery for membrane fusion (Weber et al. 1998)) to the phagosome membrane (Figure 12). The modified phagosomal membrane would thus become more fusogenic towards the plasma membrane. In fact, for the four lateral transfer events observed, three of them were followed by vomocytosis, suggesting that the phagosome membrane was highly fusible. Long-term live imaging of macrophages with fluorescent-labelled host proteins important for exocytosis could be performed to confirm the above hypothesis. Another interesting question is how much the cytoskeleton of the host cell is involved in the whole process. Alvarez et al showed that blocking actin polymerisation of the host cell led to an increase in vomocytosis rate (Alvarez and Casadevall 2006). Interestingly, timelapse imaging of GFP-labelled actin has revealed periodic accumulation of actin around the cryptococci-containing phagosome at various timepoints after phagocytosis (Johnston et al, 2009, submitted). This formation of an actin ring around the phagolysosome may represent a strategy used by the host cell to prevent pathogens from leaving. In the future, it will be interesting to study the host and pathogen factors that trigger actin re-organisation around the cryptococci-containing phagolysosome.

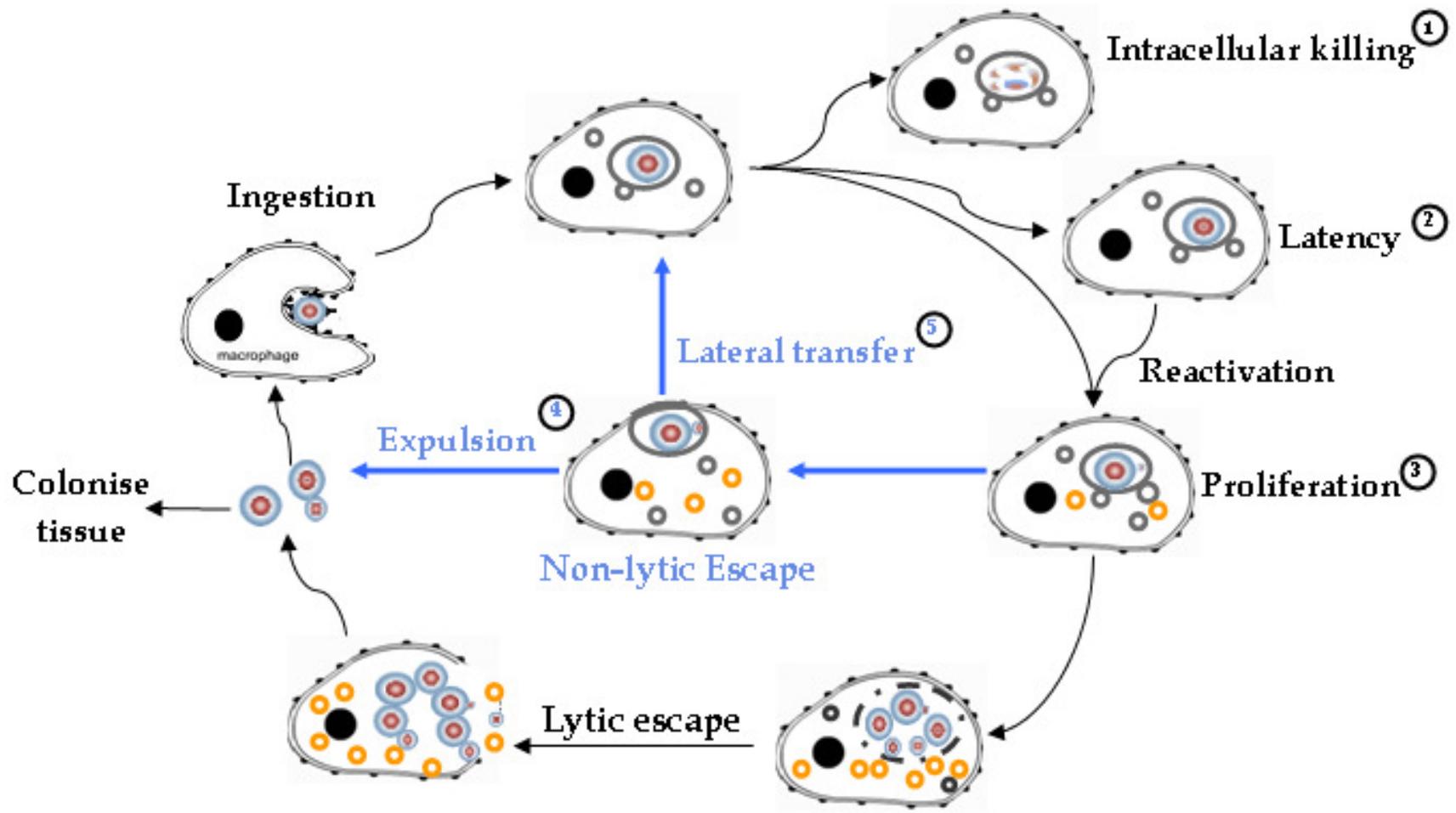


Figure 28: A summary of complex *Cryptococcus*-macrophage interaction, where both non-lytic and lytic escape pathways exist.

7.2 Intracellular proliferation and mitochondrial activity

When the host environment is less hostile, intracellular proliferation is more important for the pathogen to achieve dominance, as it leads to the fast expansion of the population size and thus rapid colonisation of local tissue. Based on our study, it seems that being able to proliferate inside macrophages is a key determinant of virulence for *Cryptococcus* and a sudden 'shift' in this ability in a clonal population can lead to disease outbreaks (e.g., VIO). More interestingly, we linked hypervirulence of VIO strains with their mitochondrial activity regulated by nuclear-encoded proteins, which probably allows more efficient respiration under the hypoxic and oxidative conditions present inside a host macrophage phagosome. The important remaining questions are how nuclear-encoded proteins regulate mitochondrial activities and what external factors induce such regulation. To answer these questions, genes involving mitochondrial fusion and tubular formation (such as *FZO1*, *MMM1*, *MDM10* and *MDM12* (Okamoto and Shaw 2005)) can be knocked out to see whether they are the mediators between the nucleus and mitochondria. Moreover, it is possible that mitochondrial tubule formation inside the phagosome may be only triggered at a particular stage of phagosome maturation. In order to find out the factors that promote tubule formation of cryptococcal mitochondria, one possibility would be subject the pathogen to simple environmental stimuli which are commonly present in phagosomes, such as osmotic shock (e.g., high salt concentration), hypoxia and the presence of oxidative molecules (e.g., H_2O_2) to reveal whether any of them is sufficient to trigger the morphological change *in vitro*. If so, then microarray experiments could be used to compare stimulated and non-stimulated cryptococci on different stimuli in order to identify common nuclear proteins with altered expression in the presence of all stimuli, as these proteins might be the mitochondrial-activity-regulators.

Interestingly, *C. neoformans* and *C. gattii* preferentially infect immunocompromised and immunocompetent individuals respectively. Therefore, one would expect significant differences between these two species in how they deal with the host adaptive immune

response. By studying the intracellular proliferation of a large collection of *C. gattii* strains, I found that most of the isolates are less capable of proliferating inside macrophages in comparison with *C. neoformans*, indicating that although they may be better at avoiding adaptive immune response, they cannot deal with the initial innate immune response. This may explain why cryptococcosis caused by *C. gattii* is far less common than that caused by *C. neoformans*. Exceptionally, the VIO strains and a few other VGII isolates show the same intracellular parasitism capacity as *C. neoformans*, which means these strains not only have the ability to deal with adaptive immune attack but also the innate immune response. Hence, it is not surprising that such *C. gattii* strains have led to an outbreak of human disease. Understanding how *Cryptococcus* interacts with the innate immune response therefore has a wide impact on cryptococcosis caused by both *C. neoformans* and *C. gattii*.

7.3 Which way to go: stay or leave

The existence of both lytic and non-lytic escape pathways means the pathogen has a 'choice'. As discussed earlier, most disseminated infection is caused by reactivation of a dormant infection acquired earlier in life rather than a primary infection. Thus the latent stage of infection can easily transfer to the active stage, where intracellular proliferation probably overtakes lateral transfer and vomocytosis, resulting in the lytic release of vast numbers of intracellular cryptococci which then establish their extracellular dominance. Voelz *et al* have recently revealed that Th2 cytokine (IL-4 & IL-13) treatment, which led to an increase in intracellular proliferation ratio, significantly reduced vomocytosis rates in both *C. neoformans* and *C. gattii* (Voelz *et al.* 2009). Interestingly, when studying *C. gattii* strains, I found a similar reverse correlation between vomocytosis and intracellular proliferation in 22 *C. gattii* isolates used for the microarray experiment (Figure 29, $P=0.0004$, linear regression). These data may suggest that if *Cryptococcus* is not 'happy' inside macrophages, it is able to trigger vomocytosis in order to find a more-'friendly' niche elsewhere in the host? Does this

mean that vomocytosis is beneficial for the pathogen but detrimental to the host? If the pathogen can actively choose one way over the other, what is involved in such “decision making”? During lytic exit, pro-inflammatory cell death facilitates pathogen escape, but also benefits the host by promoting pathogen clearance. However, some organisms have evolved to use inflammatory cell recruitment for dissemination, such as *Mycobacterium marinum* (Davis and Ramakrishnan 2009), and thus further encourage dissemination. Nevertheless, the ability to escape cells by multiple means may just present a way of ensuring efficient escape. Based on our IPR study, many cryptococcal strains are not good at proliferating inside macrophages, even in the absence of Th1 cytokines, so the chance of them experiencing lytic escape should be fairly low. Even for many good proliferators, in our *in vitro* system, lytic and non-lytic escape pathways co-exist. This is probably also the case *in vivo*, although certain environmental cues such as cytokines, O₂ availability or tissue specific molecules may favour one pathway over the other.

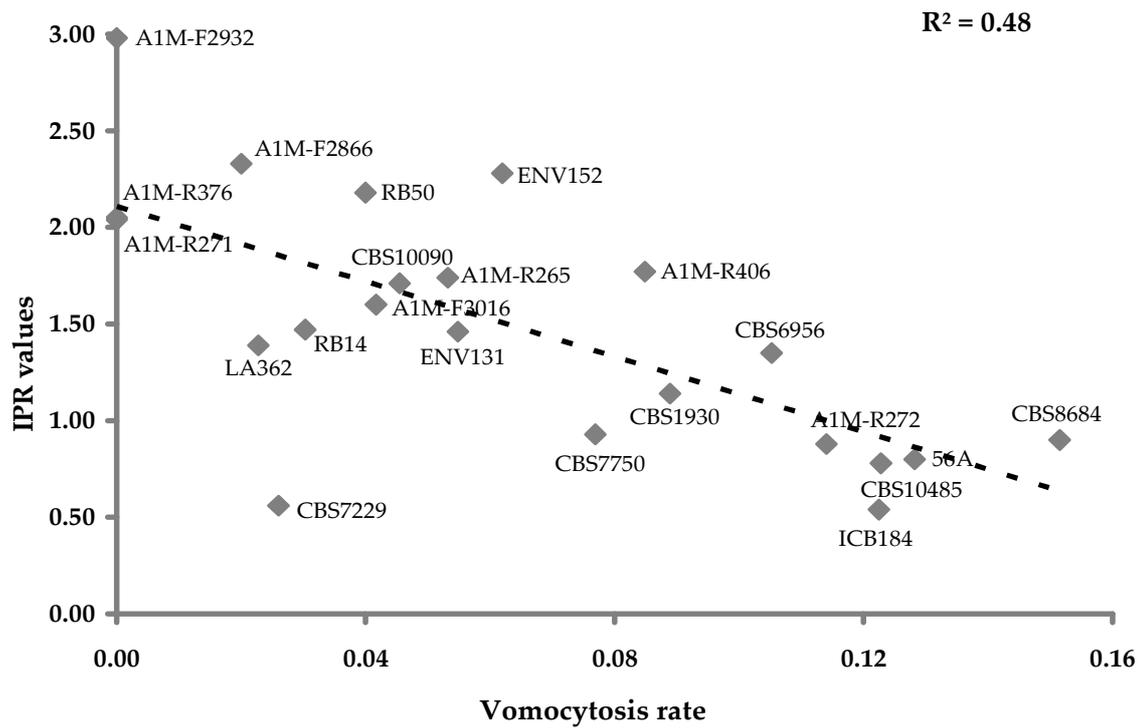


Figure 29: A reverse correlation between vomocytosis rates and IPR values of *C. gattii* strains used for microarray experiment ($P=0.0004$, linear regression, $n=22$). CBS10089 and WM276 were not included in this analysis because the IPR values for these strains were underestimated as described earlier.

7.4 Conclusion

Phagocytosis of *Cryptococcus* by macrophages is a necessary step for the elimination of the pathogen, but also increases the migratory capacity of *Cryptococcus* and promotes recrudescence infection, especially when the macrophages are not properly activated or the cryptococcal isolates are particularly virulent. Gaining an intracellular niche, even briefly, thus affords a window of opportunity for *Cryptococcus* to survive inside the host and promote disease. Various pieces of evidence have suggested that many virulence factors in *C. neoformans* and *C. gattii* are 'ready made', due to selective pressure from various existing environmental reservoirs, rather than 'specially made' in order to colonise mammalian hosts, so it is likely that being able to survive inside macrophage is an advertent feature of cryptococcal evolution. Nevertheless, co-evolution between *Cryptococcus* and its hosts is an ongoing event. The VIO is one such example, showing that *Cryptococcus* has the potential to generate new variation in traits that determine interactions with their hosts. Yet many important questions remain about how *Cryptococcus* grows within its host. The development of new biochemical and genetic tools will facilitate studies to answer these questions.

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Appendix I: A list of strains used and their intracellular proliferation ratio (IPR).

Strain name	Mating-Serotype	Genotype (AFLP)	IPR value	Source/Feature
H99	α A	1	1.42 \pm 0.15	Clinical, United States
125.91	aA	1	2.24 \pm 0.19	Clinical, Tanzania
WM714	α A	1	1.60 \pm 0.43	Cat, Australia
P152	α A	1	2.14 \pm 0.31	AIDS patient, Zimbabwe
ATCC90112	α A	1	2.85 \pm 0.19	Clinical, United States
CBS8336	α A	1	1.24 \pm 0.38	Wood of <i>Cassia grandis</i> tree, Brazil
CBS996 ^T	α A	1	1.53 \pm 0.63	Blastomycosis, Argentina
ICB105	α D	2	1.57 \pm 0.33	Non-AIDS patient, Brazil
CBS5467	α D	2	2.88 \pm 0.80	Milk of cow with mastitis, Switzerland
JEC21	α D	2	2.12 \pm 0.18	Clinical, United States
CBS7816	aD	2	2.18 \pm 0.55	Pigeon droppings, Thailand
JEC20	aD	2	1.93 \pm 0.27	HIV+ patient, United States
CBS6995	aD	2	1.26 \pm 0.29	Clinical, United States
B-3501	α D	2	1.42 \pm 0.40	Cross between NIH433 x NIH12
B-3502	aD	2	1.50 \pm 0.49	Cross between NIH433 x NIH12
BD5	α D	2	1.90 \pm 0.32	AIDS patient, France
CBS132	AD	3	2.43 \pm 0.50	Environmental, Italy
NY-40	AD	3	2.85 \pm 0.68	Clinical, United States
LPSQ308	AD	3	2.67 \pm 0.94	Clinical, Canada
CBS7229 ^T	aB	4	0.56 \pm 0.42	Meningitis, China
WM179	α B	4	0.69 \pm 0.20	Clinical, Australia
*WM276	α B	4	0.63 \pm 0.39	Environmental, Australia
CBS6992	α B	4	0.59 \pm 0.21	Patient, United States
CBS919 ^T	α B	4	0.72 \pm 0.22	Clinical, United States
CBS6290	α B	4	1.20 \pm 0.65	Clinical, Republic of Congo
56A	α B	4	0.80 \pm 0.38	Gut of a goat, Spain
CN043	α B	5	0.89 \pm 0.20	Clinical, New Zealand
WM728	α B	5	0.73 \pm 0.19	<i>Eucalyptus</i> sp., San Diego, United States

CBS6993	αB	5	1.45±0.33	CSF, human, California, United States
CBS8755	αB	5	1.32±0.31	Litter of <i>Prunus dulcis</i> tree, Colombia
384C	αC	5	1.08±0.47	Clinical, United States
B4546	αB	5	0.87±0.33	Unknown
NIH312	αB	5	0.98±0.12	Clinical
CBS7750	αB	6	0.93±0.24	<i>Eucalyptus camaldulensis</i> , United States
CBS6956	αB	6	1.35±0.35	Clinical, Seattle, United States
A1M-R265	αB	6	1.74±0.40	Human isolate, Duncan, British Columbia, Canada
CBS1930	αB	6	1.14±0.37	Sick goat, Aruba
A1M-R271	αB	6	2.04±0.48	Immunocompetent male, Nanoose Bay, British Columbia, Canada
A1M-R406	αB	6	1.77±0.43	Immunocompetent female, Nanaimo, British Columbia, Canada
A1M-F2866	αB	6	2.33±0.19	Dead wild Dall's porpoise, shores island close to Vancouver Island, Canada
A1M-F2932	αB	6	2.98±0.40	Immunocompetent patient, Kelowna, British Columbia, Canada
A1M-R376	αB	6	2.05±0.36	Non-AIDS, Vancouver Island, Canada
A1M-F3016	αB	6	1.47±0.03	Dead wild Dall's porpoise, shores island close to Vancouver Island, Canada
RB50	αB	6	1.87±0.53	Alder tree, Rathrevor Beach Provincial Park, Vancouver Island, Canada
CBS8684	αB	6	0.90±0.37	Wasp nest, Uruguay
A1M-R272	αB	6	0.88±0.25	Immunocompetent female, Ladysmith, British Columbia, Canada
RB14	αB	6	1.60±0.24	Douglas fir, Rathrevor Beach Provincial Park, Vancouver Island, Canada
*CBS10089	αB	6	0.44±0.29	Clinical, Greece
CBS10090	αB	6	1.71±0.42	Clinical, Greece
ICB180	αB	6	0.42±0.26	<i>Eucalyptus</i> sp. tree, Brazil
ICB184	αB	6	0.54±0.05	Tree, Brazil
ENV152	αB	6	2.28±0.16	Alder tree, Rathrevor Beach Provincial Park, Vancouver Island, Canada
ENV131	αB	6	1.46±0.32	Douglas fir, Rathrevor Beach Provincial Park, Vancouver Island, Canada
RB59	αB	6	1.81±0.44	Douglas fir, Rathrevor Beach Provincial Park, Vancouver Island, Canada
LA362	αB	6	1.39±0.21	Parrot liver, Jaboticabal, Brazil
CBS10485	αB	6	1.78±0.22	Danish tourist visited Vancouver Island, Canada
EJB12	αB	6	1.53±0.07	Clinical, Oregon State, United States, VGIIC subgroup
EJB14	αB	6	2.00±0.11	Feline, Oregon State, United States, VGIIC subgroup
EJB15	αB	6	1.72±0.23	Alpaca, Oregon State, United States, VGIIC subgroup
EJB18	αB	6	1.73±0.35	Clinical, Oregon State, United States, VGIIC subgroup
EJB52	αB	6	0.97±0.22	Oregon State, United States, VGIIC subgroup

EJB55	α B	6	1.39 \pm 0.14	Oregon State, United States, VGIIC subgroup
EJB74	α B	6	1.60 \pm 0.06	Oregon State, United States, VGIIC subgroup
A6M-R38	α B	6	2.02 \pm 0.19	Clinical, Oregon State, United States, VGIIC subgroup
LMM261	aB	6	1.42 \pm 0.12	Clinical, Brazil
LMM265	B	6	2.54 \pm 0.32	Clinical, Brazil
LMM645	aB	6	2.37 \pm 0.10	Clinical, Brazil
\wedge M27055	α C	7	1.09 \pm 0.83	Clinical, South Africa
\wedge CBS10101	α C	7	1.00 \pm 0.46	King Cheetah, South Africa
\wedge B5748	α C	7	1.02 \pm 0.55	Clinical, India
\wedge B5742	α C	7	1.00 \pm 0.49	Clinical, India
770616	BD	8	1.51 \pm 0.62	Clinical, the Netherlands
Progeny from CROSS I				
C1-P1			1.22 \pm 0.22	Cross between CBS10090 x NIH312
C1-P2			0.96 \pm 0.14	Cross between CBS10090 x NIH312
C1-P3			1.67 \pm 0.41	Cross between CBS10090 x NIH312
C1-P4			0.57 \pm 0.02	Cross between CBS10090 x NIH312
C1-P5			0.87 \pm 0.21	Cross between CBS10090 x NIH312
C1-P6			0.91 \pm 0.17	Cross between CBS10090 x NIH312
C1-P7			1.60 \pm 0.33	Cross between CBS10090 x NIH312
C1-P8			0.85 \pm 0.06	Cross between CBS10090 x NIH312
C1-P9			1.50 \pm 0.26	Cross between CBS10090 x NIH312
C1-P10			1.51 \pm 0.44	Cross between CBS10090 x NIH312
C1-P11			1.78 \pm 0.37	Cross between CBS10090 x NIH312
C1-P12			0.90 \pm 0.26	Cross between CBS10090 x NIH312
C1-P13			1.22 \pm 0.39	Cross between CBS10090 x NIH312
C1-P14			1.31 \pm 0.09	Cross between CBS10090 x NIH312
C1-P15			1.24 \pm 0.22	Cross between CBS10090 x NIH312
C1-P16			1.21 \pm 0.11	Cross between CBS10090 x NIH312
Progeny from CROSS II				
C2-P1			0.62 \pm 0.15	Cross between A1M-R265 x B4546
C2-P2			0.80 \pm 0.16	Cross between A1M-R265 x B4546
C2-P3			0.98 \pm 0.16	Cross between A1M-R265 x B4546
C2-P4			0.75 \pm 0.07	Cross between A1M-R265 x B4546

C2-P5			0.70±0.08	Cross between A1M-R265 x B4546
C2-P6			0.74±0.08	Cross between A1M-R265 x B4546
C2-P7			0.72±0.01	Cross between A1M-R265 x B4546
C2-P8			0.90±0.06	Cross between A1M-R265 x B4546
C2-P9			0.87±0.14	Cross between A1M-R265 x B4546
C2-P10			1.00±0.20	Cross between A1M-R265 x B4546
C2-P11			0.99±0.18	Cross between A1M-R265 x B4546
C2-P12			0.70±0.06	Cross between A1M-R265 x B4546
C2-P13			0.82±0.26	Cross between A1M-R265 x B4546
C2-P14			0.96±0.03	Cross between A1M-R265 x B4546
C2-P15			0.88±0.05	Cross between A1M-R265 x B4546
C2-P16			0.79±0.19	Cross between A1M-R265 x B4546
C2-P17			1.03±0.42	Cross between A1M-R265 x B4546
C2-P18			0.81±0.07	Cross between A1M-R265 x B4546
Mutants				
NE168d	αD	2	1.15±0.28	Strain lacking O-acetylation (cas1Δ), dull colony
NE168s	αD	2	1.22±0.35	Strain lacking O-acetylation (cas1Δ), shiny colony
KN433	αD	2	1.22	Generated by crossing NIH433a and JEC21 ^{ex}
NIH-B4131	αD	2		Acapsular mutant (cap67Δ)
CBS7931	αD	2		Acapsular mutant (cap67Δ)
NE178	αD	2		Strain lacking cas1Δ mutant
NE150	αD	2		Strain lacking usx1Δ mutant
Other yeast strains				
AH109				<i>Saccharomyces cerevisiae</i>
YES972				<i>Schizosaccharomyces pombe</i>
CCA224-??			0.40±0.20	<i>C. laurentii</i>

*indicates strains (WM276 and CBS10089) with many extracellular yeast cells attaching to macrophage cell surface at timepoint 0, which were very difficult to wash off. Therefore, the initial intracellular yeast number for these strains was over-estimated, which leads to underestimation of IPR values. Timelapse experiment confirmed underestimation of IPR values for these two strains. Strains indicated with ^ are the ones with extremely low phagocytosis rate. Since the intracellular yeast number was always low, the IPR values were vulnerable to random variations.

Appendix II: A list of media used

10% SDS, 1 litre

Dissolve 100g sodium dodecyl sulphate crystals (SDS) in 900ml dH₂O
Heat to 68°C to dissolve the crystals
Adjust pH to 7.2 with HCl (~50µl)
Adjust volume to 1 litre with dH₂O
Store at room temperature

1 x PBS, 1 litre

Dissolve 8g NaCl, 0.2g KCl, 2.68g Na₂HPO₄·7H₂O and 0.24g KH₂PO₄ in 800ml dH₂O
Adjust pH to 7.4 with HCl
Adjust volume to 1 litre with dH₂O
Sterilise by autoclaving
Store at room temperature

10 x TBE, 1 litre

Dissolve 108g Tris and 55g Boric acid in 900ml dH₂O
Add 40ml 0.5M Na₂EDTA (pH=8.0) and adjust volume to 1 litre with dH₂O
Sterilise by autoclaving
Store at room temperature
10x TBE was diluted 1/10 when required with dH₂O

YPD liquid medium, 1 litre

Dissolve 50g YPD powder in 1 litre dH₂O
Sterilise by autoclaving
Store at room temperature

YPD plate

Dissolve 10g peptone, 10g yeast extract, 20g D-glucose and 15g agar in 1 litre dH₂O
Sterilise by autoclaving
When the medium is around 55°C, add adequate amount of antibiotic (e.g., ampicillin and kanamycin) before pooling

V8 mating plate for *C. gattii* (pH=5.0), 1 litre

Mix 50ml V8 juice (TESCO) and 0.5g KH_2PO_4 with 20g agar
Adjust volume to 1 litre with dH_2O
Sterilise by autoclaving before pooling

Medium for phenotypic analysis

Medium to measure capsule size

Dissolve 10g peptone, 10g D-glucose and 15g Ox-bile to 1 litre of dH_2O
Sterilise by autoclaving
Store at room temperature

Medium to measure melanin production

Vitamin B1 solution: Dissolve 0.253g thiamine in 250ml dH_2O and sterilise through a 0.22 μm filter

L-DOPA solution: Dissolve 0.1972g L-DOPA in 100ml dH_2O by heating at 37°C and stirring. Sterilising by filtering through 0.22 μm filter.

Dopamine medium: Dissolve 2.997g glucose monohydrate, 1.204g MgSO_4 , 3.501g KH_2PO_4 , 0.975g glycine and 20g agar in 500ml dH_2O . Add dH_2O to 900ml and sterilise by autoclaving.

Add 1.0ml sterile B1 solution and 100ml L-DOPA medium when the Dopamine medium is cooled down to 50-60°C before making plates.

Medium to measure proteinase activity

Yeast nitrogen based 10x medium: dissolve 6.7g Yeast nitrogen base in 100ml of dH_2O , heat if necessary, and autoclave by filtration (0.22 μm).

Casein medium 0.75%: dissolve 7.5g casein and 5.0g glucose in 750ml dH_2O and adjust pH to 7.0 with 1.0M NaOH. Add a volume of dH_2O to a final volume of 900ml. Add 20.0g agar and autoclave for 20min

Add 100ml filter sterilised yeast nitrogen base 10x medium when the casein medium is cooled down to 50-60°C before making plates.

Medium to measure phospholipase activity

Sabourand dextrose medium: dissolve 20g Sabourand dextrose agar in 500ml dH₂O and add 58.44g of NaCl and 0.7351g CaCl₂-two hours₂O to this solution. Adjust the pH to 7.0 with NaOH. Add dH₂O to the volume of 900ml followed by autoclaving

Add 100ml sterile Bacto TM egg yolk enrichment 50% when the Sabourand dextrose medium is cooled down to 50-60°C before making plates.

Medium for DNA isolation

Lysis buffer, 100ml

TE Buffer: 10 mM Tris-Cl (pH 7.5) & 1 mM EDTA

Mix 5ml 10% w/v SDS, 5ml 10% w/v Sarkosyl to 90ml of TE (pH=7.5)
Store at room temperature

Medium for cell fixation

Moviowl

Mix 6ml glycerol and 2.4g mowiol by vortexing
Add 6ml dH₂O to the mixture and vortex
Rotate the glycerol-mowiol-dH₂O mixture for two hours
Add 12ml 10mM Tris-Cl (pH=7.5-8.5) and incubate at 50°C to dissolve
Add several grains of p-phenylenediamine
Centrifuge and aliquote the liquid layer
Store at -80°C

Permeabilisation/Wash solution: 0.1% Triton, 0.5 litre

Mix 500ml PBS with 500µl Triton-X 100
Stored at 4°C

Fixation solution: 4.0% formaldehyde, 100ml

Add 4g of PFA to 100ml of PBS
Heat at 65°C in water bath for two hours
Add drops of NaOH to enhance dissolving of PFA
Keep in 65°C water bath with occasional agitation until all PFA was dissolved
Cool down to room temperature
Aliquot and store at -20°C

Appendix III: R scripts used for microarray data analysis

- 1) Useful columns were extracted from individual microarray spreadsheets (provided by OGT) to generate smaller files (Written by Dov Stekel)

```
# which columns to extract starting at 0: i.e. A=0, B=1 etc
@wanted = (2,3,8,10,11,36,37,38,39,46,47,48,49,54,55);

# need to do the glob first otherwise glob will recursively pick up
# newly created _out files
@filelist = <data/*.txt>;
foreach $file (@filelist) {
  print "$file\n";
  open(IN,$file) || die "problem reading $file: $!";
  $fileout = $file;
  # create a new output file with _out appended to the original name just before the .txt
  $fileout = ~ s/\.txt/_out\.txt/;
  open(OUT,">$fileout") || die "problem creating $fileout: $!";

  # first 9 lines are scanner bump not data
  for ($i=0; $i<9; $i++) {
    $line = <IN>;
  }
  # 10th line contains the column headers - keep these
  $header = <IN>;
  chomp $header;
  @headers = split(/\t/, $header);
  $newheader = join("\t", @headers[@wanted]);
  print OUT "$newheader\n";

  # this is the data
  while (<IN>) {
    chomp;
    @fields = split(/\t/, $_);
    $newline = join("\t", @fields[@wanted]);
    print OUT "$newline\n";
  }
  close OUT;
  close IN;
}
```

- 2) For individual files, the gsignal and rsignal was subtracted to the backgroundsignal and and 24 new files were generated (written by Dov Stekel).

```
// GSL stuff
#include <gsl/gsl_cdf.h>
#include <gsl/gsl_randist.h>

// Some standard C++ headers

#include <iostream>
#include <fstream>
#include <cmath>

// and we use the C++ STL to allow some data structures so that I can
// upload arbitrarily sized files. This is the first time I'm using STL
// so we'll see how I get on with it.
#include <algorithm>
#include <vector>
#include <string>

// we appear to need this on Linux implementation - even though it's
// supposed to be a windoze thing. Take a look at /usr/include/iostream,
// for example, and you will see its declaration.
// similarly, if you look at http://www.yrl.co.uk/phil/stl/stl.html#Vector
// you will see this line preceded by #ifdef _WIN32
// oops! I spent a day sorting this out... don't fall into the trap
// again!!!

Using namespace std;

#define STRSIZE 100
#define BIG 1000
#define N 40
#define NUM_COLS 58 // not nice... but quick cludge until I get this sorted
#define NUM_PARS 3
#define DUMMY -9999 // dummy value for missing data
#define sigma 0.1

#define a_init 1.0
#define b_init 1.0
#define c_init 0.1 // avoids putting a singularity in the middle of the data!
#define xmin 0.0
#define xby 0.1

/*
class Data {
public:
    vector<string> headers;
    vector<Gene *> genes;
    int num_cols;

    int read_data(char *filename);
};
```

```

*/

double kooper(double mu, double xf, double sf, double xb, double sb, double sd) {

    return (gsl_ran_ugaussian_pdf((xf-mu-xb)/sd)* gsl_cdf_ugaussian_P(((xf-
mu)*sb*sb+xb*sf*sf)/(sf*sb*sd)));
}

int postmedian(double xf, double sf, double xb, double sb)
{
    double sd;
    int low, hi;
    double sum ;
    int x ;
    double z ;
    static double y[200000];

    if (sf <= 0.0 || sb <= 0.0) {
        // can't compute with no standard deviation
        return 0;
    }

    sd = sqrt(sf * sf + sb * sb);

    low = max(0, (int)(xf-xb-3*sd));
    hi = (int) (xf-xb+3*sd+1);

    // cout << low << '\t' << hi << '\n';
    if (low > 0) {
        return (int)(xf - xb);
    }

    sum = 0.0;
    for (x = low; x <= hi; x++) {
        y[x] = kooper(1.0 * x,xf,sf,xb,sb,sd);
        sum += y[x];
    }

    z = y[low] / sum;

    for (x = low+1; x<=hi; x++) {
        z += y[x] / sum;
        if (z > 0.5) {
            return x;
        }
    }
}

int normalize(string filename)
{
    char outfile[80];

    ifstream fin(filename.c_str());
    sprintf(outfile,"%s.norm",filename.c_str());
}

```

```

ofstream fout(outfilename);

vector<string> headers;
vector<string> data;

int num_cols;
int i;
char ch;
string s;
int normch1, normch2;

if (!fin) {
    cout << "cannot open " << filename << ": exiting\n";
    return 1;
}

if (!fout) {
    cout << "cannot open " << filename << ".norm: exiting\n";
    return 1;
}

do {
    fin.get(ch);
    if (ch == '\t' || ch == '\n') {
        headers.push_back(s);
        fout << s << '\t';
        s.clear();
    }
    else {
        s.push_back(ch);
    }
} while (ch != '\n');

fout << "Normch1\tNormch2\n";

num_cols = headers.size();

// now read in the data

while (!Fin.eof()) {
    fin.get(ch);
    if (ch == '\t' || ch == '\n') {
        // cout << s << ch;
        data.push_back(s);
        s.clear();
        if (ch == '\n' && ! fin.eof()) {
            normch1 =
postmedian(atof(data[5].c_str()),atof(data[7].c_str()),atof(data[9].c_str()),atof(data[11].c_str()));
            normch2 =
postmedian(atof(data[6].c_str()),atof(data[8].c_str()),atof(data[10].c_str()),atof(data[12].c_str()));

            for (i=0; i<num_cols; i++) {
                fout << data[i] << '\t';
            }
            fout << normch1 << '\t' << normch2 << '\n';
            data.clear();
        }
    }
}

```

```

    }
    else {
        s.push_back(ch);
    }

}

fin.close();
fout.close();

return 0;
}

int main(int argc, char *argv[])
{
    string s1, s2;
    // char *c;
    int err;

    ifstream fin(„filelist“);

    if (!fin) {
        cout << “Error reading filelist\n”;
        return 1;
    }

    while (!fin.eof()) {
        fin >> s1;
        cout << s1 << ‘\n’;

        err = normalize(s1);
        if (err != 0) {
            cout << “Exiting with error code “ << err << ‘\n’;
            return err;
        }
    }

    fin.close();

    return 0;
}

```

3) The script used to normalise individual files using loess (written by Dov Stekel)

```
a1=read.table("0147-BUB_P100_251786310002_S01_GE2-
v5_95_Feb07_out.txt.norm",sep="\t",header=T)
sum(a1$gIsSaturated)
sum(a1$rIsSaturated)
a1$lgnorm = log2(a1$Normch1)
a1$lnorm = log2(a1$Normch2)
plot(a1$lgnorm,a1$lnorm,pch=20)
a1$av = (a1$lgnorm+a1$lnorm)/2
a1$diff = (a1$lgnorm-a1$lnorm)
plot(a1$av,a1$diff,pch=20)
lines(c(0,20),c(0,0),col="red",lwd=2)
lmodel1 = loess(a1$diff~ a1$av)
a1$loess = predict(lmodel1,a1$av)
png("a1_koopernorm_mva.png")
plot(a1$av,a1$diff,pch=20)
lines(c(0,20),c(0,0),col="red",lwd=2)
points(a1$av,a1$loess,pch=20,col="blue")
dev.off()
a1$loessnorm = a1$diff-a1$loess
png("a1_kooperberg_final.png")
plot(a1$av,a1$loessnorm,pch=20)
lines(c(0,20),c(0,0),col="red",lwd=2)
dev.off()
write.table(a1,"a1.txt",sep="\t",col.names=T)
rm(a1)
```

- 4) The script used to extract useful columns from 24 files and combine them into a single file (written by Dov Stekel)

```

# which columns to extract starting at 0: i.e. A=0, B=1 etc
@wanted = (1,2,3,4,5,18,19,23);
for ($filenum = 1; $filenum<=24; $filenum++) {
    $file = "data/a$filenum.txt";
    print "$file\n";
    open(IN,$file) || die "problem reading $file: $!";
    $fileout = $file;
    $header = <IN>;
    if ($filenum == 1) {
        # create a new header
        chomp $header;
        @headers = split(/\t/, $header);
        $newheader = join("\t", @headers[0,1,2,3,4]);
        for ($i=1; $i<=24; $i++) {
            $newheader .= "\ta$i";
        }
    }
    # this is the data
    $line = 0;
    while (<IN>){
        chomp;
        @fields = split(/\t/, $_);
        if ($filenum == 1) {
            $newline[$line] = join("\t", @fields[1,2,3,4,5]);
        }
        $green[$filenum][$line] = $fields[18];
        $red[$filenum][$line] = $fields[19];
        $norm[$filenum][$line] = $fields[23];
        $line++;
    }
    close IN;
}
open(OUTG, ">data/green.txt") || die "problem creating data/green.txt";
open(OUTR, ">data/red.txt") || die "problem creating data/red.txt";
open(OUTN, ">data/norm.txt") || die "problem creating data/norm.txt";

print OUTG "$newheader\n";
print OUTR "$newheader\n";
print OUTN "$newheader\n";

for ($row=0; $row<$line; $row++) {
    for ($array = 1; $array <= 24; $array++) {
        if ($array == 1) {
            print OUTG "$newline[$row]";
            print OUTR "$newline[$row]";
            print OUTN "$newline[$row]";
        }
        print OUTG "\t$green[$array][$row]";
        print OUTR "\t$red[$array][$row]";
        print OUTN "\t$norm[$array][$row]";
    }
}
print OUTG "\n";
print OUTR "\n";
print OUTN "\n";

```

```
}
```

```
close OUTG;  
close OTR;  
close OUTN;
```

5) The script used to perform the linear regression and the Spearman's test

```

#linear_regression
y = c(0.56, 1.74, 0.93, 2.28, 2.98, 0.54, 1.47, 2.18, 1.71, 2.05, 1.39, 0.80, 0.88, 1.14, 1.46, 2.04, 1.77,
0.78, 1.60, 1.35, 0.63, 0.90, 2.33, 0.44)

M=read.table("norm.txt",header=T,sep="\t")
M=as.matrix(M)
row=nrow(M)
m=matrix(0, nrow=row,ncol=1)

for (i in 1:row)
{
x=as.numeric(M[i,6:29])
z=lm(y ~ x)
s=summary(z)
f=s$fstatistic

p_value=1-pf(f["value"],f["numdf"],f["dendf"])
m[i,1]=p_value
}

write.table(m, "single_linear_regression_p_alone.txt", append=T, col.name=F, row.name=F, quote=F,
sep="\t")
# add title to the single_linear_regression_p.txt
M=read.table("norm.txt",header=T,sep="\t")
m=read.table("single_linear_regression_p.txt", header=T, sep="\t")
write.table(cbind(M,m), "single_linear_regression_p.txt", append=T, col.name=F, row.name=F, quote=F,
sep="\t")

#the_spearman_test
y = c(0.56, 1.74, 0.93, 2.28, 2.98, 0.54, 1.47, 2.18, 1.71, 2.05, 1.39, 0.80, 0.88, 1.14, 1.46, 2.04, 1.77,
0.78, 1.60, 1.35, 0.63, 0.90, 2.33, 0.44)
M=read.table("norm.txt", header=T, sep="\t")
M=as.matrix(M)
row=nrow(M)
m=matrix(0, nrow=row,ncol=1)
for (i in 1:row)
{
x=as.numeric(M[i,6:29])
z=cor.test(x,y,method="spearman")
p_value=z$p.value
m[i,1]=p_value
}

write.table (m, "spearman_p_alone.txt", append=T, col.name=F, row.name=F, quote=F, sep="\t")
# add title to the spearman_p_alone.txt
M=read.table("norm.txt",header=T,sep="\t")
m=read.table("spearman_p_alone.txt", header=T, sep="\t")
write.table(cbind(M,m), "spearman_p.txt", append=T, col.name=F, row.name=F, quote=F, sep="\t")

```

- 6) The script for false positive for the p-values generated from linear regression analysis and the Spearman's test

```
#Linear regression:
M=read.table("both_analysis.txt", header=T, sep="\t")
MM=order(M$linear_p)
p=M[,30]
pp=sort(p)*243486/1:243486
q=pp
for (i in 243485:1) {q[i]=min(q[i+1],pp(i))}
#extract the first 1000 genes
write.table(cbind(M[MM[1:1000],],p[1:1000]), "linear_1000_genes.txt", col.name=F, row.name=F,
append=F, sep="\t")

#spearman:
M=read.table("both_analysis.txt", header=T, sep="\t")
MM=order(M$spearman_p)
p=M[,31]
pp=sort(p)*243486/1:243486
q=pp
for (i in 243485:1) {q[i]=min(q[i+1],pp(i))}
#extract the first 1000 genes
write.table( cbind (M [MM[1:1000],], p[1:1000]), "spearman_1000_genes.txt", col.name=F,
row.name=F, append=F, sep="\t")
```

Appendix: Publications included in the original thesis

1. **Ma H.**, Hagen F., Stekel D., Johnston S. D., Sionov E., Falk R., Polacheck I., Boekhout T., May R.C., The fatal fungal outbreak on Vancouver Island is characterized by enhanced intracellular parasitism driven by mitochondrial regulation, PNAS **106**: 12980-85 (2009)
2. **Ma H.**, and May R.C., Virulence in cryptococci, Advances in Applied Microbiology **67**: 131-90 (2009)
3. **Ma H.**, Croudace J. E., Lammas D. A., May R. C., Direct cell-to-cell spread of a pathogenic yeast, BMC Immunology **8**: 15 (2007)
4. **Ma H.**, Croudace J. E., Lammas D. A., May R. C., Expulsion of live pathogenic yeast by macrophages, Current Biology **16**: 2156-60 (2006)